A STRUCTURAL AND FUNCTIONAL ANALYSIS OF CYCLIN INTERACTIONS WITH THE RETINOBLASTOMA PROTEIN FAMILY MEMBER P130

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Abstract

pRb, p107 and p130 are structurally and functionally related polypeptides which comprise the retinoblastoma family of proteins. All three proteins are found in complexes with several cell cycle-regulating proteins, including cyclins and cyclin-dependent kinases (cdk's) which are thought to regulate the function of the pRb family members through phosphorylation. *In vivo*, p130 is observed in cyclin A/cdk2 and cyclin E/cdk2 complexes but not in complexes containing D-type cyclins and cdk4. This thesis examines these observations by identifying regions within the p130 sequence required for cyclin interactions. *In vitro* binding studies determined that D-type cyclin interactions require the majority of the "pocket domain" of p130. These interactions are disrupted upon phosphorylation of p130 by the cyclin D-associated kinase cdk4. Additional *in vitro* binding studies determined that a short sequence within the "spacer region" of p130 is required for interactions with cyclins A and E. This sequence contains an "RRL" motif which is present in several other cyclin A and cyclin E binding proteins. *In vivo*, the amino terminus of p130 is required to stabilize p130 interactions with cyclins A and E and this may be a result of inhibition of cdk2-associated kinase activity. Taken together, these results suggest that stable complexes containing p130 and cyclins A and E are not disrupted by phosphorylation of p130, perhaps because p130 inhibits the kinase activity of cdk2. In contrast, p130 interactions with D-type cyclins are disrupted *in vivo* because of cdk4-mediated phosphorylation of p130. This analysis concludes that D-type cyclin

interactions with p130 are structurally and functionally distinct from interactions between p130 and cyclins A and E and these differences may be important in the regulation of p130 during the cell cycle.

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List of Abbreviations

γ	gamma
AcNPV	*Autographa californica* nuclear polyhedrosis virus
ATP	adenosine 5' triphosphate
cDNA	complementary DNA
CT	carboxyl terminus
CTP	cytidine 5' triphosphate
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
G418	Geneticin (neomycin) (Gibco)
GST	glutathione *S*-transferase
GTP	guanosine 5' triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
IP	immunoprecipitation
iv	intravenously

IPTG	isopropyl-ß-D-galactopyranoside
kDa	kilodaltons
KLH	keyhole limpet hemocyanin
lmt	low melting temperature
mRNA	messenger ribonucleic acid
M	molar
MOI	multiplicity of infection
nt	nucleotides
$O.D._{260}$	optical density at 260 nm
pBS(KS)	Bluescript (KS) plasmid (Pharmacia)
pCMV	plasmid with cytomegalovirus promoter/enhancer sequences
pVL1392	*Autographa californica* nuclear polyhedrosis virus transfer vector
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PPO	diphenyloxazole
RAM	rabbit anti-mouse antibody
RNA	ribonucleic acid
RPM	revolutions per minute
SDS	sodium dodecyl sulphate

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TLC	thin-layer chromatography
TRIS	hydroxymethyl aminomethane
UTP	uridine 5' triphosphate

Chapter 1. Introduction

1.1 Definition of the cell cycle

The eukaryotic cell cycle is viewed as several discrete stages which culminate in the division of a cell into two genetically identical daughter cells. In the synthesis or S phase, a cell replicates its DNA and synthesizes histones to coat the chromosomes. S phase is preceded by a gap phase (G1), a functional period used by the cell to prepare for entry into S phase. Following S phase is a second gap phase (G2), during which cells continue to grow and prepare for the processes of segregation of chromosomes and division, which occur during mitosis or M phase. Cells which do not undergo growth and division may be differentiated or quiescent are said to be in "G0", a period prior to G1.

Co-ordination of cell cycle progression is regulated by a large group of genes whose products are necessary to transmit the correct signals to the nucleus to ensure replication and division occurs only in favorable conditions (reviewed in Müller *et al.*, 1993). Members of this group of genes include cyclins and cyclin-dependent kinases, the retinoblastoma family of proteins and the E2F transcription factor family, each of which is described in the sections that follow.

1.2 Discovery of maturation promoting factor

Early work on cell cycle focused on marine invertebrate oöcyte maturation. When stimulated with progesterone, fully grown frog oöcytes undergo meiosis and remain dormant until fertilization occurs. Following fertilization, the embryo undergoes many

rapid synchronous divisions referred to as embryonic cell cycles. The events which follow progesterone stimulation are characterized by breakdown of the nuclear envelope, condensation of the chromosomes and assembly of the meiosis I spindle. These events are described as meiotic maturation and early cell cycle investigators were focused on determining what factors were required following progesterone stimulation to induce maturation of oöcytes.

Studies determined that a cellular component present in mature oöcytes transferred to immature oöcytes resulted in the complete maturation of the immature eggs in the absence of hormonal stimulation (Dettlaff *et al.*, 1964; Smith and Ecker, 1971; Reynhout and Smith, 1974). The unidentified component was termed "maturation promoting factor" or MPF and was subsequently determined to be present in additional types of vertebrate as well as invertebrate cells (Kishimoto *et al.*, 1982; Sorensen *et al.*, 1985).

MPF was noted to be cyclically regulated, being present in fertilized eggs at high levels in mitosis, rapidly disappearing following mitosis, and appearing again during the following mitosis (Wasserman and Smith, 1978; Gerhart *et al.*, 1984). Around the same time, other investigations into invertebrate development identified proteins which cyclically oscillated during embryonic growth and division (Rosenthal *et al.*, 1980; Evans *et al.*, 1983). These proteins were named "cyclins" because they rapidly disappeared following mitosis and appeared again in cell lysates as mitosis began in the following embryonic cycle (Evans *et al.*, 1983). Although MPF activity and cyclin expression

correlated, only purification and identification of the components of MPF would elucidate whether there was indeed a connection.

1.3 Identification of cyclins and cyclin-dependent kinases: a working model of cell cycle control

MPF activity was crudely purified as a cell-free extract which could be used to induce nuclear envelope breakdown, chromosome condensation and spindle formation of somatic cell nuclei (Miake-Lye and Kirschner, 1985) or sperm pronuclei (Lohka and Maller, 1985) *in vitro*. Eventually MPF was purified several thousand-fold and was shown to be composed of at least two major proteins and a kinase activity capable of phosphorylating histone H1 *in vitro* (Pelech *et al.*, 1987; Lohka *et al.*, 1988).

Clues to the identity of at least one of the major proteins present in MPF came following the identification of clam and sea urchin cyclins A, B1 and B2 (Minshull *et al.*, 1989; Murray and Kirschner, 1989; Minshull *et al.*, 1990). It was demonstrated that frog oöcytes injected with mRNA encoding clam cyclins A and B matured without progesterone (Swenson *et al.*, 1986; Pines and Hunt, 1987; Westendorf *et al.*, 1989). Importantly, oöcytes injected with a combination of clam cyclin A and cyclin B mRNA's entered mitosis ahead of those injected with either cyclin alone, suggesting cyclins A and B could cooperate as inducers of mitosis in marine invertebrates. This data proved MPF contained a cyclin component and later work with frog oöcytes determined MPF was composed of cyclin A, cyclin B and a histone H1 kinase named cdc2 (Arion *et al.*, 1988;

Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Labbe *et al.*, 1988; Draetta *et al.*, 1989; Meijer *et al.*, 1989; Gautier *et al.*, 1990).

Following the identification of components comprising MPF activity, models of cell cycle control attempted to integrate the data on cyclin accumulation and destruction with kinase activity into a working model. A favored model of MPF activity suggested an increase in concentration of cyclin coupled with its association with cdc2 kinase resulted in phosphorylation of histone H1 or other substrates such as nuclear lamins and filaments (Chow *et al.*, 1990; Peter *et al.*, 1990; Ward and Kirschner, 1990). It was thought that phosphorylation of these proteins resulted in cellular changes necessary to induce cellular division. The model was useful because histone H1 kinase activity declined following degradation of cyclin, an event mediated by the ubiquitin pathway (Glotzer *et al.*, 1991). That kinase activity declined in the absence of cyclin implied that cdc2 was a cyclin-dependent kinase or "cdk" (Draetta *et al.*, 1989; Meijer *et al.*, 1989; Murray *et al.*, 1989). These results complemented the working model and taken together suggested that cyclin degradation resulted in kinase inactivation, mitosis and division.

As mentioned, MPF activity was detected in additional types of invertebrate and vertebrate cells. However, whereas early embryonic cell cycles consist of inactivation of MPF, exit from mitosis, replication of DNA in S phase and re-entry into mitosis, cell cycles in somatic cells are much longer and more complex. In the somatic cell cycle, an additional transition called "Start", which takes place in G1, must occur before the cell can initiate replication of DNA in S phase (Nurse and Bisset, 1981; Piggott *et al.*, 1982; Reed

and Wittenberg, 1990). "Start" is the term used to describe the transition in unicellular eukaryotic organisms whereas the "restriction point" is the term used for multicellular organisms (Pardee, 1974).

1.3.i Yeast cyclins and cyclin-dependent kinases

The additional complexities of somatic cell cycles were elucidated using yeast genetics. This work made use of temperature-sensitive cell division cycle (cdc) yeast mutants which arrested during the cell cycle when grown at their restrictive temperatures. Two genes able to complement these temperature-sensitive mutants encoded a 34 kDa protein kinase and a kinase regulatory subunit (cyclin). The presence of these genes in yeast predicted that cyclins and cyclin-dependent kinases were evolutionarily conserved and were universally used as a mechanism of controlling cell division (Beach *et al.*, 1982; Nurse, 1990). As in frog eggs, yeast cdc2 homologues (cdc2 in *S. pombe* and CDC28 in *S. cerevisiae*), are expressed at a constant level throughout the yeast cell cycle and become active only during their time-limited association with cyclins (Meijer *et al.*, 1989; Murray, 1989; Draetta *et al.*, 1989).

In yeast, cdc2/CDC28 kinases regulate not only the G2/M transition but also "Start". Yeast kinase activity at the G2/M phase junction is positively regulated by associations between cyclin B (the product of the cdc13 gene) in *S. pombe* (Booher *et al.*, 1989; Moreno *et al.*, 1989) and between a family of cyclin B-like proteins in *S. cerevisiae* named CLB's 1 through 6 (Ghiara *et al.*, 1991; Surana *et al.*, 1991; Schwob and Nasymth, 1993). In *S. cerevisiae*, the association of CDC28 with G1 cyclins named CLN-1, -2 and -

3 regulates passage of cells through Start and coordinates entry into S phase (Richardson *et al.*, 1989; Hadwiger *et al.*, 1989; Wittenburg, *et al.*, 1990; Tyers *et al.*, 1992). The genetic studies in yeast provided insight into somatic cell cycles by allowing the identification of families of cyclins and cyclin-dependent kinases and by revealing the higher complexity of control of cell division present in these and other eukaryotes.

1.4 Mammalian cyclins and cyclin-dependent kinases

Although much work in the field of cell cycle was initiated in marine invertebrates and continues to be extended using yeast genetics, the remainder of this introduction will focus on the mammalian cell cycle, with specific emphasis on the human proteins involved. The mammalian cell cycle is also regulated by cyclins and cyclin-dependent kinases, and as in yeast these proteins are members of large families of related molecules.

Like all eukaryotic cyclins, human cyclins share sequence similarities. These similar sequences include a conserved 100-150 amino acid domain called the cyclin box (Hunt, 1991), and mutations in the cyclin box result in loss of cdk interaction and activation (Kobayashi, 1992; Lees and Harlow, 1993). In mammals there are at least a dozen cyclins and eight cyclin-dependent kinases named to date and several more of each have been identified which are not well-characterized. The best-characterized cyclins and cyclin-dependent kinases are discussed individually below, with cyclin/cdk complexes discussed in the order they appear following cell division.

1.4.i G1 cyclins and their associated kinases

Cyclins C, D1 and E were isolated as human cDNA's which were efficient in rescuing a constructed *S. cerevisiae* strain deficient in the three CLN genes (Koff *et al.*, 1991; Lew *et al.*, 1991; Xiong *et al.*, 1991). As mentioned, yeast CLN-1, -2 and -3 are G1 cyclins which regulate the passage of cells through "Start". The experimental rationale reasoned this screen would identify the human homologues of yeast G1 cyclins. Although the cDNA's encoding cyclins C, D1 and E were efficient in rescuing yeast mutants, these cyclins have since been determined to function before, during and following G1 and their precise contribution to control of the cell cycle is still debated.

Cyclin C message levels peak in mid G1 but only by a two-fold increase over other points in the cell cycle (Lew *et al.*, 1991) and cyclin C complexes with and activates cdk8 (Tassan et. al, 1995b). A role for cyclin C/cdk8 complexes in cell cycle transitions has not been well established, but the complexes may be involved in transcriptional regulation. Cyclin C was found to associate with the large subunit of RNA polymerase II in an assay to determine cyclin C binding proteins. This subunit may be a *bona fide* substrate of this cyclin/cdk combination because the carboxyl terminal domain of RNA polymerase II is phosphorylated by cdk8 *in vitro* (Rickert *et al.*, 1996).

More data has been collected on the D-type cyclins and cyclin E, which are thought to participate in G1 control because they are expressed at some point during G1. In addition to its identification in the yeast CLN substitution experiment, cyclin D1 was

also identified as a gene (PRAD1) whose coding sequence was juxtaposed to the parathyroid hormone promoter from a break and inversion on chromosome 11 (Motokura *et al.*, 1991) and as a gene deregulated by translocation (Withers *et al.*, 1991) and amplification (Shuuring *et al.*, 1993) in several other tumor types. The presence of excess cyclin D1 levels in all of these tumors implied that like other cyclins, cyclin D1 was a regulator of cell growth and was a putative proto-oncogene. Cyclin D1 may normally function during development of eyes and breast, because mice lacking cyclin D1 show reduced numbers of retinal cells and females fail in proliferation of the breast epithelial compartment during pregnancy (Fantl *et al.*,, 1995; Sicinski *et al.*, 1995). Cyclins D2 (Inaba *et al.*, 1992; Won *et al.*, 1992; Xiong *et al.*, 1992b; Ajchenbaum *et al.*, 1993) and D3 (Inaba *et al.*, 1992; Motokura *et al.*. 1992; Won *et al.*, 1992; Xiong *et al.*, 1992b; Ajchenbaum *et al.*, 1993) are cyclin D1-related proteins and the three form a family of differentially-expressed proteins thought to be somewhat functionally redundant (Matsushime *et al.*, 1991).

Growth-factor stimulation of cells results in induction of D-type cyclin synthesis (Matsushime *et al.*, 1991; Motokura *et al.*, 1992; Won *et al.*, 1992) with cyclin D1 and D2 expression being maximal at the G1/S border of the cell cycle (Won *et al.*, 1992). Unlike cyclin D1 and D2, cyclin D3 levels begin to elevate at the G1/S boundary and peak in S phase (Motokura *et al.*, 1992). The D-type cyclins form complexes with cdk2, cdk4, cdk5 and cdk6, although the most predominant cellular complexes contain cdk4 and cdk6

(Tsai *et al.*, 1991; Meyerson *et al.*, 1992; Matsushime *et al.*, 1992; Xiong *et al.*, 1992a; Bates *et al.*, 1994a; Matsushime *et al.*, 1994; Meyerson and Harlow, 1994).

In mammalian cells, passage through the "restriction point" defines the time at which cells no longer require growth factors to enter S phase and complete the cell cycle (Pardee, 1974). Several lines of evidence show D-type cyclins can function during the restriction point. For instance, injection of anti-cyclin D antibodies into G1 fibroblasts prevents entry into S phase (Baldin *et al.*, 1993; Quelle *et al.*, 1993). The effect of cyclin D1 synthesis begins once the protein appears in mid G1 because microinjection of cells near the G1/S boundary has no effect (Baldin *et al.*, 1993). Overexpression of cyclin D1 and cyclin D2 in rodent fibroblasts decreases the time needed for cells to progress from G1 to S phase (causing a decrease in overall cell size) and results in a reduced serum requirement for growth (Quelle *et al.*, 1993). Constitutive overexpression of cyclins D2 and D3 in hematopoeitc cells results in a shortening of G1 and an increased fraction of cells in S phase (Ando *et al.*, 1993). Taken together, the data on D-type cyclins suggest they are growth factor sensors which function in the cell during a discrete interval in G1, and when present, allow progression through the restriction point.

Cyclin E levels accumulate in mid G1 and peak in late G1/S suggesting it regulates kinases following the expression of D-type cyclins (Koff *et al.*, 1991; Dulic et al, 1992; Koff *et al.*, 1992). Like cyclin A, cyclin E interacts with cdc2 and cdk2 *in vitro* (Koff *et al.*, 1991; Koff *et al.*, 1992), although the majority of cyclin E-associated kinase activity is attributed to cdk2 *in vivo* (Koff *et al.*, 1992). Inactivation of cyclin E by microinjection of

cyclin E antibodies or through expression of a dominant-negative cdk2 blocks cell entry into S phase and overexpression of cyclin E in fibroblasts shortens G1 resulting in a decrease of overall cell size and decreased dependence on serum (Pagano *et al.*, 1992; 1993; van den Heuvel and Harlow, 1993; Ohtsubo *et al.*,, 1995). Interestingly, simultaneous overexpression of cyclin D1 and cyclin E results in a further decrease in the time spent in G1, indicating that D-type cyclins and cyclin E regulate different processes during the G1 to S phase transition (Resnitzky and Reed, 1995).

1.4. ii. Mitotic cyclins and their associated cyclin dependent kinases

Cyclins A and B are viewed as mitotic cyclins, with cyclin A being the main cyclin/cdk complex present once cells have entered S phase and cyclin B/cdc2 complexes being the primary protein kinase active in mitosis. Cyclin A was originally isolated as an adenovirus E1A-associated protein in adenovirus-infected HeLa cells (Harlow *et al.*, 1986; Giordano *et al.*, 1989), suggesting it was targeted by the virus to allow viral growth and replication. Cyclin A was also identified in a hepatocellular carcinoma as a gene disrupted by integration of the hepatitis B genome (Wang *et al.*, 1990). These observations supported the notion that cyclins were important in cell division and identified cyclin A as a gene product regulating cellular growth.

Cyclin A mRNA and protein levels normally increase in the nucleus just before the onset of S phase and remain elevated until mitosis, at which point the message is degraded (Girard *et al.*, 1991; Pines and Hunter, 1990; Pines and Hunter, 1991). Cyclin A interacts

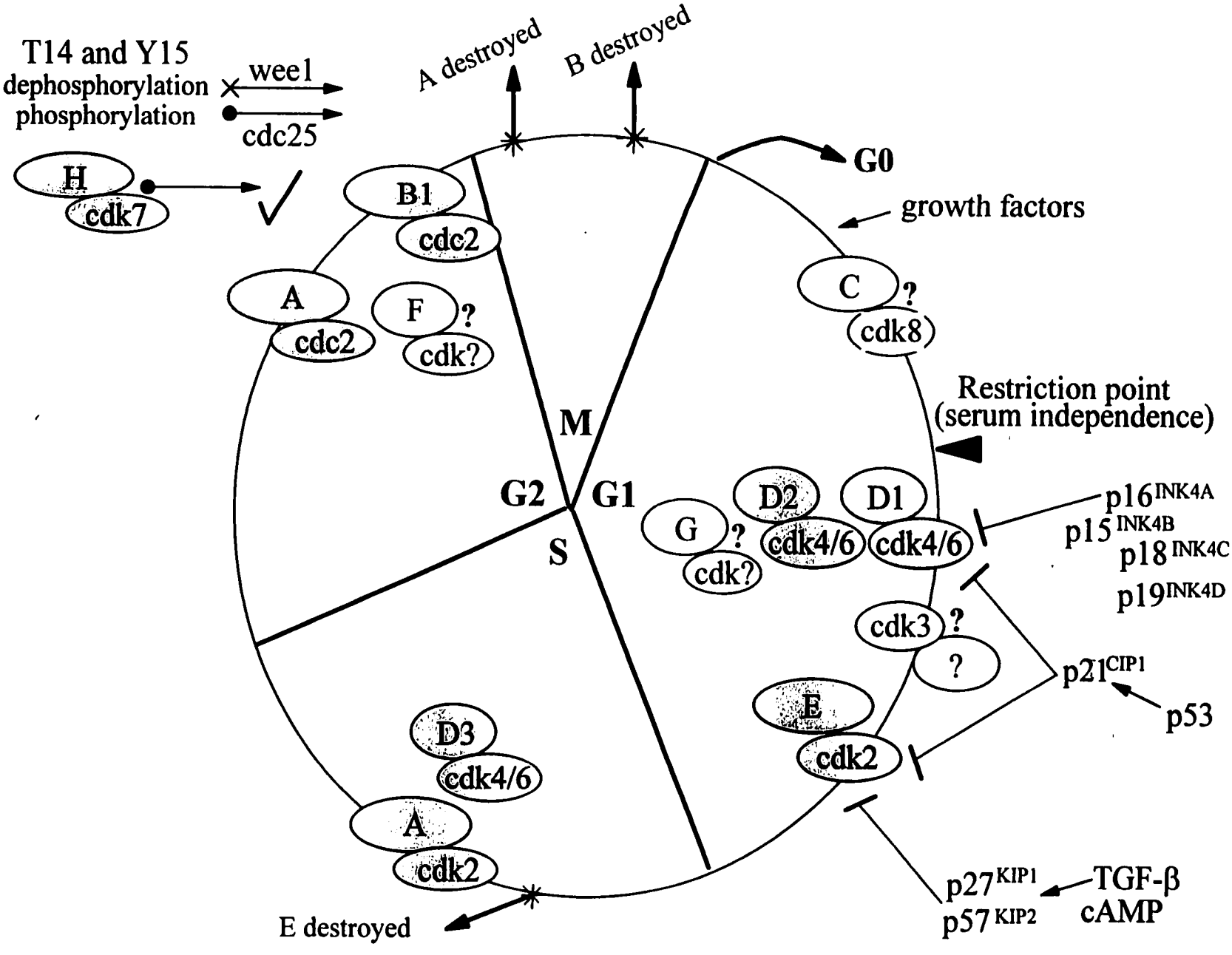

T14 and Y15
dephosphorylation
phosphorylation
wee1
cdc25
H
cdk7
A destroyed
B destroyed
G0
growth factors
B1
cdc2
A
cdc2
F
?
cdk?
C
?
cdk8
Restriction point
(serum independence)
M
G2
G1
S
D2
D1
G
?
cdk?
cdk4/6
cdk4/6
p16INK4A
p15INK4B
p18INK4C
p19INK4D
cdk3
?
?
p21CIP1
p53
E
cdk2
D3
cdk4/6
A
cdk2
E destroyed
p27KIP1
p57KIP2
TGF-β
cAMP

Figure 1.1 Cyclin/cdk complexes during the mammalian cell cycle

The cell cycle is depicted as a circle, with discrete stages labeled in the middle of the circle and G0 represented as an exit from the cycle (at the upper part of the circle). The restriction point , shown by a black arrow, is shown on the right-hand side. Cyclin/cdk complexes are placed at particular points during the cell cycle where they are thought to be active. Well-defined complexes are indicated in shaded ovals, with the cyclin component represented as a letter and the cdk component labeled on the accompanying subunit. Complexes with poorly defined roles are depicted as white ovals and their placement in the cell cycle includes a question mark (?). In addition, some cyclin components are yet to be identified and these are also represented with a (?). Stages during the cycle when cyclins E, A and B are destroyed by ubiquitination are indicated by arrows with stars. Points in the cycle where cyclin-dependent kinase inhibitors and family members are thought to act due to particular stimuli are indicated, as well as points where cyclins are thought to be degraded. Inactivating and activating phosphorylation events catalysed by wee1 and cdc25, respectively are thought to be common to all cyclin/cdk complexes and are therefore indicated in the upper left-hand corner (T14 is threonine 14 and Y15 is tyrosine 15). In addition, most cyclin/cdk complexes are thought to be activated by cyclin H/cdk7-mediated phosphorylation, shown at the upper left. This figure is somewhat generalized and serves to highlight data discussed in the text. This figure was adapted from Pines, 1995a.

not only with cdc2 (also known as cdk1), but also with cdk2 (Pines and Hunter, 1990; Elledge and Spotswood, 1991; Tsai *et al.*, 1991). Microinjection of an antisense cyclin A-expressing plasmid or cyclin A antibodies into G1 fibroblasts leads to inhibition of DNA synthesis (Girard *et al.*, 1991) and microinjection of cells in G2 results in inhibition of mitosis (Pagano *et al.*, 1992a). Additional studies have shown that cdk2 is activated prior to S phase and that this activity is required for cells to progress from G1 to S phase (Tsai *et al.*, 1993b). These results show the activity of cyclin A/cdk2 complexes is required for entry into S phase and also for continuation of the cycle into mitosis.

Human B-type cyclins (B1 and B2) were discovered by screening cDNA libraries with sequences homologous to conserved regions of cyclins from several different species (Pines and Hunter, 1989). B-type cyclin levels are difficult to detect in G1 and increase in S and G2 phases, peaking at mitosis when it is subsequently destroyed. Unlike most cyclins which are strictly nuclear proteins, cyclin B1 appears in the cytoplasm late in S phase where its levels are maintained throughout G2 until it translocates to the nucleus prior to nuclear envelope breakdown (Pines and Hunter, 1989; Pines and Hunter, 1991; Jackman *et al.*, 1995). Cyclin B2 appears to be associated with the Golgi throughout the cell cycle and unlike cyclin B1, retains a cytoplasmic distribution at M phase (Jackman *et al.*, 1995). The precise function of cyclin B2 is not well understood. Figure 1.1 is a shematic which attempts to incorporate much of the discussion on activation and function of cyclin/cdk complexes dicussed in the preceeding and following pages.

As in yeast, mammalian B-type cyclins bind and activate cdc2 (Pines and Hunter, 1989; Girard, 1991; Pines and Hunter, 1991). In contrast to microinjection of antisense cyclin A plasmids into cells, injection of antisense cyclin B plasmids does not result in inhibition of S phase (Girard *et al.*, 1991), defining the B-type cyclins as strictly mitotic. Although cyclin A, cyclin B1, and cyclin B2 are degraded during mitosis, cyclin A disappears a few hours before cyclin B, suggesting cyclin A is degraded during metaphase and cyclin B1 and B2 during anaphase of mitosis (Pines and Hunter, 1990).

1.4. iii Additional cyclin/cdk complexes and the cdk-activating kinase complex

Several other cyclins and cdk's have been identified which are not as well understood as those already described. These include three recently-identified cyclins named F, G and I. Cyclin F message levels fluctuate and peak in S and G2 phases, co-incident with cyclin A expression but disappearing well before cyclin B expression is decreased. Overexpression of cyclin F in cervical carcinoma cells results in an increased population of G2/M cells, predicting a role for cyclin F in the G2/M cell cycle transition (Bai *et al.*, 1994). The expression pattern of cyclin G shows no correlation to any specific cell cycle stage but like cyclin D expression, its mRNA levels are induced following stimulation with serum (Tamura *et al.*, 1993). Cyclin I, although cloned from a rat cDNA library, is most related to cyclin G and also shows no particular cell cycle-related expression pattern. Cyclin I message is highly expressed in postmitotic tissues including the brain but the significance of this protein in cell cycle control is not known (Nakamura

et al., 1995). In addition to the ill-understood function of these three cyclins, a kinase partner for each has yet to be identified.

Also not well characterized are at least two cdk's which share sequence similarities with other cdk family members. The activating partner of cdk5 appears not to be a cyclin but a 35 kDa protein which is expressed throughout the brain (Lew *et al.*, 1994; Tsai *et al.*, 1994). As a result, cdk5 activity is brain-specific and is present in postmitotic neurons, suggesting it may not contribute to cell cycle regulation in proliferating cells (Tsai *et al.*, 1993a, 1994; Lew *et al.*, 1992a,b; Lew *et al.*, 1994). Like cdk5, cdk3 is a poorly characterized kinase suspected of having a function in G1 in several cell types and its cyclin partner (if there is one) has yet to be identified (van den Heuvel and Harlow, 1993).

One cyclin/cdk combination yet to be mentioned is that of cyclin H/cdk7. Unlike other cdk complexes, this rather unique kinase complex is constitutively active throughout the cell cycle, implying it has no single role in cell cycle transitions *per se* (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon et al, 1993; Fisher and Morgan, 1994; Makela *et al.*, 1994; Matsuoka *et al.*, 1994; Poon et al, 1994, Tassan et al, 1995a). Instead, this complex appears to be a cdk-activating kinase or "CAK", which mediates phosphorylation events on threonine residues located on the "T loop" of cdk's (Krek and Nigg, 1991a.; Gould *et al.*, 1991; Guerol *et al.*, 1992; DeBondt *et al.*, 1993; Kato *et al.*, 1994b; Matsuoka *et al.*, 1994). This modification is known to be required for full activation of all cyclin-dependent kinases. The T loop blocks the entrance to the cdk2 catalytic cleft and

crystal structure analysis of a cyclin A/cdk2 complex reveals the T loop moves away from the active site as a result of cyclin A-induced conformational changes. This conformational change also allows the T loop to become more accessible to phosphorylation (hence activation) by CAK (Jeffrey *et al.*, 1995).

The number of cyclins and cdk's present in mammalian cells hint that cell cycle control in mammals is somewhat more advanced than the basic cell cycle mechanisms initially elucidated in marine invertebrates. The overlapping expression patterns of cyclins resulting in co-ordinated interactions with particular cdk's is subtle enough that elucidation of distinct substages within each the cell cycle transition is virtually impossible. Adding to this complexity is the existence of additional regulatory molecules which negatively regulate cyclins and cdks.

1.5 Negative regulation of cyclin/cdk complexes

In addition to the inhibition of active cyclin/cdk complexes by destruction of cyclin, there are at least two other mechanisms whereby cells negatively regulate the activity of these complexes. One mechanism is inhibitory phosphorylation on threonine and tyrosine residues in cdk's (Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren *et al.*, 1991; Russell *et al.*, 1989). Important in the negative regulation of cyclin/cdc2 complexes following mitosis, phosphorylation of these residues parallels the rise in cyclin levels occurring as cells approach M phase (Solomon *et al.*, 1990; Krek and Nigg, 1991a,b). Structural analysis of cdk2 demonstrates that phosphorylation on Thr 14 and Tyr 15 prevents ATP from entering the catalytic domain (DeBondt *et al.*, 1993). The

phosphorylation of Thr 14 and Tyr 15 is catalysed by the wee1 kinase (Featherstone and Russell, 1991; Krek and Nigg, 1991a,b; Norbury *et al.*, 1991; Parker *et al.*, 1992; McGowan and Russell, 1993), a molecule also regulated by phosphorylation (reviewed in Dunphy, 1994). Relief of this inhibition occurs upon dephosphorylation of these same residues by the cdc25 protein phosphatase (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991; Honda *et al.*, 1993).

1.5.i Inhibitors of cdk's

An additional mechanism of negative regulation of cyclin/cdk complexes involves a family of proteins named cyclin-dependent kinase inhibitors (CKI's). In mammals, several CKI's have been identified which are divided into two classes by sequence homology. One class includes $p21^{CIP1}$ (Harper *et al.*, 1993; El Deiry et al, 1993; Xiong *et al.*, 1993a; Gu *et al.*, 1993), $p27^{KIP1}$ (Polyak *et al.*, 1994a,b ; Toyoshima and Hunter, 1994) and $p57^{KIP2}$ (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). These homologous proteins selectively inhibit cyclin A/cdk2, cyclin D/cdk4 and cyclin E/cdk2 complexes and weakly inhibit cyclin B/cdc2 complexes (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993a; Polyak *et al.*, 1994a,b; Toyoshima and Hunter, 1994; Harper *et al.*, 1995). $p21^{CIP1}$ contains binding sites for both cyclins and cdk's, suggesting this inhibitor targets cyclin/cdk complexes and not cdk's individually (Chen *et al.*, 1995; Goubin and Ducommun, 1995; Luo *et al.*, 1995; Lin *et al.*, 1996). As compared to $p27^{KIP1}$ and $p57^{KIP2}$, $p21^{CIP1}$ contains an additional domain which allows it to interact with the DNA polymerase δ-subunit proliferating cell nuclear antigen (PCNA) (Xiong *et al.*, 1992a; Flores-Razas *et al.*, 1994;

Waga *et al.*, 1994). Although not completely understood, this interaction may allow $p21^{CIP1}$ to directly regulate DNA replication. $p21^{CIP1}$ induction may also be necessary to allow exit from the cell cycle, as levels of $p21^{CIP1}$ increase during terminal differentiation of several cell types (Steinman *et al.*, 1994; Jiang *et al.*, 1995; Macleod *et al.*, 1995; Poluha *et al.*, 1996).

Another class of related cdk inhibitors includes $p16^{INK4}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ which contain ankyrin repeats and bind directly to cdk6 and cdk4, although with less affinity to cdk4 (Serrano *et al.*, 1993; Guan et al, 1994; Hannon and Beach, 1994; Toyoshima and Hunter, 1994; Hirai *et al.*, 1995; Guan *et al.*, 1996). The binding of these inhibitors to cdk's occurs in the absence of cyclins and results in inhibition of cdk4 and cdk6 kinase activity.

The accumulation of the CKI's can be induced by several antiproliferative signals and this induction is thought result in negative regulation of G1 phase progression (Harper *et al.*, 1993; Xiong *et al.*, 1993a; Serrano *et al.*, 1993; Dulic *et al.*, 1994; El-Deiry *et al.*, 1994; Polyak *et al.*, 1994a,b; Toyoshima and Hunter, 1994; Deng *et al.*, 1995). For example, $p21^{CIP1}$ is transcriptionally induced by wild type p53 following exposure of cells to γ radiation, resulting in a G1 cell cycle arrest (El-Deiry *et al.*, 1993, 1994; Dulic *et al.*, 1994, Macleod *et al.*, 1995). Additionally, $p21^{CIP1}$ and $p15^{INK4B}$ levels are induced in keratinocytes when treated with TGF-ß, a growth inhibitory factor which arrests cells in G1 (Ewen *et al.*, 1993a; Hannon and Beach, 1994; Datto *et al.*, 1995). Whereas levels of $p21^{CIP1}$ and $p15^{INK4B}$ are induced following stimulation by antiproliferative signals, $p27^{KIP1}$

levels appear highest during quiescense and then decline upon mitogenic stimulation. For example, an accumulation of $p27^{KIP1}$ is observed in epithelial cells arrested in G1 as a result of high cell culture density or treatment with TGF- ß (Koff *et al.*, 1993; Polyak *et al.*, 1994a,b; Coats *et al.*, 1996) and these levels decrease when cells are released from inhibition and enter S phase. The high levels of $p27^{KIP1}$ have been shown to inhibit the activity of cyclin E/cdk2 complexes during the G1 phase. Additionally, levels of $p27^{KIP1}$ are increased in macrophages blocked in G1 due to treatment with cAMP (Kato et al, 1994; Ward *et al.*, 1996) and these levels decline when cells are treated with growth factors.

Structural analysis of $p27^{kip1}$ bound to cyclin A/cdk2 complexes reveals the inhibitor binds to the initial portion of the cyclin box of cyclin A through an Leu-Phe-Gly motif conserved in the CIP/KIP family and to a conserved region of cdk2 which is also present in cdk's 4, 5 and 6 (Russo *et al.*, 1996). The binding of $p27^{KIP1}$ to cyclin A/cdk2 results in conformational changes which prevents entry of ATP at the active site of cdk2 by sterically hindering the catalytic cleft (Russo *et al.*, 1996). In addition to the steric hindrance caused by $p27^{KIP1}$, the inhibitor prevents phosphorylation and subsequent activation of cdk4 and cdk6 by CAK (Kato *et al.*, 1994a; Polyak *et al.*, 1994a,b; Russo *et al.*, 1996), and this CAK-inhibiting activity is also shared by p18 (Aprelikova *et al.*, 1995).

$p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ were recently studied in the context of assembly of D-type cyclin/cdk complexes. Each were noted to promote the assembly of cyclin D1/cdk4 complexes in as series of titration assays (LaBaer *et al.*, 1997). Whether cdk inhibitors are

required for the assembly of D-type cyclin/cdk complexes prior to inhibiton of cdk4 activity is not well understood.

1.6 Substrates of cyclin/cdk complexes

Sequential activation of cyclin/cdk complexes during cell cycle progression supports a model whereby successive cyclin/cdk phosphorylation events on particular substrates results in transitions through distinct cell cycle checkpoints. Although histone H1 serves as an *in vitro* substrate of cyclin/cdk complexes, additional physiologic substrates of these active complexes have been identified. Currently, some of the best-characterized substrates include the retinoblastoma family members, pRb, p107 and p130, and the E2F/DP families of transcription factors. Phosphorylation regulates the activities of these substrates, and the concerted effect is thought to result in control of gene expression, providing a link between cyclic activation of kinase complexes to transitions through stages of the cell cycle.

1.6.i Identification of the retinoblastoma tumor suppressor protein

Retinoblastoma is a disease characterized by intraocular tumors which manifest in early childhood (reviewed in Vogel, 1979). It can be either a hereditary and non-hereditary disease, although less than 10% of cases are the former, suggesting the disease results spontaneously by mutational events during gametogenesis. In 1971, Knudson proposed a model to explain the occurrence of the disease. He suggested two independent rate-limiting events lead to the development of retinoblastoma. Each event was termed a "hit", such that inherited retinoblastoma would occur from the inheritence of

a germline mutation (the first hit) followed by a somatic mutational event (the second hit). Non-hereditary retinoblastoma would follow two mutational events in somatic cells (Knudson, 1971).

The RB1 gene was identified by several groups and was localized to chromosome 13q14 using positional cloning with genomic probes (Friend *et al.*, 1986; Friend *et al.*, 1987; Fung *et al.*, 1987; Lee *et al.*, 1987a). In all retinoblastomas examined, this candidate gene was absent or mutated, vindicating the predictions of Knudson's "two hit" hypothesis. In fact, in 70% of all retinoblastomas, the second "hit" is a result of loss of heterozygosity of the RB1 gene and the remaining 30% result from deletions and mutations in the functioning RB1 allele (reviewed in Zacksenhaus *et al.*, 1993).

Deletions and mutations of the RB1 gene occurs in 30% of all human tumors, including osteosarcomas and soft-tissue sarcomas (Friend *et al.*, 1986; Friend *et al.*, 1987; Mendoza *et al.*, 1988; Toguchida *et al.*, 1988; Weichselbaum *et al.*, 1988), small-cell lung carcinomas (Harbour *et al.*, 1988), bladder carcinomas (Horowitz *et al.*, 1989), breast cancers (T'Ang *et al.*, 1988), prostate cancers (Bookstein *et al.*, 1990), primary leukaemias (Furukawa *et al.*, 1990) and others. Thus although loss of pRb strongly predisposes retinoblasts to become tumorigenic, other tissues lacking pRb are also susceptible to a similar fate. As loss or mutation of the RB1 gene results in tumors, the RB1 locus is viewed as a tumor-suppressor gene.

Consistent with a role in the prevention of tumor outgrowth, replacement of missing or mutated pRb alleles by retroviral expression or transfection of a functional pRb

cDNA into some cell lines results in slower cell growth and flattening and enlargement of cells, indicative of growth arrest. These cells also show a decreased tumorigenic potential when injected into nude mice (reviewed in Zacksenhaus *et al.*, 1993). These studies concluded that pRb negatively regulates the growth characteristics of cells and in its absence this growth control is lost.

Studies in mice complement the human data but do not show identical results. The RB1 gene was inactivated in mice using homologous recombination in embryonic stem cells. Mice expressing no functional pRb die *in utero*, but mice heterozygous for pRb (one viable allele) present with pituitary tumors before the first year and show defects in differentiation of several tissue types (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). As mice have never been reported to exhibit retinoblastomas, these results suggest pRb is a tumor suppressor in other mammals but that different tissues display unique susceptibilities to loss of pRb.

1.6. ii. pRb is targeted by viral oncoproteins

During an analysis of adenovirus E1A and E1A-associated cellular proteins, a 110 kDa protein cellular protein present in E1A immunoprecipitates was noticed to be absent in pRb minus cell lines (Whyte *et al.*, 1988a). Using pRb-specific antibodies, this E1A-associated protein was shown to be pRb, a result which aided in elucidating the functional importance of pRb. Although unidentified at the time, several additional cellular proteins were shown to be associated with adenovirus E1A, and E1A mutants unable to interact with pRb or the other E1A-associated cellular proteins were inactive in a transformation

assay (Egan *et al.*, 1988; Whyte *et al.*, 1988b; Egan *et al.*, 1989; Whyte *et al.*, 1989; Giordano *et al.*, 1991; Howe and Bayley, 1992).

The E1A transforming proteins of adenovirus 5 activate transcription of both viral and cellular genes, immortalize cells in culture and transform cells in culture when in the presence of an activated oncogene such as *ras* (reviewed in Bayley and Mymryk, 1994). pRb was also found associated with simian virus 40 (SV40) large T antigen (DeCaprio *et al.*, 1988) and human papilloma virus (HPV) E7 (Dyson *et al.*, 1989a). HPV E7, like E1A, is a transcriptional transactivator and a potent oncoprotein when combined with an activated *ras* protein in primary rat kidney cells (Storey *et al.*, 1988; Phelps *et al.*, 1988). Simian virus (SV) 40 large T antigen can immortalize primary rodent cells and can promote neoplastic transformation of immortalized cell lines (reviewed in Fanning, 1992). These three small DNA tumor viruses are dependent on host cell products for viral gene replication. E1A, large T and E7 each activate cell cycle functions in quiescent cells and are thought to override these functions through direct interactions with host cell proteins.

The E1A polypeptides, SV40 large T antigen and HPV E7 share conserved amino acid motifs composed of an Leu-X-Cys-X-Glu consensus (where X = a variable amino acid) (DeCaprio *et al.*, 1988; Münger *et al.*, 1989; Whyte *et al.*, 1989; Giordano *et al.*, 1991; Dyson *et al.*, 1992) which mediates interactions between these viral proteins and pRb. Like E1A, mutations in the Leu-X-Cys-X-Glu regions which abolish oncoprotein interactions with pRb also reduce the cellular transforming activities of large T and E7 (Phelps *et al.*, 1988; DeCaprio *et al.*, 1989; Munger *et al.*, 1989). The fact that in virally-

infected cells pRb is associated with transforming viral proteins suggests that pRb is a critical *in vivo* target for the cell cycle-regulating activities of viral proteins and that removal or inactivation of pRb is critical to the transformation process.

1.6. iii Phosphorylation of pRb during the cell cycle

pRb is a 110 kDa nuclear phosphoprotein (Lee *et al.*, 1987b) displaying a constant expression level throughout the cell cycle. The phosphorylation state of the molecule is regulated during the cell cycle with pRb maintaining a low level of phosphorylation (hypophosphorylation) during G1 and becoming hyperphosphorylated as cells enter S phase. Following mitosis, pRb is re-converted into the hypophosphorylated form (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989; Ludlow et al, 1990). The hypophosphorylated form of pRb preferentially interacts with E1A (Templeton *et al.*, 1991), SV40 large T (Ludlow *et al.*, 1989) and papilloma virus E7 (Imai *et al.*, 1991), an observation which suggests that the hypophosphorylated form of pRb is the "active" form of the protein specifically targeted by viral transforming proteins.

Phosphorylation of pRb has been suggested to result in release of the protein from its nuclear environment following observations that hyperphosphorylated pRb is found predominantly in the cytoplasmic fraction of lysed cells whereas the hypophosphorylated form is found in the nuclear fraction (Mittnacht and Weinberg, 1991; Templeton, 1992). *In vitro* studies using isolated nuclei showed that hypophosphorylated pRb could be released from nuclei in high salt buffers, whereas hyperphosphorylated pRb eluted from nuclei under low salt conditions. These observations suggest hypophosphorylated pRb

interacts with the nuclear framework or some other nuclear protein which is tightly associated with the nucleus. In accordance with these observations, several naturally occurring mutants of pRb also elute from nucleii in low salt, predicting that improper cellular localization of pRb may contribute to tumorigenesis.

The dephosphorylation of pRb is a poorly understood phenomena, although protein phosphatases 1 and 2A (PP1 and PP2A) have been implicated (Durphee *et al.*, 1993; Ludlow *et al.*, 1993). When injected into the cytoplasm or nucleus of rat cells, PP1 and PP2A were active in modifying pRb such that at the G1/S boundary it remained associated with the nucleus instead of dissociating upon phosphorylation. (Alberts *et al.*, 1993).

pRb appeared to be important in cell cycle regulation when it was shown to regulate progression through G1 of the cell cycle. Injection of synchronized pRb minus osteosarcoma cells with purified wildtype or amino-terminally truncated pRb early in G1 inhibited progression into S phase, and this effect was antagonized by SV40 large T (Goodrich *et al.*, 1991). Injection of cells with either forms of pRb in late G1 or at the G1/S boundary had no effect on S phase or at later points in the cell cycle. In addition, cells transfected with wildtype pRb or amino terminally truncated pRb stopped growing and arrested in early G1 (Templeton *et al.*, 1991; Hinds *et al.*, 1992; Qin *et al.*, 1992). Other investigators using different systems have suggested expression of pRb following S phase arrests cells in G2 (Karantza *et al.*, 1993), although the significance of this observation is not clear.

As well as a role in negative regulation of the G1 phase of the cell cycle, the activity of pRb may also be important in the process of differentiation. As mentioned, RB1 knockout mice die *in utero* at days 12-16 and the embryos also display defects in differentiation of hematopoetic cells and neuronal cells (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Related observations showed that in hematopoetic cell lines induced to differentiate, pRb phosphorylation decreased incrementally during the differentiation process (Akiyama and Toyoshima, 1990), suggesting hypophosphorylated pRb was involved in the process of differentiation. Taken together, these studies suggest pRb is required during development and may be specifically required to execute the processes of differentiation.

The regions of pRb necessary to suppress growth of cell lines correspond to the same regions necessary to interact with E1A, SV40 large T and E7 (Qin *et al.*, 1992; Stirdivant *et al.*, 1992). This domain of pRb is termed the "pocket" domain and encompasses amino acids 379-792 of the 928 amino acid protein. The pocket is composed of two domains, A and B, separated by a short segment (Figure 1.2). The A and B domains are responsible for interactions with viral proteins (Hu *et al.*, 1990; Huang *et al.*, 1990; Kaelin *et al.*, 1990). Importantly, several naturally-occurring pRb mutants isolated from tumors show deletions and mutations in this region of the protein.

1.6. iv. Interactions between pRb and the D-type cyclins

The cell-cycle dependent phosphorylation of pRb was initially proposed to be the result of the kinase activity of cdc2 (Lees *et al.*, 1991; Lin *et al.*, 1991; Hu *et al.*, 1992)

and has subsequently been shown to be mediated by cyclin A/cdk2, cyclin E/cdk2, cyclin D1/cdk4, cyclin D2/cdk4 and cyclin D3/cdk4 complexes (Hinds *et al.*, 1992: Dowdy *et al.*, 1993; Ewen *et al.*, 1993b). In the presence of these complexes, pRb becomes phosphorylated and no longer functions as a growth suppressor (Hinds *et al.*, 1992; Ewen *et al.*, 1993b). Several serine and threonine residues in pRb have been shown to be phosphorylated *in vivo* and *in vitro* by cdc2 or a cdc2-like kinase activity (Figure 1.2) and additional amino acids may also be phosphorylated by other kinases (Lees *et al.*,, 1991).

Unlike cyclin A and cyclin E, the D-type cyclins form stable complexes with pRb both *in vivo* and *in vitro* (Dowdy *et al.*, 1993; Ewen *et al.*, 1993b; Kato *et al.*, 1993) and phosphorylation of pRb by the cyclin D-associated kinase cdk4 appears to dissociate the pRb/cyclin D complex (Kato *et al.*, 1993). The pocket domain of pRb appears to mediate interactions with cyclins D1, D2 and D3 *in vitro* (Dowdy *et al.*, 1993; Ewen *et al.*, 1993b; Kato *et al.*, 1993), implying that viral oncoproteins and D-type cyclins interact with a similar region on pRb. Interestingly, D-type cyclins interact with pRb through the same Leu-X-Cys-X-Glu motif located in their N-termini, which when mutated in cyclin D1 results in a cyclin unable to interact with pRb *in vitro* (Dowdy *et al.*, 1993).

The interactions between pRb and the D-type cyclins is further substantiated by studies in mice. Mouse retinas express high levels of cyclin D mRNA and mice deficient in cyclin D1 show, among other phenotypes, incomplete retinal development (Fantl *et al.*,, 1995; Sicinski *et al.*, 1995). The phenotypes of these mice and of the RB1 knockout mice suggest that pRb and cyclin D are both important for retinal cell proliferation and

differentiation. Further data supporting the connection between pRb and cyclin D came from observations that pRb minus cell lines contained normal levels of cdk4 and cdk6 but that these kinases were not found in complexes with D-type cyclins (Xiong et al, 1993b; Bates *et al.*, 1994b; Tam *et al.*, 1994a,). This was explained when many of these cell lines were shown to express high levels of $p16^{INK4}$, the cdk4 and cdk6 inhibitor. High levels of $p16^{INK4}$ bound to the kinases and prevented their association with the D-type cyclins (Parry *et al.*, 1995). These studies and others predicted an inverse correlation between the pRb status and the expression of $p16^{INK4}$ in tumor samples and cell lines (Okamoto *et al.*, 1994). In agreement with this prediction, a survey of 55 small cell lung carcinomas showed 6 samples contained mutant $p16^{INK4}$ expression but expressed wildtype pRb, while the majority of the remaining samples lacked pRb but expressed wildtype $p16^{INK4}$ (Otterson *et al.*, 1994). Microinjection of $p16^{INK4}$ into normal diploid cells results in a G1 arrest when cells are injected before late G1 (Lukas *et al.*, 1995) and a similar result is noted following microinjection of cells with antibodies to cyclin D1.

As cdk4/cdk6-mediated phosphorylation of pRb results in pRb functional inactivation during late G1, $p16^{INK4}$ was also envisioned as a tumor suppressor protein because it inactivates cdk4/cdk6 activity. This appears to be plausible, as re-introduction of $p16^{INK4}$ into U2OS cells (a $p16^{INK4}$ minus osteosarcoma cell line) by transfection or microinjection results in a G1 arrest dependent upon the presence of pRb (Lukas *et al.*, 1995; Koh *et al.*, 1995). Similar results were obtained in U2OS cells following transfection of p18, a $p16^{INK4}$ family member (Guan *et al.*, 1994), suggesting $p16^{INK4}$

family members may also be potential tumor suppressers. Interestingly, additional data indicates pRb functions as a negative regulator of the $p16^{INK4}$ promoter (Li *et al.*, 1994). Combined, the data predict that loss or mutation of pRb, overexpression of D-type cyclins and loss or mutation of $p16^{INK4}$ define a common pathway to tumor formation.

1.6.v. pRb interactions with cyclin A/cdk2 and cyclin E/cdk2 complexes

As noted, cyclin A/cdk2 and cyclin E/cdk2 complexes also contribute to the phosphorylation of pRb. Like the D-type cyclins, constitutively expressed cyclin A and cyclin E rescues pRb-mediated growth suppression in an pRb minus osteosarcoma cell line SAOS-2 (Hinds *et al.*, 1992) and this effect is attributed to the resulting phosphorylation of pRb. Based on the timing of expression of cyclin/cdk complexes, the current model envisions the initial phosphorylation (and subsequent inactivation) of pRb occurring following activation of D-type cyclins and their associated kinases, with the phosphorylation of pRb being maintained by cyclin E/cdk2 in late G1 followed by cyclin A/cdk2 throughout S phase and through to mitosis. Stable complexes between pRb and cyclins A and E have not been detected *in vivo* or *in vitro.*

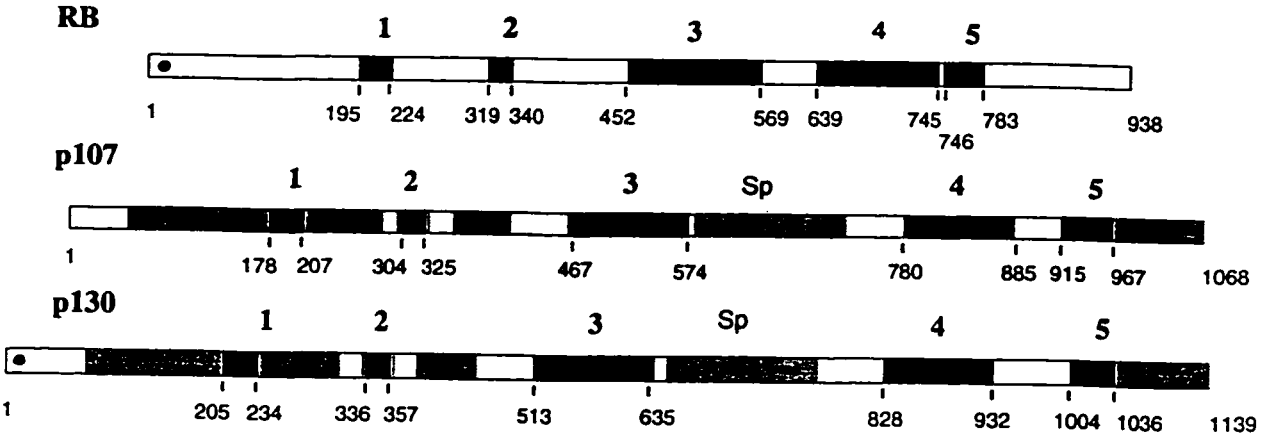
1.7 pRb family members p107 and p130

p107 and p130 (named according to their molecular weights) are proteins which like pRb, were first noted to be present in association with E1A in adenovirus-infected HeLa cells (Yee and Branton, 1985; Harlow *et al.*, 1986). This association aided in the purification of the proteins from 293 cells which contain high levels of E1A (Graham *et al.*, 1977). The E1A proteins were isolated from 293 cells by incubation with E1A-

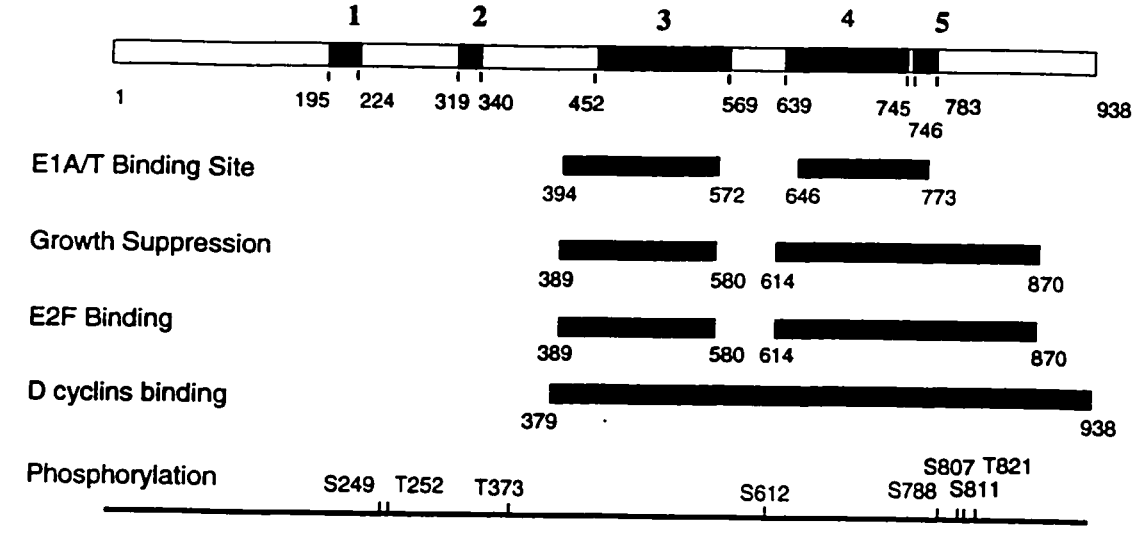
specific monoclonal antibodies and the p107 and p130 bound to E1A in these co-immunoprecipitations were resolved by SDS-PAGE, isolated and digested with trypsin and the tryptic peptide fragments sequenced. The peptide fragments served as templates for the design of degenerate oligonucleotides which were used to screen cDNA libraries (Ewen *et al.*, 1991; Li *et al.*, 1993). In addition, p130 was also identified following a yeast two-hybrid screen as a cdk2-interacting protein (Hannon *et al.*, 1993) and by PCR using primers homologous to p107 and pRb (Mayol *et al.*,, 1993).

Both proteins display a high degree of structural homology with pRb but are more related to one another than to pRb (Figure 1.2). This homology includes the presence of the "pocket" regions which, like pRb, are targeted by viral oncoproteins including E1A, SV40 large T and HPV E7 in infected cells (Dyson *et al.*, 1989b; Ewen *et al.*, 1991; Giordano *et al.*, 1991; Dyson *et al.*, 1992; Davies *et al.*, 1993; Hannon *et al.*, 1993; Mayol *et al.*,, 1993; Wolf *et al.*, 1995). Additional regions are conserved in each of the family members, and these also are depicted in Figure 1.2. PRb and p130 share approximately 20-30 % homology whereas p107 and p130 share approximately 50% (Hannon *et al.*,, 1993; Li *et al.*,, 1993; Mayol *et al.*,, 1993). p107 and p130 contain spacer regions located between the A and B domains of the pocket, and this region harbours four clusters of similar amino acid sequences. pRb and p130 share a proline/alanine-rich sequence at their N-termini although the nature of this sequence has yet to be determined. Interestingly, a similar proline/alanine sequence is not present in the N-terminus of p107.

A. Homology of pRB, p107 and p130



B. Domains of pRB



C. Domains of p107

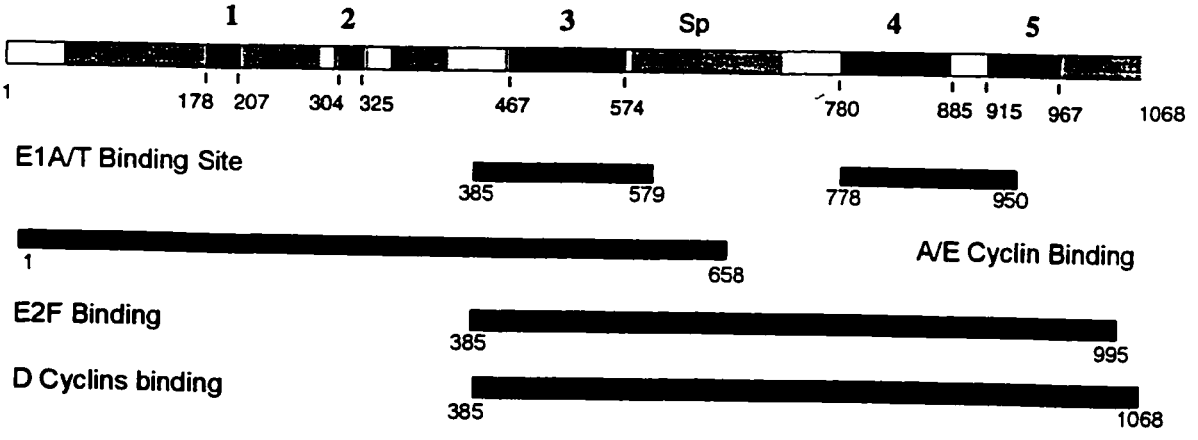


Figure 1.2 Comparison of structural and functional homology among the pRb family members

A. pRb, p107 and p130 are shown schematically and numbers below each protein represent amino acids. Regions conserved among all three members are indicated by black shading whereas conserved regions between p107 and p130 are indicated by gray shading. Regions 3 and 4 represent domains A and B respectively, which make up the "pocket domain " present in each of the three proteins. The A and B domains are separated by a spacer region ("Sp") in p107 and p130. The location of an alanine/proline-rich sequence in pRb and a proline/alanine-rich sequence in p130 are indicated by a small black dot near in the amino termini of these proteins. **B.** Representations of the known and/or predicted functional domains and the mapped serine (S) and threonine (T) phosphorylation sites of pRb. **C.** Representations of the known and/or predicted functional domains within p107. Some domains shown in B and C were determined *in vitro* and others *in vivo*. Several regions are approximate because not all domains have been extensively defined. This figure is a modification of one presented in Whyte, 1995.

p107 maps to chromosome 20q11.2 (Ewen *et al.*, 1991), a region found deleted in a small number of myelogenous leukemias and p130 to 16q12.2, a region noted to be deleted or mutated in several types of cancers including hepatocellular, breast and prostate (Li *et al.*, 1993; Hannon *et al.*, 1993). Despite these inferences, there have been no reports of loss or mutation of p107 or p130 in tumors or tumor cell lines.

1.7.i. Regulation of p107 and p130 by cyclin/cdk complexes

p107 and p130 are also substrates of cyclin/cdk complexes, and like pRb, they undergo cell cycle-dependent phosphorylation which is thought to regulate their activity (Beijersbergen *et al.*, 1995; Mayol *et al.*, 1995; Xiao *et al.*, 1996). In quiescent cells, p107 and p130 are hypophosphorylated. Following serum stimulation, p107 and p130 remain hypophosphorylated until mid G1 when they gain phosphate and remain as hyperphosphorylated proteins thoughout S phase. The mid-to-late G1 phosphorylation of p107 and p130 roughly co-incides with the phosphorylation of pRb (Mayol *et al.*, 1995; Xiao *et al.*, 1996).

The initial phosphorylation of p107 and p130 most closely correlates with the expression of cyclin D1 and D2, suggesting D-type cyclin/cdk4 complexes initiate the phosphorylation of this protein (Beijersbergen *et al.*, 1995; Mayol *et al.*, 1995; Xiao *et al.*, 1996). Like pRb, p107 and p130 can be detected in stable complexes with D-type cyclins *in vivo* and p107 serves as an *in vitro* substrate of cyclin D-associated kinase activity (Ewen *et al.*, 1993; Hannon *et al.*, 1993; Li et al, 1993). This interaction is presumably mediated through the Leu-X-Cys-X-Glu motif present in the D-type cyclin amino acid

sequences. It is not known if phosphorylation of p107 by D-type cyclin-dependent kinases results in a release of the p107/cyclin D interaction as has been shown for pRb but work contained within this thesis shows that phosphorylation of p130 by D-type cyclins and cdk4 does result in destabilization of the p130 cyclin D complex.

Unlike pRb, p107 and p130 form stable complexes with cyclin A/cdk2 and cyclin E/cdk2 complexes (Ewen *et al.*, 1992; Faha *et al.*, 1992; Lees *et al.*, 1992; Faha *et al.*, 1993; Hannon *et al.*, 1993; Li *et al.*, 1993; Peeper *et al.*, 1993) and in p107 these interactions are mediated through the "spacer" domain (Ewen *et al.*, 1992; Faha *et al.*, 1992; Zhu *et al.*, 1993; Zhu *et al.*, 1995a,b). p130 also contains a homologous spacer domain and this thesis presents evidence that a small region within the p130 spacer region does in fact mediate interactions with cyclins A and E. Unlike p107 and p130, pRb does not contain a spacer domain and this is presumably why stable complexes between pRb and cyclins A and E have not been detected.

1.8. Identification of the E2F transcription factor family

Studies aimed at identifying cellular protein counterparts to viral proteins which targeted pRb revealed the association of pRb with a cellular transcription factor termed E2F (Bandara and LaThangue, 1991; Bagchi *et al.*, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991). E2F was first identified as a cellular DNA binding protein necessary for the transcription of the adenoviral E2 gene during infection (Kovesdi *et al.*, 1986) and later as a transcription factor whose DNA binding activity was up-regulated following serum stimulation of quiescent fibroblasts (Mudryj *et al.*, 1990). Adenovirus

E1A, SV40 large T antigen and HPV E7 all competed for pRb when pRb was complexed to E2F, suggesting the viral proteins were competing with endogenous E2F for interactions with pRb (Bandara and LaThangue, 1991; Bagchi *et al.*, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991; Stirdivant *et al.*, 1991) and this disruption was mediated by viral protein sequences also required for interactions with pRb, immortalization and transformation (Bandara and LaThangue, 1991; Bagchi *et al.*, 1991; Chellappan *et al.*, 1991; Raychaudhuri *et al.*, 1991; Stirdivant *et al.*, 1991).

Identification and cloning of the E2F-1 sequence (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992) was an important step in elucidating the molecular details of this transcriptional activity. Additional proteins were subsequently identified as E2F family members and include E2F-2 (Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993), E2F-3 (Lees *et al.*, 1993), E2F-4 (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Sardet *et al.*, 1995) and E2F-5 (Hijmans *et al.*, 1995; Sardet *et al.*, 1995). Additional cellular proteins identified as components of E2F activity are the DP proteins, DP-1 (Girling *et al.*, 1993; Helin *et al.*, 1993a) and DP-2 (Wu *et al.*, 1995; Zhang and Chellappan, 1995). The DP proteins heterodimerize with the E2F family of proteins and enhance E2F DNA binding and transactivation (Bandara *et al.*, 1993; Helin *et al.*, 1993a; Huber *et al.*, 1993; Krek *et al.*, 1993).

1.8.i Expression patterns of the E2F family of proteins

Several of the E2F family members display a cell cycle-dependent expression pattern. In quiescent cells, expression of E2F-1, -2, -3, and -5 is very low and E2F-4 is

the predominant family member detectable by western blot (Kaelin *et al.*, 1992; Slansky *et al.*, 1993; Ginsberg *et al.*, 1994; Johnson *et al.*, 1994; Sardet *et al.*, 1995; Moberg *et al.*, 1996). Following stimulation with growth factors or the appropriate chemical stimuli, E2F-3 protein expression is up-regulated just prior to the G1/S boundary and this is followed closely by the increase in expression of E2F-1 and E2F-5. E2F-3 and E2F-1 levels remain elevated throughout the remaining hours of the cell cycle whereas E2F-5 decreases to low levels again as cells enter S phase and complete the cell cycle. E2F-4 appears to remain at a steady-state level before, during and following growth factor stimulation, implying it is not a growth-responsive gene.

1.8.ii. E2F expression and control of the cell cycle

E2F transcription factors define major "endpoints" of cell cycle-related pathways because of their direct influence on gene expression. In the case of E2F-1, entry into S phase can be directly induced in quiescent fibroblasts by overexpression of the protein (Johnson *et al.*, 1993; Beijersbergen *et al.*, 1994). Additionally, E2F-1, -2 or -3 are each able to overcome a GST-$p16^{INK4}$-induced G1 arrest in fibroblasts when overexpressed in these cells (Lukas *et al.*, 1996) and are also effective in overriding G1 arrests induced by expression of $p21^{CIP1}$, $p27^{KIP1}$ or exposure of cells to gamma-irradiation, respectively (DeGregori *et al.*, 1995b). E2F-1, -2 and -3 retrovirus-infected fibroblasts display anchorage-independent growth (as determined by soft agar colony formation assays), a hallmark of transforming capability of a particular gene product (Xu *et al.*, 1995) and

overexpression of E2F-1 and E2F-4 plus *ras* results in transformation (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Singh *et al.*, 1994).

E2F consensus binding sites are present in the promoters of several genes which are regulated in a cell cycle-dependent manner. These genes include dihydrofolate reductase (DHFR), c-myc and N-myc (Blake and Azizkhan, 1989; Hiebert *et al.*, 1989; Mudryj *et al.*, 1990), DNA polymerase α (Pearson *et al.*, 1991), cyclin D (Herber *et al.*, 1994), cdc 2 (Dalton, 1992), cyclin A and cyclin E (Henglein *et al.*, 1994; Ohtani *et al.*, 1995; Geng *et al.*, 1996), pRb and p107 (Hamel *et al.*, 1992; Shan *et al.*, 1994; Zhu *et al.*, 1995c) and several others. Many of these genes appear to be *bona fide* targets of E2F activity - for instance, overexpression of E2F-1 in cells results in a large increase in DHFR promoter activity (Slansky *et al.*, 1993) and microinjection of E2F-1 or infection of quiescent fibroblasts with an adenovirus expressing E2F-1 induces expression of the above genes as well as cyclin A, cyclin E and B-myb (DeGregori *et al.*, 1995a).

Additionally, E2F binding sites are found in the promoter of E2F-1 itself (Johnson *et al.*, 1994; Neuman *et al.*, 1994). The kinetics of E2F-1 gene expression appear to be regulated by the presence of E2F sites within this promoter, implying E2F-1 (and possibly the other E2F family members) operates through a feedback loop (Johnson *et al.*, 1994). The fact that E2F activity stimulates genes whose products will ultimately regulate its activity (discussed in sections 1.7.iii, iv and v) suggests that E2F autoregulates itself through a feedback circuit.

1.8. iii. The regulation of E2F by pRb

E2F sites appear to function in both the activation and repression of gene transcription. For example, the presence of E2F sites in the adenovirus E1A promoter or another simple promoter construct correlates with inhibition of expression of a reporter gene (Weintraub *et al.*, 1992). pRb, p107 and p130 are thought to influence the type of effect E2F has on positive or negative transcriptional regulation. E2F complexed to pRb behaves as an inhibitory complex when bound to E2F sites whereas uncomplexed E2F behaves as an activator of transcription (Weintraub *et al.*, 1992; Zamanian and LaThangue, 1992; Flemington *et al.*, 1993; Heibert *et al.*, 1993). As shown using pRb minus cell lines, uncomplexed E2F behaves as a constitutive activator of transcription, and inhibitory pRb-complexed E2F can be reconstituted by transfection of pRb into these cells. *In vivo*, pRb preferentially interacts with E2F-1, E2F-2 and E2F-3 (Lees *et al.*, 1993; Wu *et al.*, 1995), although pRb has also been detected in complexes with E2F-4 (Vairo et al, 1995; Ikeda *et al.*, 1996; Moberg *et al.*, 1996).

As a result of their affinity for pRb, viral oncoproteins such as E1A and SV40 large T disrupt pRb/E2F complexes, resulting in an uncomplexed, transcription-activating E2F pool (Bandara *et al.*, 1991; Mudryj *et al.*, 1991; Chellappan *et al.*, 1991; Weintraub *et al.*, 1992; Zamanian and LaThangue, 1992; Flemington *et al.*, 1993). In addition to dissolution of pRb/E2F complexes, it is possible that the presence of E1A or SV40 large T prevents the initial association of pRb with E2F.

There is evidence that pRb - once bound to E2F on DNA - is able to block adjacent transcription factors preventing their association with the basal transcription complex (Weintraub *et al.*, 1995). This data implies pRb is a general repressor of transcription when coupled with E2F. Interestingly, E2F sites within the pRb promoter are repressive elements which can be regulated by pRb itself, suggesting the transcription of pRb is autoregulated through E2F (Neuman *et al.*, 1994; Shan *et al.*, 1994).

pRb appears to interact directly with the E2F transactivation domain, disabling E2F-mediated transactivation of target genes (Hamel *et al.*, 1992; Zamanian and LaThangue, 1992; Weintraub *et al.*, 1992; Flemington *et al.*, 1993; Helin *et al.*, 1993b). This interaction only occurs when pRb is hypophosphorylated, and requires the pRb pocket region plus additional carboxyl-terminal sequences (Hiebert *et al.*, 1993). As hypophosphorylated pRb binds to E2F, phosphorylation of pRb by cyclin A/cdk2, cyclin D1/cdk4 or cyclin E/cdk2 is sufficient to disrupt pRb complexes with E2F-1, -2, and -3 (Hamel *et al.*, 1992; Kato *et al.*, 1993; Dynlacht *et al.*, 1994; Suzuki-Takahashi *et al.*, 1995). This phosphoryaltion event is presumed to be the cause of an increase in E2F activity at the G1/S phase transition (Mudryj *et al.*, 1991; Shridokar *et al.*, 1992; Schwartz *et al.*, 1993).

In summary, release of E2F transcription factors from pRb as a result of pRb phosphorylation is though to result in transcriptionally active E2F. Although this idea was discussed for several years, it may oversimplify the *in vivo* situation. Current data shows that even following the phosphoryaltion of pRb at the G1/S boundary, pRb/E2F

complexes remain (section 1.7.vi) and the existence of pRb/E2F complexes may also be dependent on the phosphorylation state of E2F itself (section 1.7 v).

1.8. iv. The regulation of E2F by p107 and p130.

In addition to interactions with pRb, the E2F transcription factor family is thought to be regulated by p107 and p130. p107 and p130 are detected in E2F complexes (Cao *et al.*, 1992; Shirodkar *et al.*, 1992; Cobrinik *et al.*, 1993) preferentially containing E2F-4 and E2F-5 (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Hijmans *et al.*, 1995; Sardet *et al.*, 1995), although interactions between p107 and p130 with E2F-1 have also been detected (Ikeda *et al.*, 1996). Similar to pRb, only hypophosphorylated p107 interacts with E2F-4, suggesting p107 interacts with E2F proteins mainly in early to late G1 when it is found in this form (Chellappan *et al.*, 1991; Beijersbergen *et al.*, 1995; Xiao *et al.*, 1996). Like pRb, p107 and p130 suppress E2F-mediated transcription by directly binding and inhibiting the E2F transactivation domain (Schwarz *et al.*, 1993; Zhu *et al.*, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Hijmans *et al.*, 1995; Johnson *et al.*, 1995; Smith and Nevins 1995; Vairo *et al.*, 1995), and in the case of p107, sequences homologous to those present in pRb (the pocket region plus additional C-terminal residues) are required for this interaction (Zhu *et al.*, 1995a). Of note is the fact that the p107 promoter contains two tandem E2F sites, each of which appears to contribute to the expression of p107 (Zhu *et al.*, 1995c). This situation, like that with the pRb promoter, is likely to result in a p107 feedback loop which autoregulates its own synthesis.

As a result of the regions of p107 and p130 required to interact with E2F, viral oncoproteins compete with E2F for interactions with p107 and p130 (Zhu *et al.*, 1993; Smith and Nevins, 1995; Wolf *et al.*, 1995; Starostik *et al.*, 1996). Although not completely understood, interactions of SV40 large T or E1A with p107 and p130 do not completely abrogate the repressive effects these molecules have on E2F activity, unlike the situation with pRb. Phosphorylation of p107 and p130 by cyclin D1/cdk4 complexes releases these members from E2F-4 and E2F-5-containing complexes, releasing active E2F (Beijersbergen *et al.*, 1995; Johnson *et al.*, 1995; Hateboer *et al.*, 1996; Xiao *et al.*, 1996). Active E2F may have a short half-life however, as uncomplexed E2F proteins become susceptible to ubiquitin-mediated proteolysis (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996).

Like pRb, p107 also demonstrates general repression effects on transcription, implying that it too may interact with other required transcriptional activators while in a complex with E2F (Starostik *et al.*, 1996).

1.8.v. The regulation of E2F by cyclin/cdk complexes

E2F family members are also substrates of cyclin/cdk complexes, resulting in both positive and negative regulation of E2F activity. Cyclin A interacts directly with E2F-1 through a small domain present within the N-terminus of E2F-1 (Krek *et al.*, 1994; Peeper *et al.*, 1995) and during its expression, cyclin A can be detected in S phase E2F complexes along with cdk2 (Bandara *et al.*, 1991; Mudryj *et al.*, 1991; Pagano *et al.*, 1992b; Shridokar *et al.*, 1992; Dynlacht *et al.*, 1994; Krek et al, 1994). Interestingly, the E2F-1

cyclin A binding domain is conserved in E2F-2 and -3 but is absent in E2F-4 and -5. The E2F interaction with cyclin A/cdk2 complexes results in the cdk2-mediated phosphorylation of E2F-1 and DP-1, resulting in the release of E2F/DP heterodimers from DNA (Dynlacht *et al.*, 1994; Krek *et al.*, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995). Similar results might be expected with E2F-2 and E2F-3 but there is as yet no available data. As a result of the release of E2F from DNA following these phosphorylation events, the expression of cyclin A in late S phase is thought to negatively regulate the activity of E2F during this stage of the cell cycle. Interestingly, these studies show cyclin E does not interact directly with E2F-1 and as a result, E2F-1 is not a substrate of cyclin E/cdk2. Presumably only E2F-1, -2 and -3 are influenced by phosphorylation events resulting from stable interactions with cyclin A, because the cyclin A binding domain located in the N-terminus of these proteins is absent in E2F-4 and -5.

Cdk3 has been shown to activate E2F-1 and E2F-2 (and to a lesser extent E2F-3) by a mechanism linked to direct binding of cdk3 to E2F-1/DP-1 complexes (Hofmann and Livingston, 1996). It is not clear if this activation is due to phosphorylation events on E2F and/or DP or on other proteins required for initiation of transcription, and if these events do occur, the activating cyclin for cdk3 is not known.

E2F-1 is phosphorylated *in vivo* on several residues including serine 332, serine 337 and serine 375, residues located in a region of E2F-1 which is not involved in direct interactions with pRb (Fagan *et al.*, 1994; Peeper *et al.*, 1995). When these residues are phosphorylated in late G1, E2F-1 has less affinity for pRb and acts as an activator of

transcription. Although the precise cyclin/cdk complexes responsible for the phosphorylation of Ser 332 and 337 appear to be cyclin A/cdc2 and cyclin B/cdc2 *in vitro*, it is not clear what cyclin/cdk complex may be responsible *in vivo*. Whatever the kinase complex, phosphorylation of E2F-1 by this complex may preclude pRb/E2F-1 complex formation. Taken together, this data suggests the phosphorylation status of E2F itself adds an additional mechanism of control over formation of pRb/E2F complexes (and possibly p107, p130 complexes).

In summary, the cyclin/cdk-mediated phosphorylation status of E2F and E2F/DP complexes plays an important role in both the activation and repression of E2F activity and differential phosphorylation of E2F family members may significantly influence their relative interactions with pRb, p107 and p130.

1.8.vi. E2F complexes during the cell cycle

E2F is found in higher order complexes throughout the cell cycle owing to the varied timing of expression of the E2F family members, the pRb family members and cyclins (Figure 1.3). The predominant E2F complexes detected in quiescent fibroblasts and quiescent primary human T cells are composed of E2F-4/p130 (Lees *et al.*, 1992; Shridokar *et al.*, 1992; Chittenden *et al.*, 1993; Cobrinik *et al.*, 1993; Vairo *et al.*, 1995; Wolf *et al.*, 1995; Moberg *et al.*, 1996), although minor E2F complexes containing pRb and p107 have also been reported. Following serum or mitogen stimulation, E2F-4/p130 complexes are also prominent during early to mid G1 until they are outnumbered by E2F complexes containing p107. Due to its presence in G0/early G1 E2F complexes, p130

may be an important negative regulator of E2F-dependent transcription in quiescent cells and during entry into the cell cycle. Interestingly, the regression of E2F-4/p130 complexes coincides with the cyclin D/cdk4-mediated phosphorylation of p130 (Mayol *et al.*, 1995).

Following the disappearance of the majority of E2F-4/p130 complexes, free E2F-4 activity is released from p130 which is then detected in complexes with p107. Predominant complexes containing E2F-4/p107/cyclin E/cdk2 appear in late G1 and then E2F/p107/cyclin A/cdk2 complexes predominate at the G1/S border (Cao *et al.*, 1992; Devoto *et al.*, 1992; Lees *et al.*, 1992; Pagano *et al.*, 1992b; Shridokar *et al.*, 1992). It is possible that E2F-5 is also present in a complex with p107, but it may be less easily detected by gel shift assays. Low levels of E2F-4/p130 complexes can also be detected in late G1, and these, like the complexes with p107, contain cyclin E and cdk2 or cyclin A and cdk2 (Cobrinik *et al.*, 1993).

pRb first appears in E2F-1 complexes at the G1/S boundary, and the levels of these complexes peak during S phase and decline over the remainder of the cell cycle (Mudryj *et al.*, 1991; Shridokar *et al.*, 1992; Chittenden *et al.*, 1993; Schwartz *et al.*, 1993; Moberg *et al.*, 1996). E2F-2/pRb complexes appear low in abundance but are detectable at G1/S and all but disappear later in the cell cycle (Moberg *et al.*, 1996). E2F-3 appears in pRb complexes just before the appearance E2F-1 complexes, these become more abundant in S phase and then regress to lower levels throughout the remainder of the cell cycle (Moberg *et al.*, 1996). The presence of pRb in G1/S and early S phase complexes suggests it

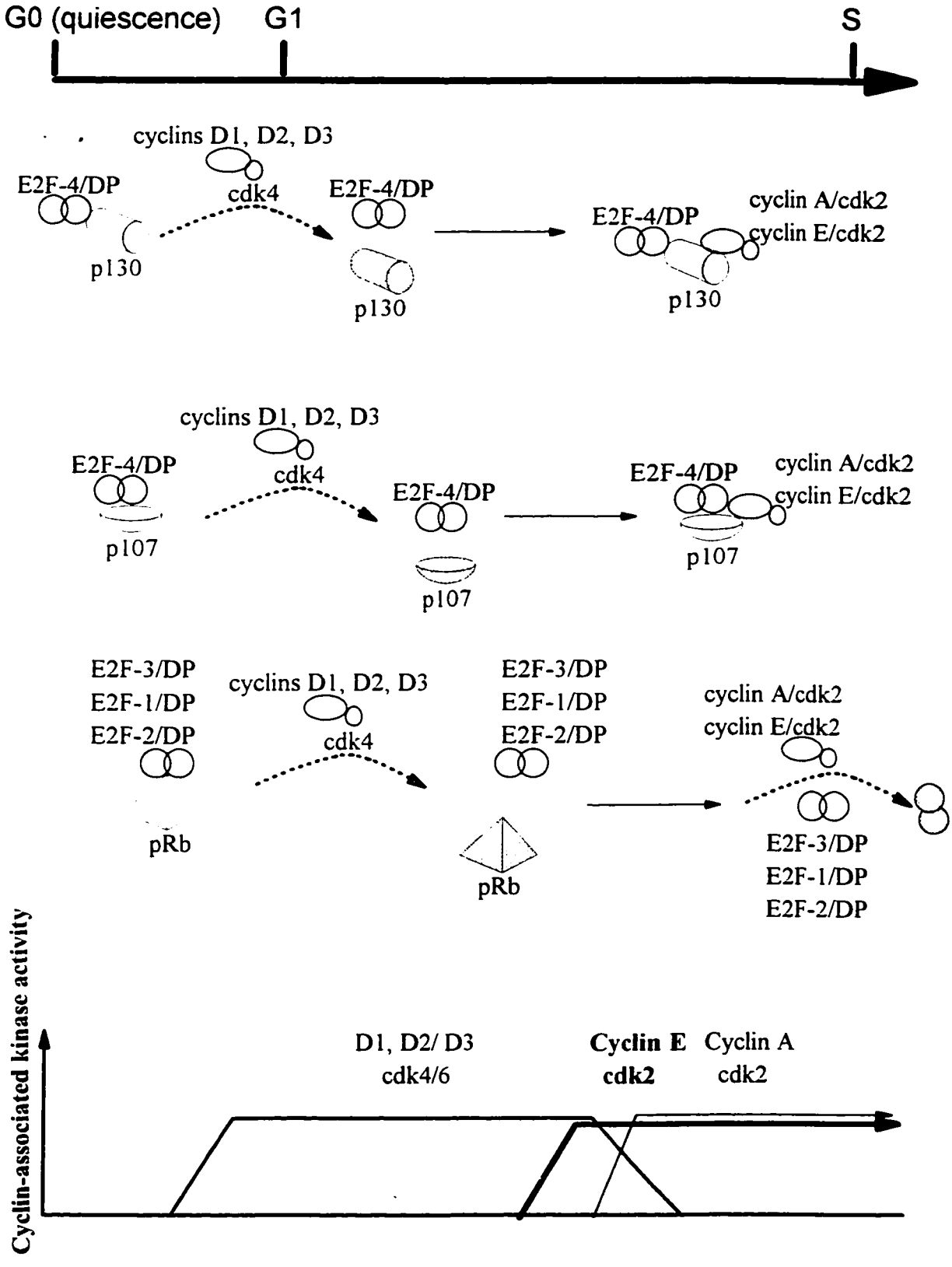

G0 (quiescence)
G1
S
cyclins D1, D2, D3
E2F-4/DP
cdk4
E2F-4/DP
p130
p130
E2F-4/DP
cyclin A/cdk2
cyclin E/cdk2
p130
cyclins D1, D2, D3
E2F-4/DP
cdk4
E2F-4/DP
p107
p107
E2F-4/DP
cyclin A/cdk2
cyclin E/cdk2
p107
E2F-3/DP
E2F-1/DP
E2F-2/DP
cyclins D1, D2, D3
cdk4
pRb
E2F-3/DP
E2F-1/DP
E2F-2/DP
pRb
cyclin A/cdk2
cyclin E/cdk2
E2F-3/DP
E2F-1/DP
E2F-2/DP
Cyclin-associated kinase activity
D1, D2/ D3
cdk4/6
Cyclin E
cdk2
Cyclin A
cdk2

Figure 1.3 E2F complexes during the cell cycle

A timeline of the cell cycle is shown at the top to align the relative timing of detection of E2F complexes shown below. E2F complexes with p130 are detectable in G0 before complexes containing p107 and pRb are detectable. Each pRb family member is represented as a different shape, and each interacts with a subset of E2F family members as shown. Shaded pRb family members represent the phosphorylated species which are unable to complex with E2F's. Curved dotted arrows represent phosphorylation events whereas solid horizontal arrows represent the continuation of the cell cycle and synthesis of new E2F/pRb family members in complexes observed later in the cell cycle. Phosphorylated E2F/DP heterodimers are shown at the bottom right hand corner and represent a transcription complex unable to bind to DNA. Along the bottom of the diagram is a timeline indicating peaks of cyclin/cdk activity. This figure is somewhat oversimplified and serves only to summarize some of the more comprehensive information on the subject to date. This figure is adapted from Beijersbergen and Bernards, 1996.

controls the levels of E2F accumulation prior to and during the entry into S phase. Importantly, although the E2F/pRb complexes are thought to be disrupted upon pRb phosphorylation, E2F/pRb complexes remain detectable during S phase (Helin *et al.*, 1992; Shridokar *et al.*, 1992; Weintraub *et al.*, 1992; Schwartz *et al.*, 1993). One possibility is that pRb phosphorylation at the G1/S boundary prevents any future interactions of pRb with E2F and this preserves the pool of active E2F.

The number of E2F-containing complexes and the fairly narrow window of the cell cycle in which they are thought to be most important in regulation presents researchers with a complicated picture of cell cycle control by this transctiption factor. Although not yet appreciated, different E2F complexes may have functionally distinct effects on target genes, perhaps through subtle differences in E2F binding site preferences.

1.9 The pRb family members: subtle differences or redundancy?

p107 and p130 also appear to negatively regulate growth when overexpressed in Rb minus cell lines as compared with cell lines containing pRb which are for the most part refractory to the effects of p107 and p130 (Zhu *et al.*, 1993; Claudio *et al.*, 1994; Smith and Nevins, 1995). Growth suppression mediated by each of these proteins results in a late G1 arrest of the cell cycle, similar to that shown with pRb (Zhu *et al.*, 1993; Smith and Nevis, 1995; Zhu *et al.*, 1995a) but in contrast to pRb, the p107-mediated growth suppression can not be relieved by co-expression of cyclins A and E (Zhu *et al.*, 1993). The inability of these cyclins to overcome p107-mediated growth suppression may in part be due to the fact that only cyclin D1/cdk4-mediated phosphorylation events on p107 release it from E2F-4

(Beijersbergen *et al.*, 1995; Xiao *et al.*, 1996). This subtle difference may correlate with the timing of expression of the various pRb members and the cyclin/cdk complexes, with D-type cyclins targeting p107 and cyclins A and E targeting pRb. It has not been reported if p130-mediated growth suppression can be rescued by co-expression of cyclins.

It has been suggested that the presence of the spacer domain in p107 confers an additional mechanism of growth suppression resulting from stable interactions with cyclin A and cyclin E. It is possible that in addition to inhibiting the activity of free E2F, it may also sequester cyclins needed for cell cycle progression (Zhu *et al.*, 1993; Zhu *et al.*, 1995a). It is not known if a similar hypothesis is plausible for the effect of p130 on growth. Whatever the mechanism(s), p107 and p130 do not share equivalent roles in differentiated cells. E2F/p130 complexes are abundant in differentiated muscle cells, whereas E2F/p107 complexes are relatively minor, suggesting maintenance of G0 and differentiation require p130 specifically (Shin *et al.*, 1995).

Functional differences between pRb, p107 and p130 have been borne out in mouse knockout studies. Mice engineered to be deficient in expression of p107 or p130 displayed no abnormal phenotypes associated with the loss of either of the two gene products, suggesting neither gene product alone is required for development (Cobrinik *et al.*, 1996; Lee *et al.*, 1996). Mice lacking both proteins died at birth or shortly thereafter as a result of incomplete bone development (Cobrinik *et al.*, 1996). Embryos displayed increased chondrocyte density in cartilage, shortened limbs and defective endochondral bone development. A similar study examined the effects of loss of pRb in combination

with loss of p107 expression. These mice, like pRb null mice, are not viable and appear to die two days earlier than the pRb null mice. Mice heterozygous for pRb expression (one functional RB1 allele) and null for p107 expression do survive but are growth retarded and many die shortly after birth. (Lee *et al.*, 1996).

These mouse studies suggest that unlike pRb, p107 and p130 act in concert during limb development in the mouse but may not be important in controlling the outgrowth of tumors. It is possible that bone differentiation and formation is more dependent on p107 and p130 protein levels than pRb levels whereas other tissues are more dependent on pRb.

1.10 Statement of purpose

The interactions of pRb, p107 and p130 with cyclin/cdk complexes and the E2F family of transcription factors are documented in this introduction. The details of these interactions are well-studied in the case of pRb, but less so in the case of p107 and even poorer in the case of p130. Specifically, the function of p130 is not well understood nor it is not known how cyclin/cdk interactions affect the regulation or function of p130. This thesis describes work aimed at understanding why and how cyclins A, D1, D2, D3 and E interact with p130 and if these interactions serve to regulate the function of p130. It is hoped that data from this work will contribute to the overall understanding of the function of p130 as a cell cycle regulator.

The objectives of this work include the following:

1. To determine if complexes containing cyclins A, D1, D2, D3 and E , their respective cdk's and p130 exist in cells.

2. To reproduce these potential interactions *in vitro* by mixing individual protein components.

3. To isolate specific minimal regions of p130 required to mediate interactions with these cyclins both *in vitro* and *in vivo*.

4. To mutate and/or delete these cyclin-binding regions from the p130 protein and to test the mutant(s) for gain or loss of function.

Chapter 2. General Methods

2.1 Cell Culture

2.1.i Cell lines

Human cell lines (osteosarcomas 143B, SOAS-2 and U2OS, cervical carcinoma C33A and breast carcinoma MDA MB157) were obtained from the American Type Culture Collection. NS-1 cells are a murine myeloma kindly provided by E. Harlow (Massachusetts General Hospital). SF9 cells are insect cells derived from *Spodoptera fragiperda* kindly provided by D. Johnson (McMaster University).

2.1.ii Mammalian cells

Mammalian cell lines were cultured in Dulbecco's modified Eagle media (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Sigma), 250 U/mL penicillin/streptomycin (Gibco) and 5 μg/mL fungisone (Gibco) at 37^0C, 5% CO_2 in a humidified incubator. Adherent cells were detached from dishes by addition of trypsin/EDTA (0.05%) (Gibco) and gentle agitation.

2.1.iii Insect cells, infection and metabolic labeling

SF9 cells were cultured in Grace's insect cell media (Gibco) supplemented with 10% fetal bovine serum (Sigma), 250 U/mL penicillin/streptomycin (Gibco) and 5μg/mL fungisone (Gibco), 0.07 g/L yeastolate and lactalbumin (provided as a 50X stock by Gibco) at 27^0C in a humidified incubator. SF9 cells were typically infected at a multiplicity of infection (M.O.I.) of 5 plaque forming units (p.f.u.)/cell by addition of virus

in a total volume of 1.5 mL/6 cm dish for two hours, then 2.5 mL additional media was added and the cells allowed to incubate for 48 hours. In the case of D-type cyclins and cdk4, cells were infected at an M.O.I of 15 p.f.u./cell.

Following infection, most infected cells remain loosely adherent to the dish but a percentage float in the media. For metabolic labeling, media containing floating SF9 cells was saved in a centrifuge tube, while remaining adherent cells (in 6 cm dishes) were rinsed once with Grace's methionine minus media (Sigma). The media used to rinse the adherent cells was combined with the additional media containing the floating cells and the tube centrifuged at 1000 RPM for 5 minutes. Media was aspirated from the cells, and 1.5 mL Grace's methionine minus media was added to the cell pellet. Cells were resuspended and added to the adherent cells remaining on the dish. To the 1.5 mL total, 11 μCi ^{35}S methionine (DuPont) was added and cells were allowed to incubate at 27^0C for 2 hours. Infected cells were gently squirted off the plate and centrifuged at 1000 RPM following which the supernatant was removed and the cell pellet lysed in 500 μL E1A buffer (50 mM HEPES pH 7.0, 250 mM $NaCl_2$, 0.1% NP40, 2mg/mL aprotinin, 1 mg/mL leupeptin). Lysates were centrifuged in an Eppendorf centrifuge for 10 minutes and supernatants removed to a fresh tube containing 50 μL glycerol and stored at -20^0C.

2.2 Titration and amplification of baculoviruses

Viruses were titered by plaque assays which were performed in triplicate. Viral supernatants were serially diluted (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) and allowed to infect 6 cm dishes containing 2.3 X 10^6 SF9 cells in a total volume of 1 mL. Media were aspirated and 3 mL

of a 1% low melting temperature (lmt) agarose (Seaplaque) solution made up in Grace's media and cooled to 40^{0}C was overlaid onto cells. Plaques were visualized after 10 days by addition of a 1% lmt agarose solution containing 0.05% trypan blue (Sigma). Following solidification of the trypan blue agarose, plaques (which stain blue) were counted and viral titers calculated and expressed as plaque forming units (p.f.u.) per mL.

Viruses were amplified in 15 cm dishes by infection of cells at MOI of 0.7 p.f.u./cell. Virus was added in a total volume of 5 mL and allowed to incubate for two hours, then 15 mL additional media was added and the cells allowed to incubate for 4 days. Supernatants were removed from plates and centrifuged at 4000 RPM for 10 minutes. Supernatants were titrated, aliquoted and stored at -20^0 C.

2.3 Immunoprecipitations and *in vitro* kinase assays following immunoprecipitations

Mammalian or insect cells were lysed on ice in E1A buffer and cellular debris was removed by a 10 minute centrifugation in an Eppendorf centrifuge at 14000 RPM. Supernatants were immunoprecipitated by addition of the appropriate antibody, rabbit anti-mouse (in the case of monoclonals) and protein A Sepharose (Sigma). Reaction volumes were made up to 500 µL with E1A buffer and rocked gently for 1 hour at 4^{0}C. Immune complexes collected on protein A Sepharose were washed three times in E1A buffer by sequential rounds of a 1 minute centrifugation in an Eppendorf centrifuge followed by aspiration of buffer. To resolve immune complexes, protein A Sepharose was resuspended in 60 µL of 1X Laemmli sample buffer (0.0625 M TRIS-HCl (pH 6.8), 2%

SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue) (Laemmli, 1970), heated to 80^{0}C for 3 minutes and loaded onto an SDS-PAGE gel.

For *in vitro* kinase assays, following the final round of washing with E1A buffer, Sepharose was resuspended in 40 μL kinase buffer (50 mM TRIS pH 7.4, 10 mM $MgCl_2$) plus 0.5 μCi gamma^{32}P-ATP, 0.1 mM ATP and incubated at room temperature for 30 minutes. Reactions were stopped by addition of 10X Laemmli sample buffer followed by heating to 80^0 C and phosphoproteins were resolved by SDS-PAGE.

For *in vitro* kinase assays using crude insect cell lysates and GST-p130 fusion proteins, aliquots of SF9 cell lysates were combined with 1-2 μg (or the amounts indicated in each figure) of GST-p130 fusion protein plus 0.5 μCi gamma^{32}P-ATP and 0.1 mM ATP. Reaction volumes were made up to 60 μL with E1A buffer containing $MgCl_2$ at a final concentration of 10 mM. Reactions were incubated at room temperature for 30 minutes and the reaction terminated by addition of 10X Laemmli sample buffer. Reactions were heated to 80^{0}C for 3 minutes and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

2.4 *In vitro* kinase assays with SF9 cell lysates and phosphopeptide mapping

SF9 cells were infected with the indicated baculoviruses at an MOI of five p.f.u./cell for 48 hours followed by lysis in E1A buffer plus protease inhibitors (250 mM $NaCl_2$, 50 mM HEPES pH 7.0, 0.1 % NP40, 2 mg/mL aprotinin, 1 mg/mL leupeptin). 10 μg of glutathione Sepharose-bound GST-p130 (593-698) or mutant versions of it (ΔRRL, ΔRYSSP, ΔRYSSP/RRL) were incubated with 18 μg of cyclin A/cdk2-containing SF9

cell lysate or 24 μg of cyclin E/cdk2-containing lysate. Each reaction contained 10 μCi gamma ^{32}P-ATP, 10 mM $MgCl_2$ and 0.5 μM ATP in a total volume of 40 μL. Samples were incubated at room temperature for 40 minutes, centrifuged to isolate Sepharose and washed once in E1A buffer. Samples were processed for electrophoresis exactly as described below in Immunoprecipitation and immunoblot analysis, except following electrophoresis gels were rinsed for 10 minutes in water prior to drying. Following overexposure of the gel, the autoradiograph was aligned with the gel and bands corresponding to GST-p130 (593-698) and the RRL mutant were excised. Isolation and digestion of the proteins with TPCK-trypsin (Worthington Biochemical, NJ) was performed according to Boyle *et al.* 1991. 5000 cpm of digested protein was spotted onto a TLC glass-backed plate and separated in two dimensions. In the first dimension, TLC plates were subjected to horizontal electrophoresis on a Hunter thin-layer electrophoresis system (HTLE-7000; CBS Scientific, Del Mar, CA) at 1500 V for 30 minutes in pH 1.9 buffer (1:3:36 formic acid (88%): glacial acetic acid: water). In the second dimension, plates were placed in an equilibrated chromatography tank containing phospho chromatography buffer (6.5:5:1:4 *n*-butanol: pyridine: glacial acetic acid: water) for 9 hours. Plates were dried and exposed for autoradiography.

2.5 Fluorography and autoradiography

To process SDS-PAGE gels for fluorography, gels were soaked in destain (7% acetic acid, 15 % methanol in water) with gentle agitation for 30 minutes followed by a ten minute soak in dimethylsulphoxide (DMSO). Gels were then soaked in PPO (2,5-

diphenyloxazole)/DMSO (10% PPO w/v) for 30 minutes followed by a 30 minute rinse in continuously flowing tap water. Gels were placed onto filter paper and dried prior to exposure to film (Kodak). For autoradiography (in the case of ^{32}P-labeled proteins) gels were rinsed briefly in water at room temperature, placed on filter paper and dried. During exposure, gels were in the presence of intensifying screens (Kodak) to amplify the signal. In both cases gels were stored at -80^{0}C during exposure.

2.6 Immunoblot (Western) analysis

Following resolution of proteins by SDS-PAGE, gels were wet-blotted onto nitrocellulose in western transfer buffer (380 mM glycine, 50 mM TRIS, 10% methanol) at 36 V for 4 hours. Blots were blocked in 5% w/v nonfat milk in PBS for 1.5 hours and incubated in primary antibody diluted in PBS overnight. Blots were washed three times in PBS plus 0.5% Tween-20 for 25 minutes each time, following which the blots were rinsed once in PBS alone. Blots were incubated with horseradish peroxidase-conjugated secondary antibody diluted 1/6000 (Signal Transduction Laboratories) for 1.5 hours and were washed exactly as described after the primary antibody incubation. Blots were developed by addition of ECL substrate (Amersham), covered in plastic wrap and exposed to film.

2.7 Purification of plasmid DNA

Plasmids were propagated in E. coli strain DH5α in Luria-Bertani medium (LB) (Gibco) plus ampicillin (0.1 mg/ml) (Sigma). Saturated overnight bacterial cultures were centrifuged at 5000 RPM for 10 minutes at 4^0 C, the supernatant removed and the cell

pellet lysed using the alkaline lysis method (Birnboim and Doly, 1979). Following neutralization, supernatants were precipitated with 1 volume isopropanol and allowed to sit for 30 minutes before a 10 minute centrifuge at 7000 RPM. Pellets were resuspended in water and CsCl added to a final concentration of 1 mg/mL. Solutions were loaded into ultracentrifuge tubes (Beckman) and ethidium bromide added to a final concentration of 0.5 mg/mL. Tubes were centrifuged in a Beckman ultracentrifuge at 55 000 RPM in a Vti65.1 rotor for 16 hours, after which plasmid bands were extracted by a syringe and needle. Ethidium bromide was removed from DNA by several rounds of water-saturated butanol extraction and DNA was precipitated by addition of 1/10 volume of 3M sodium acetate and 2 volumes ethanol. DNA was collected by centrifugation at 5000 RPM for 10 minutes and resuspended in water. DNA was quantitated by a spectrophotometer reading at $O.D._{260}$.

2.8 *In vitro* translations

pBS-cyclin E was constructed by isolation of cyclin E (nt 1-1509) from pLXSN-cyclin E (see 2.2.i) by digestion with Eco RI and Xho I and ligation into pBSKS digested with Eco RI and Xho I. 10 μg of pBS-cyclin E was transcribed *in vitro* by addition of 5 units T7 RNA polymerase in transcription buffer (40 mM TRIS-HCl pH 7.9, 6mM $MgCl_2$, 2 mM spermine, 10 mM DTT, 0.5 mM each UTP, CTP, GTP, ATP) for 3 hours at 37^0C. RNA was phenol/chloroform (24:1 v/v) extracted, ethanol precipitated and resuspended in 50 μL DEPC-treated water. One tenth of the precipitated RNA was added to a rabbit

reticulocyte translation reaction (Gibco) containing ^{35}S-methionine for 1 hour at 30^{0}C. The translation products were resolved by SDS-PAGE and detected by fluorography.

2.9 Stable and transient transfection of cells

Stable and transient transfections of mammalian cells were performed using the calcium phosphate precipitation technique (Graham and Van Der Eb, 1973). For stable transfections using pCMV-Bam-Neo plasmids, 10 μg of each plasmid was mixed with 1 mL HEBS (25 mM HEPES, 140 mM $NaCl_2$, 0.7 mM Na_2HPO_4) and 50 μL 2.5 M $CaCl_2$ was slowly bubbled into the mixture. Precipitates were allowed to stand for 20 minutes before their addition to 3-5 x 10^5 cells/10 cm dish containing 9 mL cell culture media. Cells were incubated in the presence of DNA-calcium phosphate precipitates for 15 hours. Cells were then washed twice with PBS and incubated with media containing 425 μg/mL G418 (Geneticin; Gibco, BRL). The pCMV-Bam-Neo expression vector contains a neomycin resistance gene which allows for selection of transfected cells in the presence of neomycin (Geneticin - Gibco). Cells which integrate the expression plasmid are Geneticin resistant and often overexpress the cDNA sequence carried by the plasmid. C33A cells were split 1:5 before addition of G418-containing media. Media were aspirated and replaced with fresh G418-containing media every three days for three weeks. Surviving colonies were fixed in 10% buffered formalin phosphate and stained with 4% Giemsa in PBS prior to counting. For transient transfections, cells were incubated in the presence of a DNA-calcium phosphate precipitate exactly as for stable transfections but were re-fed with DMEM plus 10% FBS and lysed 24 hours later.

Several cell lines expressing p130 or p130 mutants (p130 (Δ620-697) and 336C) were produced by stably expressing the corresponding cDNA in U2OS cells. These cDNAs were ligated into the pCMV-Bam-Neo expression vector and transfected into U2OS cells exactly as described above for stable transfections. Fresh media containing 425 μg/mL Geneticin was added to cells and was aspirated and replaced every 4 days for 17 days or until colonies began to emerge. Once colonies were visible, clonal populations were expanded following trypsinization of the colonies using cloning rings. Cells were transferred to a 96 well dish, then slowly expanded until several 10 cm dishes could be frozen as stocks. U7 cells overexpress wildtype p130, UΔ620 cells overexpress the p130 (Δ620-697) mutant and U336C cells overexpress the p130 336C truncation mutant.

2.10 PCR

Primers were synthesized and purified by The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. Every PCR reaction described in this work used purified plasmid DNA as a template. Typically, the annealing temperature used during PCR was 3^0 C below the lowest melting temperature calculated for each primer (forward and reverse). PCR conditions typically used a 3 minute denaturation step at 94^0 C, an annealing step at the calculated temperature for 30 seconds, an extension step at 72^0 C for 1 minute and a 30 cycle loop which repeated each of these steps. Following PCR, a sample of the product was analyzed on a 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

2.11 Expression and purification of GST-cyclin E and GST-p130 fusion proteins

All GST fusion proteins were expressed in and purified from DH5α *E.coli* as follows. Isolated colonies were grown in 50 mL LB plus ampicillin overnight at 37°C with shaking and expanded into 500 mL cultures the next morning. Cultures were allowed to grow to an $O.D._{600}$ of 0.6 and were then induced by addition of 0.5 mM isopropyl-ß-D-galactopyranoside (IPTG) (Gibco) for 3 hours. Cells were harvested by centrifugation at 5000 rpm for 10 minutes and resuspended in 5 mL NTEN buffer (100 mM $NaCl_2$, 20 mM Tris pH 8.0, 1 mM DTT, 1mM EDTA, 1% NP40, 1 mg/mL leupeptin and 2 mg/mL aprotinin). Resuspended cells were sonicated two times on ice, 15 seconds each. Supernatants were cleared by centrifugation in an Eppendorf centrifuge for 10 minutes. Supernatants were mixed with 300 µL of glutathione Sepharose (10%w/v, Pharmacia) (equilibrated in NTEN buffer) and allowed to rock for 1.5 hours at 4°C. Unbound bacterial proteins were washed away by resuspending the Sepharose in 10 mL NTEN buffer followed by gentle agitation. Sepharose was collected by centrifugation at 500g in a tabletop centrifuge and the supernatant removed by aspiration. The washing procedure was repeated three more times. To elute fusion proteins from glutathione Sepharose, beads were packed into a mini column and eluted by addition of 4 mL 15 mM reduced glutathione (Calbiochem) in 50 mM Tris pH 8.0. Eluates were dialyzed twice in 4 L PBS. Proteins were analyzed by SDS-PAGE and visualized by staining with Coomassie brilliant blue (CBB). Protein concentration was determined by comparison with a known quantity of BSA analyzed by SDS-PAGE and stained with CBB. GST fusion proteins remaining

bound to Sepharose (used in *in vitro* binding assays) were stored in NTEN buffer plus 10% glycerol at -20^{0}C following washing of the Sepharose.

2.12 *In vitro* binding assays

1μg of each GST-p130 fusion protein or 10 μg of GST alone (all bound to glutathione Sepharose) was mixed with 100 μg of cyclin A-containing ^{35}S-labeled SF9 cell lysates or 20 μg of cyclin E-, cdk2- or cyclin E/cdk2- containing ^{35}S-labeled lysates (varying levels of lysates were used owing to the different expression levels of the indicated baculoviruses). The mixtures were incubated for 1 hour at 4^0 C and complexes collected by centrifugation. Sepharose beads were washed three times in high salt E1A buffer (500 mM $NaCl_2$, 50 mM HEPES pH 7.0, 0.1% NP40, 1 mg/mL leupeptin and 2 mg/mL aprotinin) and re-suspended in 60 μL Laemmli sample buffer, heated to 80^0 C for three minutes and the supernatants analyzed by SDS-PAGE. Gels were processed for fluorography by addition of PPO, dried and exposed to film.

2.13 M13 oligonucleotide-directed mutagenesis

M13Mp18 DNA and bacterial strains MV1190 and CJ236 were kindly provided by J. Capone (McMaster University). An 1131 nt Xba I/Sph I p130 fragment was cloned into the Xba I/Sph I site of M13Mp18 and mutagenesis carried out according to the method of Zoller and Smith with the modification of Kunkel (Zoller and Smith, 1984; Kunkel, 1985). The replicative form of this p130-containing viral DNA (p130 RF) was propagated in the MV1190 strain of E.coli following transformation of cells and plaque purification. To produce uracil-containing single-stranded M13 phage (p130 SS), a 500

mL culture of E. coli strain CJ236 (dut^-, ung^-) was transformed with p130 RF and grown in the presence of 0.25 µg/ml of uracil for 6 hours. Single-stranded phage was isolated by ethylene glycol precipitation and viral proteins removed by multiple rounds of phenol/chloroform extraction. Uracil-containing single-stranded template was added to a reaction mixture with primer 86 (5' GGTAGTGCTGGCTGGATCGTATAATGTGGT 3') to delete p130 nt's 2074-2088 (ΔRYSSP) or primer 87 (5' GCTATCATTCTCAACAAATCTGG TAGTGCTGGC 3') to delete p130 nt's 2107-2115 (ΔRRL). Each reaction mixture also contained the M13 universal reverse primer (5' GTAAAACGACGGCCAGT 3'). After allowing the polymerization/ligation reaction to proceed, the DNA was transformed into MV1190 cells and phage DNA from individual plaques was screened for the correct deletion by sequencing. A double mutant (ΔRYSSP/ RRL) was produced by sequential rounds of mutagenesis using the two mutagenesis primers described above. Following mutagenesis, an XbaI-SphI restriction fragment of the p130 sequence which contained the mutation was isolated from replicative form M13 DNA, blunted with Klenow and ligated into the pGEX 2T vector.

Chapter 3. Production of monoclonal and polyclonal antibodies to study cyclin interactions with p130

3.1 Introduction

As discussed in Chapter 1, the pRb family members are found associated with cyclins. Although phosphorylation of pRb by cyclin A/cdk2 and cyclin E/cdk2 complexes has been reported in mammalian cells, pRb forms stable complexes only with the D-type cyclins in the absence of cdk4 (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993b; Kato *et al.*, 1993). These complexes have been detected in mammalian cells (Dowdy *et al.*, 1993) and the interactions have been reproduced in SF9 insect cells infected with baculoviruses expressing pRb, D-type cyclins and cdk4 (Dowdy *et al.*, 1993; Kato *et al.*, 1993). When co-expressed in the insect cell system, pRb serves as a substrate of D-type cyclin-associated cdk4. Like pRb, p107 can be detected in stable complexes with D-type cyclins, cyclin A and cyclin E in mammalian cells (Lees *et al.*, 1992; Ewen *et al.*, 1993b; Li *et al.*, 1993). These interactions were detected by co-immunoprecipitation of p107 with the D-type cyclins, cyclin A and cyclin E. Following co-immunoprecipitation of p107 with these cyclins in mammalian cells, p107 served as an *in vitro* substrate of cdk2 and cdk4-associated kinase activity (Lees *et al.*, 1992; Li *et al.*, 1993). Identical results were noted in the insect cell system when p107, cyclin A and cdk2 were co-expressed in insect cells (Peeper *et al.*, 1993). The pocket domain and carboxyl terminus of both pRb and p107 is required for interactions with D-type cyclins (Ewen *et al.*, 1993b; Kato *et al.*,

1993) and the spacer domain of p107 mediates interactions with cyclin A *in vitro* (Ewen *et al.*, 1992; Faha *et al.*, 1992).

Following the cloning of a p130 cDNA in this lab and another (Hannon *et al.*, 1993; Li *et al.*, 1993; Mayol *et al.*, 1993), sequence comparisons of p107 and p130 revealed the two pRb family members share approximately 50% homology at the amino acid level, defined by several conserved regions including the spacer domain. These structural similarities prompted a study to determine if p130 also associated with cyclin A/cdk2, cyclin E/cdk2 and cyclin D/cdk4 complexes *in vivo*. To begin this study, antibodies to cyclin A, cyclin E, the D-type cyclins and cdk2 were required. Monoclonal antibodies to cyclin A (C160) (Giordano *et al.*, 1989) were available in the lab and monoclonal antibodies to cyclins D1, D2 and D3 (Lukas *et al.*, 1994) were the generous gift of J. Bartek, Danish Cancer Society. Antibodies to cyclin E and cdk2 were not available when these studies were initiated and the production of these antibodies is described below.

3.2 Production of GST-cyclin E as an immunogen

To immunize animals against cyclin E, a plasmid encoding a GST-cyclin E fusion protein was constructed using a pGEX expression vector. pGEX expression vectors (Pharmacia) direct the synthesis of proteins in bacteria as fusions with the C-terminus of glutathione *S*-transferase (GST), a 27 kDa protein encoded by the parasitic helminth *Schistosoma japonicum* (Smith and Johnson, 1988). pGEX vectors are available in several variations (pGEX 1, pGEX 2T, pGEX 3X), all encoding identical genes but having

modified multiple cloning sites depending on the reading frame required. GST fusion protein synthesis is regulated by the *tac* promoter (a trp-lac hybrid), which is repressed by the plasmid-encoded $lacI^q$ (lac repressor) gene and induced in the presence of isopropyl-ß-D-galactopyranoside (IPTG). In most cases, GST fusion proteins (following their induction in the presence of IPTG) are soluble in aqueous buffer and can be affinity purified on immobilized glutathione.

3.2.i Construction of the GST-cyclin E expression vector

pGEX-cyclin E was constructed by first isolating the cyclin E cDNA from pLSXN-cyclin E (kindly provided by J. Roberts, Fred Hutchison Cancer Research Center). LXSN-cyclin E contains a blunted Hind III fragment of the cyclin E cDNA (nt's 1-1509) ligated into the Hpa I site of pLSXN (the coding region of the cyclin E cDNA is nt's 26-1213). A portion of the cyclin E cDNA (nt's 100-1509) was removed from this vector by digestion with Bam HI and cloned into pGex 1 cut with Bam HI. The resulting GST-cyclin E fusion protein consists of cyclin E amino acids 26-395 fused in-frame to glutathione-S-transferase (GST).

3.3 Production of cyclin E antibodies in rabbits

GST-cyclin E was expressed in and purified from *E.coli* strain DH5α as described in General Methods (Chapter 2.11). Purified GST-cyclin E was used as an antigen for immunization of rabbits to produce polyclonal sera. New Zealand white rabbits were injected subcutaneously in two separate regions at the back of the neck once every three to four weeks with an emulsion composed of 200 µg GST-cyclin E (diluted in PBS to a

final volume of 500 μL) and 500 μL Freuds adjuvant (Gibco) (complete adjuvant was used in the first injection and subsequent injections used Freuds incomplete). One week following the third injection, 50 mL of blood was obtained by ear bleed. Blood was allowed to coagulate by overnight refrigeration, centrifuged for 10 min at 6000 x g (4^0C) and the sera carefully removed to a fresh tube which was re-centrifuged to remove contaminating erythrocytes. TRIS-HCl pH 7.6 was added to a final concentration of 0.1 M and sodium azide to a final concentration of 0.02% before storage at -20^0C. Three to four weeks following each bleed, rabbits were re-injected with antigen and bled.

3.3.i. Characterization of polyclonal cyclin E antisera

Crude antisera obtained from ear bleeds of immunized rabbits were characterized in several assays including immunoprecipitation assays and Western blotting. ^{35}S-labeled cyclin E was produced in cyclin E baculovirus-infected SF9 insect cells and by *in vitro* transcription and translation of the cyclin E cDNA (as described in Materials and Methods, Chapter 2). In addition, cyclin E was isolated from a breast carcinoma cell line (MDA MB157) noted to contain high levels of cyclin E protein (Keyomarsi and Pardee, 1993). The crude antisera immunoprecipitated ^{35}S-labeled cyclin E from infected SF9 insect cell lysates (Figure 3.1A, lane 1). Cyclin E produced in these cells appears as a doublet migrating from 47-55 kDa. The heterogeneous nature of the protein on SDS-PAGE gels is a result of phosphorylation of the protein (Koff *et al.*, 1991). The antisera also immunoprecipitated *in vitro* transcribed and translated cyclin E from rabbit reticulocyte lysates (Figure 3.1B). The *in vitro* transcribed and translated product of the

cyclin E cDNA appears as a series of ^{35}S-labeled bands due to multiple translational start sites present within the cyclin E cRNA. The authentic translational start site in the cRNA does not contain a Kozak consensus sequence (Kozak, 1989) which is required to promote efficient translation of mRNA *in vitro* and *in vivo*. In addition, the antisera detected cyclin E on Western blots of whole cell lysates of MDA MB157 cells (Figure 3.1C). A summary of the profile of this antisera is shown in Table 3.1.

3.3.ii. Production of cyclin E monoclonal antibodies

The production of monoclonal antibodies is based on a technique devised by Köhler and Milstein (1975). In this technique, a B-cell tumor (myeloma) cell is fused to an antibody secreting (plasma) cell to form a hybrid cell or hybridoma. The hybridoma cells are immortal and may constitutively secrete homogeneous monospecific antibodies. To produce the hybridoma, plasma cells (derived from the spleen of an immunized mouse) and NS-1 myeloma cells are fused in the presence of ethylene glycol and hybridomas are selected for by drug selection.

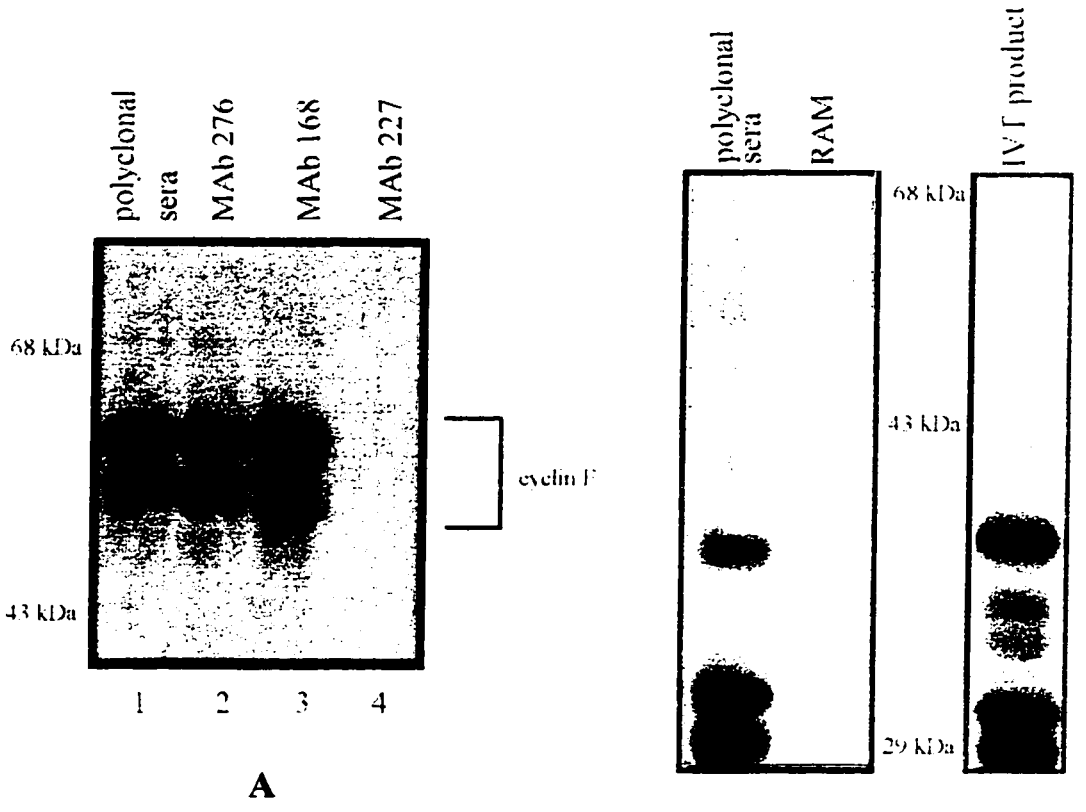
Hybridoma selection is accomplished by manipulation of two inherent variables. Firstly, unfused mouse splenocytes will fail to proliferate and die in tissue culture. Secondly, unfused NS-1 cells are selected against by addition of azaserine, a chemical which blocks *de novo* purine biosynthesis. Azaserine is an analogue of glutamine which inhibits amidotransferases required in the first stages of purine biosynthesis. The *de novo* purine biosynthetic pathway is vital to these cells because they contain a mutated hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene, making them deficient in

the nucleotide salvage pathway. Thus, selection forces NS-1 cells to utilize a pathway that is non-functional and allows only fused cells (which contain a viable nucleotide salvage pathway due to complementation) to grow in the selection media.

3.3.iii Injection of mice and isolation of splenocytes

To prepare GST-cyclin E for injection of mice, 200 μg GST-cyclin E (50 μg/mouse) was diluted in PBS to a final volume of 350 μL and emulsified with 350 μL Freuds adjuvant (Gibco). Freuds complete adjuvant was used in the first injection and subsequent injections used Freuds incomplete. Four Balb/C mice were injected intraperitoneally every three weeks for a total of 4 injections. Test bleeds were obtained from mouse orbitals and tested for response to GST-cyclin E by dot blotting.

To prepare dot blot membranes, PBS-diluted solutions of GST-cyclin E or GST alone (both 10 μg /mL) were incubated with nitrocellulose squares for two hours at room temperature. Following incubation, squares were blocked in 5% (w/v) milk powder in PBS for 1.5 hours. Squares were maintained in a moist environment at room temperature and gridded off into cells by pencil markings. To each cell, 5 μL of mouse serum (diluted 1/10, 1/50 and 1/100) was "dotted" onto the blocked nitrocellulose and allowed to incubate in a moist environment for 45 minutes. Nitrocellulose squares were rinsed twice in PBS-Tween (PBS plus 0.5% Tween-20) and once in PBS alone for 15 minutes each. Horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody was added for 1 hour and rinsed as described for the primary incubation. Nitrocellulose squares were developed by addition of ECL substrate (Amersham) and exposed to film.


polyclonal sera
MAb 276
MAb 168
MAb 227
68 kDa
43 kDa
cyclin E
1
2
3
4
A
polyclonal sera
RAM
IVT product
68 kDa
43 kDa
29 kDa
B

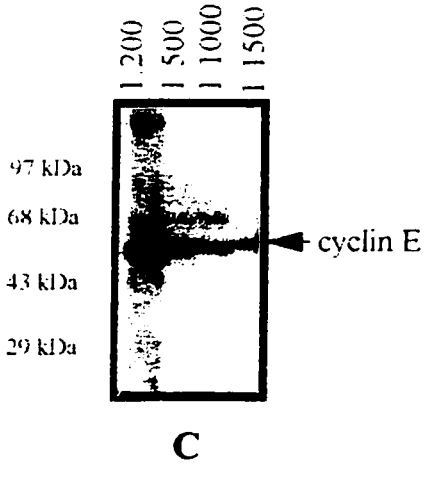

1:200
1:500
1:1000
1:1500
97 kDa
68 kDa
43 kDa
29 kDa
cyclin E
C

Figure 3.1 Characterization of cyclin E polyclonal antibodies

A. Immunoprecipitation of baculovirus-expressed cyclin E

SF9 cells infected with the cyclin E baculovirus (at an M.O.I of 5) were metabolically labeled with ^{35}S-methionine and lysed. 20 μg of total cell protein was immunoprecipitated with 10 μL polyclonal (lane 1) and 100 μL monoclonal (lanes 2-4) antibodies to cyclin E as indicated. 5 μL rabbit anti-mouse antisera (RAM) was used as a negative control. Immune complexes were resolved by SDS-PAGE and the gel processed for fluorography.

B. Immunoprecipitation of *in vitro* translated cyclin E

Following *in vitro* transcription and translation of cyclin E, the ^{35}S-labeled products from rabbit reticulocyte lysates were resolved by SDS-PAGE (IVT product). The same products were immunoprecipitated with polyclonal cyclin E antisera. Rabbit anti-mouse antisera (RAM) was used as a negative control. Immune complexes were resolved by SDS-PAGE and the gel processed for fluorography.

C. Western blotting of cyclin E by polyclonal antibodies

An MDA MB157 cell lysate was resolved in one large lane by SDS-PAGE and blotted to nitrocellulose. The blot was divided into 4 separate strips, each strip incubated in a different dilution of crude cyclin E antisera as shown. The strips were realigned prior to development with ECL substrate.

One week following the 4th injection of all mice, the best responding animal (as judged by dot blot) was injected intravenously (iv) with 200 µL of PBS containing 20 µg GST-cyclin E. Three days later, the mouse was sacrificed and the spleen aseptically removed and placed in DMEM prewarmed to 37^{0}C. Extraneous tissue was trimmed away and the spleen dissected and teased to release splenocytes. Material was transferred to a 50 mL conical tube and the contents allowed to settle for 2 minutes. Splenocytes were removed from sediment, transferred to a fresh 50 mL tube and rinsed twice by addition of 10 mL DMEM followed by centrifugation at 1000 RPM for 5 minutes. Concurrently, 1x 10^7 NS-1 cells were rinsed once in DMEM. Following the rinses, the splenocyte and NS-1 cell pellets were combined by resuspension of the two cell types in DMEM followed by centrifugation.

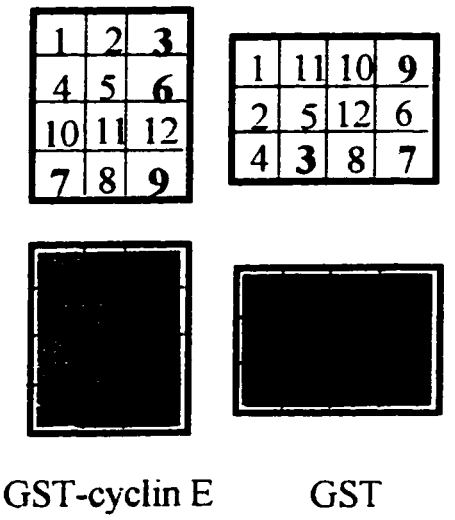
3.3.iv Fusion of splenocytes and NS-1 myeloma cells

To fuse the cells in the final cell pellet, 1 mL of 50% PEG (polyethylene glycol, molecular weight 1300-1600, Sigma) diluted in DMEM was slowly added to the combined cell pellet by gentle stirring and the reaction slowly diluted by addition of 10 mL DMEM over 3 minutes of continuous stirring. Fused cells were centrifuged to remove PEG and resuspended in 200 mL azaserine-hypoxanthine (AH) media (DMEM plus 20% fetal bovine serum, 0.015 mg/mL oxaloacetate, 5 mg/mL sodium pyruvate, 20 IU/mL bovine insulin, 0.58 mM azaserine, 10 mM hypoxanthine). Resuspended cells were aliquoted into 20 96-well microtitre plates, 100 µL/well.

3.3.v Screening and evaluation of cyclin E hybridomas

Screening of approximately 580 hybridoma supernatants was initiated 9 days after fusion and consisted of removal of 5 μL of tissue culture supernatant followed by dot-blotting on GST and GST-cyclin E nitrocellulose. Samples positive on both membranes were discarded and only samples positive on GST-cyclin E and not GST were expanded (Figure 3.2A). Following screening, positive clones were single-cell cloned using a mouth-pipetting device and plated in 96-well microtitre dishes containing splenocyte feeder cells obtained from an non-immunized mouse. Clones arising from single cells were tested by dot blot, expanded and frozen as stocks. Colonies were assumed to be clonal if 90% or more of the isolated single cells produced positive colonies. Three monoclonal antibodies to cyclin E were generated in this manner: MAb 168, MAb 227 and Mab 276. All three monoclonals were isotyped as IgG2a using a commercial kit (Serotec).

MAb's 168, 227 and 276 were collected and utilized in experiments without further purification. Each monoclonal antibody was subsequently analyzed for the ability to recognize cyclin E in immunoprecipitations and on Western blots. All three monoclonal antibodies Western blotted GST-cyclin E fusion protein but not GST alone, confirming the results of the initial dot blots (Figure 3.2B). MAb's 168 and 276 immunoprecipitated cyclin E produced in SF9 cells infected with a cyclin E baculovirus (Figure 3.1A, lanes 2 and 3) but Mab 227 did not immunoprecipitate cyclin E (Figure 3.1A, lane 4) and was not characterized further. MAb's 168 and 276 also detected endogenous levels of cyclin E in U2OS and C33A cells by Western blot (Figure 3.2C). Cyclin E appears to be more highly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4 5 6
10 11 12
7 8 9
1 11 10 9
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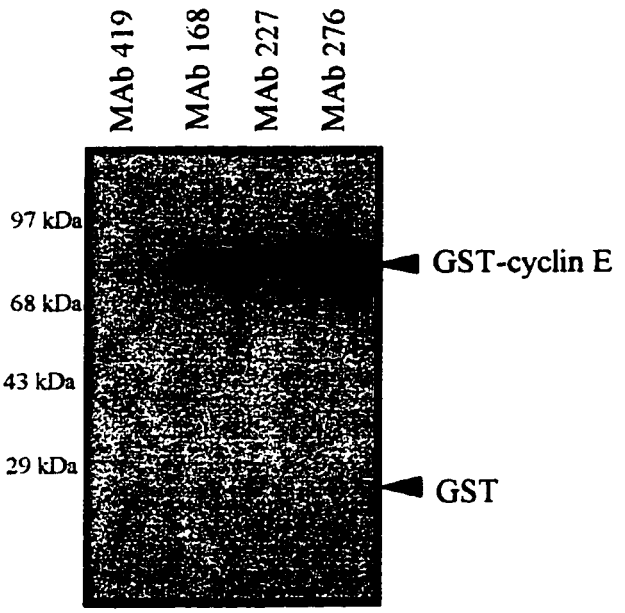
4 3 8 7
GST-cyclin E
GST

A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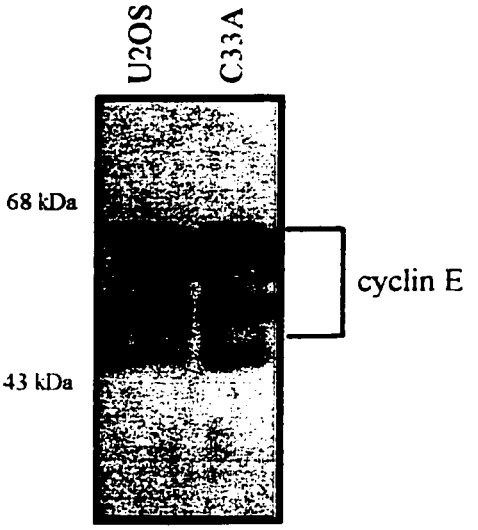
MAb 168
MAb 227
MAb 276
97 kDa
68 kDa
43 kDa
29 kDa
GST-cyclin E
GST

B


U2OS
C33A
68 kDa
43 kDa
cyclin E

C

Figure 3.2 Characterization of cyclin E monoclonal antibodies

A. Dot blots of cyclin E hybridoma supernatants

Samples of supernatants from hybridomas were spotted onto GST-cyclin E nitrocellulose (left side) or GST nitrocellulose (right side), incubated briefly and washed to elute unbound antibody. Blots were exposed to secondary antibody and developed by addition of ECL substrate. The numbered grids above the dot blots indicate which samples were added to each square. Only samples positive on GST-cyclin E nitrocellulose and not GST nitrocellulose were considered as hybridomas secreting cyclin E antibody. Approximately 600 hybridoma supernatants were screened in this manner.

B. Western blotting of GST-cyclin E by monoclonal antibodies

A mixture of GST-cyclin E and GST was resolved in one large lane by SDS-PAGE and blotted to nitrocellulose. The blot was divided into 4 separate strips, each strip incubated in a different cyclin E monoclonal antibody or Mab 419 recognizing SV40 large T as a negative control. As signified by the absence of detectable signal where GST migrates, the cyclin E monoclonals are specific for cyclin E and not for GST.

C. Western blotting of cyclin E by monoclonal antibodies

Whole-cell lysates from U2OS and C33A cells were resolved by SDS-PAGE, blotted to nitrocellulose and probed with a mixture of monoclonal antibodies 276 and 168 (diluted 1:2000). Following addition of secondary antibody, the blot was developed by addition of ECL substrate.

phosphorylated in these cell types than in MDA MB157 cells and the reason for this has not been established. A summary of the profile of the three monoclonal antibodies is shown in Table 3.1.

3.4. Commercial synthesis of cdk2 peptide as an immunogen

As a source of cdk2 antigen, a peptide containing the carboxyl terminal-most 12 amino acids was commercially synthesized by solid phase peptide synthesis. This peptide sequence had previously been shown to produce quality anti-cdk2 antibodies in rabbits (Tsai *et al.*, 1991). The cdk2 peptide is YCG***QDVTKPVPHLRL***, where the bolded amino acids represent residues 287-298 of the 298 amino acid human cdk2 sequence. The additional amino terminal residues mediate conjugation to keyhole limpet hemocyanin (KLH), a protein carrier molecule. This protein carrier molecule is coupled to the cdk2 peptide (which on its own is non-immunogenic) to make the peptide a more antigenic immunogen.

To conjugate the peptide, KLH (at a final concentration of 5 mg/mL) was combined with 50 μg cdk2 peptide in 2 mL borate buffer (100 mM NaCl, 100 mM borate pH 9.0) and the peptide crosslinked to KLH through its amino terminal tyrosine residue by addition of 400 μL bis-diazobenzidine (BDB) solution (BDB solution was prepared by dissolving 5 mg benzidine-HCl (Sigma) and 35 mg $NaNO_2$ in 0.2 M HCL with stirring for 1 hour at 4^0C). Crosslinking was performed by rocking reactions at 4^0C for one hour.

The additional amino terminal cysteine in the cdk2 peptide was used to prepare a peptide column using Sulpholink coupling gel (Pierce). This matrix is composed of

iodoacetyl groups linked to the matrix by twelve atom spacer arms. The iodoacetyl groups preferentially react with the free sulfhydryl group of the cysteine residue present in the peptide, thus covalently binding the cdk2 peptide to the matrix. 50 μg cdk2 peptide was added to a 1.5 mL bed volume of Sulpholink coupling gel (Pierce) equilibrated in column buffer (50 mM TRIS, 5 mM EDTA pH 8.5) by rocking for 30 minutes. The column was washed with column buffer and remaining free sulfhydryls on the matrix were blocked by addition of 1.5 mL of 50 mM cysteine solution in column buffer. Following a 30 minute incubation, the column was washed with 1M NaCl and then equilibrated in PBS.

3.4.i Antipeptide cdk2 antibodies

Rabbits were injected and bled exactly as described for the production of cyclin E antisera in section 3.3. Before characterization, crude cdk2 antisera obtained from ear bleeds of immunized rabbits was affinity purified on a cdk2 peptide column. 15 mL rabbit sera was diluted 1:1 with PBS and poured through the cdk2 affinity column twice, the column washed with 10 bed volumes of PBS and the bound antibodies eluted with 0.1 M glycine pH 2.8. Five 1 mL eluates were collected and neutralized in 1.5 mL Eppendorf tubes containing 50 μL 1M TRIS-HCl pH 8.0. Combined eluates were dialyzed extensively against PBS and the final antibody concentration was determined by titering.

The affinity-purified antisera was characterized by testing its performance in immunoprecipitation assays and western blots using cdk2 baculovirus-infected SF9 cells. cdk2 antisera recognized cdk2 present in infected insect cell lysates by western blot

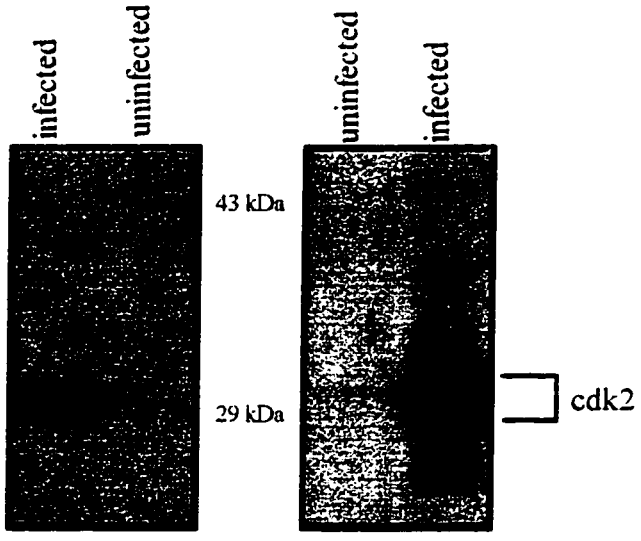

infected
uninfected
43 kDa
29 kDa
uninfected
infected
cdk2

Figure 3.3 Detection of cdk2 by antipeptide antibodies

(Left panel) Uninfected SF9 cells and cells infected with cdk2 baculovirus were metabolically labeled with ^{35}S-methionine and lysed. 20 μg of total cell protein was immunoprecipitated with affinity-purified cdk2 antisera and immune complexes were resolved by SDS-PAGE. The gel was processed for fluorography. (Right panel) Uninfected or cdk2 baculovirus-infected SF9 cells were lysed and 20 μg of total cell protein was resolved by SDS-PAGE, blotted to nitrocellulose and probed with affinity-purified cdk2 antibodies (diluted 1:2000). Following addition of secondary antibody, the blot was developed by addition of ECL substrate.

(Figure 3.3 left side) and immunoprecipitated ^{35}S-labeled cdk2 from the same cells (Figure 3.3 right side). A summary of the profile of the affinity-purified cdk2 antipeptide antisera is shown in Table 3.2

3.5 Concluding remarks

The antibodies described in this chapter are utilized throughout this thesis and in combination with additional antibodies were important in studying various interactions between p130, cyclin E and cdk2.

Table 3.1 Characterization of cyclin E-specific polyclonal antisera and monoclonal antibodies

Source of cyclin E detected	Crude polyclonal sera	MAb 168	MAb 276	Mab 227
Detection of GST-cyclin E by dot blot	✓	✓	✓	✓
Immunoprecipitation of *in vitro* translated cyclin E and baculovirus-expressed cyclin E in SF9 cells	✓	✓	✓	✗
Detection of endogenous levels of cyclin E and overexpressed cyclin E by western blotting	✓	✓	✓	not thoroughly tested

Table 3.2 Characterization of cdk2-specific antipeptide antisera

Source of cdk2 detected	Affinity-purified antipeptide antisera
Immunoprecipitation of baculovirus-expressed cdk2 in SF9 cells	✓
Detection of baculovirus-expressed cdk2 by western blotting	✓

Chapter 4. Identification of p130 interactions involving cyclins A, D1, D2, D3 and E.

4.1 Introduction

As a result of the sequence conservation between p107 and p130, it was important to determine if p130 was associated with cyclins *in vivo*. The association of p130 with cyclins would imply that p130 has a role in regulation of the cell cycle. The analyses of these potential interactions required antibodies to cyclins and cdk2, and these were obtained or generated as described in Chapter 3. Additionally, a p107 antibody, several p130 antibodies and an affinity-purified anti-peptide antisera to cyclin D were already available in this laboratory. These antibodies were utilized in co-immunoprecipitation assays to visualize protein:protein interactions which naturally exist in a cell. Once detected, the interactions were reproduced and more thoroughly analyzed by purifying the components and reproducing the interactions *in vitro*.

4.2 Association of p130 with cyclin A and cyclin E by *in vitro* kinase assay

A sensitive method to detect interactions between substrates and cyclin/cdk complexes is co-immunoprecipitation of cyclins followed by *in vitro* kinase assays. Following isolation of cdk complexes on Sepharose beads, beads are incubated in the presence of magnesium ions and ATP, allowing phosphorylation of any substrates associated with the kinase in the immune complex. The substrates appear as radioactively-

labeled phosphorylated proteins when resolved by SDS-PAGE owing to the fact that Υ^{32}P-ATP is also present as an ATP source.

Following immunoprecipitation of cyclin A, cyclin E and cyclin D from 143B cells, an *in vitro* kinase assay was performed to determine if any cyclin-associated proteins were substrates of the active cyclin/cdk2 complexes (Figure 4.1). Several phosphorylated proteins appeared, and several migrated at approximately 100-140 kDa (lanes 4-6). To determine if these proteins were p107 and/or p130, control immunoprecipitations were resolved on the same gel. In one control, E1A was immunoprecipitated from 293 cells with an E1A-specific monoclonal antibody M73 (lane 2). Following an *in vitro* kinase assay, p107 and p130 appeared as substrates of E1A-associated kinase activity (the bands corresponding to p107 and p130 are illustrated by arrows) exactly as presented in Herrmann *et al.*, 1991. As an additional control, p107 was immunoprecipitated with an affinity-purified antipeptide antisera from 143B cells and subjected to an *in vitro* kinase assay (lane 3). Also included in the gel is a negative control immunoprecipitation with Mab 419 which recognizes SV40 large T (lane 1). Mab 419 did not detect any proteins with inherent kinase activity, owing to the fact that 293 cells do not contain SV40 large T.

The *in vitro* kinase assay suggested that both p107 and p130 were present in immune complexes containing E1A, cyclin A and cyclin E and in addition, p107 was also present in complexes containing cyclin D. The antipeptide antisera used to immunoprecipitate cyclin D detects cyclins D1 and D2 but not D3, owing to the fact that

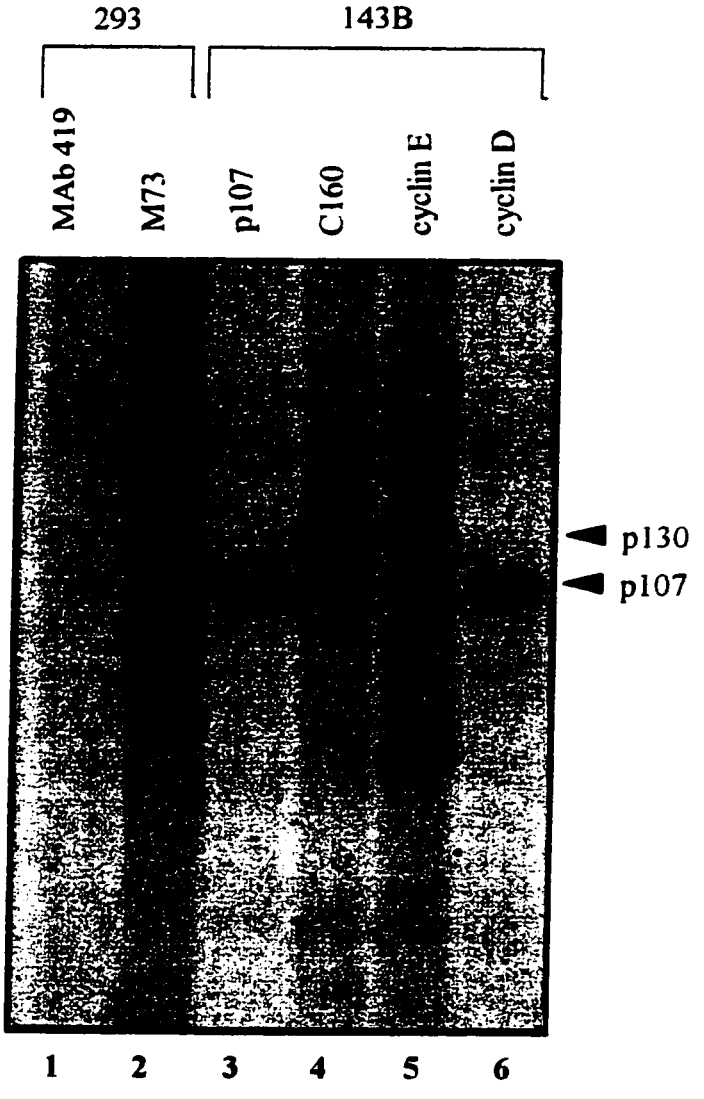

293
143B
MAb 419
M73
p107
C160
cyclin E
cyclin D
p130
p107
1
2
3
4
5
6

Figure 4.1 *In vitro* kinase assays following immunoprecipitation of cyclins

Immunoprecipitations were performed in 293 or 143B cells as indicated at the top of the figure. Antibodies used are noted directly under the cell lines. Mab 419 recognizes SV40 large T antigen, M73 recognizes E1A and C160 recognizes cyclin A. Antibodies to p107, cyclin D and cyclin E are described in Chapter 3. Following immunoprecipitation, immune complexes were resuspended in kinase buffer containing Υ^{32}P-ATP and allowed to incubate for 30 minutes. Complexes were resolved by SDS-PAGE and compared to control lanes (1, 2 and 3) for identification of proteins. The proteins shown to be p107 and p130 are indicated to the right of the figure.

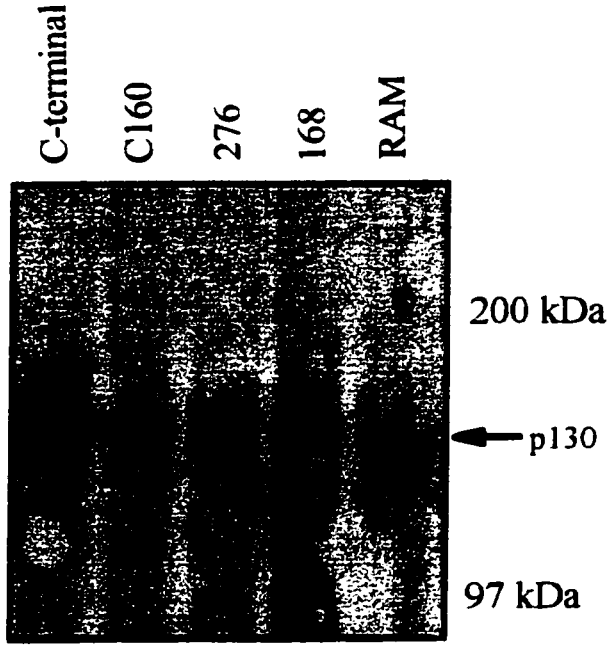

C-terminal
C160
276
168
RAM
200 kDa
p130
97 kDa

Figure 4.2 p130 interactions with cyclins A and E *in vivo*

Immunoprecipitations were performed in U7 cells, a U2OS-based cell line which constitutively overexpresses p130. Antibodies used in immunoprecipitations are noted directly above the figure. C-terminal is a p130 C-terminal antibody, C160 recognizes cyclin A, 276 and 168 are cyclin E monoclonal antibodies described in Chapter 3 and RAM is rabbit anti-mouse, a negative control. Following immunoprecipitation, complexes were resolved by SDS-PAGE, blotted onto nitrocellulose, and probed with C20, a p130 C-terminal antibody (Santa Cruz Biotechnology).

it was raised against a C-terminal peptide of the cyclin D1 sequence. This peptide sequence is partially conserved amongst the three D-type cyclin family members. As a result of the dual-specificity of this antipeptide antisera, it is difficult to determine if p107 is interacting with one or both of these cyclins. Several additional bands are detectable in the in vitro kinase assays and the identity of these proteins is not known. They may be proteins which cross-react with the C160 antibody or are unidentified proteins which interact with cyclin/cdk complexes.

The identification of p130 in cyclin A and cyclin E complexes is a novel finding that was confirmed by analysis of the same immunoprecipitations by western blotting with Z11, a p130 monoclonal antibody recognizing both p107 and p130 (Li *et al.*, 1993). This analysis confirmed the two indicated phosphorylated bands in Figure 4.1 were in fact p107 and p130 (Li *et al.*, 1993) and these observations are extended in a different cell line in the following section.

4.2.i. Association of p130 with cyclin A and cyclin E by western blot

Endogenous levels of p130 are difficult to detect in many cell lines. To compensate for this, a U2OS-based cell line was generated to constitutively overexpress p130. U2OS cells contain pRb but lack $p16^{INK4A}$ (Lukas *et al.*, 1995; Koh *et al.*, 1995). As a result, the growth characteristics of these cells are unaffected by overexpression of pRb, p107 and p130 (data not shown). The cell line, named U7, was used in further experiments to demonstrate the stable association of p130 with cyclins A and E.

In Figure 4.2, p130 produced in U7 cells is detectable by immunoprecipitation followed by western blotting with a carboxyl-terminal affinity-purified antipeptide antisera ("C-terminal"). p130 also co-immunoprecipitates with cyclin A and cyclin E, and it is present when either cyclin E monoclonal antibody is used. p130 does not co-immunoprecipitate with affinity-purified rabbit anti-mouse antisera (RAM). These studies indicate that cyclins A and E stably associate with p130 (and p107) *in vivo* and that p130 (and p107) serve as *in vitro* substrates of the cyclin A and cyclin E-associated kinase (Figure 4.1).

4.3. Production of GST-p130 fusion proteins

To further study the interactions of p130 with cyclins A and E, each protein (cyclin A, cyclin E, cdk2 and p130) was purified and combined *in vitro*. Cyclin A, cyclin E and cdk2 were produced in insect cells and combined *in vitro* with several GST-p130 fusion proteins. It was of interest to determine if GST fusion proteins containing different regions of p130 could be phosphorylated by cyclin A/cdk2 and cyclin E/cdk2 activity *in vitro*. In preparation for these studies, fifteen GST-p130 fusion proteins were constructed, each containing a portion of the p130 amino acid sequence in frame with GST. These fusion proteins retain all or portions of the "spacer" domain of p130, defined as amino acids 616-828. The pGEX expression plasmids (described in Chapter 3.2) were used to construct the fusion proteins, several of which were used in the studies described in this Chapter and in studies described in Chapter 5. These fusion proteins are depicted in Figure 4.3.

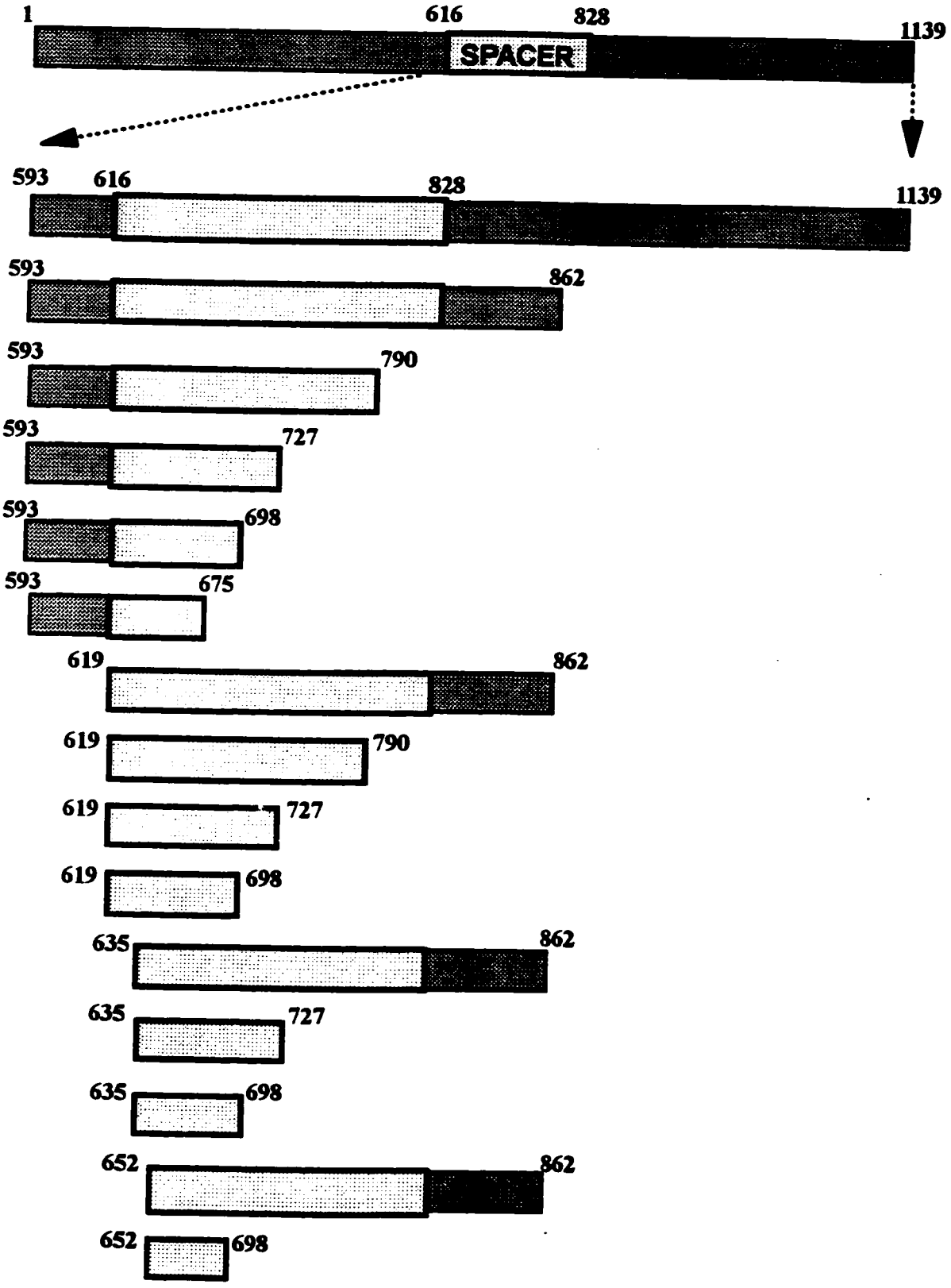
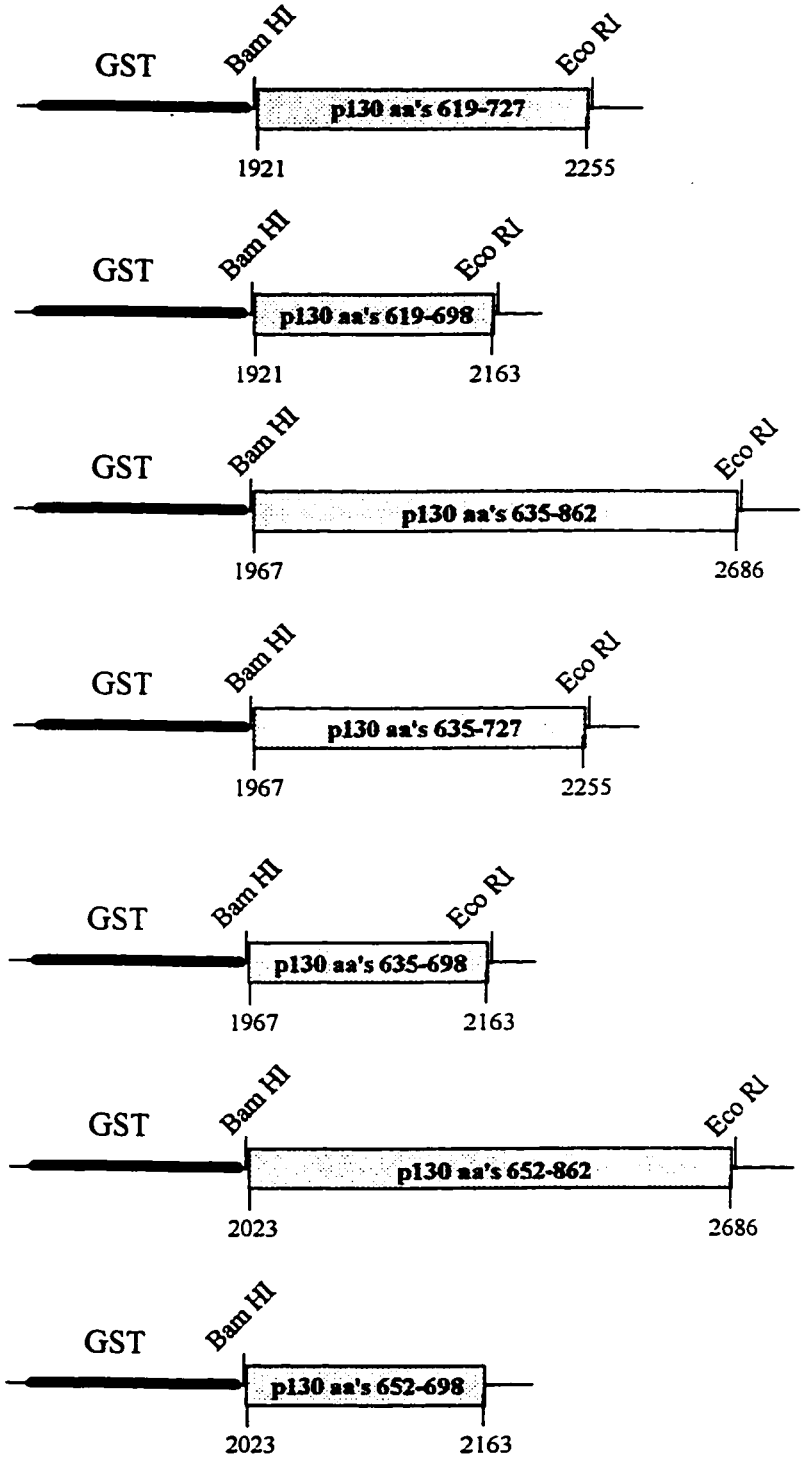

1
616
828
1139
SPACER
593
616
828
1139
593
862
593
790
593
727
593
698
593
675
619
862
619
790
619
727
619
698
635
862
635
727
635
698
652
862
652
698

Figure 4.3 Schematic diagram of GST-p130 fusion proteins

GST-p130 fusion proteins are depicted underneath the full length protein shown at the top of the diagram for reference where numbers indicate amino acids. The spacer region is highlighted in light gray throughout the diagram with all other regions of p130 indicated in darker gray. The fusion proteins encompass all or portions of the latter half of the protein which has been magnified as shown by the arrows.


GST
Bam HI
Eco RI
p130 aa's 619-727
1921
2255
GST
Bam HI
Eco RI
p130 aa's 619-698
1921
2163
GST
Bam HI
Eco RI
p130 aa's 635-862
1967
2686
GST
Bam HI
Eco RI
p130 aa's 635-727
1967
2255
GST
Bam HI
Eco RI
p130 aa's 635-698
1967
2163
GST
Bam HI
Eco RI
p130 aa's 652-862
2023
2686
GST
Bam HI
p130 aa's 652-698
2023
2163

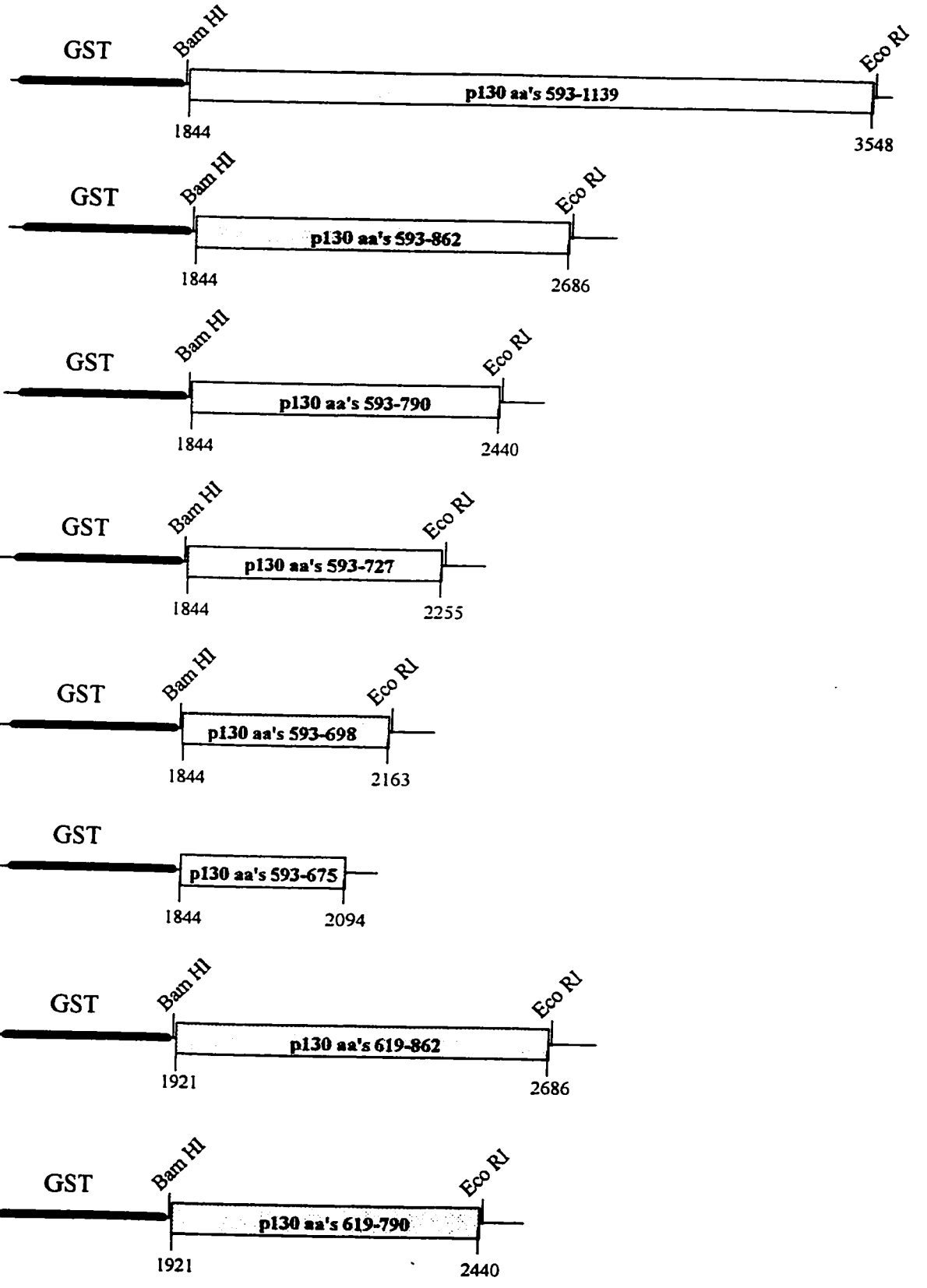

GST
Bam HI
Eco RI
p130 aa's 593-1139
1844
3548
GST
Bam HI
Eco RI
p130 aa's 593-862
1844
2686
GST
Bam HI
Eco RI
p130 aa's 593-790
1844
2440
GST
Bam HI
Eco RI
p130 aa's 593-727
1844
2255
GST
Bam HI
Eco RI
p130 aa's 593-698
1844
2163
GST
p130 aa's 593-675
1844
2094
GST
Bam HI
Eco RI
p130 aa's 619-862
1921
2686
GST
Bam HI
Eco RI
p130 aa's 619-790
1921
2440

Figure 4.4 Linear plasmid maps of GST-p130 fusion proteins

GST-p130 fusion proteins shown in Figure 4.3 are depicted as they were constructed using pGex vectors as described in the text. The p130 coding sequence present in each construct is shown in light gray, and the amino acids present in each construct indicated in the gray box. At the 5' and 3' ends of each p130 insert are numbers which represent nucleotides. The remainder of each plasmid is implied to be intact as no other manipulations were made except for those indicated.

4.3.i. Construction of GST-p130 fusion proteins

GST-p130 fusion proteins depicted in Figure 4.3 are named according to the p130 amino acids present within them. The exact nucleotides contained within each construct are depicted in Figure 4.4. The first 5 GST-p130 fusion proteins (593-1139, 593-862, 593-790, 593-727, 593-698) share the same 5' terminus derived from a blunted Bgl II/Xba I fragment (nt 1844-3548) ligated into the Sma I site of pGEX-2T. The remaining 4 fusion proteins contain a different 3' terminus as a result of digestion of the first plasmid with Eco RI (nt 2686), Sac I (nt 2440) plus Eco RI, Nco I (nt 2255) plus Eco RI or Sph I (nt 2163) plus Eco RI. Following each digestion, the vector was separated by agarose gel electrophoesis, isolated and religated to complete the deletion of 3' sequences.

pGST-130 (593-675) was generated by PCR using the forward primer (5' GTTCCTTACATGATG 3') and the reverse primer (5' GACTTAAGCCGACCACCC CTCGACAT 3') using full length p130 cDNA as a template. This 250 nt PCR product (nt 1844-2094) was digested with Bgl II and Eco RI and blunted with Klenow. The blunted PCR product was ligated into the blunted Bam HI, Eco RI sites of pGEX 2T. The construction of this plasmid destroys the Bam HI and Eco RI sites in pGEX 2T.

Four additional GST-p130 fusion proteins (619-862, 619-790, 619-727, 619-698) share the same 5' terminus derived from a blunted Afl III/Eco RI fragment ligated into the Sma I site of pGEX-2T. The additional fusion proteins were generated exactly as for the first 5 fusion proteins described above. Three additional fusions (635-862, 635-727, 635-698) share the same 5' terminus derived from a blunted Apo I/Eco RI fragment ligated

into the Sma I site of pGEX-2T. Deletions in the 3' region of this construct were likewise generated as for the first 5 fusions described above. Finally, two additional fusion proteins (652-862 and 652-698) were generated by PCR using a forward primer (5' TACAAATGGATAGGGA 3'). This 1157 nt PCR product (nt 2023-3180) was digested with Bam HI and Eco RI and ligated into the Bam HI/Eco RI site of pGEX 1X. Fusion (652-698) was generated by deletion of 3' sequences as a result of digestion of the plasmid with Sph I and Eco RI followed by re-ligation as described above. All GST-p130 fusions were analyzed by expression and purification analysis.

4.4 *In vitro* kinase assays using baculovirally-produced cyclins and cdk's

As an *in vitro* source of cyclins and cdk's, the baculoviral system of protein expression was utilized. Baculoviruses are a family of double-stranded DNA viruses which infect many species of insect cells. The *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome can accommodate foreign DNA, making it a suitable vector for expression of recombinant proteins in the host insect cell line SF9 (*Spodoptera frugiperda*) (reviewed in Kidd and Emery, 1993). Recombinant baculoviruses are generated upon co-transfection of AcNPV DNA and a transfer vector which contains homologous viral sequences and the heterologous gene of interest. Recombination between the homologous sequences results in transfer of the heterologous gene from the transfer vector into the AcNPV genome. Upon infection with AcNPV, SF9 cells synthesize recombinant mRNA and protein at a high rate, owing to host gene expression

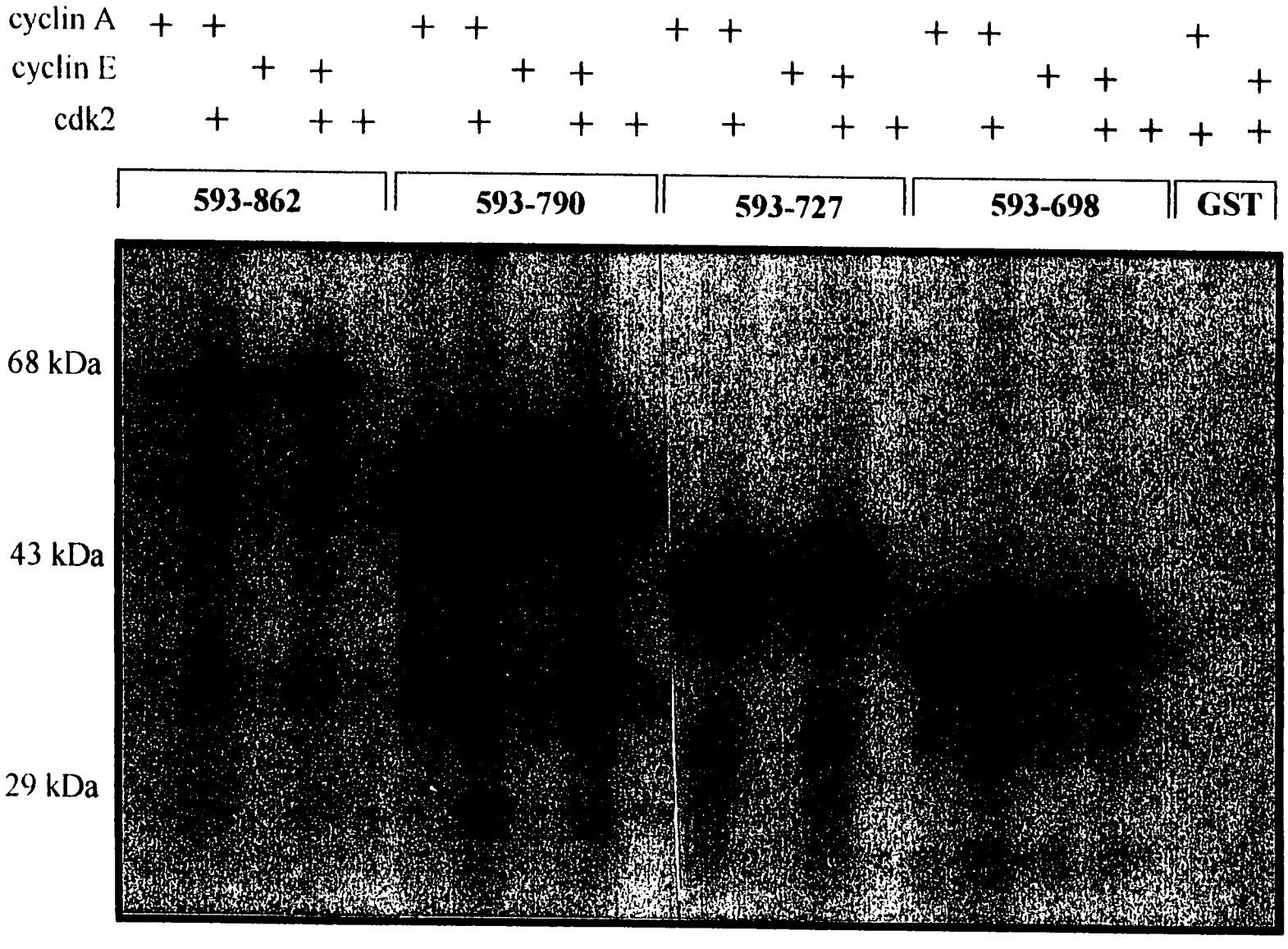

cyclin A + + + + + + + + + +
cyclin E + + + + + + + + +
cdk2 + + + + + + + + + + + + + + +
593-862
593-790
593-727
593-698
GST
68 kDa
43 kDa
29 kDa

Figure 4.5 *In vitro* kinase assays with GST-p130 fusion proteins

SF9 cell lysates infected with the indicated cyclin and cdk2 (at top of figure) were mixed with glutathione Sepharose-bound GST-p130 fusion proteins (indicated in numbers) or GST alone (last two lanes). Reactions were performed in the presence of kinase buffer containing Υ^{32}P-ATP and were allowed to incubate for 30 minutes. Phosphorylated fusion proteins were resolved by SDS-PAGE followed by autoradiography. GST-p130 (593-862) migrates at 66 kDa, GST-p130 (593-790) migrates at 50 kDa, GST-p130 (593-727) migrates at 42 kDa and GST-p130 (593-698) migrates at 37 kDa.

shut-off following infection. The baculoviral system is a popular one for expression of foreign genes because SF9 cells often produce high levels of functionally active, post-translationally modified protein. Additionally, SF9 cells co-infected with two or more viruses are useful for studying protein:protein interactions.

Several baculoviruses are described throughout this thesis. Viruses encoding cyclin A, cyclin E and cdk2 were generously provided by D. Morgan (University of California at San Francisco). Viruses encoding cyclin D1, cyclin D2, cyclin D3 and cdk4 were generously provided by C. Sherr (St. Jude Children's Research Hospital). A p130 baculovirus encoding the latter two thirds of the p130 protein (amino acids 372 to 1139 of the 1139 amino acid protein) named 372C was kindly constructed and provided by Yun Li. To construct 372C, a 2319 nt PCR product of the p130 cDNA (nt 1182 to 3504) was created using a forward primer (5' CCGCGGATCCGAGCATGGGTTCAGGAACA GAGAC 3') and reverse primer AB4022. The PCR product was ligated into the Bam HI site of transfer vector pVL1392 and used in a co-transfection assay with linearized wildtype pAcNPV DNA. Following homologous recombination, rescued viruses were plaque purified, amplified and titered (see General Methods, Chapter 2).

4.4.i. Phosphorylation of GST-p130 fusion proteins by cyclin A/cdk2 and cyclin E/cdk2

To determine if GST-p130 fusion proteins could be phosphorylated by cyclin/cdk complexes *in vitro*, a series of *in vitro* kinase assays were completed. Four GST-p130 fusion proteins (593-862, 593-790, 593-727 and 593-698) or GST alone were incubated

in the presence of SF9 cell lysates containing cyclin A, cyclin A/cdk2, cyclin E, cyclin E/cdk2 and cdk2 (Figure 4.5). Following the incubation, the reaction was terminated and phosphorylated proteins resolved by SDS-PAGE.

GST-p130 fusion proteins appeared as phosphorylated proteins which migrated at the correct molecular weight in each case. In contrast, the 27 kDa GST protein did not serve as a cdk2 substrate (last two lanes) indicating that cdk2-mediated phosphorylation was directed to the p130 moiety of the GST-p130 fusion proteins. Several bands appear to be phosphorylated in these kinase assays. In all cases, the uppermost band in each lane corresponds to the correct molecular weight of each fusion protein. The additional lower molecular weight species are most likely due to degradation of the fusion protein during purification. Additionally, a subsequent quantitative assessment of the fusion protein preparations revealed that unequal quantities of each fusion protein were present in these assays, explaining why some fusion proteins appear more abundant than others.

Weak kinase activity appeared to be present in SF9 cells infected with cyclin A alone, cyclin E alone or cdk2 alone, suggesting insect cell proteins may substitute for cyclins A and E and cdk2. Association between cyclins and cyclin-dependent kinases are known to be promiscuous, for example yeast CDC28 associates with at least four B-type cyclins (reviewed in Pines, 1995b) and D-type cyclins can activate both cdk2 and cdk4 when co-expressed in SF9 cells (Ewen *et al.*, 1994b; Matsushime *et al.*, 1992). In uninfected SF9 cells, no phosphorylation of GST-p130 fusion proteins (or GST alone) could be detected (data not shown).

These results indicated that several GST-p130 fusion proteins which contain the spacer region of p130 are substrates of cyclin A/cdk2 and cyclin E/cdk2-mediated phosphorylation events *in vitro*. Other data indicates GST-p130 fusion proteins containing the amino terminus of p130 (GST-p130 (1-320)) or containing regions between the amino terminus and the spacer (GST-p130 (339 to 595) are not substrates of cyclin A/cdk2 or cyclin E/cdk2 complexes (data not shown), suggesting phosphorylation by cdk2 is directed towards residues within the spacer and C-terminus. These observations are extended in studies aimed at identifying regions of p130 required for interactions with cyclin A/cdk2 and cyclin E/cdk2 complexes (Chapter 5).

4.5 p130 interactions with D-type cyclins

Studies examining the interactions with pRb and D-type cyclins noted stable interactions between these two proteins were difficult to detect *in vivo*. This was explained when studies using purified pRb and D-type cyclins in the baculoviral expression system revealed that upon phosphorylation by cyclin D-associated cdk4 or cdk6 activity, the pRb/cyclin D interaction was abolished (Kato *et al.*, 1993). Figure 4.1 shows that like pRb, p130 does not form stable complexes with D-type cyclins. To determine if these interactions existed but were difficult to detect because of phosphorylation events, the D-type cyclins and p130 were expressed in the baculoviral system to study interactions by co-immunoprecipitation assays.

4.5.i. Co-immunoprecipitation of p130 with D-type cyclins

To investigate potential p130/cyclin D interactions, one or more of the described viruses (372C, cyclin D1, cyclin D2, cyclin D3 and cdk4) were used to infect SF9 cells. Following infection, cells were metabolically labeled with ^{35}S-methionine and subjected to immunoprecipitation analyses (Figure 4.6). Cells infected with the indicated viruses (top of figure) were immunoprecipitated with the indicated antibody ("ip" - at top of figure). A C-terminal p130 antibody (CT) and the monoclonal antibodies to the D-type cyclins (D1, D2, D3) were used in the immunoprecipitations which were resolved by SDS-PAGE. Unfortunately, a cdk4 antibody was unavailable.

The 372C protein migrates as a 95 kDa band and the D-type cyclins migrate from 32-40 kDa as indicated to the left of the figure. In cells infected with 372C and the D-type cyclins, 372C was seen to co-immunoprecipitate with the D-type cyclins in each case. Cyclin D1 appears less abundant in SF9 cell lysates than does cyclin D2 and D3 and this may be because of unequal expression or isotopic labeling. In contrast, in cells co-infected with 372C, cyclin D1 and cdk4 or 372C, cyclin D3 and cdk4, the 372C interaction with cyclin D1 and cyclin D3 was abolished. Interestingly, in cells co-infected with 372C, cyclin D2 and cdk4, the interaction between 372C and cyclin D2 was only weakly disrupted, although a shift in the 372C band had occurred, most likely due to its phosphorylation by cdk4. These results suggest that interactions between D-type cyclins and p130 do occur *in vivo* and that phosphorylation of 372C by cyclin D1/cdk4 and cyclin D3/cdk4 disrupts these complexes. It is not clear why phosphorylation of 372C by cyclin

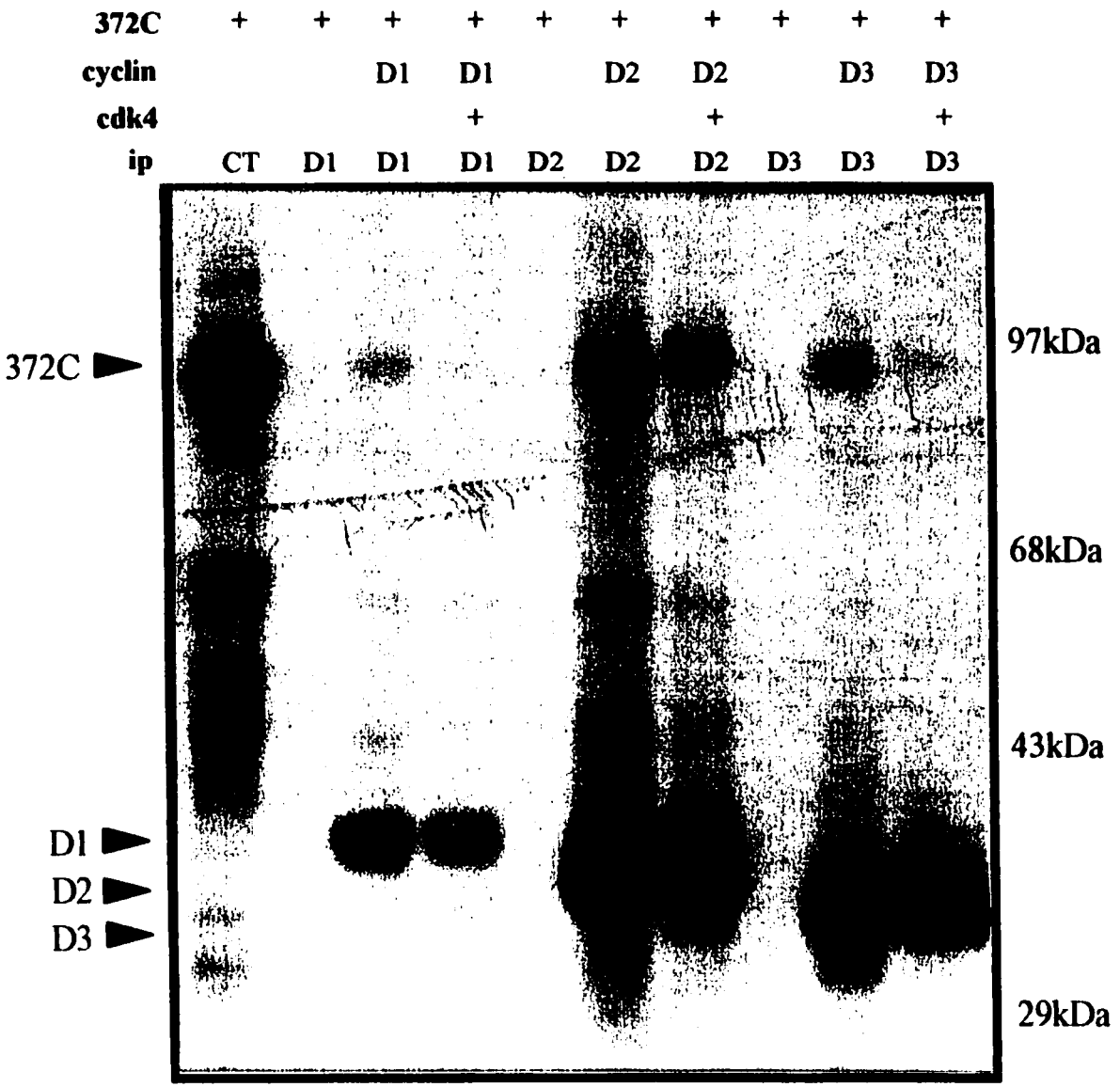

372C + + + + + + + + + +
cyclin D1 D1 D2 D2 D3 D3
cdk4 + + +
ip CT D1 D1 D1 D2 D2 D2 D3 D3 D3
372C
D1
D2
D3
97kDa
68kDa
43kDa
29kDa

Figure 4.6 D-type cyclin interactions with p130

SF9 cells infected with the indicated baculoviruses (top of figure) were metabolically labeled with ^{35}S-methionine and lysed. Lysates were subjected to immunoprecipitation analyses using the indicated antibody ("ip") at the top of the figure. CT is a p130 C-terminal antibody, D1, D2 and D3 are monoclonal antibodies recognizing cyclin D1, cyclin D2 and cyclin D3 respectively. Following immunoprecipitation, complexes were resolved by SDS-PAGE and the gel processed for fluorography.

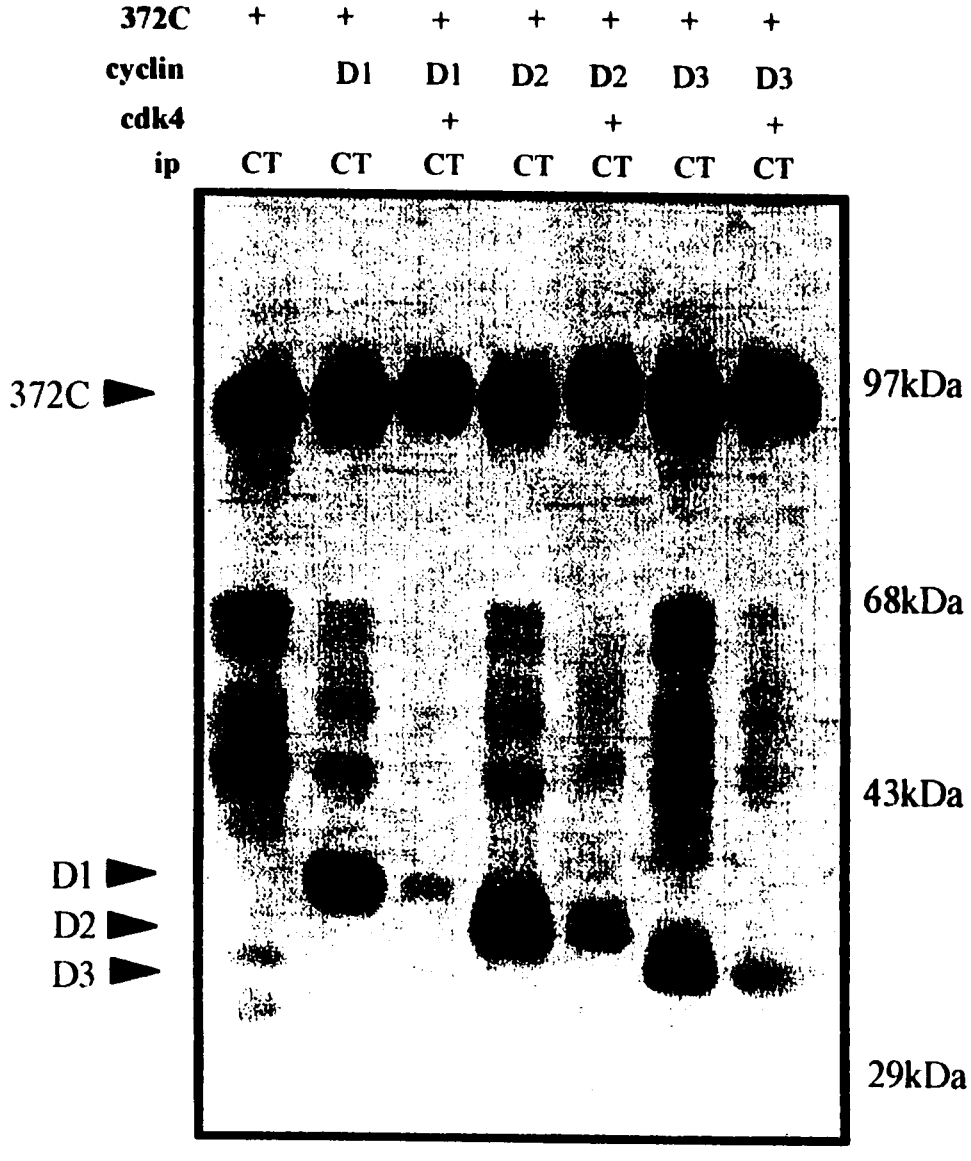

372C + + + + + + +
cyclin D1 D1 D2 D2 D3 D3
cdk4 + + +
ip CT CT CT CT CT CT CT
372C
D1
D2
D3
97kDa
68kDa
43kDa
29kDa

Figure 4.7 p130 interactions with D-type cyclins

SF9 cells infected with the indicated baculoviruses (top of figure) were metabolically labeled with ^{35}S-methionine and lysed. All lysates were subjected to immunoprecipitation analyses using the p130 C-terminal antibody. Following immunoprecipitation, complexes were resolved by SDS-PAGE and the gel processed for fluorography. The p130 N-terminal deletion mutant 372C is indicated at the left side of the figure and migrates at 95 kDa. The position of the D-type cyclins are also indicated on the left side and these migrate from 32-40 kDa.

D2/cdk4 does not disrupt the complex or why, if these complexes do exist, they are difficult to detect *in vivo*. It is a possibility that the association of p107 with D-type cyclins in Figure 4.1 was artefactual or that p107 complexes preferentially with cyclin D2/cdk4 and like p130, does not release from these complexes upon phosphorylation.

The same experiment was repeated in Figure 4.7 using the indicated viruses and the p130 C-terminal antibody (top of figure). This assay was designed to determine whether D-type cyclins could co-immunoprecipitate with 372C, the reciprocal experiment to Figure 4.6. Comparable results were obtained in this experiment as in Figure 4.6, with all D-type cyclins binding to 372C in the absence of cdk4. In the presence of cdk4, 372C appeared as a slightly slower-migrating band and the interactions with cyclins D1 and D3 were significantly hindered. The interaction with cyclin D2 also appeared to be negatively affected, although slightly more cyclin D2 remained bound to 372C in the presence of cdk4 than cyclins D1 and D3. These results confirm the results in Figure 4.6 and reveal that 372C interactions with the D-type cyclins are negatively regulated by phosphorylation of 372C by cdk4. The variances noted in Figure 4.7 may reflect the different antibodies used in the immunoprecipitations where antibody binding may sterically hinder some interactions while encouraging others.

4.6 p130 sequences required to interact with D-type cyclins

Following the results of Figures 4.6 and 4.7, it was apparent that the amino terminus of p130 (amino acids 1-371) was not required to mediate interactions with D-type cyclins in the baculoviral system. To more precisely determine which region(s) of

p130 mediate interactions with D-type cyclins, GST-p130 fusion proteins were used in an *in vitro* binding study using D-type cyclins produced in SF9 cells. Four GST-p130 fusion proteins were used, two of which (593-1139 and 593-862) were described in Chapter 4.3. Additionally, the 372C deletion mutant was fused in-frame with GST to produce GST-p130 (372C). This fusion protein (kindly constructed and provided by Yun Li) was made by ligating the 372C PCR product (Chapter 4.4) into the Bam HI site of pGex-1. Also included in the binding studies, a GST-p130 fusion protein containing amino acids 787-1139 was constructed by Yun Li by ligation of a blunted Sst I/Xba I fragment (nt 2440-3548) into the Sma I site of pGex 1.

To perform the *in vitro* binding studies, all GST-p130 fusion proteins were expressed and purified on glutathione-Sepharose beads, the beads washed extensively to remove unbound bacterial proteins and the GST-p130 fusion proteins allowed to remain bound to Sepharose. An aliquot of Sepharose-bound GST-p130 fusion protein was added to SF9 cell lysates containing ^{35}S-metabolically labeled D-type cyclins in the presence or absence of cdk4. ^{35}S-labeled cyclins bound to GST-p130 Sepharose were revealed following resolution of the complexes by SDS-PAGE.

The GST-p130 fusion proteins used in the *in vitro* binding assays are indicated in numbers at the top of Figure 4.8. Although initial experiments were completed with all three D-type cyclins, the interactions with GST-p130 (372C) were quite poor *in vitro*, and the best interaction was observed with cyclin D3. In addition, unlike the co-immunoprecipitation results in SF9 cells, GST-p130 (372C) did not appear to have less

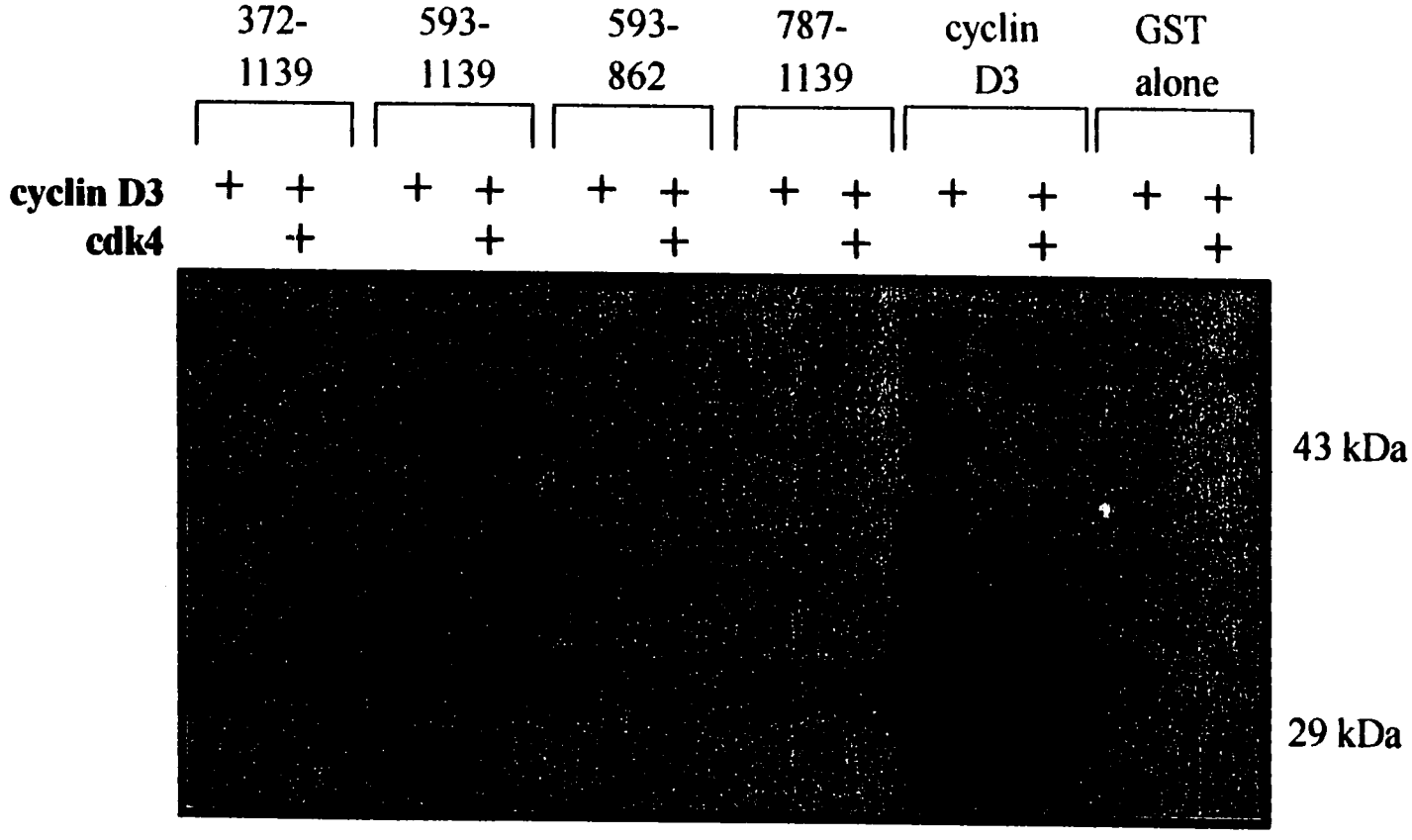

372-
1139
593-
1139
593-
862
787-
1139
cyclin
D3
GST
alone
cyclin D3
cdk4
43 kDa
29 kDa

Figure 4.8 Delineation of p130 sequences required to interact with cyclin D3

SF9 cells infected with cyclin D3 or cyclin D3 and cdk4 (as indicated at the top of the figure) were metabolically labeled with ^{35}S-methionine and lysed. Lysates were mixed with 2μg of the indicated GST-p130 fusion protein bound to glutathione-Sepharose (numbers represent p130 amino acids present in each fusion protein). The presence of ^{35}S-labeled cyclin D3 indicates an interaction with the indicated fusion protein. The last four lanes are controls, including an immunoprecipitation of cyclin D3 (using a cyclin D3 monoclonal antibody) from infected lysates and an *in vitro* binding assay using 2 μg GST alone as a negative control (last two lanes).

preference for cyclin D3 in the presence of cdk4 in the *in vitro* assay. This is not unexpected because the *in vitro* assay is not performed in the presence of ATP or magnesium. The GST-p130 fusion proteins used in each binding assay are listed at the very top of the figure and the SF9 cell lysates used (containing cyclin D3 or cyclin D3 and cdk4) are indicated just below. The last four lanes represent positive controls (immunoprecipitations with a cyclin D3-specific monoclonal antibody) and negative controls (GST protein alone).

The results indicate that GST-p130 (372C) and GST-p130 (593-1139) are both able to interact with cyclin D3 or cyclin D3/cdk4, suggesting that amino terminal p130 residues 1-592 are not required for this interaction. Fusion proteins containing p130 amino acids 593-862 and 787-1139 appeared to interact poorly with cyclin D3 and cyclin D3/cdk4. These results suggest that deletion of C-terminal sequences 863-1139 or deletion of amino terminal sequences up to amino acid 787 has a negative effect on p130 interactions with cyclin D3, although as noted, the *in vitro* binding assay was not as representative as the assays completed in the baculoviral system. At least two possibilities may account for these observations. Fusion of the 27 kDa GST protein to the indicated p130 sequences may have caused improper protein folding, resulting in a functionally altered p130. Additionally, post-translational modifications required for activity of p130 may not have occurred in the bacterial expression system, unlike those which occur in the insect cell system.

4.7. Conclusion

In vivo, p130 can be found stably associated with cyclin A/cdk2 and cyclin E/cdk2 complexes. As a component of these complexes, p130 serves as substrate of cdk2, and GST-p130 fusion proteins containing the spacer region of p130 are also substrates of cdk2 *in vitro*. Although interactions between p130 and D-type cyclins are difficult to detect *in vivo*, an amino-terminally truncated version of p130 (372C), when co-expressed with D-type cyclins in SF9 cells, does interact with D-type cyclins in the absence of cdk4. Cdk4-mediated phosphorylation of 372C appears to result in a disruption of the 372C/cyclin D interaction.

The region of p130 required to interact with D-type cyclins *in vitro* was determined to encompass amino acids 593-1139, although this was not narrowed to a more precise region. These results compare favorably with data which shows a similar region of pRb and p107 is required for interactions with cyclin D1 *in vitro* (Figure 1.2) (Ewen *et al.*, 1993b). It was also important to define the region of p130 which mediates interactions with cyclins A and E and this work is described in detail in the next chapter.

Taken together, this data suggests p130 interacts with most cyclin/cdk complexes thought to act prior to, during and following S phase. The function of these multiple interactions is not well understood, but possibilities may include regulatory events which occur during or following these interactions, including phosphorylation events which may positively or negatively regulate the function of p130.

Chapter 5. Identification of a p130 domain mediating interactions with cyclin A/cdk2 and cyclin E/cdk2 complexes

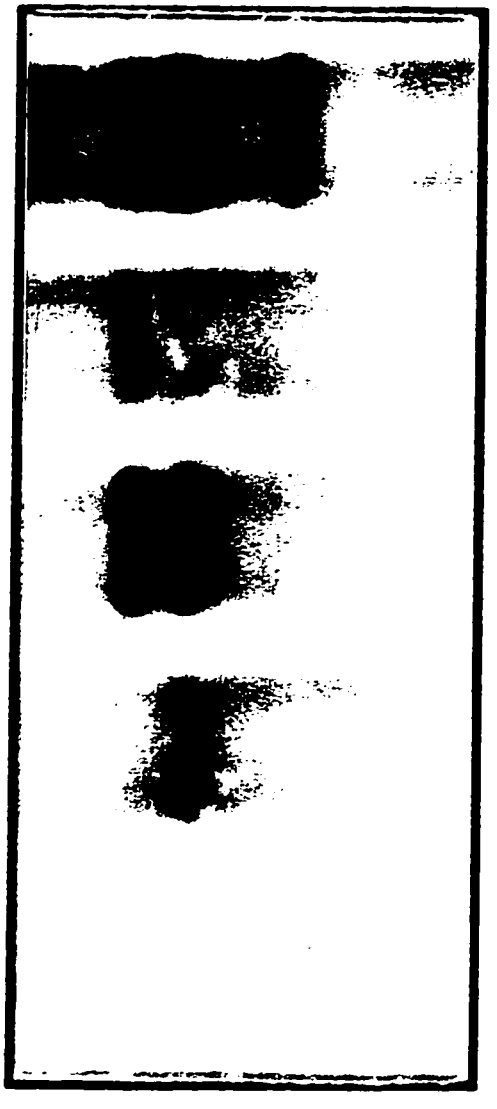
5.1 Introduction

Following the observation that p130 forms stable interactions with cyclin A and cyclin E *in vivo*, it was important to understand what functions were positively or negatively affected as a result of these interactions. The fact that these complexes remain in the presence of cdk2 suggests that phosphorylation of p130 does not affect its interactions with these cyclins. Additionally, p130 bound to cyclin/cdk complexes may be present in an underphosphorylated form. To better understand the stable association of cyclins A and E with p130, an analysis of the p130 domain required for interactions with these cyclin/cdk2 complexes was performed *in vitro* using GST-p130 fusion proteins and cyclin A/cdk2 and cyclin E/cdk2 complexes produced in SF9 cells. These components were combined in an *in vitro* binding assay exactly as described for the GST-p130 fusion proteins and cyclin D3 in Chapter 4.6. Much of this work is published as: **Lacy, S. and P. Whyte.** 1997. Identification of a p130 domain mediating interactions with cyclin A/cdk2 and cyclin E/cdk2 complexes. Oncogene **14**:2395-2406.

5.2 Results

5.2.i Cyclin A/cdk2 and cyclin E/cdk2 complexes interact with distinct forms of p130.

When cyclin A or cyclin E is immunoprecipitated under the appropriate conditions p130 co-precipitates with the cyclin complex (Fig 5.1; Hannon *et al.*, 1993; Li *et al.*, 1993). Several differentially migrating forms of p130 were immunoprecipitated with an antibody against the C-terminus of p130. The two faster migrating bands have been confirmed to be p130 using other antibodies to different regions of p130 (data not shown). The most slowly migrating form is of undetermined origin and may represent either an altered form of p130 or a cross reacting protein. Consistent with previous observations, p130 was detected in complexes immunoprecipitated with monoclonal antibodies to cyclins A and E (Fig 4.1.). In this experiment and those that follow, two previously undescribed monoclonal antibodies, 168 and 276 were used to immunoprecipitate cyclin E. Both of the faster migrating forms of p130 were present in immunoprecipitations using the cyclin E antibody 276; however, only the slower migrating form of p130 was present in immunoprecipitates using cyclin E antibody 168. The reason for this difference is not clear but binding of antibody 168 to cyclin E may be sterically incompatible with binding of one of the p130 species. Only the faster migrating form of p130 was detected in complexes immunoprecipitated with the antibody to cyclin A.



p130 (CT)

cyc A

cyc E (276)

cyc E (168)

RAM

Figure 5.1 Interaction of p130 with cyclins A and E *in vivo*.

Immunoprecipitates from C33A cells using an antipeptide antisera to the carboxyl terminus of p130 (p130 CT), monoclonal antibodies to cyclin A (C160) or cyclin E (168 and 276) and rabbit anti-mouse (RAM) serum as a negative control were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with C20, an anti-serum raised to a p130 peptide.

5.2.ii Interaction of the p130 spacer region with cyclins A and E *in vitro*.

To assay the ability of different regions of p130 to interact with cyclin/cdk complexes, an *in vitro* system using baculovirus produced cyclins and cdk2 was established. Baculoviruses expressing human cyclin A, cyclin E or cdk2 were used to produce these proteins in insect SF9 cells and complexes containing cyclin A and cdk2 or cyclin E and cdk2 were produced by co-infecting cells with the respective baculoviruses. Baculoviruses encoding cyclin A, cyclin E and cdk2 were generously provided by D. Morgan, UCSF (Desai *et al.*, 1992; Koff *et al.*, 1992). Proteins of the expected sizes were readily detectable following immunoprecipitation from ^{35}S-radiolabeled extracts of infected SF9 cells (Fig. 5.2, lanes 1-3 and 5-9) but not from extracts of uninfected cells (Fig. 5.2, lanes 4 and 10-12). Cyclin A and cyclin E migrate as doublets and both bands are recognized by the appropriate antibodies. Cyclin A/cdk2 and cyclin E/cdk2 complexes were detected in extracts from co-infected cells; however, the proportion of each of the proteins participating in the complex formation was somewhat lower than we expected. Nonetheless, these complexes proved suitable for studying interactions with p130, as described below.

To define the region(s) of p130 mediating the interactions with cyclin A and cyclin E, four plasmids encoding GST-p130 fusion proteins were constructed. Each p130 fusion protein is named according to which p130 amino acids are fused in frame with GST (Fig. 5.3). The four GST-p130 fusion proteins together represent the full length of p130.

A. **SF9 cells infected with :**

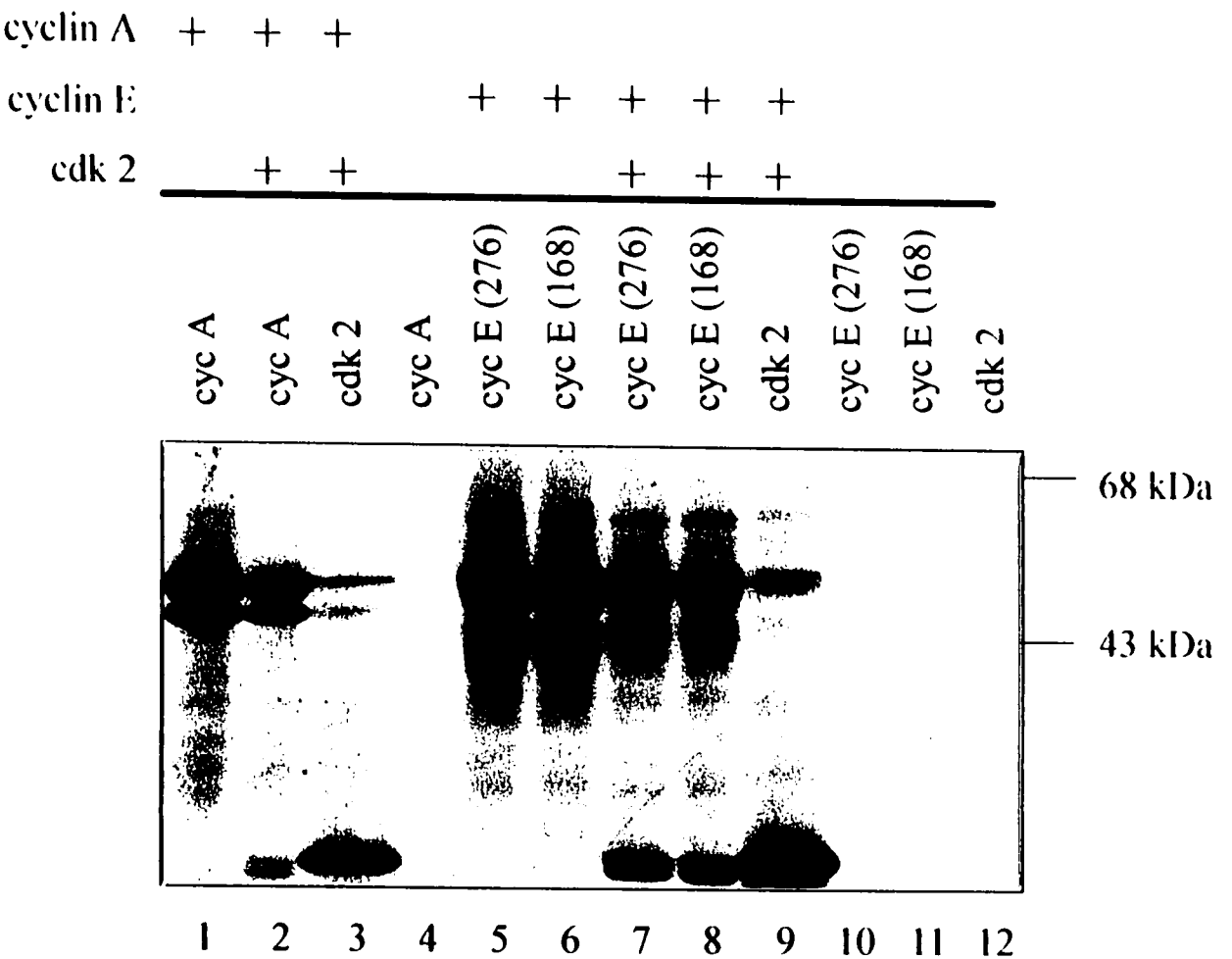


Figure 5.2 Expression of cyclin A, cyclin E and cdk2 in SF9 cells

SF9 cells were infected alone or in combination with baculoviruses expressing cyclin A, cyclin E, and cdk2 as indicated and the cells labeled with ^{35}S-methionine. Cyclin A, cyclin E and cdk2 were immunoprecipitated from cell lysates using monoclonal antibody C160 for cyclin A (lanes 1, 2 and 4), monoclonal antibodies 276 (lanes 5, 7 and 10) and 168 (lanes 6, 8 and 11) for cyclin E and an anti-peptide antibody to cdk2 (lanes 3, 9 and 12).

To determine which GST-p130 fusion proteins could mediate interactions with the SF9-produced cyclin/cdk2 complexes, an *in vitro* binding assay was performed. Lysates from ^{35}S-labeled SF9 cells that were infected with individual baculoviruses expressing cdk2, cyclin A, or cyclin E or from cells infected with combinations of baculoviruses were mixed with each GST-p130 fusion protein or GST alone. GST alone, GST-130 (1-320), (339-595) and (787-1139) each failed to interact with either cyclin A/cdk2 or cyclin E/cdk2 complexes (Fig 5.4. lanes 1-9). In contrast, GST-p130 (593-1139) interacted specifically with both cyclin A/cdk2 and cyclin E/cdk2 complexes (Fig. 5.4, lanes 11,13). This result suggested that p130 residues 593-1139 contain a cyclin binding domain. Although fusion proteins 593-1139 and 787-1139 both contain the same p130 carboxyl-terminal sequences, 593-1139 contains additional sequences which include the spacer region. These results imply that sequences within amino acids 593-786 are necessary for the interactions with cyclin/cdk2 complexes.

When lysates from co-infected cells were used, both the cyclin and cdk2 were present in the complexes formed with GST-130 (593-1139) (Fig. 5.4, lanes 11, 13). Interestingly, cyclin E was efficiently precipitated from lysates containing either cyclin E alone or cyclin E and cdk2 (Fig. 5.4, lanes 12, 13). In contrast, only a low level of cyclin A bound to GST-p130 (593-1139) when a lysate containing only cyclin A was used. Substantially more cyclin A was bound when the cyclin A/cdk2 co-infected lysate was used (Fig.5.4, compare lanes 10 and 11). This raised the question of whether cyclins complexed to cdk2 interacted more efficiently than uncomplexed cyclins.

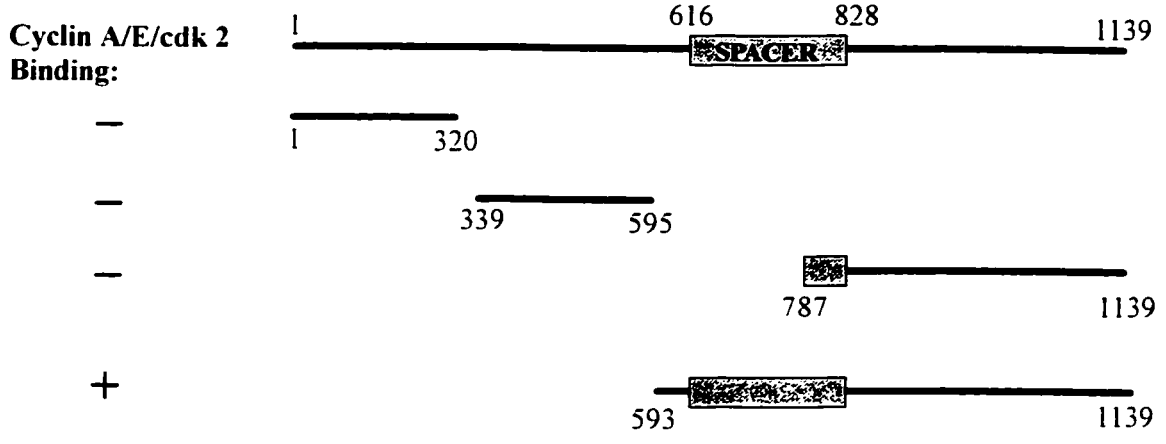

Cyclin A/E/cdk 2
Binding:
1
616
828
1139
SPACER
–
1
320
–
339
595
–
787
1139
+
593
1139

Figure 5.3 GST-p130 fusion proteins

A schematic diagram of p130 amino acid sequence is represented on the top (where numbers indicate amino acids) and the regions expressed as GST fusion proteins are shown.

C. **SF9 cells infected with:**

cyclin A	+		+		+		+	+			+		+				
cyclin E		+		+		+			+	+		+		+			
cdk 2	+	+	+	+	+	+	+	+	+		+		+	+	+	+	+	+

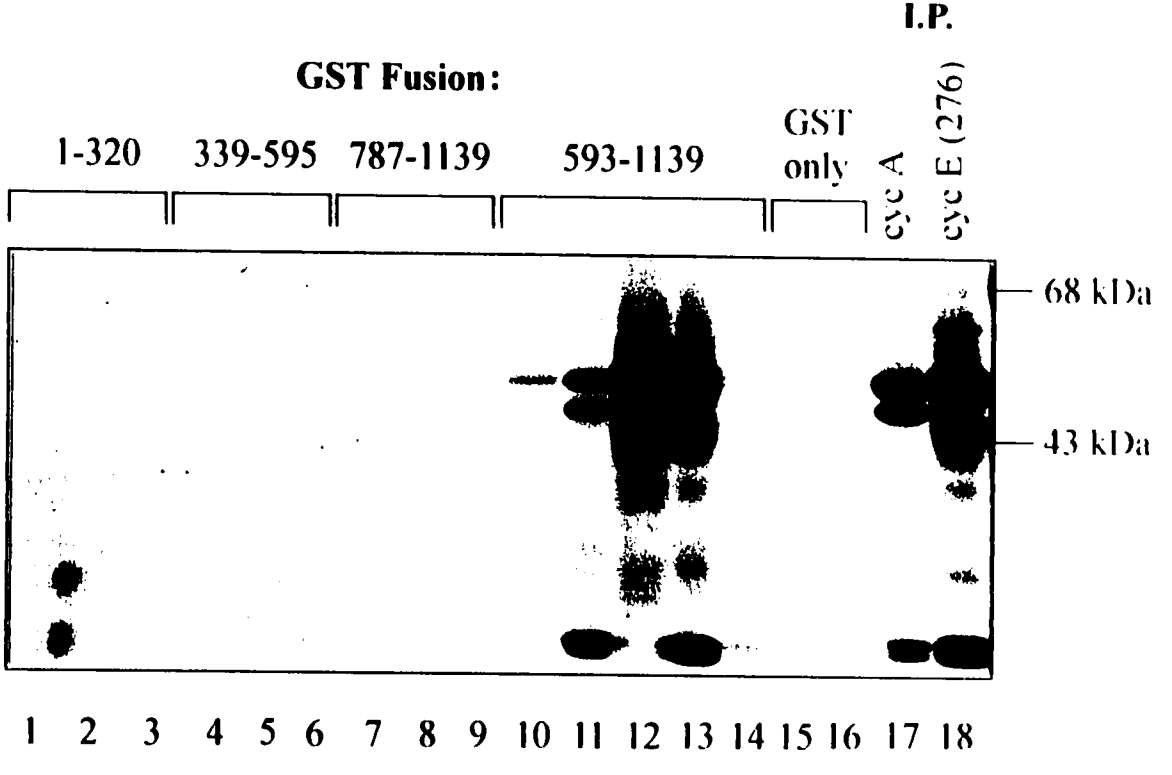


Figure 5.4 *In vitro* interactions of baculovirus cyclin A, cyclin E and cdk2 with GST-p130 fusion proteins

In vitro association of GST-p130 fusion proteins with cyclins and cyclin/cdk2 complexes was assayed using ^{35}S-labeled SF9 cell lysates containing cyclin A, cyclin A/cdk2, cyclin E or cyclin E/cdk2. Lysates were mixed with each of the four Sepharose-bound GST-p130 fusion proteins as indicated (lanes 1-14) and GST alone (lanes 15 and 16). Immunoprecipitations using antibodies to cyclin A and cyclin E were included as controls (lanes 17 and 18). Complexes bound to the glutathione Sepharose beads were resolved by SDS-PAGE and detected by autoradiography (lanes 17 and 18).

5.2.iii. Cyclin A, but not cyclin E, requires cdk2 to bind GST-p130.

A series of titration experiments were conducted to determine if the interactions between cyclin A or cyclin E and GST-p130 (593-1139) were dependent upon the presence of cdk2. Constant amounts of GST alone, GST-p130 (593-1139) or anti-cdk2 antisera were mixed with increasing amounts of cdk2-containing SF9 lysate. In the absence of a cyclin, cdk2 did not interact detectably with either GST alone or the GST-p130 (593-1139) fusion protein indicating that this region of p130 does not detectably interact with cdk2 (Fig 5.5 lanes 1-12). A similar experiment conducted with increasing amounts of cyclin E-containing SF9 lysate indicated that cyclin E binds the fusion protein in the absence of cdk2 (Fig 5.6 lanes 7-12). Under the conditions of this experiment, the interaction between the GST-p130 fusion protein and cyclin E was linearly dependent on the amount of cyclin E added and the GST-130 fusion protein bound cyclin E as efficiently as the 276 monoclonal antibody. When cdk2 was present with cyclin E in the lysate it neither enhanced nor inhibited the interaction with the GST-p130 fusion protein (data not shown). These results suggest that cyclin E can bind efficiently to amino acids 593-1139 of p130 in the absence or presence of cdk2.

In contrast to the results obtained for cyclin E, GST-p130 (593-1139) did not efficiently interact with cyclin A even when cyclin A was present in high quantities (Fig 5.7 lanes 7-12). To investigate whether the cyclin A/p130 interaction was dependent on the presence of cdk2, increasing amounts of cdk2-containing lysate were mixed with a constant amount of cyclin A-containing lysate and incubated with GST-p130 (593-1139).

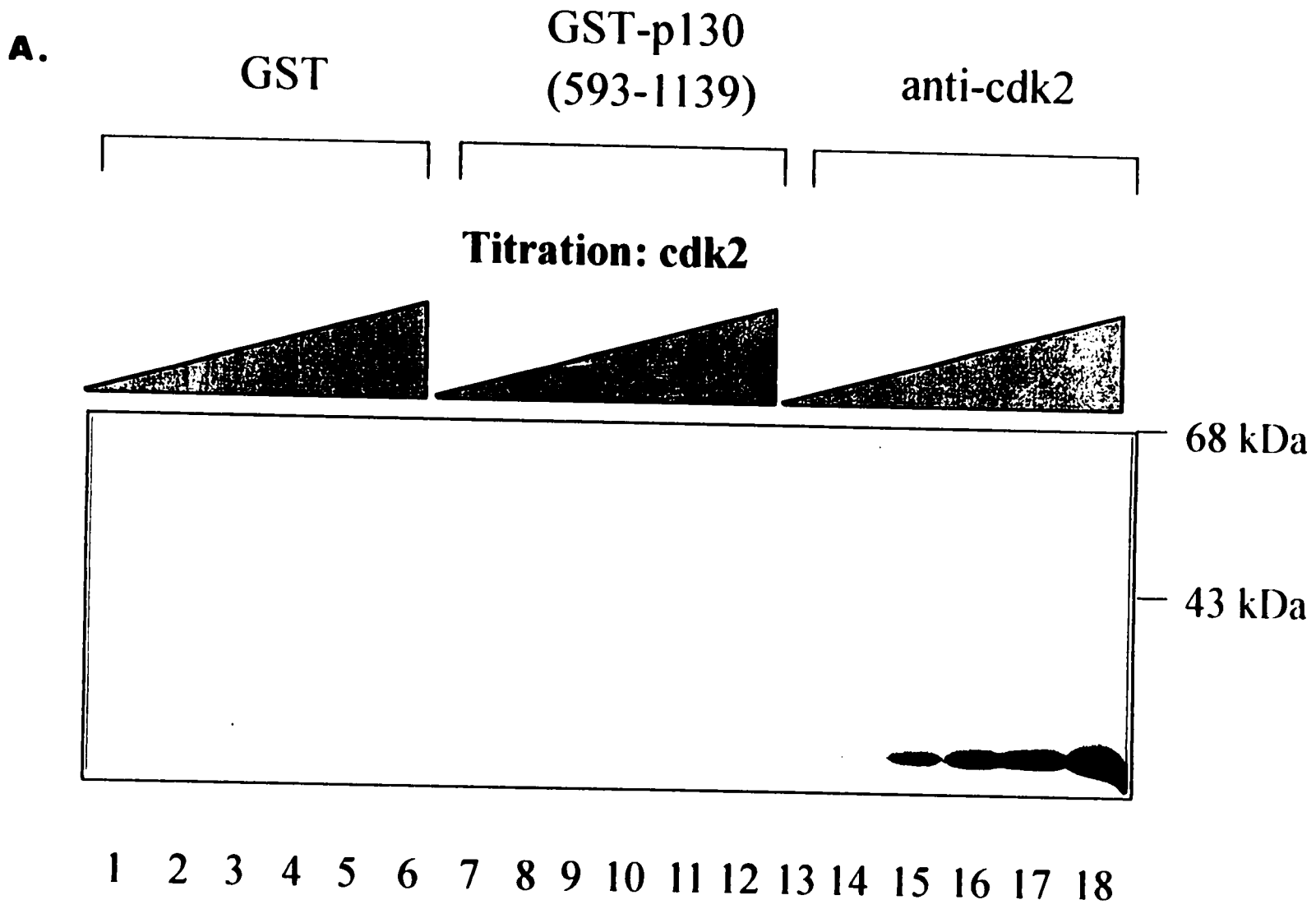

A.
GST
GST-p130
(593-1139)
anti-cdk2
Titration: cdk2
68 kDa
43 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.5 Cdk2 binding studies using a GST-p130 fusion protein

1 μg of GST-p130 (593-1139) or 10 μg of GST alone was incubated with 0, 0.5, 1.5, 3.5, 7 and 15 μg of cdk2-containing SF9 cell lysate (lanes 1-12). Immunoprecipitations of equivalent amounts of cdk2-containing lysate are included as controls (lanes 13-18).

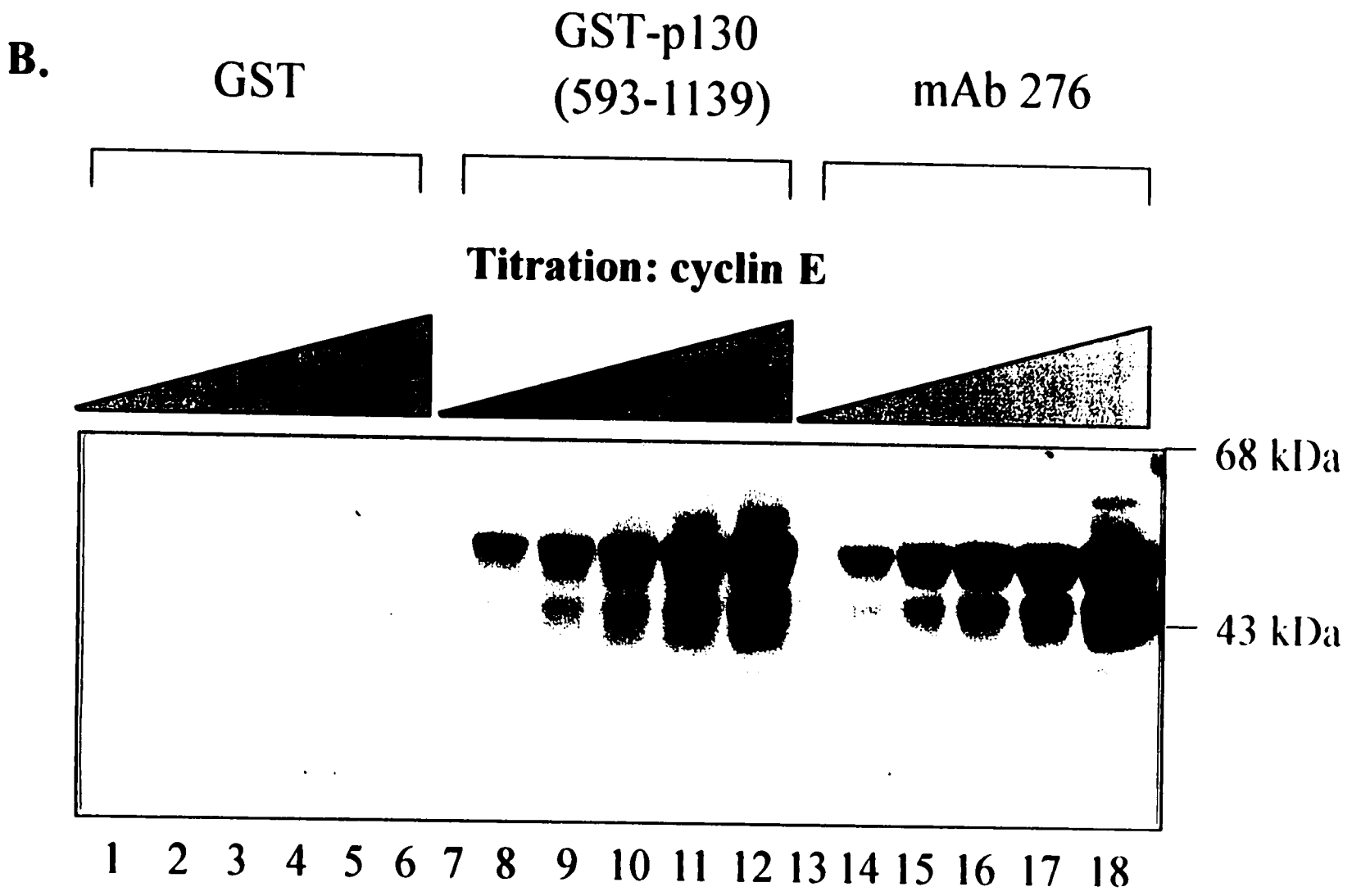

B.
GST
GST-p130
(593-1139)
mAb 276
Titration: cyclin E
68 kDa
43 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.6 Cyclin E binding studies with a GST-p130 fusion protein

Titrations of cyclin E binding to GST-p130 (593-1139) were performed as described in Figure 5.5 except with 0, 1.2, 2.5, 5, 10 and 20 μg of cyclin E-containing lysates added to GST or GST-p130 (593-1139) (lanes 1-12). Immunoprecipitations of equivalent amounts of cyclin E-containing lysate using monoclonal antibody 276 are included as controls (lanes 13-18).

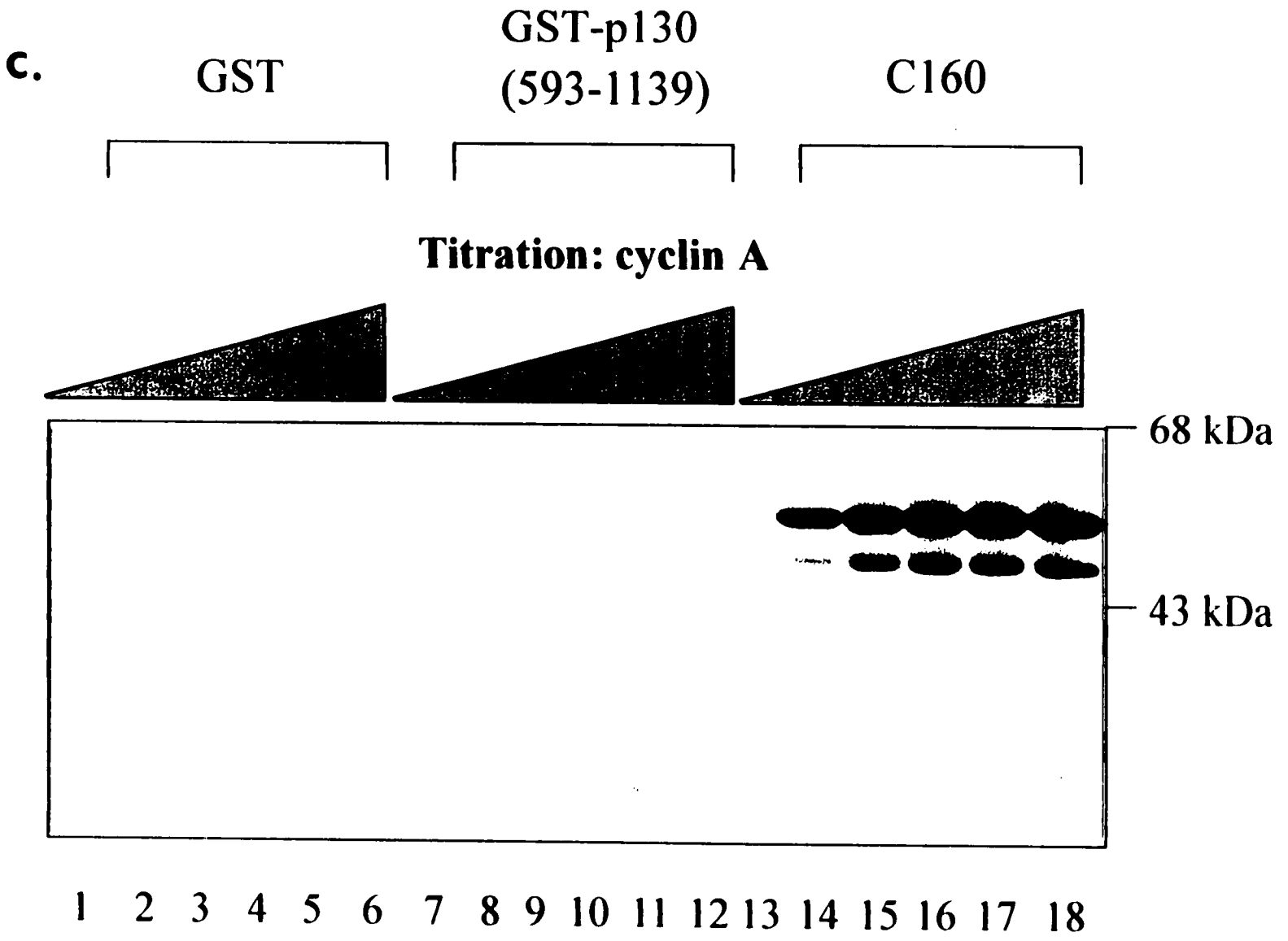

c.
GST
GST-p130
(593-1139)
C160
Titration: cyclin A
68 kDa
43 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.7 Cyclin A binding studies with a GST-p130 fusion protein

A titration for cyclin A binding was performed exactly as in Figure 5.5 except with 0, 3, 10, 25, 60 and 140 μg of cyclin A-containing lysate incubated with GST or GST-p130 (593-1139). Immunoprecipitations using monoclonal antibody C160 are shown in the lanes 13-18.

D.

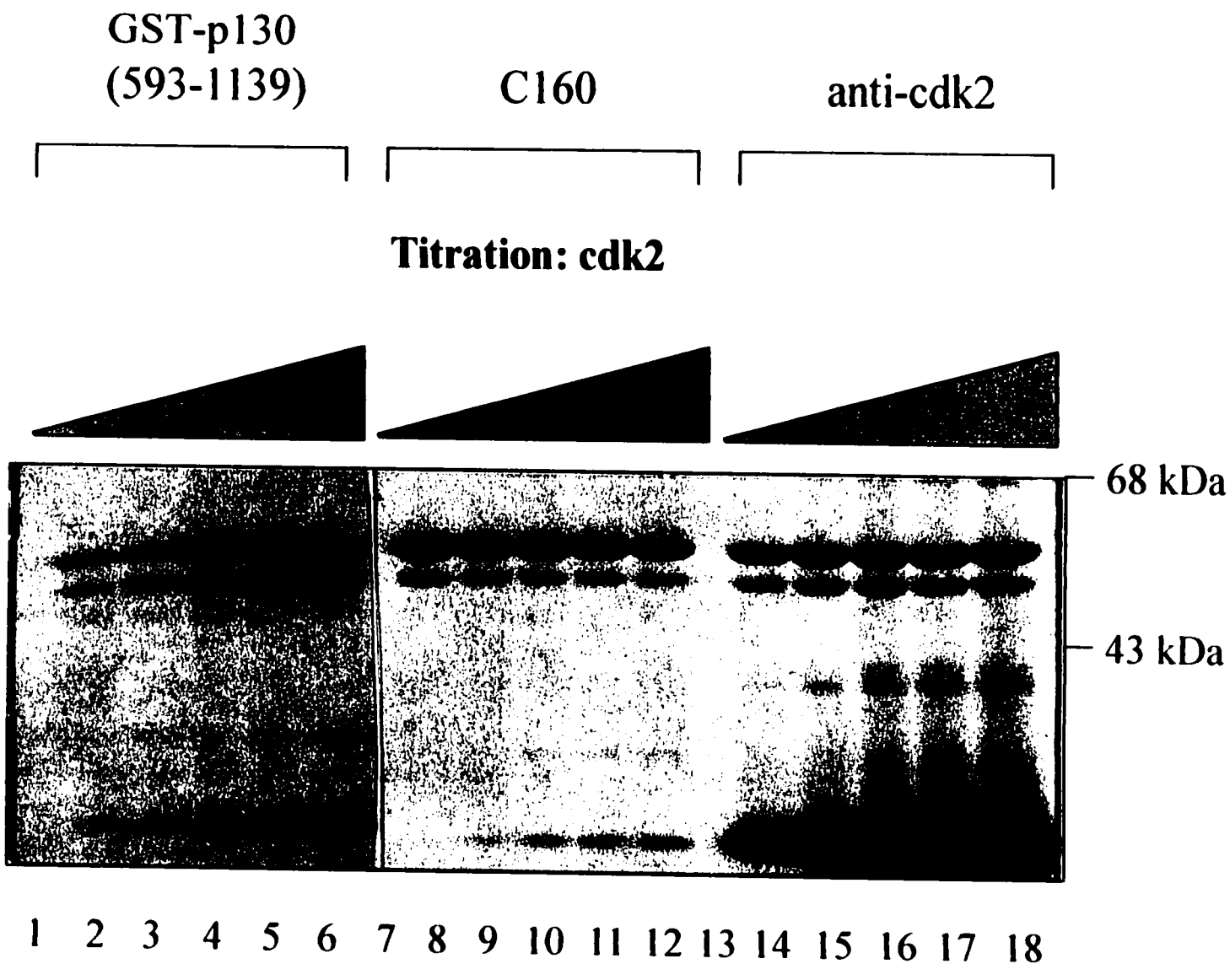

GST-p130
(593-1139)
C160
anti-cdk2
Titration: cdk2
68 kDa
43 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.8 Role of cdk2 in cyclin A binding to a GST-p130 fusion protein

100 μg of cyclin A-containing SF9 cell lysate plus 1 μg of GST-p130 (593-1139) were mixed with 0, 3, 9, 20, 30 and 75 μg of cdk2-containing lysate (lanes 1-6). Immunoprecipitations of equivalent amounts of cyclin A and cdk2-containing lysates are shown in lanes 7-18.

The addition of cdk2-infected SF9 lysate greatly enhanced the efficiency of complex formation between the p130 fusion protein and cyclin A (Fig 5.8). As levels of cdk2 increased, so did binding of cyclin A to the p130 fusion protein (Fig 5.8 lanes 1-6). Levels of cyclin A and cdk2 present in lysates were monitored in parallel by immunoprecipitations with anti-cyclin A and anti-cdk2 antibodies (Fig 5.8 lanes 7-18). This data demonstrates that in contrast to cyclin E, cyclin A can efficiently interact with GST-p130 (593-1139) only when complexed with its kinase partner, cdk2. In this and other experiments, we observed a slight increase in levels of cdk2 complexed to cyclin A and cyclin E in the *in vitro* binding assay using GST-p130 (593-1139) as compared to the interactions detected by co-immunoprecipitation. One possibility is that GST-p130 (593-697) stabilizes cyclin/cdk2 complexes.

To ensure that cyclin E was not interacting with GST-p130 (593-1139) due to the presence of endogenous insect cdk's, $p13^{suc\ 1}$ beads were used to pre-clear cdk-type molecules from the cyclin E-infected SF9 cell lysate. $p13^{suc\ 1}$ is a subunit of *S. pombe* $p34^{cdc2}$ and binds cdc2 and cdk2 with high affinity (Brizuela *et al.*, 1987). Preclearing cyclin E-infected SF9 cells by incubation with $p13^{suc1}$ beads did not reduce the interaction of cyclin E with GST-p130 (593-1139) (data not shown). This suggests that insect cell cdk's do not significantly contribute to the GST-p130/cyclin E interaction.

5.2.iv. Identification of p130 sequences required for cyclin interactions.

The above data suggests that p130 contains a cyclin binding region within amino acids 593-1139 and that amino acids 593-786 are required for this interaction (Fig. 5.4).

To further delineate which residues of p130 mediate the interaction between p130 and cyclins A and E, plasmids encoding five additional GST-p130 fusion proteins were constructed. These fusion proteins encoded p130 amino acids 593-790, 593-698, 635-698, 652-698 and 593-675 (Fig 5.9). Each p130 fusion protein was mixed with ^{35}S-labeled SF9 cell lysates containing cyclin A/cdk2 or cyclin E/cdk2 complexes and assayed for its ability to bind to the cyclin/cdk2 complexes. All of the fusion proteins except GST-p130 (593-675) were capable of interacting with both cyclin A/cdk2 and cyclin E/cdk2 (Fig. 5.10). The smallest GST-p130 fusion protein capable of interacting with cyclin A/cdk2 or cyclin E/cdk2 complexes contained p130 amino acids 652-698, indicating that this region of p130 is sufficient for interacting with either of the cyclin complexes. A fusion protein containing p130 amino acids 593-675 failed to interact with either cyclin/cdk2 complex further suggesting that residues 652-675 are not sufficient for cyclin binding and that residues 676-698 are critical for the interactions. As observed previously, all GST-p130 fusion proteins which retained the ability to interact with cyclin/cdk2 complexes efficiently interacted with cyclin E (but not cyclin A) in the absence of cdk2 (data not shown).

Alignment of p130 amino acids 652-697 with the homologous region of p107 (amino acids 576 to 787) revealed two clusters of conserved amino acids (Fig 5.11). P130 residues 669-673 (RYSSP) and residues 679-685 (RRRLFVE) are relatively well-conserved within p107 sequences raising the possibility that these regions might be important for the interactions with the cyclin complexes. To address this possibility, the

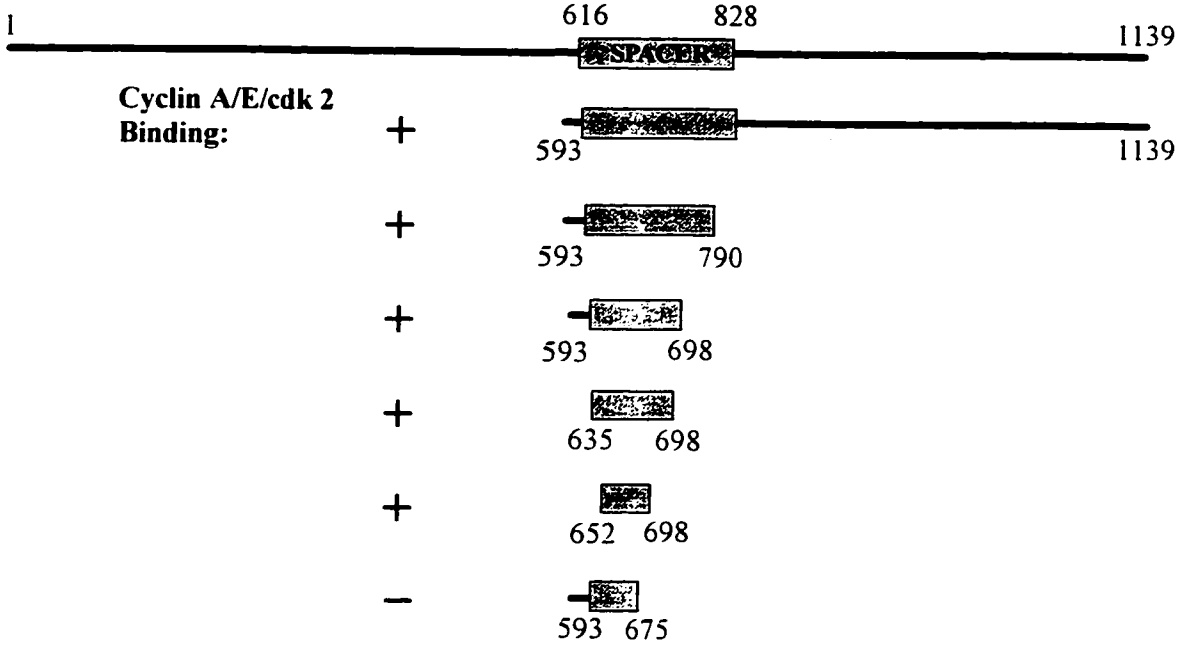

1
616
828
1139
SPACER
Cyclin A/E/cdk 2
Binding:
+
593
1139
+
593
790
+
593
698
+
635
698
+
652
698
−
593
675

Figure 5.9 Additional GST-p130 fusion proteins

Schematic representation of regions of p130 present in additional GST fusion proteins.

B.

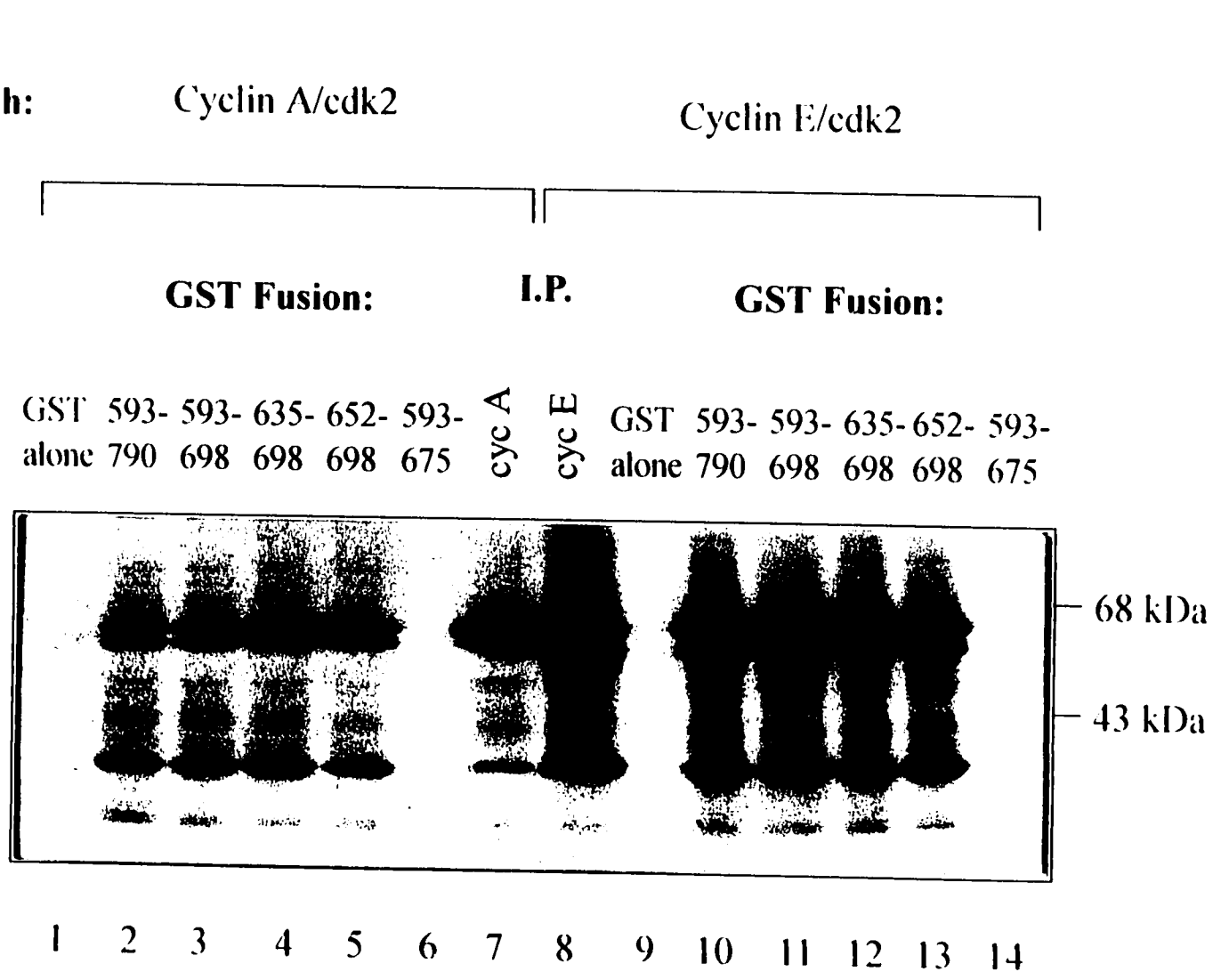

SF9 cells
infected with:
Cyclin A/cdk2
Cyclin E/cdk2
GST Fusion:
I.P.
GST Fusion:
GST alone
593-790
593-698
635-698
652-698
593-675
cyc A
cyc E
GST alone
593-790
593-698
635-698
652-698
593-675
68 kDa
43 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14

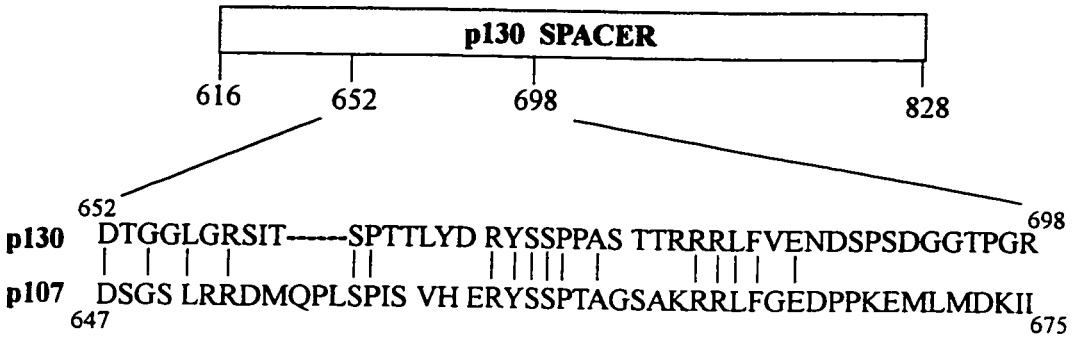
Figure 5.10 Delineation of a minimal cyclin binding region using GST-p130 fusion proteins

In vitro binding assays of GST-130 fusion proteins to cyclin/cdk2 complexes were used to determine the cyclin binding region of p130. ^{35}S-methionine labeled SF9 cells infected with the indicated baculoviruses were lysed and mixed with the indicated GST-p130 fusion protein (lanes 2-6, 10-14) or GST alone (lanes 1 and 9). Complexes were analyzed as described in Figure 5.4 and compared to immunoprecipitations of cyclin A and cyclin E from infected SF9 cells with antibodies C160 and 276, respectively (lanes 7 and 8).

p130 nucleotides encoding the residues RYSSP and RRL were deleted from the p130 sequence using M13 oligonucleotide-directed mutagenesis. Three additional GST-p130 (593-698) fusion proteins were constructed, containing deletions of amino acids 669-673 (ΔRYSSP), amino acids 680-682 (ΔRRL) or both deletions (ΔRYSSP/RRL). The three fusion proteins were then tested for binding to cyclin A/cdk2 and cyclin E/cdk2 complexes (Fig 5.12). The ΔRYSSP mutant retained the ability to bind both cyclin A and E complexes in a manner equivalent to the non-mutated GST-p130 (593-698) (Fig. 5.12 lanes 4-5), demonstrating that this conserved region is not critical for the interactions with cyclin/cdk2 complexes. In contrast, the ΔRRL and ΔRYSSP/RRL mutants each failed to form complexes with cyclin A/cdk2 and cyclin E/cdk2 complexes (lanes 1-3 and 7-9). These observations suggest that the conserved region centered around the RRL residues plays a major role in the interactions with cyclin A/cdk2 and cyclin E/cdk2.

5.2.v. P130 amino acids interacting with cyclins are conserved in other cyclin-binding proteins.

Conservation in p130 and p107 of the small region which apparently mediates the p130/cyclin interactions raised the possibility that these sequences might be present in other proteins known to interact with cyclin/cdk complexes. An examination of the sequences of various other cyclin/cdk interacting proteins revealed sequences similar the p130 RRRLFVE motif in several other proteins including E2F-1,2 and 3, $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$ (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992; El-Deiry *et al.*, 1993;



GST-p130 (593-698) deletion mutants: △ **RYSSP** (669-673) △ **RRL** (680-682)

p130	PASTTRRRLFVE
p107	TAGSAKRRLFGE
E2F-1	GRPPVKRRLDLE
E2F-2	GRLPAKRKLDLE
E2F-3	GGPPAKRRLELG
p21	CGSKACRRLFGP
p27	PKPSACRNLFGP
p57	VRTSACRSLFGP

Figure 5.11 Conservation of the p130 cyclin binding region in p107 and other cyclin binding proteins

Amino acid sequence alignment of p130 amino acids 652-698 with the homologous region of p107 (amino acids 647-675). The p130 amino acids which are deleted in the ΔRYSSP and ΔRRL mutations are shown directly below the alignment. In the lower part of the diagram, a comparison of similar putative cyclin binding regions from p130, p107, E2F-1, -2, -3, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. Amino acids present within each of these regions are as follows: p130 (674-685), p107 (652-663), E2F-1 (84 to 95), E2F-2 (81 to 92), E2F-3 (128 to 140), $p21^{cip1}$ (13-24); $p27^{kip1}$ (24-35), $p57^{kip2}$ (25-36).

B. **SF9 cells infected with:**

cyclin A	+		+		+		+		+							
cyclin E		+		+		+								+		+
cdk 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

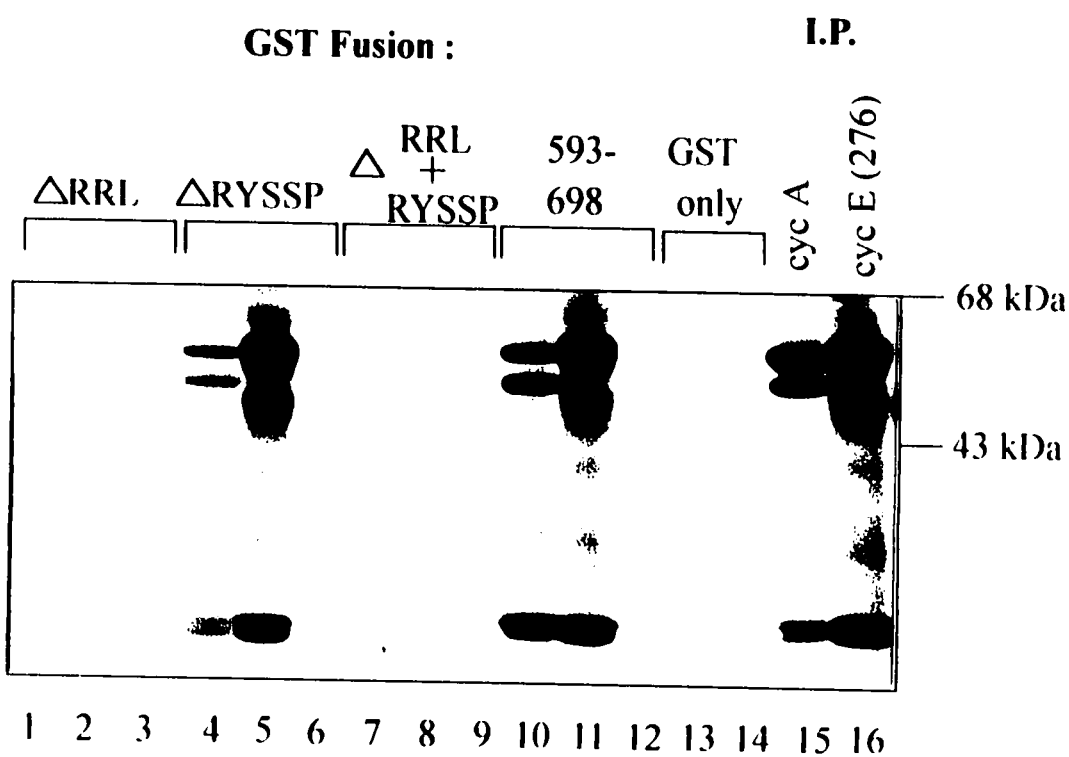


Figure 5.12 *In vitro* binding studies using GST-p130 deletion mutants

The effect that loss of p130 amino acids 669-673, 680-682 has on cyclin binding *in vitro* was examined by incubating GST-p130 (593-675), ΔRYSSP, ΔRRL and ΔRYSSP/RRL with SF9 cell lysates containing cyclin A/cdk2 or cyclin E/cdk2 complexes exactly as in Figure 5.4.

Gu *et al.*, 1993; Harper *et al.*, 1993; Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993; Xiong *et al.*, 1993a; Polyak *et al.*, 1994a; Toyoshima and Hunter, 1994; Lee *et al.*, 1995; Matsuoka *et al.*, 1995) (Fig. 5.11). As discussed below, each of these proteins has been shown to be present in complexes containing cyclin A or E. In some cases, this particular region has been implicated in the cyclin interactions.

5.2.vi GST-p130 (593-698) ΔRRL is a poor substrate of cyclin/cdk2 complexes.

To understand the consequences of the ΔRRL mutation on phosphorylation by cyclin/cdk2 complexes, *in vitro* kinase assays were performed. GST-p130 (593-698) and the ΔRRL, ΔRYSSP, ΔRRL/ΔRYSSP derivatives were incubated in the presence of uninfected-, cyclin A/cdk2- or cyclin E/cdk2- infected SF9 cell lysates and gamma ^{32}P-ATP. Phosphorylated proteins were then analyzed by SDS-PAGE. None of the fusion proteins were detectably phosphorylated by uninfected SF9 cell lysates (Fig. 5.13 lanes 1-5). GST-p130 (593-698) and the ΔRYSSP mutant were efficiently phosphorylated by cyclin A/cdk2 and cyclin E/cdk2 complexes produced in infected SF9 cells (Fig. 5.13 lanes 7, 9, 12 and 14). In contrast, GST fusion proteins containing the ΔRRL deletion alone or in combination with the ΔRYSSP mutation were very poor substrates for both cyclin/cdk2 complexes (lanes 8, 10, 13 and 15). The data from the kinase assays could be explained by at least two possibilities: 1) p130 fusion proteins lacking RRL were poor cdk2 substrates because a stable interaction with cyclin/cdk2 complexes did not occur or 2) deletion of RRL resulted in alteration of a cdk2 phosphorylation site. To determine whether removal of RRL resulted in alteration of a cdk2 phosphorylation site, cyclin

A.

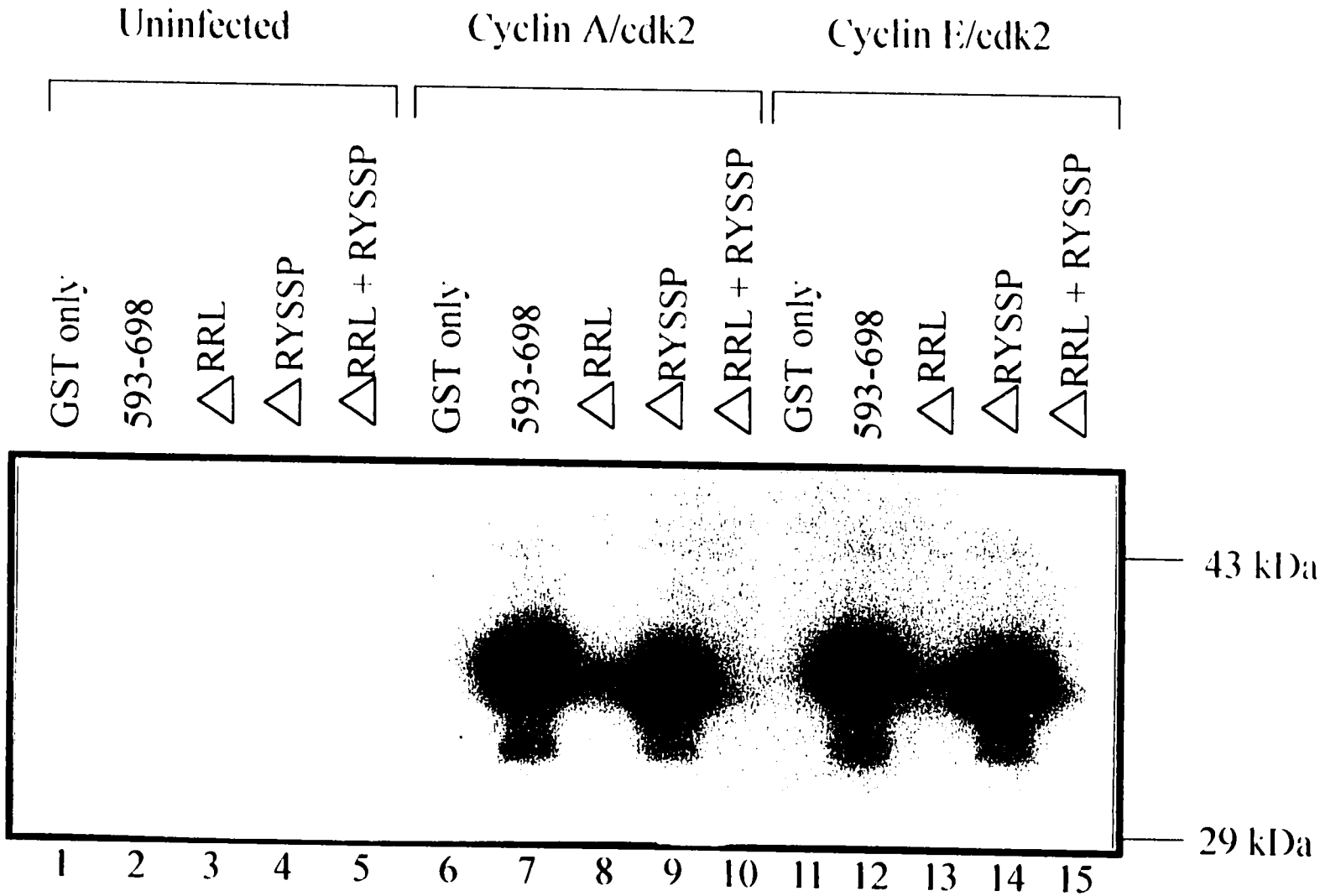

Uninfected
Cyclin A/cdk2
Cyclin E/cdk2
GST only
593-698
△RRL
△RYSSP
△RRL + RYSSP
GST only
593-698
△RRL
△RYSSP
△RRL + RYSSP
GST only
593-698
△RRL
△RYSSP
△RRL + RYSSP
43 kDa
29 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 5.13 Phosphorylation of p130 fusion proteins by cyclin/cdk2 complexes

Lysates from uninfected SF9 cells (lanes 1-5) and cells co-infected with cyclin A/cdk2 (lanes 6-10) and cyclin E/cdk2 (lanes 11-15) were used to phosphorylate GST-p130 (593-698) and the three deletion mutants derived from it (ΔRRL, ΔRYSSP and ΔRYSSP/RRL), as described in Materials and Methods section 2.13.

B.

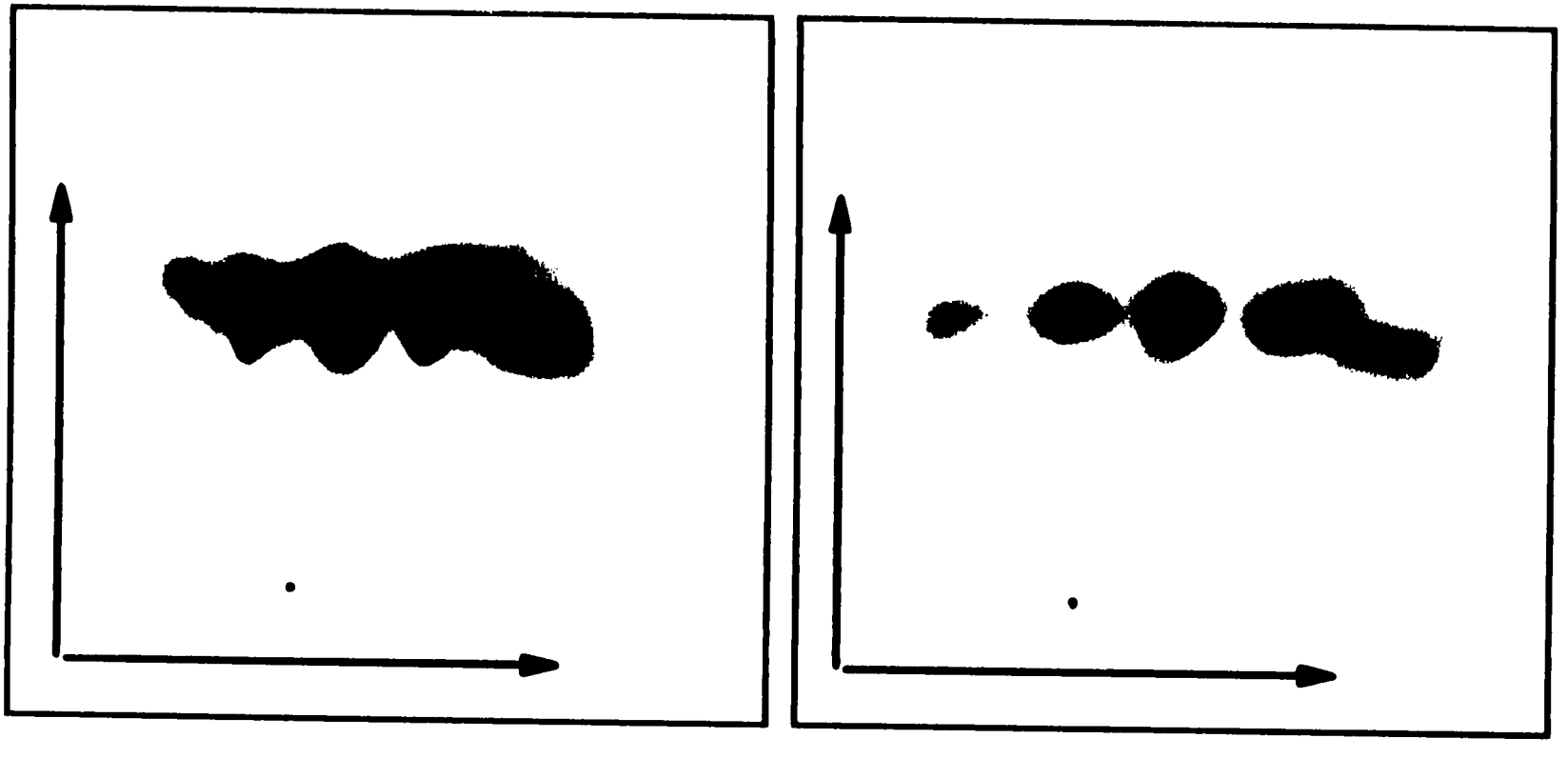


Figure 5.14 Phosphopeptide analysis of GST-p130 fusion proteins

Protein bands corresponding to cyclin A/cdk2-phosphorylated GST-p130 (593-698) and the ΔRRL mutant were excised and subjected to phosphopeptide analysis. Phosphopeptide maps of GST-p130 (593-698) (left) and the ΔRRL mutant (right) following electrophoresis (horizontal) and ascending chromatography (vertical) are shown. The origin is represented as a spot at the bottom left-hand corner of each figure.

A/cdk2-phosphorylated GST-p130 (593-698) and the ΔRRL mutant were excised from the gel and subjected to phosphopeptide analysis. Each fusion protein was digested with TPCK-trypsin followed by separation of the peptide fragments by electrophoresis in the first dimension and chromatography in the second dimension (Fig. 5.14). The analysis revealed the presence of five major phosphopeptides which were conserved between GST-p130 (593-698) and the ΔRRL mutant. This data suggested that deletion of RRL did not result in the destruction of a cdk2 consensus site. We conclude that poor phosphorylation of ΔRRL mutants resulted from a failure of the mutants to form stable interactions with cyclin/cdk2 complexes. In the absence of this stable interaction, cyclins A and E were unable to efficiently target cdk2 to the GST-p130 substrate.

5.4.vii. P130 Δ620-697 is unable to bind cyclins *in vivo*.

To determine if p130 residues surrounding the RRL motif mediate cyclin A/cdk2 and cyclin E/cdk2 binding *in vivo*, a p130 mutant was constructed containing a deletion of amino acids 620-697. The p130 mutant, p130 (Δ620-697), was expressed under the control of the human CMV early enhancer/promoter. The ability of this p130 mutant to interact with cyclins A and E was tested by transfecting the plasmid into C33A cells and immunoprecipitating with antibodies to p130, cyclin A and cyclin E. P130 was immunoprecipitated from cells transfected with pCMV-Bam-Neo (Fig. 5.15, lanes 7-9) or with the same vector expressing a full length p130 cDNA (pCMV-130) (lanes 4-6). P130 (Δ620-697) was immunoprecipitated, in addition to the endogenous p130, from

mutant p130
wt p130
1 2 3 4 5 6 7 8 9
cyc A
cyc E (276)
p130 (CT)
pCMV -130 Δ620-697
cyc A
cyc E (276)
p130 (CT)
pCMV-130
cyc A
cyc E (276)
p130 (CT)
pCMV-Bam-Neo
110 kDa
170 kDa

Figure 5.15 P130 (Δ620-697) is unable to interact with cyclins A or E *in vivo*.

C33A cells were transiently transfected with 10 ug of pCMV-Bam-Neo, pCMV-130 and pCMV-130 (Δ620-697), the cells lysed and immunoprecipitated with p130 C-terminal antibody (p130 CT), cyclin E antibody 276 or cyclin A antibody C160. Rabbit anti-mouse (RAM) was used as a negative control. Samples were resolved by SDS-PAGE and transferred to nitrocellulose. The immunoblot was probed with C20, a p130 C-terminal antibody.

transfected C33A cells using a p130 C-terminal antibody. The p130 (Δ620-697) mutant protein migrated as a 120 kDa band (lane 3) and was easily distinguished from the endogenous p130. Levels of expression of transfected p130 and p130 (Δ620-697) were comparable (Fig. 5.15, lanes 3, 6). In contrast to the normal p130, the Δ620-697 mutant did not co-immunoprecipitate with either cyclins A or E (lanes 1 and 2). This indicates that the region of p130 required for cyclin A/cdk2 and cyclin E/cdk2 interactions *in vitro* is also necessary for similar interactions *in vivo*.

5.2. viii. P130 (Δ620-697) is comparable to wildtype p130 in suppressing cell growth.

When overexpressed, p130 can suppress the growth of certain cell lines (Claudio *et al.*, 1994; G. Culp and P. Whyte, unpublished). To determine if binding to cyclin A and cyclin E complexes is critical to its growth suppressing capacity, p130 and the Δ620-697 mutant were assayed for growth suppression in C33A and SAOS-2 cells. p130, p130 (Δ620-697), p107 and pRb along with a neomycin resistance marker (neo^r) were transfected into cells and G418-resistant colonies were selected over a three week period. pCMV-Rb and pCMV-107 have been described previously (Qin *et al.*, 1992; Zhu *et al.*, 1993). pCMV-130 contains the complete p130 cDNA cloned into the Bam HI site of pCMV-Bam-Neo (Baker *et al.*, 1990) and was provided by G. Culp. The number of colonies observed for cells transfected with each of the plasmids is expressed as a percentage of neo^r (Fig 5.16). PRb and p107 each reduced colony formation to 8-10% in SAOS-2 cells and to 2-3% in C33A cells as compared to neo^r alone. On average,

wildtype p130 suppressed colony formation to 43% in SAOS-2 cells and to 25% in C33A cells. In comparison with wildtype p130, p130 (Δ620-697) suppressed growth to 9% in SAOS-2 cells and to 18% in C33A cells. Although it is not clear why the p130 mutant appears to suppress growth more effectively in SAOS-2 cells than wildtype p130, the data suggests that a p130 mutant deleted in a region mediating cyclin binding *in vivo* retains its capacity to negatively regulate growth and may, in fact, be more potent than p130 in suppressing growth of these cells.

5.2.ix p130 (Δ620-697) interacts with E2F-4

The CMV-p130 (Δ620-697) mutant described in this publication is a mutant version of p130 unable to interact with cyclin A/cdk2 and cyclin E/cdk2 complexes. To determine if this mutant is altered in its ability to interact with other cellular proteins known to bind to wildtype p130, the mutant was tested for its ability to complex with E2F-4. For this analysis, CMV-p130 and CMV-p130 (Δ620-697) plasmids were transiently co-transfected into C33A cells along with a CMV-E2F4-HA expression plasmid (kindly provided by K. Helin, University of Milano). The E2F-4 protein produced contains an influenza hemagglutinin (HA) epitope tag fused in frame with E2F-4 at the amino terminus. As a result of the HA tag, transfected E2F-4 can be immunoprecipitated with an anti-hemagglutinin monoclonal antibody called 12CA5 (Niman *et al.*, 1983), kindly provided by J. Hassell (McMaster University).

Following transient transfections, C33A cells were immunoprecipitated with a

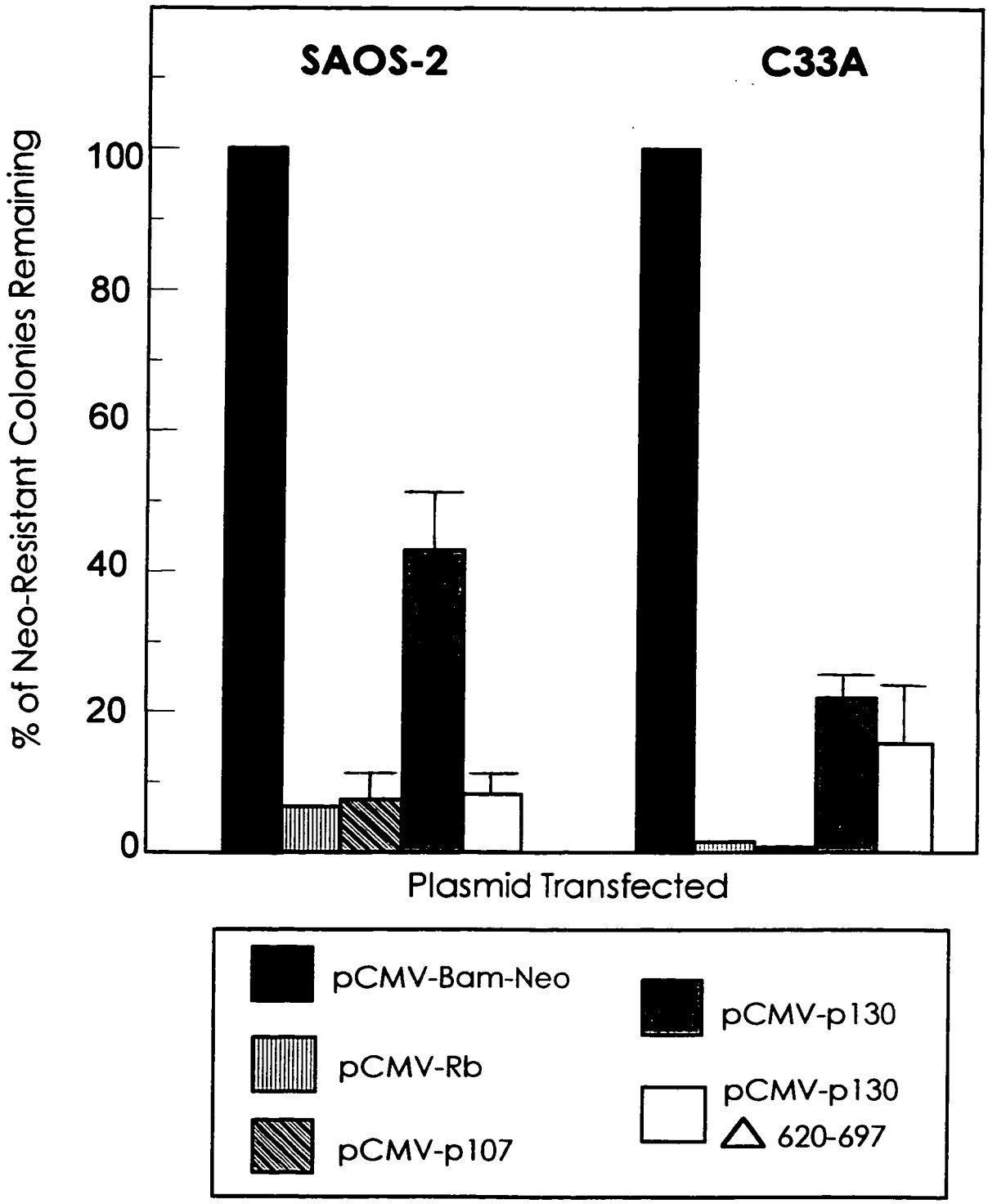

SAOS-2
C33A
% of Neo-Resistant Colonies Remaining
100
80
60
40
20
0
Plasmid Transfected
pCMV-Bam-Neo
pCMV-Rb
pCMV-p107
pCMV-p130
pCMV-p130
△ 620-697

Figure 5.16 Suppression of colony formation by p130 and p130 (Δ620-697)

SAOS-2 and C33A cells were stably transfected with 10 μg of pCMV-Bam-Neo, pCMV-Rb, pCMV-p107, pCMV-p130 or pCMV-p130 (Δ620-697) as indicated and colonies counted after three weeks. The results presented are a combination of three independent experiments. The standard deviations of 1% or less are not indicated. 100% represents 212 colonies/plate for SAOS-2 cells and 423 colonies/plate for C33A cells.

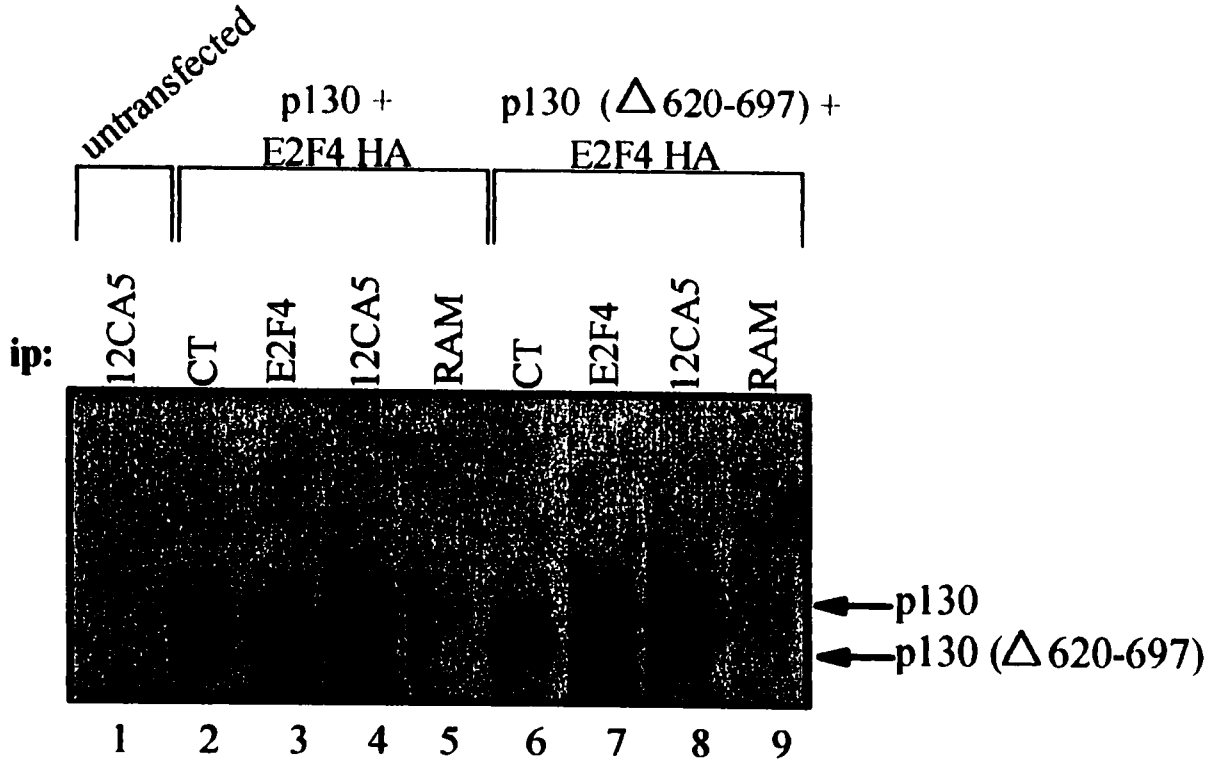

untransfected
p130 +
E2F4 HA
p130 (Δ620-697) +
E2F4 HA
ip:
12CA5
CT
E2F4
12CA5
RAM
CT
E2F4
12CA5
RAM
p130
p130 (Δ620-697)
1 2 3 4 5 6 7 8 9

Figure 5.17 Wildtype and mutant p130 interactions with E2F-4

Lysates from untransfected C33A cells or cells transiently transfected with 10 μg of each of the indicated plasmids were immunoprecipitated with antibodies to p130 (CT), E2F-4 (E2F-4) and hemagglutinin (12CA5) as indicated. Following resolution of the immune complexes by SDS-PAGE and transfer of the gel to nitrocellulose, the blot was probed with a commercial p130 antibody (Santa Cruz Biotechnology). The positions of wildtype p130 and p130 (Δ620-697) are shown to the right of the gel.

p130 antibody, an E2F-4 polyclonal antisera (Santa Cruz Biotechnology) and 12CA5 (Fig. 5.17). The presence of p130 in the immunoprecipitates was detected by western blot analysis with a p130 C-terminal antibody (Santa Cruz Biotechnology). Cells transfected with wildtype p130 plasmid expressed high levels of the protein (lane 2) which was detected in complexes containing E2F-4 (lanes 3 and 4). Interestingly, cells transfected with p130 (Δ620-697) expressed high levels of the mutant protein (lane 6) which also appeared to co-immunoprecipitate with E2F-4 (lanes 7 and 8). Neither wildtype or mutant p130 immunoprecipitated with a negative control antibody, rabbit anti-mouse (lanes 5 and 9) and untransfected cells showed no p130 protein interacting with the 12CA5 monoclonal antibody (which only detects the hemagglutinin-tagged E2F-4) (lane 1).

This data indicates that the p130 (Δ620-697) is not deficient in its ability to interact with E2F-4, suggesting that this mutant is only defective in its ability to interact with cyclin A and cyclin E.

5.2.x. p130 (ΔRRL) and suppression of growth in SAOS-2 and C33A cells

The results presented in section 5.2.iv show that deletion of p130 amino acids 680-682 (ΔRRL) abolishes interactions between p130 and cyclins A and E *in vitro*. To determine if the same mutation introduced into the full length protein would have a similar effect *in vivo*, a p130 (ΔRRL) mutant was constructed. The Xba I/Sph I fragment of p130 carrying a deletion of nucleotides 2107-2115 (generated by M13 oligonucleotide mutagenesis described in section 2.13) was substituted for a wildtype Xba I/Sph I

fragment present in the hemagglutinin-tagged p130 cDNA (kindly provided by D. Cobrinik, Columbia University). The hemagglutinin tag is present at the carboxyl terminus of the cDNA and was retained to allow identification of this mutant over endogenous wildtype p130 when the mutant was expressed in cells. The resulting cDNA (containing the ΔRRL deletion and the hemagglutinin tag) was cloned into the Bam HI sites of the pCMV-Bam-Neo expression vector and used in transient and stable transfection assays. The p130 (ΔRRL) mutant appeared as a 130 kDa band when immunoprecipitated from transfected cells with the p130 C-terminal antibody, however the mutant was not immunoprecipitated by the 12CA5 antibody (data not shown). It is possible that the carboxyl terminal location of the tag prevented it from being accessible to the antibody and that an amino terminal location may have been more useful. As a result of these technical difficulties, expression data on the pCMV-p130 (ΔRRL) mutant is not presented.

To determine the effect of deletion of these amino acids on the function of p130 in colony formation assays, the CMV-p130 (ΔRRL) mutant was stably transfected into SAOS-2 and C33A cells exactly as described for the p130 (Δ620-697) mutant in section 5.2.viii, however only three expression vectors were compared - pCMV-Bam-Neo, pCMV-p130 and pCMV-p130 (ΔRRL) (Fig. 5.18). Following the three week selection period, G418-resistant colonies were counted and expressed as a percentage of neo^r. Wildtype p130 reduced colony formation to an average of 30% in SAOS-2 cells and to 20% in C33A cells as compared to neo^r alone. These results are comparable to those

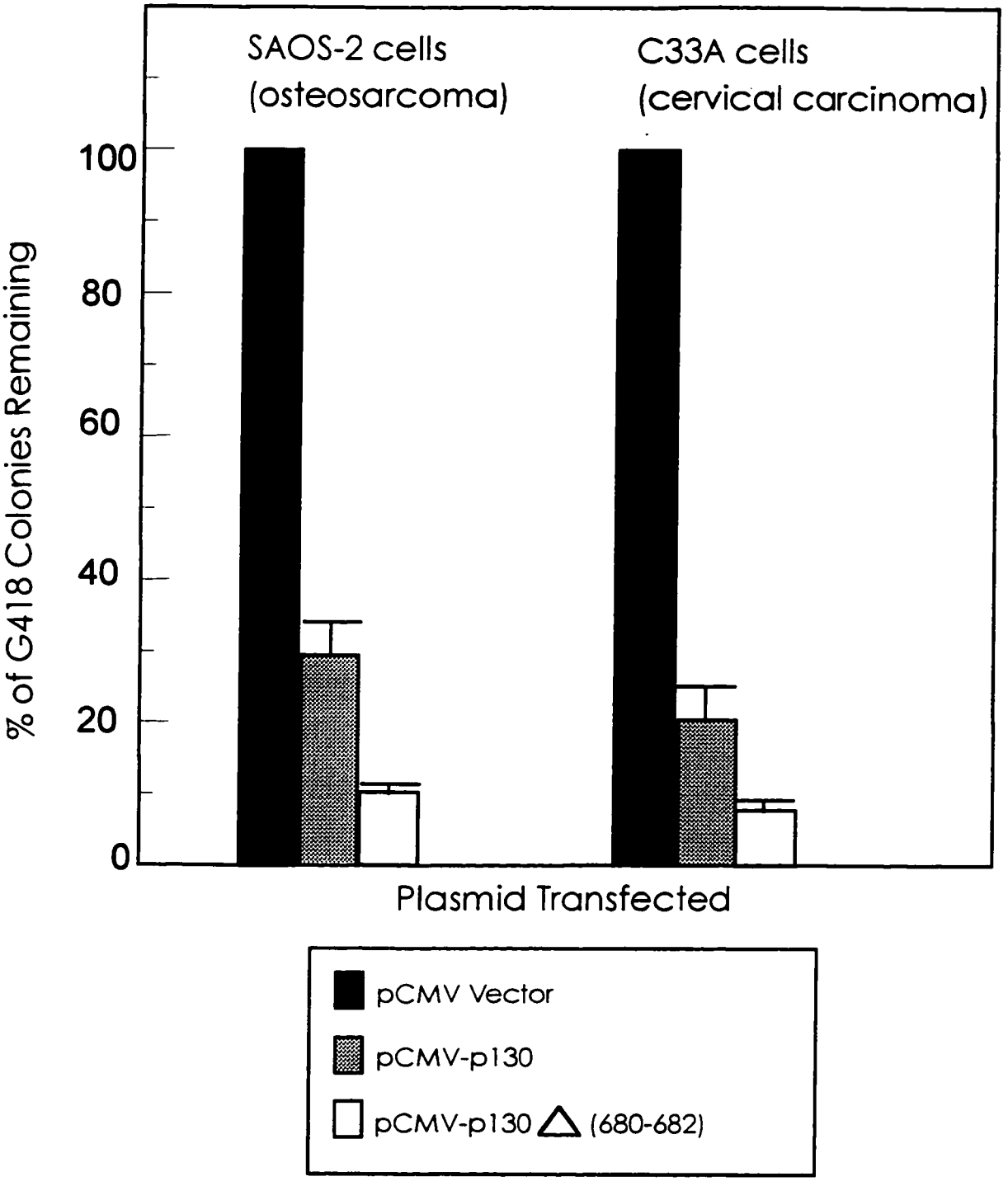

SAOS-2 cells
(osteosarcoma)
C33A cells
(cervical carcinoma)
100
80
60
40
20
0
% of G418 Colonies Remaining
Plasmid Transfected
pCMV Vector
pCMV-p130
pCMV-p130 △ (680-682)

Figure 5.18 Suppression of colony formation by p130 and p130 (ΔRRL)

SAOS-2 and C33A cells were stably transfected with 10 μg of pCMV-Bam-Neo, pCMV-p130 or pCMV-p130 (Δ680-682) (ΔRRL) as indicated and colonies counted after three weeks. The results presented are a combination of three independent experiments. The standard deviations of 1% or less are not indicated. 100% represents 190 colonies/plate for SAOS-2 cells and 370 colonies/plate for C33A cells.

shown in Figure 5.16. In comparison with wildtype p130, p130 (ΔRRL) suppressed colony formation to 10% in SAOS-2 cells and to 4% in C33A cells. These results suggest that p130 (ΔRRL) suppresses colony formation as well if not more effectively than wildtype p130, suggesting that the RRL deletion has a similar effect on the function of p130 as does the larger deletion of amino acids 620-697.

5.3 Conclusions

Using GST-p130 fusion proteins representing various regions of p130, baculovirus-produced cyclin A/cdk2 and cyclin E/cdk2 complexes were found to interact with residues within a part of p130 known as the spacer region. Cyclin E was able to bind the p130 spacer region in the presence or absence of cdk2 whereas cyclin A binding was dependent upon the presence of cdk2. This observation suggests that cyclin A requires cdk2 as a stabilizing component when interacting with p130 *in vitro*. The smallest p130 fusion protein sufficient to interact with cyclin A/cdk2 or cyclin E/cdk2 complexes contained p130 amino acids 652-698 and deletion of p130 amino acids 680-682 abolished binding to both of the cyclin/cdk2 complexes. When overexpressed in C33A cells, a p130 mutant containing a deletion of amino acids 620-697 was unable to form complexes with either cyclin A or cyclin E. When tested in colony formation assays using C33A or SAOS-2 cells, p130 (Δ620-697) and p130 (ΔRRL) were at least as active as wild type p130 in suppressing the growth of G418 resistant colonies. In light of this result, it appears that p130 is not regulated by direct interactions with cyclins A and E. p130

(Δ620-697) is not impaired in its ability to form stable complexes with E2F-4 *in vivo*, implying that this mutant is only impaired in its ability to interact with cyclin A/cdk2 and cyclin E/cdk2 complexes.

Chapter 6. p130 N-terminal deletion mutants

6.1 Introduction

As discussed in Chapter 5, amino acids within the spacer region of p130 mediate interactions with cyclins A and E and a similar region is also present in p107. In both proteins, the spacer regions contain conserved sequences (the "RRL" motifs) which have been shown to be required for interactions with cyclins A and E. A recent study of regulatory domains of p107 showed that in addition to these conserved sequences, the amino terminus of p107 was also necessary for the full length molecule to interact with cyclins A and E *in vivo* (Zhu *et al.*, 1995a). A p107 N-terminal deletion mutant containing amino acids 385-1068 was unable to co-immunoprecipitate cyclins A and E when the p107 mutants were transiently transfected into cells. This data suggested that sequences within the spacer region of p107 are necessary but not sufficient for mediating interactions with cyclins A and E *in vivo*.

This study prompted an investigation of p130 N-terminal mutants, to understand if like p107, the N-terminus of p130 is also required to maintain interactions with cyclins A and E *in vivo*. To undertake these studies, the p130 N-terminal mutant (372C) described in Chapter 4.4 plus an additional N-terminal p130 mutant (336C) were tested for their ability to interact with cyclins A and E *in vivo*.

6.2 Construction of p130 N-terminal deletion mutants

In addition to the 372C baculovirus (expressing the p130 N-terminal mutant 372C), sequences encoding 372C were ligated into the pCMV-Bam-Neo expression vector to transiently transfect mammalian cells (this plasmid was kindly provided by Greg Culp). The 372C fragment contains the pocket region of p130 (denoted as regions 3, spacer and 4 in Figure 1.2) plus an intact carboxyl terminus, sequences which are highly conserved amongst the three pRb family members.

To determine if additional amino-terminal sequences might be necessary for interactions with cyclins A and E *in vivo*, an additional N-terminal p130 mutant was constructed which contains region 2 plus the pocket and intact carboxyl terminal sequences (see Figure 1.2). Importantly, region 2 is also conserved amongst the three pRb family members. This mutant contains amino acids 336 to 1139 and is denoted as 336C.

The 336C sequence was derived by PCR amplification of the p130 cDNA with a forward primer (5' CGGGGTACCGGATCCAGCATGGGCAAGGCCTATGAGGAGT AT 3') and a reverse primer (5' CTGGATCCAGGGACAGCATTGACTAG 3'). The forward primer contained a Kpn I site, a Bam HI site and a Kozak consensus sequence translational start site. The 1.1 kb fragment containing primer sequences plus p130 nucleotides 1075-2192 was digested with Kpn I and Sph I resulting in a fragment containing primer sequences plus p130 nucleotides 1075-2163. This fragment replaced the 5' sequences present in pBSKS 372C (kindly provided by Greg Culp) by substitution

of the Kpn I/Sph I fragment in 372C with the new PCR sequence following the digestion of pBSKS 372C with Kpn I/Sph I. Following ligation of the extended 5' fragment into pBSKS 372C (to produce the plasmid pBSKS 336C) the entire N-terminally truncated cDNA (nucleotides 1075-3504) was excised from pBSKS 336C with Bam HI. The Bam HI fragment was subsequently ligated into the Bam HI site of pCMV-Bam-Neo to produce pCMV-336C.

6.3 Cyclin binding studies with 372C and 336C in mammalian cells

To determine if N-terminal mutant versions of p130 were able to interact with cyclin A/cdk2 and cyclin E/cdk2 complexes *in vivo*, C33A cells were transiently transfected with 372C and lysates subjected to immunoprecipitation analysis with antibodies to the C-terminus of p130, cyclin A and cyclin E (Figure 6.1). The resolved complexes were blotted onto nitrocellulose and probed with a p130 antibody. When immunoprecipitated with a p130 C-terminal antibody, 372C migrated as a heterogeneous series of bands, 93-95 kDa in apparent molecular weight. In contrast, 372C was absent in co-immunoprecipitations with antibodies to cyclin E (Mab 276 and Mab168) or to cyclin A (C160). Endogenous levels of wildtype p130 (indicated by an arrow) were evident on the blot, which was exposed for only a short time due to the overexpression of the 372C mutant. Neither 372C nor p130 co-immunoprecipitated with a negative control antibody, rabbit anti-mouse. These results suggested that *in vivo*, 372C was unable to complex with cyclins A or E. Indeed, not even a small quantity of the highly overexpressed protein appeared to be associated with the cyclins.

To determine if an N-terminal mutant with an extended amino terminal sequence containing the homologous domain "2" (shown in Figure 1.2) was able to interact with cyclins A and E *in vivo*, 336C was stably expressed in U2OS cells. The 336C cell line is similar to the U7 cell line established and described in Chapter 4.2.i. As a control, another stable U2OS cell line (UΔ620) overexpressing p130 (Δ620-697) was also used in this study, as p130 (Δ620-697) has been shown to be unable to interact with cyclins A and E *in vivo* (Chapter 5.4.vii).

Following the lysis of cells form each of the three cell lines (U7, UΔ620-697, U336C) immunoprecipitations were performed with the p130 C-terminal antibody, antibodies to cyclin E (276+168), cyclin A (C160) and an E2F-4 antibody (Figure 6.2). Wildtype p130 (overexpressed in U7 cells) was revealed following immunoprecipitation with the C-terminal antibody (lane 2) as was the Δ620-697 mutant (lane 7) and the 336C mutant (lane 12). Like 372C, 336C migrated as a series of heterogeneous bands from 97 to 100 kDa. Immunoprecipitated 336C appeared identical following transient and stable expression (in C33A cells and U336C cells respectively) (data not shown). As expected, wildtype p130 co-immunoprecipitated with cyclin A (lane 3) and cyclin E (lane 4) but did not with rabbit anti-mouse (lane 1). Also as expected, p130 (Δ620-697) did not co-immunoprecipitate with either cyclin as shown in Chapter 5 (lanes 8 and 9). 336C, like 372C, did not appear in co-immunoprecipitations with cyclins A (lane 13) or E (lane 14), suggesting that an extended amino terminus (beyond amino acid 336) is required to form stable interactions with the cyclins *in vivo*.

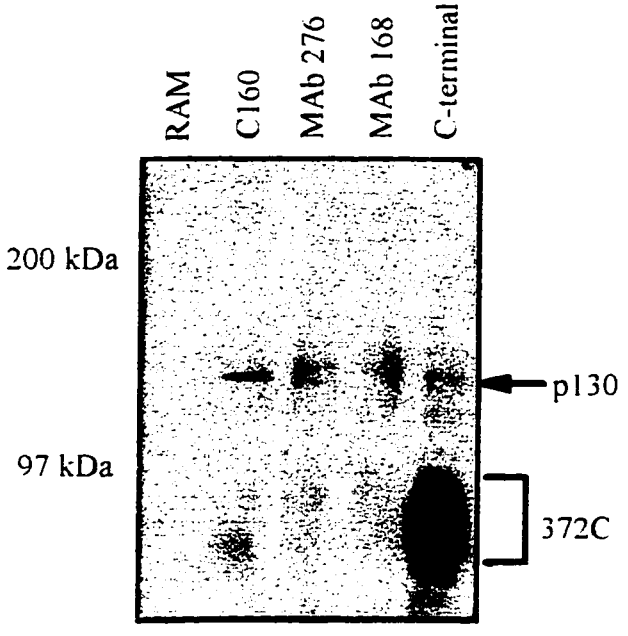

RAM
C160
MAb 276
MAb 168
C-terminal
200 kDa
97 kDa
p130
372C

Figure 6.1 Expression of 372C in C33A cells

C33A cells were transiently transfected with 10 μg pCMV-372C and cell lysates immunoprecipitated with antibodies to the C-terminus of p130 (C-terminal), cyclin E (276 and 168), cyclin A (C160) and rabbit anti-mouse (RAM) as a negative control. Immune complexes were separated by SDS-PAGE and the gel blotted to nitrocellulose. The blot was probed with a commercial C-terminal p130 antibody (Santa Cruz Biotechnology).

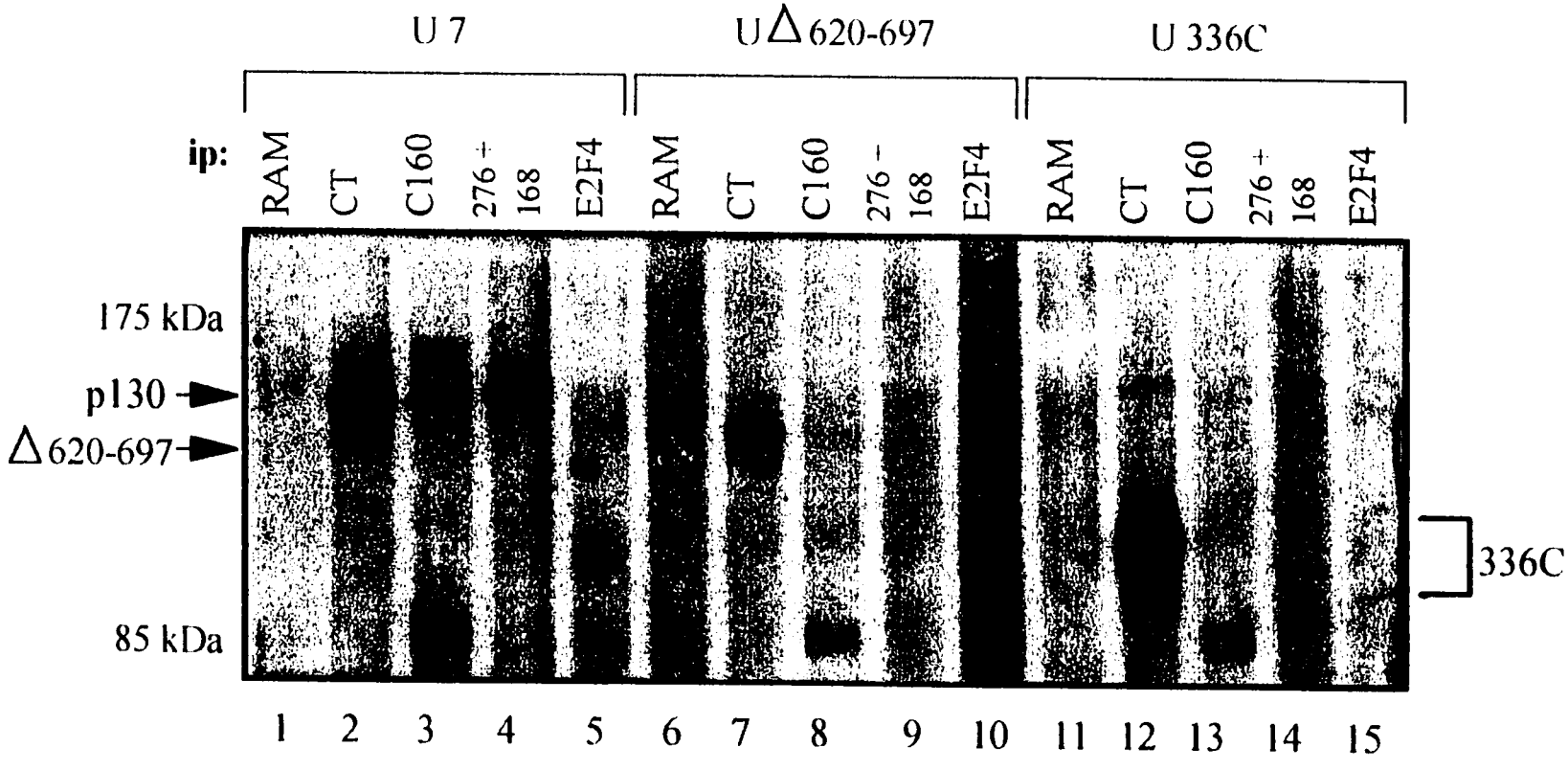

U 7
U Δ620-697
U 336C
ip:
RAM
CT
C160
276 + 168
E2F4
RAM
CT
C160
276 − 168
E2F4
RAM
CT
C160
276 + 168
E2F4
175 kDa
p130
Δ620-697
85 kDa
336C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 6.2 Expression of 336C in U2OS cells

Stable cell lines overexpressing wildtype p130 (U7), p130 (Δ620-697) and 336C (U336C) were lysed and subjected to immunoprecipitation analyses with antibodies to E2F-4, cyclin E (a mixture of Mab's 276+168), cyclin A (C160), the C-terminus of p130 (CT) and rabbit anti-mouse (RAM). Immune complexes were resolved by SDS-PAGE and the gel blotted to nitrocellulose. The blot was probed with a commercial C-terminal p130 antibody (Santa Cruz Biotechnology).

Interestingly, none of the three proteins (wildtype p130, p130 (Δ620-697) or 336C) co-immunoprecipitated with E2F-4 in these cell lines. This result is most likely due to the fact that $p16^{INK4A}$ is absent in U2OS cells (Koh *et al.*, 1995; Lukas *et al.*, 1995). In the absence of this cdk4 and cdk6 inhibitor, p130 and p130 N-terminal mutants remain in a hyperphosphorylated form, unable to complex stably with the E2F family members. This possibility implies that hyperphosphorylation of p130 in U7 cells does not affect the association of cyclin A and cyclin E *in vivo*, because these associations are easily detected by co-immunoprecipitations (Figure 3.2). The interactions between E2F-4 and p130 may be more similar to the interactions of the D-type cyclins and p130 which are disrupted upon phosphorylation of p130 by cyclin D-associated kinases.

The results of the co-immunoprecipitation studies strongly suggest that, as is the case for p107, the amino terminus (or portions thereof) of p130 is important for formation of stable complexes with cyclin A and cyclin E in mammalian cells *in vivo*.

6.4 Cyclin binding studies with 372C in insect cells

To determine if, in addition to overexpression in mammalian cells, 372C was deficient in cyclin A and E binding in the baculoviral system, SF9 cells were co-infected with 372C in the presence or absence of cyclin A, cyclin E and cdk2 (Figure 6.3). Following ^{35}S-metabolic labeling of infected cells, lysates were subjected to immunoprecipitation analyses with the p130 C-terminal antibody, a cyclin E antibody (276), the cyclin A antibody C160, the cdk2 antipeptide antisera and rabbit anti-mouse as a negative control.

In contrast to the situation in mammalian cells, in SF9 cells containing 372C plus cyclin E or cyclin E and cdk2, 372C co-immunoprecipitated with cyclin E (lanes 1 and 5) and cdk2 (lane 2). In addition, in cells containing 372C and cyclin A and cdk2, low levels of 372C co-immunoprecipitated with cyclin A (lane 10) and higher levels co-immunoprecipitated with cdk2 (lane 11). In cells containing 372C and cyclin A, no 372C was detectable in co-immunoprecipitations with cyclin A (lane 8). Levels of 372C in these cells was quite high as shown by immunoprecipitation with the p130 C-terminal antibody (lanes 4, 7, 9 and 12), suggesting not all the 372C present in these cells was complexed to cyclins A or E. This may be due to the fact that the stoichiometry of the proteins is unequal, and not as much cyclin was present in the cells as 372C. That cyclin A interacted more efficiently with 372C in cells containing cdk2 is reminiscent of results which showed cdk2 was required for GST-p130 fusion proteins to interact stably with cyclin A (Chapter 5.4.iii). Interestingly, more 372C is apparent in co-immunoprecipitations with the cdk2 antisera, possibly because the C160 antibody interferes with the cyclin A/372C interaction.

These results imply that cyclin interactions that occur between p130 N-terminal mutants in SF9 cells are distinct from those which occur in mammalian cells. Mammalian cells may contain additional cellular proteins which normally compete for regions of p130 or cyclins necessary for interactions and these proteins may be absent in insect cells. Additionally, a negative regulatory modification on cyclins or p130 may not occur in insect cells, providing an opportunity for these proteins to interact in the absence of these

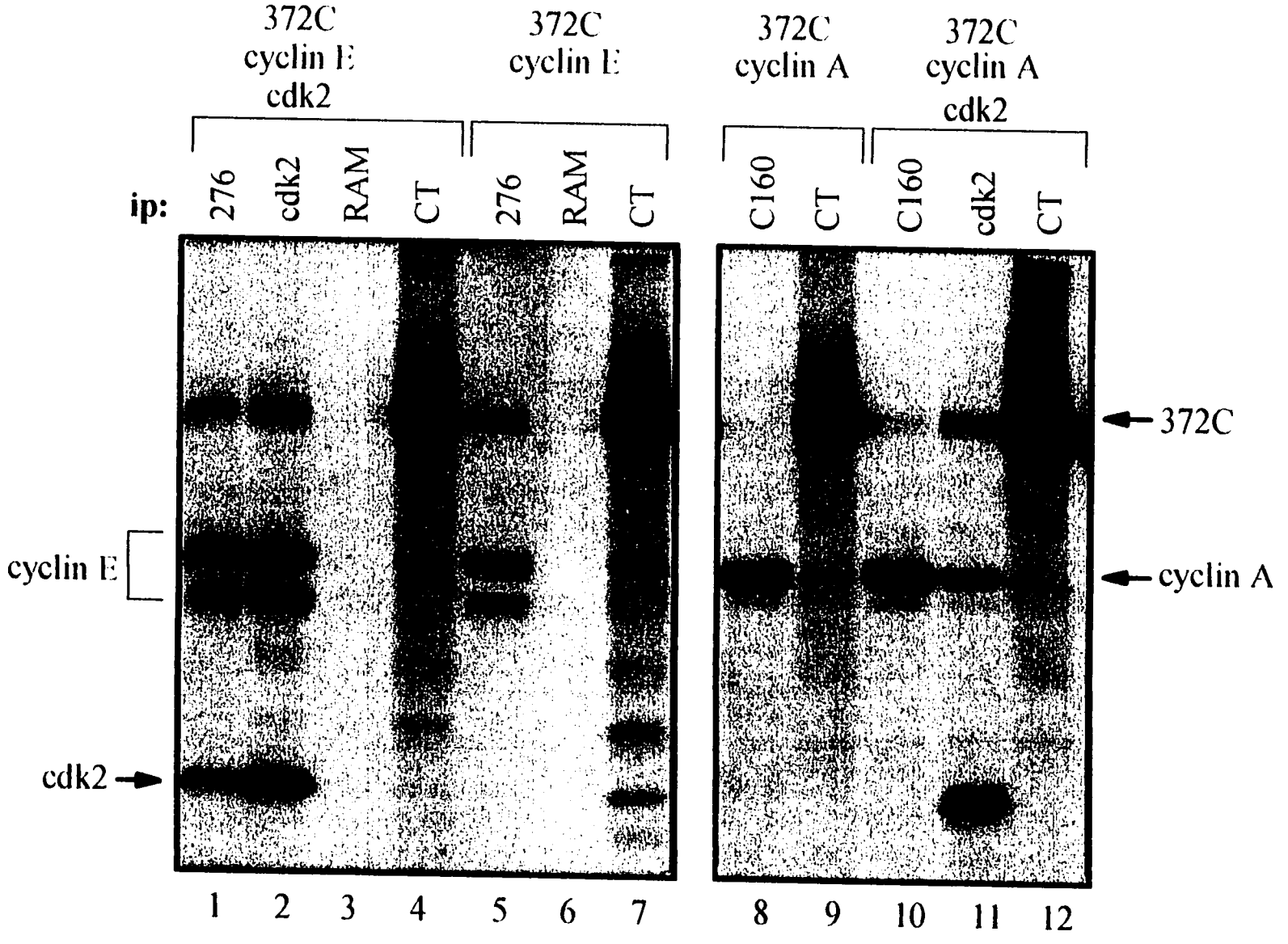

372C cyclin E cdk2
372C cyclin E
372C cyclin A
372C cyclin A cdk2
ip:
276
cdk2
RAM
CT
276
RAM
CT
C160
CT
C160
cdk2
CT
372C
cyclin A
cyclin E
cdk2
1 2 3 4 5 6 7 8 9 10 11 12

Figure 6.3 Co-immunoprecipitation of 372C with cyclins A and E in SF9 cells

SF9 cells were co-infected with 372C plus combinations of cyclin E, cyclin A and cdk2 where indicated at the top of the diagram. Cells were metabolically labeled with ^{35}S-methionine, lysed and immunoprecipitated with antibodies to cyclin E (276), cdk2, rabbit anti-mouse, cyclin A (C160) and the C-terminus of p130 (CT) as indicated in each lane. Following separation of the immune complexes by SDS-PAGE, the gel was processed for fluorography.

modifications. Whatever the case, these results suggest that in mammalian cells, the p130 amino terminus plus the RRL motif are required for stable interactions with cyclins A and E as opposed to insect cells, where the amino terminus of p130 appeared to be dispensable.

6.5 The amino terminus of p130: a potential inhibitor of cyclin-dependent kinase activity

The contrasting data obtained from the studies in sections 6.3 and 6.4 prompted further analysis of the potential function of the p130 amino terminus. Discussions within this laboratory resulted in a hypothesis that the amino terminus of p130 was stabilizing cyclin A and cyclin E interactions by inhibiting the activity of cdk2. Once inhibited, cdk2-mediated phosphorylation events would be prevented and stable interactions between 130 and cyclins A and E would be maintained.

To determine if such an effect could be assayed *in vitro*, a GST-p130 fusion protein containing amino acids 1-320 was kindly provided by C. Mandalfino. This fusion protein was described and used in cyclin binding studies discussed in Chapter 5. As a source of cyclin E and cdk2, SF9 cells were co-infected with cyclin E and cdk2 baculoviruses and the lysates immunoprecipitated with cyclin E monoclonal antibody 276. Following immunoprecipitation, *in vitro* kinase assays were performed in the presence of the immobilized cyclin E/cdk2 immune complexes, varying amounts of GST-p130 (1-320) and GST-pRb as a substrate. GST-pRb contains pRb amino acids 706-938 (Figure 1.2) and was constructed with an Nde I fragment of the pRb cDNA (nt 2268-3576). This

blunted fragment was ligated into the Sma I site of pGex 1 and the plasmid encoding this 55 kDa fusion protein was kindly provided by C. Mandalfino. GST-pRb was chosen as a substrate due to the fact that it is a physiological substrate of cyclin/cdk2 complexes *in vivo*, although additional substrates (histone H1, GST-p130 fusion proteins) could also have been used.

Kinase assays were performed in the presence of increasing amounts of GST-p130 (1-320) or GST alone to determine if the presence of the N-terminus of p130 affected the activity of cdk2 (Figure 6.4). As assessed by the *in vitro* kinase assay, cyclin E/cdk2 kinase activity was not altered in the presence of increasing concentrations of GST. In contrast, kinase activity declined rapidly as concentrations of GST-p130 (1-320) were increased. An identical result was observed with kinase assays performed with immobilized cyclin A/cdk2 complexes (data not shown). Interestingly, neither GST (which migrates at 27 kDa) nor GST-p130 (1-320) (which migrates at 65 kDa) is a substrate of cdk2 activity.

These results suggest that amino-terminal sequences of p130 are somehow able to negatively influence the kinase activity of cdk2. Although the mechanism of this negative regulatory event is not yet understood, it is interesting to note that like p130, the amino terminus of pRb contains a proline/alanine-rich sequence in addition to other conserved regions which may be important in the regulation of cdk2 activity.

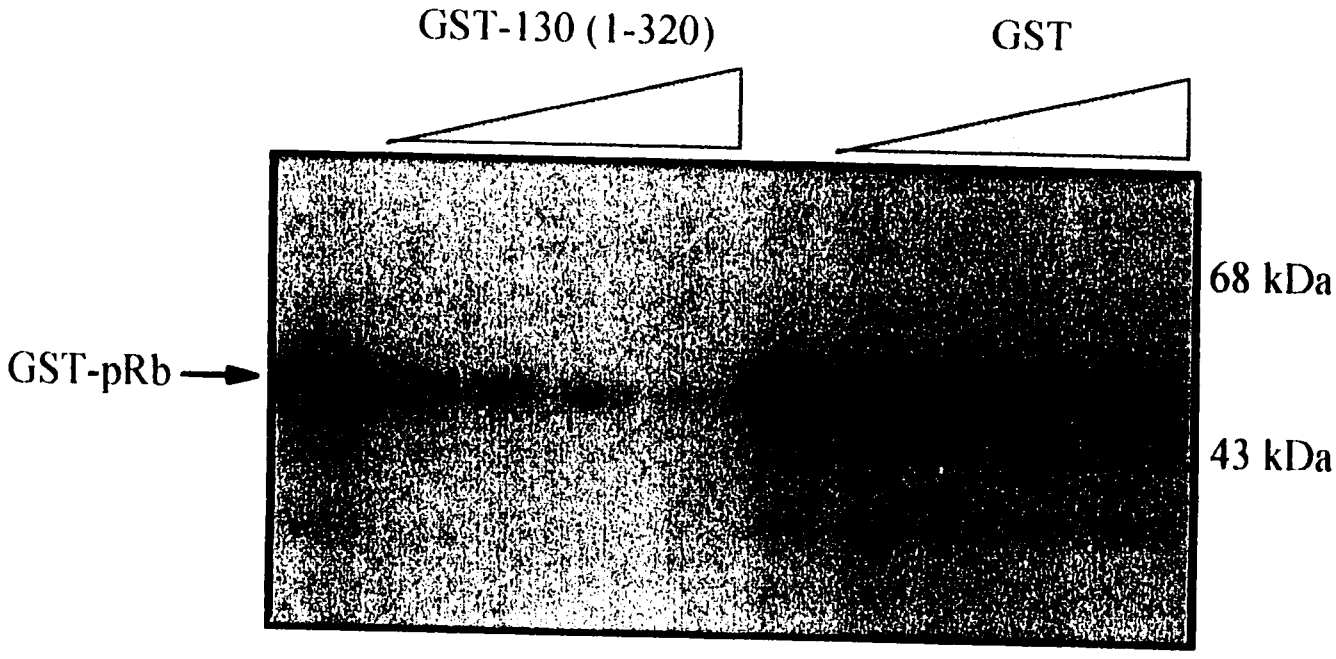

GST-130 (1-320)
GST
GST-pRb
68 kDa
43 kDa

Figure 6.4 p130 N-terminal inhibition of cdk2 activity *in vitro*

In vitro kinase assays were performed following immunoprecipitation of SF9 cell lysates infected with baculoviruses expressing cyclin E and cdk2. Kinase assays contained immobilized immune complexes, kinase buffer, 0.5 μg GST-pRb and 0, 1.25, 2.5, 3.75, and 5 μg GST-p130 (1-320) or GST alone as indicated at the top of the diagram. Kinase assay were allowed to incubate at room temperature for 30 minutes prior to separation of proteins in the reaction by SDS-PAGE. The gel was dried and processed for autoradiography.

6.6 Conclusion

This chapter highlights the necessity of an intact p130 amino terminus in maintaining stable interactions with cyclins A and E in mammalian cells. Specifically, amino terminal sequences present before p130 amino acid 336 are required. These results are in contrast to those obtained in SF9 insect cells co-infected with the 372C amino terminal mutant and cyclin A/cdk2 and cyclin E/cdk2 complexes. In this system, cyclin E, cyclin E/cdk2 and cyclin A/cdk2 interacted with 372C as shown by co-immunoprecipitation studies. These results could be due to the high levels of protein overexpression obtained in these cells following infection and/or could be a result of the distinct nature of insect cells as compared to mammalian cells. As discussed, mammalian cells may contain proteins which naturally compete for interactions with p130 and/or cyclins A and E, and these proteins may be absent in insect cells. *In vitro* kinase assays revealed the amino terminus of p130 (amino acids 1-320) when fused in frame with GST was a inhibitor of cyclin A/cdk2 and cyclin E/cdk2 kinase activity. It is not clear if this region functions in a similar manner when present in the full length protein or why such an activity would be required in mammalian cells and not insect cells. A further investigation of which amino acids within the 1-320 region are specifically required for the inhibition of cdk2 activity may help elucidate the nature of this function.

Chapter 7. Conclusions and Discussion

Following the identification of a cDNA encoding the 130 kDa E1A-associated protein, this laboratory was interested in understanding the function and regulation of p130. The sequence of p130 classified it as the third member of the retinoblastoma family of proteins. This thesis addresses the function of p130 through determination of the structural domains which mediate interactions with cyclins A, D1, D2, D3 and E and how phosphorylation of p130 by cyclin-dependent kinases affects these interactions.

7.1 p130 interactions with D-type cyclins

Co-expression of 372C and the D-type cyclins by baculoviral infection of SF9 cells revealed that 372C interacts with cyclins D1, D2 and D3 in these cells. In contrast, interactions between the D-type cyclins and 372C were reduced in the presence of cdk4, albeit less so in the case of cyclin D2. A marked shift in molecular weight accompanied the disruption of the interaction between D-type cyclins and 372C, suggesting phosphorylation of 372C by cdk4 disrupted the complex. Similar studies in SF9 cells co-infected with pRb and D-type cyclins (Kato *et al.*, 1993) showed cdk4 phosphorylation of pRb disrupts pRb/cyclin D complexes. This suggests that D-type cyclin interactions with pRb and p130 are unstable in the presence of cdk4, explaining why ternary complexes containing these proteins are difficult to detect *in vivo*. Phosphorylation of pRb, p107 and p130 by cyclin D/cdk4 complexes may result in conformational changes in these proteins, releasing them from D-type cyclin interactions. p130 complexes containing E2F are

predominant in G0 and early to mid G1. Cyclin D/cdk4 complexes peak in mid to late G1, suggesting that D-type cyclins may regulate the function of p130 by phosphorylation early in the cell cycle. Given this scenario, D-type cyclins and E2F likely bind to hypophosphorylated p130 *in vivo*. In U7 cells which overexpress hyperphosphorylated p130 (due to the absence of the cdk4 inhibitor $p16^{INK4}$), p130 did not co-immunoprecipitate with E2F-4 nor did it not co-immunoprecipitate with cyclins D1, D2 and D3 (data not shown). This suggests in the absence of $p16^{INK4}$, cyclin D/cdk4 kinase activity is constitutively acitve and p130 remains in a hyperphosphorylated state, unable to interact with D-type cyclins or E2F.

In vitro binding studies with GST-p130 fusion proteins and cyclin D3 produced in insect cell lysates revealed that p130 residues 593-1139 (of the 1139 amino acid sequence) were required to mediate interactions with cyclin D3 *in vitro*. This region of p130 includes the spacer and B domain, suggesting the A domain (which in combination with the spacer region and the B domain comprises the pocket region of p130) is not required for p130 interactions with cyclin D3. Previous *in vitro* studies have determined intact pocket regions of p107 and pRb are necessary for interactions with D-type cyclins *in vitro* (Ewen *et al.*, 1993), although there has been no further data presented on whether this entire region is required. From the data presented in this thesis, it appears that all three pRb family members utilize the spacer and B domain to mediate D-type cyclin interactions and pRb and p107 also require the A domain.

7.2 p130 interactions with cyclin A and cyclin E

7.2.i. Stable formation of p130 complexes with cyclin A and cyclin E

p130 complexes containing cyclin A and cyclin E can be detected *in vivo* by co-immunoprecipitation of p130 with these cyclins. In SF9 cells, interactions between cyclins A and E and 372C were stable in the presence of cdk2, in fact interactions between cyclin A and 372C appeared to require the presence of cdk2. In contrast, D-type cyclins (with the possible exception of cyclin D2) are not found stably associated with p130 *in vivo* due to the presence of cdk4. Interestingly, p130 is detectable in stable complexes with cyclin A and cyclin E in U7 cells. Due to the absence of the cdk4/cdk6 inhibitor $p16^{INK4}$ p130 is thought to be in a hyperphosphorylated state in these cells. This implies that cdk4/cdk6-mediated phosphorylation events do not affect p130 interactions with cyclin A and cyclin E. From this data it can be inferred that the conformational change which results from cdk4/cdk6-mediated phosphorylation of p130 disrupts interactions with E2F and D-type cyclins but does not result in disruption of cyclin A and cyclin E complexes.

Several considerations may help to explain this observation. As described in Chapter 4, p130 is a substrate of cyclin A and cyclin E-associated kinase activity *in vitro*, however it is not clear if it is also a substrate *in vivo*. If p130 is an *in vivo* substrate of cdk2, phosphorylation of p130 by this kinase might result in conformationally distinct changes in the overall structure of p130 as compared to phosphorylation by cdk4. Cyclin D/cdk4 complexes may not phosphorylate identical residues as cyclin A/cdk2 and cyclin E/cdk2 complexes, especially in light of the fact that each complex interacts with distinct

portions of p130. The identification of the sites of cdk4 and cdk2 phosphorylation in p130 may help to verify this possibility. Although it is also not clear if p130 is a substrate of cdk4 *in vivo*, the *in vivo* phosphorylation of p130 correlates with the timing of formation of cyclin D/cdk4 complexes rather than cyclin E/cdk2 and cyclin A/cdk2 complexes. During the cell cycle, cyclin D/cdk4 activity is thought to act before cyclin E/cdk2 activity and cyclin A/cdk2 activity, suggesting that the majority of p130 is present in a phosphorylated state following late G1. It is possible that phosphorylated p130 interacts with cyclin E/cdk2 and then cyclin A/cdk2 complexes later in the cell cycle and that once formed, p130 remains associated with these cyclin complexes during late G1 and throughout S phase. Another possibility is that p130 binds to cyclin E/cdk2 and cyclin A/cdk2 complexes and acts as a cdk2 inhibitor, as described in Chapter 6. In this scenario, p130 maintains cyclin/cdk2 complexes in an inactive state (discussed in section 7.4). It is difficult to explain what function p130/cyclin A and p130/cyclin E complexes might have during these points in the cell cycle and there is little data to support any model.

7.2.ii The p130 region required to interact with cyclins A and E

Binding studies defined regions of p130 important in mediating interactions with cyclins *in vitro* and *in vivo*. In contrast to cyclin D3, cyclin A and cyclin E interact with a region present in the amino terminal half of the spacer domain and these interactions are dependent on the RRRLFVE sequence region (which will be referred to as the cyclin binding region throughout this section). Several other cellular proteins shown to stably interact with cyclin/cdk complexes also contain a region that is homologous to the p130

cyclin binding region, including p107, the cdk inhibitors $p21^{CIP1}$, $p27^{KIP1}$, $p57^{KIP2}$ (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993a; Polyak *et al.*, 1994a; Toyoshima and Hunter, 1994; Lee *et al.*, 1995; Matsuoka *et al.*, 1995) and E2F-1 (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992). A similar sequence is found in E2F-2 and -3; however, these proteins have not been demonstrated to interact with the cyclin/cdk complexes (Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993). The apparent conservation of a the cyclin binding region suggests that it serves as a mechanism for interactions of different proteins with cyclin/cdk complexes.

Studies on some of the above mentioned proteins have indicated that the residues homologous to the p130 cyclin binding region are directly involved in cyclin/cdk interactions. p107 utilizes this region in interactions with cyclins A and E (Ewen *et al.*, 1992; Faha *et al.*, 1992; Zhu *et al.*, 1995a; Zhu *et al.*, 1995b) as do $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$. Several reports show the amino terminus of $p21^{CIP1}$ (which contains the cyclin binding domain) is necessary and sufficient to inhibit cdk activity (Chen *et al.*, 1995; Goubin and Ducommun, 1995; Luo *et al.*, 1995; Nakanishi *et al.*, 1995; Zukut and Givol, 1995) and substitution of $p21^{CIP1}$ amino acids 21 and 24 severely disrupts its *in vitro* interactions with cyclin D and cyclin E (Lin *et al.*, 1996). Both of these residues are within the region homologous to the p130 cyclin binding region. Similarly, studies on mutants of $p27^{KIP1}$ have implicated the amino terminal region as being essential for interactions with cyclin E/cdk2 complexes (Luo *et al.*, 1995) and substitution of amino acids within the cyclin binding region abolished these interactions (Luo *et al.*, 1995).

Evidence for the function of the conserved domain in cyclin binding has come from structural studies of a 69 amino acid fragment of $p27^{KIP1}$ bound to cyclin A and cdk2 (Russo *et al.*, 1996). The structure of this complex, as determined by x-ray crystallography, implicated the LFG residues of the $p27^{KIP1}$ cyclin binding region as contact residues mediating the interaction with cyclin A while other residues in $p27^{KIP1}$ interacted with and inhibited the activity of cdk2. E2F-1 also has been shown to utilize its cyclin binding region to interact with cyclin A (Krek *et al.*, 1994; Xu *et al.*, 1994). In this case the function of the interaction is to phosphorylate the E2F-1/DP1 complex and downregulate DNA binding (Dynlacht *et al.*, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995; Krek *et al.*, 1995). Although the above-mentioned proteins all contain a conserved cyclin binding domain, they appear to target cyclins for different purposes.

Presently, it is not apparent how specificity of the cyclin/cdk interaction is determined. The results presented in this thesis imply that identical or very similar p130 sequences mediate the interactions with both cyclin A- and cyclin E- containing complexes. Studies on E2F-1 interactions with cyclins indicate that although E2F-1 contains a cyclin binding motif that is similar to p130, it is capable of interacting only with cyclin A and not cyclin E (Dynlacht *et al.*, 1994; Krek *et al.*, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995). Further diversity of cyclin interaction is observed with the cyclin binding regions of $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$ (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993a; Polyak *et al.*, 1994a; Toyoshima and Hunter, 1994; Lee *et al.*, 1995; Matsuoka *et al.*, 1995). These proteins have a broader specificity

range and can interact with complexes containing cyclins A, B and E and the D cyclins. It is possible that the sequence variability within the cyclin binding regions is responsible for the specificity of the cyclin/cdk interactions.

The conservation of cyclin binding regions in several different proteins raises the possibility of competition for interactions with the cyclin/cdk complexes. P21 has been shown to compete with both p107 and p130 for interactions with cyclin A/cdk2 and cyclin E/cdk2 (Zhu *et al.*, 1995; Shiyanov *et al.*, 1996). The competition for cyclin/cdk interactions may extend to kinase substrates which do not form stable interactions with cyclin/cdk complexes if this activity requires the same binding site occupied by p130, p107, $p21^{cip1}$ and $p27^{kip1}$.

7.3 Amino terminal mutants of p130

Co-immunoprecipitation assays in mammalian cells expressing two amino-terminally truncated p130 mutants (336C and 372C) revealed that these proteins were unable to interact with cyclins A and E *in vivo*, suggesting the loss of the amino terminus was detrimental to cyclin interactions. 336C contains amino acids up to and including those in domain 2, implying that residues amino terminal to this region (specifically domain 1 and the proline/alanine-rich region) are necessary in the context of the full length protein to allow stable interactions with cyclins A and E *in vivo*. This result implies that the intact cyclin binding domain (containing the RRRLFVE motif) is necessary for cyclin interactions *in vitro* but not sufficient for cyclin interactions *in vivo*. These observations are in contrast to the results showing co-immunoprecipitation of 372C with cyclins A and

E in SF9. In these cells, 372C co-immunoprecipitated with cyclin E in the presence and absence of cdk2 and with cyclin A only in the presence of cdk2. Although the interaction between cyclin A/cdk2 and 372C was poor, it was a positive result. Cyclins A and E interacted with 372C in a cdk2-dependent and independent manner respectively, a result reminiscent of those obtained in the *in vitro* binding studies. This suggests the baculoviral system may be more like an *in vitro* system in the sense that the protein components are highly overexpressed in cells which are fundamentally distinct from transformed mammalian cells.

Why is the amino terminus of p130 not required to mediate cyclin A and E interactions *in vitro* or in SF9 cells? It is possible that in mammalian cells the N-terminal deletion mutants are less stable than those with an intact amino terminus, but the high expression level of each mutant as determined by immunoprecipitation assays would argue against this. The amino terminus may also be necessary for the correct folding of the protein but this does no appear to be a factor in assays using GST-p130 fusion proteins or in SF9 cells, suggesting the folding of the spacer region is correct. One hypothesis is that the amino terminus of p130 is required to interfere with the interactions of a unidentified cellular protein present in mammalian cells. This unidentified protein might compete for interactions with the cyclin-binding domain in mammalian cells but would be absent in insect cells or where the components are purified in an *in vitro* system. The amino terminus of p130 might inhibit the binding of this cellular factor, preventing it from competing with cyclins A and E for the cyclin-binding region. Another hypothesis is that

the amino terminus of p130 inhibits cdk2 activity when in a complex with cyclins A and E. If this is true, cyclin A/cdk2 and cyclin E/cdk2 complexes are inactive when bound to p130, explaining why the complexes are stable *in vivo*. This hypothesis also suggests that the amino terminus of p130 is not active in inhibiting cyclin D/cdk4 complexes, explaining why D-type cyclins interact with 372C and why these complexes are disrupted upon phosphorylation by active cdk2. Evidence for this possibility was suggested in an assay which determined that the amino terminus of p130 appears to contain a functional inhibitory domain.

7.4 The inhibitory function of the p130 amino terminus

Chapter 6 presents an experiment to determine whether the p130 amino terminus is capable of inhibiting the kinase activity of cyclin A/cdk2 and cyclin E/cdk2 complexes. It was determined that GST-p130 (1-320) was an inhibitor of cyclin A and cyclin E-associated cdk2 activity by *in vitro* kinase assays using GST-pRb as a substrate. Unlike GST-p130 fusion proteins which contain portions of the spacer region and amino terminal sequences, GST-p130 (1-320) is not a substrate of cdk2 *in vitro*. This may be a result of direct cdk2 inhibition or may be a consequence of an absence of cdk2 phosphorylation sites within this region of p130. Interestingly, GST-p130 (1-320) does not bind to cyclin A/cdk2 and E/cdk2 complexes *in vitro*, suggesting that stable association of cyclins might be a prerequisite for phosphorylation by cdk2. It is not mechanistically evident how the amino terminus of p130 is able to inhibit cdk2 activity, however several scenarios are possible including competitive and non-competitive inhibition. As a competitive inhibitor,

the amino terminus of p130 would maintain cdk2 in an inhibited state until levels of substrate outcompeted the levels of p130 present. As a non-competitive inhibitor, the amino terminus of p130 would maintain cdk2 complexes in an inhibited state regardless of the concentration of substrate present. Preliminary data not presented in this thesis suggested the p130 kinase inhibitory domain acts as a non-competitive inhibitor.

Discovery of a functional domain within the amino terminus of p130 is a novel finding which will be important in future studies of this protein. Interestingly, in a recent communication from Jim Roberts' laboratory (Fred Hutchison Cancer Center), p130 was found to be an inhibitor of cyclin E/cdk2 complexes in mouse embryo fibroblasts derived from $p27^{KIP1}$ knockout mice (Kiyokawa *et al.*, 1996; Fero *et al.*, 1996). This data followed data presented by Koff *et al.*, 1993, Polyak *et al.*, 1994a,b and Coats *et al.*, 1996 where it was shown that levels of $p27^{KIP1}$ accumulated as a result of treatment of epithelial cells with TGF-ß. High levels of $p27^{KIP1}$ inhibited cyclin E/cdk2 activity in these cells, but cyclin E/cdk2 complexes also remained inhibited in $p27^{KIP1}$ knockout mouse fibroblasts, suggesting another cellular protein was replacing $p27^{KIP1}$ as the cyclin E/cdk2 kinase inhibitor. Data from the Roberts' lab concluded the substituting cellular protein was p130. It is not clear why both $p27^{KIP1}$ and p130 are active in inhibiting cyclin E/cdk2 complexes in these cells. The artificial situation created by the inactivation of the $p27^{KIP1}$ gene may have resulted in the substitution of p130 for $p27^{KIP1}$. If both proteins are active in suppressing cyclin E/cdk2 activity, their functions may be redundant or different cell types may utilize one protein or the other depending on the cellular stimuli. Cyclin E/cdk2

complexes held in an inactive state by p130 may normally be low in abundance during G0, explaining why these complexes were not previously identified. Although the cyclin-binding regions of p130 and $p27^{KIP1}$ are conserved, the p130 inhibitory domain (amino acids 1-320) shows no homology to other regions of $p27^{KIP1}$. Further investigation into the function of the amino terminus of p130 and the specific residues required to mediate cdk2 inhibition may help elucidate the potential functions of p130 during the cell cycle.

7.5 Insights into the function of p130

The studies completed in this thesis help to elucidate the function and regulation of p130. Unfortunately, there are very few functional assays which are capable of analyzing the effects of wildtype or mutant p130 on cellular growth. One of the few assays is the colony formation assay, which measures the effect growth-suppressing proteins have on colony formation. Overexpression of pRb, p107 or p130 in C33A and SAOS-2 cells results in growth suppression, with p130 being the least potent member of the pRb family in these assays. Importantly, overexpression of any of the pRb family members in U2OS cells does not result in suppression of colony formation, suggesting the genetic background of the cells used in these assays is a consideration and that the pRb family members do not possess a non-specific growth suppressing capability. It is thought that U2OS cells are unaffected by the overexpression of the pRb family members because pRb and p107 remain hyperphosphorylated by cdk4 and cdk6 (hence inactive) throughout the cell cycle. Specifically, the fact that p130 can be overexpressed in these cells without

reducing colony formation implies that constitutive cdk4 and cdk6-mediated phosphorylation of p130 is inhibitory to its function.

Deletion of p130 amino acids 620-697 eliminates p130 interactions with cyclins A and E *in vivo*. Importantly, this mutant retains the amino terminus of p130 and is still able to complex with E2F-4 *in vivo*. These structural features imply that p130 (Δ620-697) is deficient only in its ability to interact with cyclins A and E, although it is possible that interactions between p130 and other cellular protein(s) are also affected by this deletion. The colony formation assay results suggest that this p130 mutant is not compromised in its ability to suppress growth in C33A and SAOS-2 cells. In fact, even fewer C33A colonies remained in assays using the Δ620-697 mutant as compared to wildtype p130. Taken together, the Δ620-697 mutant appears to be as efficient if not slightly more efficient in suppressing growth of certain pRb minus cell lines, and this effect is not attributed to failure of the mutant protein to interact with E2F-4. If phosphorylation of p130 by cyclin A/cdk2 or cyclin E/cdk2 complexes is a negative regulatory event, a slight increase in growth-suppressing capacity might be expected for a mutant unable to interact with these cyclins. Whatever the effect, the mechanism by which p130 suppresses growth does not appear to be mediated through its interactions with these cyclins.

As a result of studies with the Δ620-697 mutant, the ability of p130 to suppress growth appears to be due to its interactions with the E2F transcription factor family, or with other cellular proteins. Additionally, the ability of p130 to complex with D-type cyclins might also be important to its growth-suppressing ability. The fact that the D-type

cyclins utilize all or most of the pocket domain of pRb, p107 and p130 makes a mutagenesis strategy more difficult because the majority of each protein would have to be deleted in order to produce such a mutant and these deletions are likely to affect E2F interactions with each pRb family member. A more detailed analysis of the D-type cyclin binding site in p130 and identification of the E2F binding site in p130 are first steps to constructing additional mutants of p130 which could then be tested in colony suppression assays. The definition and manipulation of additional functional domains within p130 will aid the further understanding of the function and regulation of p130.

Chapter 8. References

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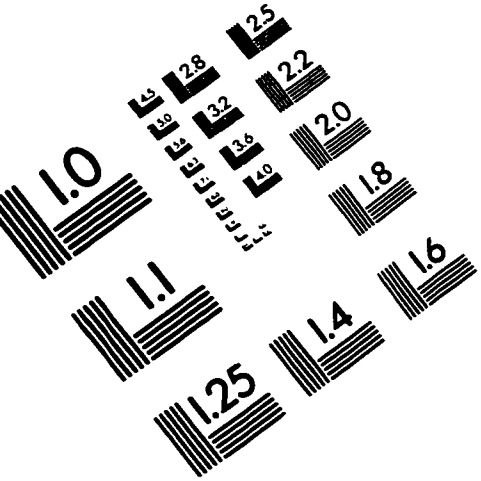
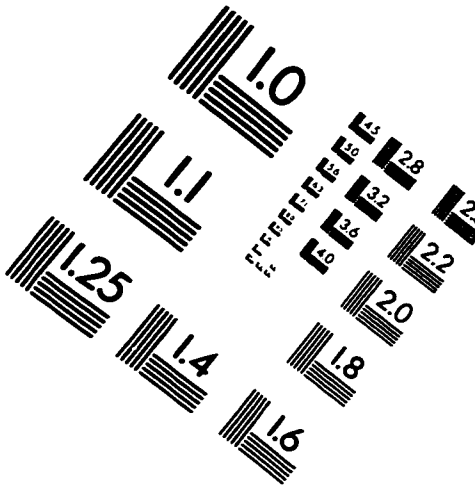
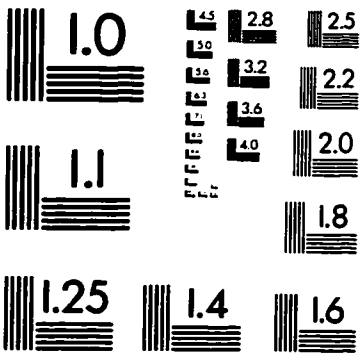
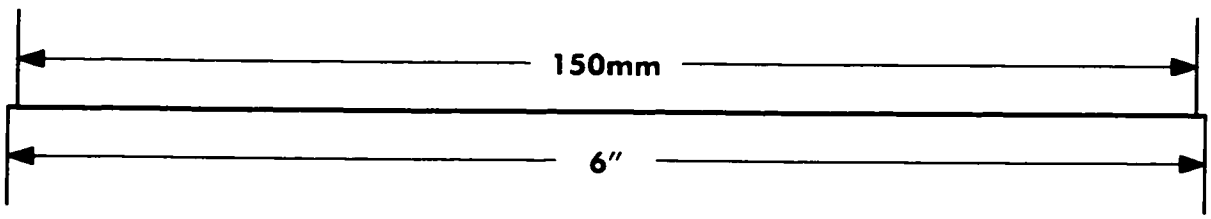
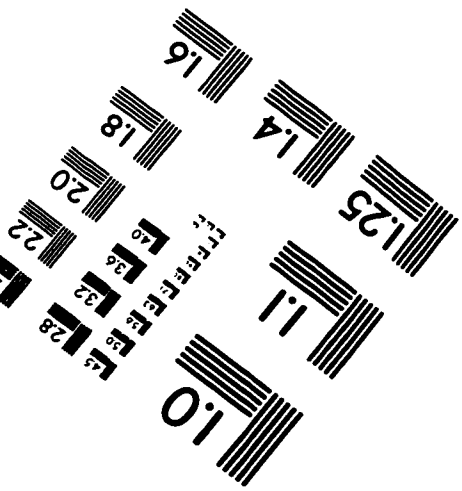
IMAGE EVALUATION TEST TARGET (QA-3)

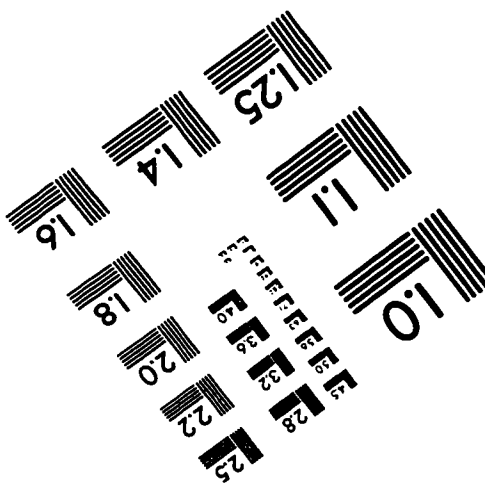












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