THE CHARACTERIZATION OF THE p34-NEU/ERBB2 INTERACTION

THE CHARACTERIZATION OF A NOVEL PUTATIVE SIGNALLING PROTEIN AND ITS INTERACTION WITH TYROSINE 1253 OF THE NEU/ERBB2 RECEPTOR TYROSINE KINASE

By

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ABSTRACT

The Neu/ErbB2 receptor tyrosine kinase has been implicated in the induction of mammary tumourigenesis. Both Ras-dependent and independent signalling downstream of activated Neu is believed to mediate cellular signalling that contributes cellular transformation in oncogenic Neu. Signalling from five *bona fide* autophosphorylation sites (termed sites A through E) in the carboxyl tail of the receptor mediate these signals to the cytosol. Of the four positive regulatory sites (B, C, D, E), only three (B, C, D,) signal through known signalling molecules. Site E, (Y1253) in Neu, though known to interact with DOKR, is essentially an orphan site. The discovery of a 34kD protein capable of associating to peptides corresponding to site E prompted investigations into the nature of site E signalling. Mass spectrometric analyses revealed that the 34kD protein is 2,4-dienoyl-CoA reductase (DECR1), a lipid metabolism protein typically localized to the mitochondria.

Investigations into this protein reveal that DECR1 is capable of associating with Neu site E in a tyrosine phosphorylation and sequence specific manner. Furthermore, analyses with site E second-site mutants (YE[APEY], YE[DPEY], YE[NAEY] and YE[NDEY]) show that DECR1 associates in a manner consistent to a PTB domain-containing protein. Analyses of amino acid sequence demonstrate the presence of a putative Bcl-domain in DECR1 suggestive of a role in apoptosis. Experiments into the apoptotic activity of DECR1 proved inconclusive. The examination of sub-cellular localization of Neu and DECR1 showed that the two proteins are found in both the mitochondrial and plasma membrane fractions. These results demonstrate that DECR1 may be a veritable binding partner for Neu Y1253.

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LIST OF ABBREVIATIONS USED

Bcl	B-cell lymphoma
BSA	bovine serum albumin
DECR1	2,4-dienoyl CoA reductase
DOKR	downstream of kinase related
D'IT	dithiothreitol
EC	enzyme commission
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
erbB	erythroblastoma beta
Erk	extracellular regulated kinases
FGF	fibroblast growth factor
Grb	growth factor receptor bound
GST	glutathione S-transferase
Her2	human ErbB2
IB	immunoblot
IP	immunoprecipitations
IPTG	isopropyl-P-β-thio-galactopyranoside
IRS	insulin receptor substrate
HRP	horseradish peroxidase
LTR	long terminal repeat
mAbs	monoclonal antibodies
MAPK	mitogen activated kinase
MMTV	murine mammary tumour virus
neu	neuroblastoma
NRG	neuregulin
nt	nucleotide(s)
P13'K	phosphatidylinositiol 3'kinase
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PDGFR	platelet derived growth factor
PLCγ	phospholipase C gamma
PMSF	phenylmethylsulfonyl fluoride

PPAR	peroxisomal proliferator activator receptor
PTB	protein tyrosine binding
PTyr	phosphotyrosine
PVDF	polyvinylidene diflouride
RTK	receptor protein tyrosine kinase
SH2	Src homology 2
Shc	SH2 and collagen homology proteins
SOS	son of sevenless
TBS	tris-buffered saline
TNE	tris, NP-40, EDTA
TUNEL	transferase-mediated dUTP nick-end labelling

CHAPTER 1

INTRODUCTION

1.1 Neu/ErbB2 in Mammary Tumourigenesis

Mammary cancer is a serious and prevalent disease in North American women. One in nine women will develop this disease in their lifetime. Naturally, the development of therapeutics requires the understanding of the basic molecular nature of breast tumourigenesis. Over the past several decades, multiple molecular/genetic signalling components have been identified in the genesis of primary mammary tumours. Of particular interest have been tyrosine phosphorylation cascades and the mitogenic pathway. The apex of these pathways includes receptor tyrosine kinases (RTKs). These membrane-bound molecules mediate extracellular signals to the cytosol. Subsequent signalling via phosphorylation cascades leads to the activation of the mitogenic signalling pathways and ultimately to cellular proliferation.

Of special importance to breast cancer is the epidermal growth factor receptor (EGFR) related protein Neu/ErbB2*. The Neu oncogene, possessing a naturally occurring activating point mutation in the transmembrane region, was originally isolated from rat neuroblastoma (Bargmann et al., 1986). Elevated expression of the human Neu/ErbB2 orthologue, Her2, is found in 20-30% of all human mammary carcinomas (Slamon et al., 1989). Furthermore, amplification and consequent over-expression of Her2 indicates a

* Neu is the rat orthologue of the mouse ErbB2 protein. Both proteins are identical in amino acid sequence.

poor clinical prognosis in breast cancer patients (Hynes and Stern, 1994; Mansour et al., 1994). Moreover, elevated ErbB2 expression is detected in many human invasive ductal carcinomas, but is infrequently observed in benign breast disorders such as hyperplasias and dysplasias implying that ErbB2 over-expression leads to aggressive tumour formation (reviewed in (Mansour et al., 1994)). Indeed, as interest in this gene has increased, therapeutic agents targeting Neu specifically have been developed. The monoclonal antibody Herceptin (Genentech) is one such proprietary agent that has been created to target Neu and demonstrates the importance attributed to the human orthologue, Her2, in human mammary cancer. However, it is from evidence obtained from transgenic mice that a large body of evidence implicating Neu in mammary cancers has been derived.

In mice, tumour development in mammary epithelium can be recapitulated by the mammary gland-specific over-expression of oncogenic Neu by using the mouse mammary tumour virus (MMTV) promoter to drive Neu transcription (Guy et al., 1992; Muller et al., 1988). Although histologically comparable human breast tumours demonstrate an over-expression of Neu, these tumours do not possess homologous oncogenic mutations found in the rat orthologue (Cardiff and Muller, 1993; Zoll et al., 1992). Indeed, mice over-expressing wild-type Neu develop focal tumours morphologically similar to human Her2 positive tumours and exhibit a longer onset than tumours generated by activated Neu (Guy et al., 1992). The observation that both tumour and normal mammary epithelium in these mice express similar levels of protein, and the long tumour latency suggests that over-expression is not a sufficient mechanism for tumourigenesis (Guy et al., 1992). Indeed, analyses of these tissues showed hyperphosphorylation in the tumour-bound Neu, and not in

the epithelial Neu (Guy et al., 1992) suggesting the acquisition of activating mutations during tumourigenesis. Consistent with this, somatic mutations have been shown to arise in the majority of tumours derived from these wild-type *neu* over-expressing mice (Siegel et al., 1994). Furthermore, these mutations represent in-frame deletions, insertions and point-mutations in the juxtamembrane region of Neu believed to be involved in its homo- and heterodimerization with other ErbB family members (Siegel et al., 1999). Interestingly, physiological levels of expression of these mutated forms of Neu can also reconstitute tumour development at an onset that is more rapid than wild-type over-expression alone (Siegel et al., 1999). Although no similar mutations have been yet been found in human Her2, the discovery of an alternate transformation-inducing Her2 splice variant possessing a 16 amino acid exon exclusion (Kwong and Hung, 1998) demonstrates strong similarity to the activating somatic mutations developed in mice (Siegel et al., 1999). Furthermore, recent transgenic experiments have shown that tumour formation can be induced without the concomitant over-expression of Neu. Knock-in mice generated to express activated Neu under endogenous transcriptional regulation in only the mammary epithelium are shown to develop tumours after long latencies (Andrechek et al., 2000). More importantly, tumour formation is accompanied by a variant 2-22 fold amplification and subsequent over-expression of the *neu* gene (Andrechek et al., 2000). Interestingly, this amplification event exhibits a similarity to *her2* amplification seen in human breast tumours (Slamon et al., 1987), suggesting these mice offer an accurate model of the genetic events preceding Neu-mediated tumour formation in humans.

Although it is widely acknowledged that Neu/ErbB2 can contribute to tumour formation by mediating disparate Ras-dependent and independent signals (Dankort et al., 2001a; Dankort et al., 2001b; Dankort et al., 1997; Luttrell et al., 1994), the issue becomes more complicated since Neu can act synergistically with other ErbB molecules by dimerizing with them (Sweeney and Carraway, 2000). Though Neu itself does not have a corresponding ligand, over-expression of known EGFR- or ErbB3/ErbB4-specific ligands have a potentiating effect on Neu dependent signalling (Sweeney and Carraway, 2000). Expression of the EGFR-specific ligand, EGF, and expression of Neuregulins specific for ErbB3 and ErbB4 leads to the transphosphorylation of Neu (Karunagaran et al., 1996). Conversely, the activation of Neu has effects upon the other ErbB members. In one study, ErbB3 has been shown to undergo up-regulation at the translational level during the induction of MMTV activated-Neu tumours (Siegel et al., 1999). Given ErbB3 plays a positive role in the recruitment and activation of PI3'K, (Soltoff et al., 1994) it can be inferred that ErbB3 is involved in the PFB/AKT survival pathway, contributing to Neu-mediated tumour progression by inhibiting apoptosis. Indeed, over-expression of both Neu and ErbB3 in bi-transgenic animals leads to accelerated tumour formation (Gillgrass, 2001).

Although other ErbB family members play an important role in Neu mediated tumour formation, it is Neu that factors in centrally. Ablation of Neu activity by mutagenic truncation of the carboxyl-terminus or sequestering Neu to the cytosol by single-chain antibody targeting suppresses MAPK activation by EGF and Neuregulins (Graus-Porta et al., 1995; Qian et al., 1994). Conversely, mutation of EGFR does not disrupt Neu

transphosphorylation or Neu-mediated mitogenic activation (Spivak-Kroizman et al., 1992; Wong et al., 1999).

1.2 Structure and Mechanism of Signalling by Neu and the EGFR Family

Neu/ErbB2 is a type I receptor tyrosine kinase belonging to the EGFR family of receptors comprising ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4 (Sweeney and Carraway, 2000). The domain structure within this receptor group follow a similar order but differ significantly in their exact placement and sequence. The extracellular amino-terminal domain consists of four defined regions: two ligand-binding domains (regions I and III), and two furin-like cysteine-rich regions (II and IV). Carboxyl-terminal to the extra-cellular domain is a hydrophobic region spanning the membrane through a single-pass alpha helix (Gullick et al., 1992). Downstream of this is the highly conserved tyrosine kinase domain, and following that is the carboxyl-terminal effector domain. Dimerization of the receptors occurs through regions I and III and is thought to be stabilized by the furin-like regions II and IV (Sweeney and Carraway, 2000). In Neu, mutations in these cysteine-rich regions, either by deletion or addition of cysteines, or through in-frame deletions can lead to constitutive activation of the receptor by ligand-independent homo-aggregation via the formation of intermolecular di-sulphide bonds (Siegel et al., 1994; Siegel and Muller, 1996).

The kinase region of the EGFR family is highly conserved (63-83% amino acid homology) and is involved in the ligand-dependent transphosphorylation of coupled receptors. Interestingly, all the ErbB members other than ErbB3 are known to possess kinase activity (Guy et al., 1994). Although the reason for this is not known, it is believed that

ErbB3 serves as a platform for tyrosine phosphorylation and subsequent docking for signalling molecules through heterodimerization with other ErbB members (Guy et al., 1994). Activation of these RTKs is thought to occur via ligand-dependent dimerization resulting in the rearrangement of individual monomers in order to transphosphorylate the activation loop of the opposed receptor's kinase domain (Hubbard et al., 1998). Phosphorylation of this loop lifts the stearic barrier inhibiting access to the kinase active site and increases the enzymatic efficiency of the kinase domain to allow transphosphorylation of tyrosines on the carboxyl terminus (C-term) of the trans receptor. It is through the phosphorylation of these C-term tyrosines that the receptor signalling is defined.

Although still poorly understood, differential phosphorylation of the effector domain mediates signal specificity. Furthermore, it is through differential ligand binding that this effect is achieved (Tzahar et al., 1997). Ligand binding specificity can be categorized into several classes based on preferred binding; EGFR (EGF, TGFα, amphiregulin), EGFR and ErbB4 (HB-EGF, BTC, epiregulin), ErbB3 and ErbB4 (NRG1, NRG2), and ErbB4 alone (NRG3, NRG4) (reviewed in (Sweeney and Carraway, 2000). Neu has no known ligand. Although each ligand has a preferred specifity, it is also believed that EGFR family ligands have a secondary broader specificity and in this way exhibit receptor bivalency (Tzahar et al., 1997). It is through this mechanism that preferred ErbB2 receptor heterodimerization (Graus-Porta et al., 1997) is shown to occur. In fact, differential ligand binding not only induces differential receptor heterodimerization but also affects the phosphorylation of specific tyrosines in the effector domain (Olayioye et al., 1998) of EGFR and ErbB2, thereby affecting the repertoire of signalling molecules that can be recruited to the

receptor complex. Similar effects have been seen in ErbB4 homodimers activated by various NRG ligands (Sweeney et al., 2000). Thus, receptor dimerization is a necessary step in receptor activation. Receptor activation in turn leads to differential phosphorylation of the effector domain tyrosines enabling Neu to mediate disparate signals downstream.

The phosphorylation of specific tyrosines in the effector domain (dubbed autophosphorylation sites) creates docking sites with specificities for subsets of signalling molecules. These residues are part of larger consensus regions that bind signalling proteins containing modular phospho-tyrosine-binding SH2 and PTB domains. Generally, PTB domains bind phosphotyrosines with a specificity that is dictated by the sequence one to five amino acids preceding the tyrosine. Typically, the consensus sequence is XΨXNPpY, where Ψ is a hydrophobic residue and pY represents phosphotyrosine (Margolis, 1999). The PTB domain is further sub-divided into the Shc-type PTB domain, which recognizes the consensus domain with high specificity, and the IRS-type PTB domain whose specificity is broader. Although the Shc-type domain is well characterized, the IRS-type domain is frequently degenerate and may be hard to identify by sequence homology (Margolis, 1999).

In contrast to the PTB domain, SH2 domain specificity is determined by residues one to five amino acids carboxyl-terminal to the phosphotyrosine. Although the SH2 domains themselves are highly conserved, the specificity with which they bind is broader. There appear to be three distinct consensus motifs: pYhhI/P (Abl, Nck), pYΨXΨ (p85 of

PI3'K, PLCγ), and pYEEI (Src family), where h indicates a hydrophilic residue (Songyang et al., 1993).

The binding of PTB/SH2-containing signalling molecules to the effector domain of RTKs has various effects. In some cases it offers a way to alter a protein's subcellular localization (Sos), to recruit adapter proteins (Grb2), to phosphorylate targets (PLCγ), or to activate enzymatic activity (Pawson, 1995). Thus, through activation and consequent recruitment and/or activation of various signalling molecules at its effector domain, Neu mediates cellular signalling.

1.3 Neu Signals Through Distinct Autophosphorylation Sites

As mentioned previously, receptor activation leads to the phosphorylation of various tyrosines located in the effector domain. Variable tyrosine phosphorylation states within the carboxyl tail function to potentiate, or attenuate, mitogenic signaling through the binding of downstream molecular targets such as Grb2, and Shc (Batzer et al., 1994). It is believed that the ability of Neu/ErbB2 to form both homo- and heterodimers with other EGFR family members is key to the inherent heterogeneity of ErbB2 phosphorylation states (Schlessinger, 2000). However, despite the extensive progress made to understand Neu/ErbB2 signaling, the some particulars of the structure/function relationship remain to be resolved.

Analyses of the Neu/ErbB2 carboxyl-terminus has led to the discovery and partial characterization of five *bona fide* tyrosine phosphorylation sites (Dankort et al., 1997), Y1028, Y1144, Y1201, Y1226/7, and Y1253 (termed sites A through E). Interestingly, cell culture studies have revealed that ablation of individual sites within oncogenically activated

Neu (NeuNT; V664E) does not result in a significant difference in transforming potential (Dankort et al., 1997). However, the removal of all five phosphorylation sites in NeuNT, the so-called Neu tyrosine phosphorylation deficient (NYPD) mutant, reduces the receptor's transformation capacity to 8% of NeuNT (Dankort et al., 1997). Restoration of individual phosphorylation sites (the so-called add-back mutants) reveals the disparate signaling nature of the individual sites. Of the five autophosphorylation sites, B through E act as potentiators of mitogenic signaling. Each corresponding add-back mutant demonstrates equal or greater transformation activity (Dankort et al., 1997) relative to NeuNT. The remaining site, Y1028 attenuates transforming activity (Dankort et al., 1997).

Unlike the other potentiating sites, site C is unique insofar as it is weakly transforming (108% relative to NeuNT) and that it signals independently of Ras (Dankort et al., 2001a). Experiments conducted by this lab have shown that signalling through site C is not ablated by the Ras antagonist Rap1A (Dankort et al., 2001a) and that it likely signals through the CrkII adapter molecule. Of the remaining sites that signal through Ras, B and E are particularly interesting. Both add-back mutants (YB, and YE) show increased transformation potential compared to NeuNT alone (Dankort et al., 1997). YB and YE mutants also demonstrate aberrant, possibly metastatic, cellular morphology in soft agar assays (Dankort et al., 1997). Furthermore, YB transgenic mice have been shown to undergo rapid metastasis, a characteristic rarely observed in NeuNT mice (Dankort et al., 2001b). The YE mutant, demonstrating similar morphology and transformation capacity to YB in cell culture (Dankort et al., 2001b; Dankort et al.,

1997), may likewise reveal itself to be an equally potent activator of metastatic tumours in transgenic mice.

Interestingly, experiments performed by our lab have shown that proliferative signaling downstream of sites B and D signal through Grb2 and Shc adapter molecules respectively (Dankort et al., 2001b). Mutation of certain critical residues (the +2 position in Y1144; -3 in Y1227, corresponding to SH2 and PTB consensus motifs respectively) surrounding sites B and D ablate binding of the cognate signaling molecules despite intact tyrosines (Dankort et al., 2001b). Furthermore, transformation efficiency in these 'second-site' mutants is down-regulated significantly, suggesting Grb2 and Shc mediate proliferative signaling downstream of these sites. Similar experiments using DOKR, a 56kDa docking protein of the insulin-regulated substrate/downstream of kinase (IRS/DOK) family, as a binding partner for site E have failed to show transformation-dependent binding in the case of the transformation impaired site E mutants [DPEY] and [APEY] (Dankort et al., 2001b). Indeed, DOKR has been shown to play a negative regulatory role in EGFR signaling (Jones and Dumont, 1999) consistent with the observation that DOKR binding does not correlate with cellular proliferation.

1.4 Tyrosine 1253 Binds p34 in a Fashion Coincident with Neu-mediated Transformation

What differentiates site E from other mitogenically activating phosphorylation sites in Neu/ErbB2 is that hitherto Y1253 remains an essentially orphan site in the context of cellular proliferation. Thus far, no well-characterized signaling proteins have been shown to

interact or mediate signalling through this site in a transformation-dependent manner. Despite this, YE cell-lines transform at 114% of NeuNT levels or higher, and YE transgenic mice develop palpable tumours as early as six weeks (Dankort and Muller, unpublished). Both these observations suggest that site E is a significant player in Neu/ErbB2 mediated transformation. Since mitogenic signaling must be physically mediated through the cytosol, this necessitates that site E transduce through at least one intracellular effector.

In order to address this question, an attempt to affinity purify a site E specific signalling molecule was undertaken (Dankort et al., 2001a). To this end, previous experiments conducted by our lab showed that the association of an unidentified 34kDa protein with peptides corresponding to site E [biotin-FEGTPTAENPE(pY)LGLDVPV] is positively correlated with transformation in rat fibroblasts (Dankort et al., 2001a). Additionally, experiments using dephosphorylated peptides and peptides containing second-site mutations in the PTB-binding consensus domain of site E showed that p34 binding is phospho- and sequence-specific (Dankort et al., 2001a). Subsequent mass-spectrometric analysis of p34 has revealed a protein heretofore uncharacterized within the context of signal transduction. This protein, 2,4-dienoyl-CoA reductase (EC 1.3.1.34; referred to herein in its abbreviated form DECR1), is a mitochondrial/peroxisomal lipid reductase typically involved in linoleic acid degradation (Hirose et al., 1990). DECR1 is an auxiliary enzyme involved in the beta-oxidation of even numbered double-bonds in saturated fatty acids. In particular, p34 converts 2,4-dienoyl-CoA into 3-trans-enoyl-CoA (Kimura et al., 1984), (reviewed in Hiltunen et al., 1993). In its active form, DECR1 is found as a homotetramer localized to mitochondria, and possibly the peroxisome,

although peroxisomal reductase activity has not yet been conclusively attributed to the *decr1* gene product (Hakkola et al., 1989; Kimura et al., 1984).

1.5 Experimental Rationale

Although the protein in question is unknown within the context being investigated, the fact that *in vitro* association of p34 correlates with transformation from site E, suggests that p34 is an excellent candidate molecule for mediating signalling from site E. Characterization of the association and signalling downstream of this molecule could provide important insight into the function of Neu/ErbB2 mediated signalling and subsequently the mode of action of Neu-mediated tumour progression. Furthermore, the characterization of this putative signalling molecule would provide an unprecedented look at what appears to be a unique example of signal transduction that, from a strictly scientific perspective, justifies investigation.

To achieve this goal, three main lines of experimentation were carried out. The first objective was to clone DECR1 and confirm that the association between p34 and site E satisfies the condition in the intact Neu receptor. Further, the directness of the association had to be determined. Second, the possibility of an artefactual association had to be eliminated. To prove this, co-localization studies had to be carried out in order to show that Neu and p34 localize to the same sub-cellular compartment. Finally, the question of biological relevance of the association between p34 and Neu had to be answered. Clues to a potential function of this molecule were found in the amino acid sequence. The revelation that p34 possesses a putative Bcl domain (discussed in section 3.3) implies a

role in the apoptotic machinery of the cell. Investigations to resect this were undertaken. Together, these three lines of research were undertaken to address the role of tyrosine 1253 in intracellular signalling.

CHAPTER 2

MATERIALS AND METHODS

2.1 DECR1 cDNA isolation

Initial DECR1 cDNA isolation was performed by reverse-transcription (RT). RNA was isolated from Rat1 fibroblasts by trizol (Invitrogen) extraction (according to manufacturer's protocols), isopropyl alcohol precipitated, washed three times with 70% ethanol and resuspended in diethylene pyrocarbonate (DEPC)-treated water. 5μg of total RNA, and a 2.5μM final concentration of reverse primer (AB16903; primer sequences shown in table 2.1) were used in a standard RT procedure (Sambrook, 1989). One μl of RT reaction (approximately $1/20^{th}$ volume) was used to PCR amplify the reverse-transcribed target. Target was amplified using the high-fidelity thermophilic DNA polymerase Accurase (DNAmp) using primers AB16901 and AB17221 at a final concentration of 40nM. After 25 rounds of amplification, DNA was run out on a low-melting point agarose gel. A gel fragment corresponding to the estimated size of cDNA migration (approximately 1.1 kb) was then excised and melted at 65°C. Two μl of DNA-containing agarose (approximately $1/50^{th}$ volume) was re-amplified by the same PCR. DNA band corresponding to desired cDNA was cloned by TOPO/TA cloning into the pEF6/V5-His vector (Invitrogen) in strict accordance with the manufacturer's protocol. Clones were sequenced to verify presence of DECR1 cDNA.

After multiple attempts to clone DECR1 by the former method, it was revealed that the process was introducing numerous mutations into the cDNA desired. In order to remedy this issue, a cDNA library approach was employed to isolate a mutant-free DECR1 cDNA. Plaque lifts were performed using the UniZapXR rat liver cDNA library (Stratagene) according to the manufacturer's protocol. To identify positive clones, the 1.1kb RT-generated DECR1 cDNA (BamH1/Not1 fragment) was used as a random-primed ^{32}P dCTP labelled probe (probe labelled according to Sambrook, 1989). After four rounds of plaque lifting, six positive phage clones were circularised into the pBluescriptSK phagemid via *in vivo* excision and sequenced (as per manufacturer's instruction). Of the six clones, four contained the entire cDNA and all contained at least one mutation.

2.2 DNA constructs

To clone DECR1 into an expression vector, the cDNA was engineered via PCR-directed mutagenesis to include a 5' BamH1 and 3' Not1 restriction site using AB16901 and AB17221. The template used was the afore-excised pBluescriptSK phagemid containing the DECR1 cDNA. The following cDNA was then subcloned in frame with an amino terminal V5-His epitope tag residing in the pEF1/V5-His(C) plasmid (Invitrogen). Mutations present in the cDNA were corrected by a three-way subcloning procedure using cDNA fragments from multiple DECR1 clones. Specifically, two sequentially correct fragments (BamH1/ClaI and ClaI/NotI) from two different clones were simultaneously cloned back into pEF1/V5-His(C). For the generation of DECR1

expression vector for use in stable cell-line derivation, 10ng of the corrected DECR1 cDNA was amplified by five cycles PCR (to minimize mutation) and cloned by the TOPO/TA cloning technique into the pEF6/V5-His TOPO expression vector. The former constructs are referred to herein as pEF1/DECR1 and pEF6/DECR1 respectively. For reciprocal tagging assays, DECR1 cDNA was cloned in-frame into the pcDNA4/TO-Myc-His vector (Stratagene) as a BamH1/NotI fragment.

DECR1 deletion mutants were designed by a combined site-directed mutagenesis/restriction digest approach. Three deletion mutants were created; a 5' truncation, Bcl-domain mutant and 3' truncation. To generate the 5' mutant, a BamH1 restriction site was introduced into position 290 of the DECR1 cDNA. This position delineates the start of the region coding for the putative Bcl-domain within the cDNA. Introduction of the restriction site was followed by restriction digest using BamH1. The remaining construct was gel purified and self-ligated to generate a 5' deletion mutant. The 3' mutant was similarly generated by introduction of a NotI site at position 570 corresponding to the terminus of the region coding for the putative Bcl-domain. The Bcl-domain mutant was derived by generating two SpeI sites at positions 290 and 570 (demarcating the start and stop of the Bcl-domain coding region), excising the intervening sequence and then self-ligating the remaining construct. All restriction enzyme insertions were generated by PCR mutagenesis (primers listed in table 2.1). These mutants are referred to herein as DECR1Δ5', DECR1Δ3' and DECR1ΔBcl.

The pEF1/DOKR plasmid was created by excising the DOKR cDNA from pcDNA3.1/DOKR (Dankort et al., 2001a) as an EcoR1/NotI fragment and cloning it into

pEF1/V5-His. All neu cDNA constructs used herein were generated by David L. Dankort (Dankort et al., 1997) with the exception of NeuNTYE(A-3) (NTYE[APEY]) and NeuNTYE(D-3) (NTYE[DPEY]) which were re-derived. NeuNTYE(A-3) and NeuNTYE(D-3) were generated by site-directed mutagenesis using the QuikChangeXL mutagenesis kit (Stratagene) following manufacturer's guidelines. The template used for mutagenesis was pJ4Ω/NTYE (Dankort et al., 1997). Mutants were generated and then subcloned into pcDNA3.1 (Stratagene). All remaining activated *neu* add-back cDNA's were subcloned into pcDNA3.1 via HindIII/EcoR1 ligation. pcDNA3.1 *neu* add-back plasmids (i.e. pcDNA3.1/NeuNTYA, pcDNA3.1/NeuNTYB) are referred to in the following manner: pcNTYA, pcNTYB; and so forth in accordance with the appropriate designation for the specified add-back site. All newly generated mutants and clones were sequenced to ensure correct genetic sequence. T3 and T7 oligonucleotides were used to prime all sequencing reactions for all pEF-based plasmids. All pcDNA based constructs were sequenced using T7 and pcDNA3.1/BGH reverse primer. Sequencing was performed by Brian Allore and Alison Cowie at the MOBIX central facility at McMaster University, Hamilton Ontario, Canada. A list of all oligonucleotides used for mutagenesis, PCR amplification and reverse transcription is shown in Table 2.1.

2.3 Transient transfection of 293T cells

Cells were seeded-out $2x10^5$ cells per 60mm dish, or $5x10^4$ cells per 35mm dish or single well of a 6-well dish. Cells were allowed to grow overnight in 10% FBS/DMEM. Media was subsequently aspirated and cells were washed gently with phosphate-buffered

Table 2.1 List of oligonucleotides used.

	DECR1			HindIII.EcoR1
AB16901	forward	CCC**GGATCC**AAGCTTAACATGGCGCTGCTGGCCCGTGCG		
AB16903	RT reverse	CCC**AGATCT**GAATTCTCCATGTATATAGTCCCTAAAC		Bgl2. EcoR1
AB17221	reverse	CGCGGCCGCACCGGAGCCTTTTGTCTTTCTGAT		NotI
	DECR1 deletion mutants			
AB29257	5' BamH1	GCGAGCAGGAATATT**GGATCCATG**AAAGCTACTGCAGAAG	Sense	BamH1
AB29258	Insertion	C TTCTGCAGTAGCTTTCATGGATCCAATATTCCTGCTCGC	antisense	
AB29259	5' SpeI insertion	GCAGGAATATTGATGTACTAGTAGCTACTGCAGAAGAG	Sense	SpeI
AB29260		CTCTTCTGCAGTAGCTACTAGTACATCAATATTCCTGC	antisense	
AB29261	3' SpeI insertion	GCTAATTAAAGCACAGAAACTAGTAGCCTTTCTTGCTATC	Sense	SpeI
AB29262		GATAGCAAGAAAGGCTACTAGTTTCTGTGCTTTAATTAGC	antisense	
AB29263	3' NotI insertion	CAGAAAGGAGCTGCCGCGGCCGCTATCACTACGATC	Sense	NotI
AB29264		GATCGTAGTGATAGCGGCCGCGGCAGCTCCTTTCTG	antisense	
	Neu siteE second-site add-backs			
AB25381		AGGCCTAGGTACTCAGGGTCCTCTGC	Antisense	
AB25382	NeuNTYE(D-3)	GCAGAGGACCCTGAGTACCTAGGCCT	Sense	N→D
		A E **D** P E Y L G		
AB25383		AGGCCTAGGTACTCAGGGGCCTCTGC	Antisense	N→A
AB25384	NeuNTYE(A-3)	GCAGAGGCCCCTGAGTACCTAGGCCT	Sense	
		A E **A** P E Y L G		

saline (PBS; 140mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4, pH7.6). Following, cells were transfected using 6μg total DNA and 36μl lipofectamine (Invitrogen) per transfection per 60mm dish or 3μg total DNA and 9μl lipofectamine per 35mm/single well of a six-well dish. Preparation of liposomes was carried-out according to manufacturer's protocols. Cells were incubated with liposomes for 4-6 hours then aspirated and replaced with 10% FBS/DMEM. Cells were split into 100mm dishes the following day and harvested on the third day following initial transfection.

2.4 Generation of stable cell lines

In order to generate stable DECR1 expressing and control fibroblasts, Rat1 cells were transfected with pEF6/DECR1 and pEF6/LacZ. Rat1 cells were seeded-out at $2x10^5$ cells per 60mm dish and grown overnight in 10% FBS/DMEM. Media was subsequently aspirated and cells were washed twice with PBS. Cells were transfected using 1μg total DNA and 3μl lipofectamine per transfection and incubated for two hours. Preparation of liposomes was carried-out according to manufacturer's protocols. After two hours, media was aspirated and replaced with 10% FBS/DMEM. On the third day following transfection, 10% FBS/DMEM containing 4μg/ml blasticidin replaced normal growth media. Selection was continued for two weeks, replacing selection media every two days. Twenty-four clones were picked and seeded into a 24-well dish. Selection was continued for another five days or until cells were confluent. Surviving clones were split into duplicate 60mm dishes one of which was used to assay transgene expression by protein blotting.

2.5 Protein extraction

Media was aspirated from culture dishes and cells were washed twice with PBS. Cells were harvested with a cell scraper and resupended in 500µl PBS. Tubes were spun for 1 minute at 1000g to pellet cells. Cells were lysed on ice for 20 minutes in either TNE (50mM Tris-HCl [pH8.0], 150mM NaCl, 10mM NaF, 2mM EDTA, 0.1% Nonidet-P-40) or PLCγ (50mM HEPES [pH 7.5], 150mM NaCl, 10% Glycerol, 1.5mM $MgCl_2$, 1mM EGTA, 10mM NaP_2O_7, 10mM NaF, 1% Triton-X100) lysis buffer supplemented with 1mM sodium orthovanadate (Na_3VO_4), 10µg/ml aprotinin/leupeptin. Cellular debris was precipitated by micro-centrifugation and supernatant was removed for processing or short-term storage at –20°C. Protein concentration was determined by the Bradford assay (Bio-Rad).

2.6 Immunoprecipitation

250-500 µg of protein were immunoprecipitated by addition of 0.2-0.5 µg desired antibody and rotated at 4°C 2h or overnight. One to two hours before washing, 30µl of 1:1 protein G sepharose bead slurry (Amersham-Pharmacia) was added. Five ice-cold washes (500-1000µl volumes) were conducted using lysis buffer in which precipitation was carried-out. All samples were incubated on ice during all intervening steps. Residual wash buffer was removed by aspiration using a 25gauge needle and beads were

resuspended in 20µl 2X SDS-PAGE loading buffer (125mM Tris-HCl [pH6.8], 4% SDS, 20% glycerol, 2mM DTT, 0.1% bromophenol blue).

For immunoprecipitations using biotinylated peptides a peptide pre-blocking procedure was employed. One µg of peptide was pre-bound to 10µl equilibrated/compacted streptavidin-sepharose for 30 minutes in TBST at 4°C. Pre-bound peptides were washed three times in TBST then blocked with 1ml of 1mM biotin, for 30min. Peptides were washed thrice again and then blocked with 1ml of 5mM streptavidin. Peptides were washed and used to precipitate 20µl of reticulocyte lysate (containing protein of interest). Reaction volume was 500µl of PLCγ lysis buffer.

2.7 Protein blotting

Protein samples were denatured in 2X SDS-PAGE buffer (immunoprecipitations) or in 1x SDS-PAGE buffer (1-20µg straight lysate) 5-10 minutes at 100°C. Samples were allowed to cool and spun briefly to collect condensed water from sample. Samples were loaded into vertical slab gel apparatus and run through denaturing polyacrylamide gel (125mM Tris-HCl [pH 8.8], 1%SDS, 8-10% acrylamide depending on resolution desired) at 150 V. Protein was transferred and immobilized to polvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) by wet-transfer method for 1-2 hours at 1.36 mA in transfer buffer (25mM Tris, 190mM glycine, 20% methanol). Membranes were rinsed in TBS and blocked for an hour in either 3% BSA in TBS or 3% skim milk in TBS depending on antibody used. Antibody incubations were done at room temperature for two hours or overnight at 4°C. Membranes were rinsed in TBS, and washed thrice for 10

minutes in TBS or TBST (TBS, 0.1-0.2% Tween-20) and subsequently incubated in secondary antibody for one hour. Membranes were rinsed and washed as before. Visualization of target proteins was conducted via enhanced chemiluminescence (ECL, Amersham) and exposed to Kodak X-OMAT AR film.

2.8 Antibodies

A list of primary antibodies and their dilutions is located in Table 2.2. All secondary antibody incubations for protein blotting were done using either anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Jackson Labs). Secondary antibodies for protein blotting were diluted 1:2500 in Tris-buffered saline (TBS; 20mM Tris-HCl [pH 7.6], 150 mM NaCl), 3% skim milk and 0.1% Tween-20 (TBST-milk). Secondary antibody for immunofluorescence was anti-mouse IgG conjugated to rhodamine (Sigma) and diluted 1:400 in 3% BSA in PBS.

2.9 *In vitro* translation and recombinant protein purification

For *in vitro* translation, the TNT coupled transcription/translation reticulocyte system (Promega) was used. One μg of target plasmid was used to translate protein. The reaction was carried-out using the manufacturer's protocol. Translated protein was run on SDS-PAGE and immunoblotted for the V5 epitope to verify presence of DECR1 protein.

For recombinant protein purification, two 60mm plates of 293T cells were transfected with pEF1/DECR1, split into a 150mm dish and grown for three days in 10% FBS/DMEM. Cells were harvested mechanically, washed twice in PBS and lysed in

Table2.2 List of antibodies used.

Protein/Epitope	Antibody	Dilution/Block Buffer
Bad	Mouse mAb (BD Transduction Labs)	1:500/TBST-milk
Bax	Mouse mAb (BD Transduction Labs)	1:500/TBST-milk
Bcl-2	Mouse mAb (BD Transduction Labs)	1:500/TBST-milk
Bcl-x	Mouse mAb (BD Transduction Labs)	1:500/TBST-milk
β-gal	Rabbit pAb (Invitrogen)	1:5000/TBS-milk
Cytochrome C	Rabbit pAb (Clonetech)	1:500/TBST-milk
DokR	Rabbit pAb H-192 (Santa Cruz)	1:1000/TBS-milk
Grb2	Rabbit pAb C-23 (Santa Cruz)	1:2000/TBS-milk
Hsp90	Mouse mAb F-8 (Santa Cruz)	1:1000/TBS-milk
Myc-tag EQKLISEEDLN	Mouse mAb (Invitrogen. clone 9E10.2)	1:5000/TBS-BSA
Neu	Mouse mAb Ab3 (Oncogene Science)	1:1000/TBS-milk
	Mouse mAb Ab4 (Oncogene Science) – used for immunoprecipitation exclusively	N/A
	Rabbit mAb C-18 (Santa Cruz)	1:1000/TBS-milk
PARP	Mouse mAb (Biomol. clone C-2-10)	1:2000/TBST-BSA
Phospho tyrosine	Mouse mAb PY20 (Transduction Labs)	1:1000/TBST-BSA
V5-tag GKPINPLLGLDST	Mouse mAb (Invitrogen) for protein blot	1:5000/TBS-BSA
	Mouse mAb (Invitrogen) for immunofluorescence	1:250/PBS-BSA

500µl of native lysis buffer (PBS containing 300mM NaCl, 10mM imidazole, 1% Triton X-100, protease inhibitors and 1mM sodium orthovanadate) for twenty minutes at 4^0C. Cellular debris was spun out and supernatant was incubated with 80µl of 1:1 slurry of lysis buffer-equilibrated Ni-NTA-sepharose beads for 2hours at 4^0C. Beads were washed on ice in five volumes of wash buffer (PBS containing 300mM NaCl, 20mM imidazole and 1% Triton X-100). All wash volumes were kept for protein analysis. Recombinant DECR1 was eluted first in elution buffer-250 (PBS containing 300mM NaCl, 250mM imidazole and 1% Triton X-100) and second in elution buffer-500 (PBS containing 300mM NaCl, 500mM imidazole and 1% Triton X-100). Eluates and remaining beads were kept for protein analysis. The expression of DECR1 was determined by immunoblotting and by either Coomassie Blue or silver nitrate staining (Ausubel, 1998).

2.10 Direct blotting

Purified His-tagged DECR1 (150ng) was spotted onto nitrocellulose membrane and air-dried. Membranes were blocked in 3% BSA in 1% TBS-Tween (TBST-BSA) for one hour. Membranes were then rinsed twice with TBST and incubated with peptides (1µg/ml) in TBST-BSA overnight at 4°C. Blots were washed four times for 10 minutes with TBST and incubated with a 1:1000 dilution of streptavidin-HRP (Amersham) in TBST-BSA for an hour. Blots were washed again, dried and exposed. Peptide sequences were as follows: pYE, [biotin-FEGTPTAENPE(pY)LGLDVPV], pYE[DPEY], [biotin-FEGTPTAE**D**PE(pY) LGLDVPV]; pYC, [biotin- FAFGGAVENPE(pY)LVPREGT].

Dephosphorylation of peptides was catalysed by calf intestinal alkaline phophatase (CIAP) treatment (as per Ausubel, 1998). Briefly, 10μg of peptide was incubated with 30 units of CIAP at 30°C for 30 minutes in dephosphorylation buffer (50Mm HEPES[pH7.5], 1mM $MgCl_2$,). Reaction was stopped by the addition of EDTA to a final concentration of 5mM and incubating peptides at 65°C for 15 minutes.

2.11 PARP-cleavage apoptosis assay

Rat1-derived stable and transiently transfected cells were seeded-out at $2x10^5$ cells per 60mm dish and grown overnight in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS). Media was aspirated and cells were washed with PBS and treated with either 500nM staurosporine or 40μM etoposide in 0.1%FBS/DMEM for varying lengths of time. Both suspended and adherent cells were harvested mechanically at regular intervals, spun-down for 1 minute at 1000g and washed twice with ice cold PBS. Remaining supernatant was removed and cells were lysed with boiling 2X SDS-PAGE loading buffer supplemented with protease inhibitors and incubated at 100°C for 5 minutes. Lysate was passed through a 26 gauge needle five times to shear DNA. 20-30μl of lysate was loaded into an SDS-PAGE gel and processed according to standard protein blotting procedure. Anti-PARP antibody was used to assess PARP cleavage.

2.12 Immunofluorescence

Rat1-derived stable cells were grown to 50% confluence on glass cover slips and fixed for 15 minutes in 3% paraformaldehyde in PBS. Cells were washed three times with PBS and permeabilized in 0.2% Triton X-100 in PBS for 10 minutes. Cells were washed with PBS again and blocked in 1% goat serum in PBS for 30 minutes. Cells were washed again and incubated with primary antibody (anti-V5) for one hour at room temperature of overnight at 4°C. Cells were washed three times in PBS and then incubated in the dark with secondary antibody for an hour at room temperature. Cells were mounted onto a slide with mounting medium containing 0.6% 1,4-diazabicyclo[2.2.2]octane to prevent photo-bleaching. Cells were viewed under U.V. light on a Zeiss immunofluorescence scope.

2.13 Sub-cellular fractionation

Cells were harvested mechanically, washed in twice in PBS, and split into two equal volumes. One volume was used for mitochondrial fractionation and the second for plasma membrane isolation.

Mitochondrial isolation was performed in accordance with the ApoAlert Cell Fractionation Kit (Clontech). Briefly, cells were incubated 10 minutes at 4°C in cell fractionation buffer (proprietary) containing protease inhibitors, DTT and 1mM sodium orthovanadate. Cells were homogenized using 50 strokes of a 2ml Dounce homogeniser and aliquotted into microfuge tubes. Homogenate was centrifuged at 700g for 10

minutes. Supernatant was collected and re-spun. The second supernatant was then further spun at 10,000g for 25 minutes. The supernatant containing the cytosol was collected for assaying and the pellet was re-suspended in lysis buffer (TNE or PLCγ) containing protease inhibitors and 1mM sodium orthovanadate.

Plasma membrane fractionation was carried-out as follows: Cells were incubated 10 minutes at 4°C in hypotonic cell-lysis buffer (10mM HEPES [pH8], 10mM NaCl, 1mM KH_2PO_4, 5mM $NaHCO_3$, 1mM $CaCl_2$, 0.5mM $MgCl_2$,) supplemented with 5mM EDTA, 10μg/ml protease inhibitors and 1mM sodium orthovanadate. Cells were homogenized using 50 strokes of a 2ml Dounce homogeniser and aliquotted into microfuge tubes Homogenate was centrifuged at 10,000g for 10 minutes to clear nuclei. Supernatant was re-suspended in sucrose buffer (10mM Tris-Hcl [pH7.6], 300mM sucrose, 1mM EDTA) and spun in an SW41 rotor at 25,000 rpm for 30 minutes. Pellet was re-suspended in 50μl lysis buffer (TNE or PLCγ) containing protease inhibitors and sodium orthovanadate.

CHAPTER 3

RESULTS

3.1 Introduction

As discussed earlier, Neu is a potent mitogenic activator. Specifically, by signalling through four of five autophosphorylation sites (B-E), Neu is capable of both Ras-dependant and independent mitogenic potentiation (Dankort et al., 2001a). Further, it has been shown that three of these transformation-inducing sites (sites B, C, D) signal via known signalling components, Grb2, CrkII and Shc respectively. One site however remains elusive in its mode of signal transduction (Dankort et al., 2001a). Tyrosine 1253, site E, is known to associate with the docking protein p56DOKR (Dankort et al., 2001a), a protein shown to down-regulate mitogenic activity in EGFR (Jones and Dumont, 1999). The revelation that DOKR does not associate with Neu in a transformation dependent manner and the failure to show that it associates with Neu directly (Dankort et al., 2001a) prompted this lab to search for novel effector proteins for Y1253. Through whole-cell lysate peptide-affinity screens, a potential binding partner, p34, was revealed (Dankort et al., 2001a). p34, now identified to be 2,4 dienoyl-CoA-reductase precursor protein (EC 1.3.1.34) provides the starting point for the next generation of Neu/ErbB2 research.

Although unusual, precedent has been set regarding metabolically associated

enzymes exhibiting a dual metabolic/signaling function. Recently, the mitochondrial oxidoreductase, *apoptosis inducing factor* (AIF) has been shown to induce apoptosis in cell culture through its interaction with the pro-apoptotic proteins of the Bcl-2 family (Susin et al., 1999). Furthermore, upon activation by apoptosis inducing agents, AIF has been shown to localize to the cytoplasm where it has been correlated with the induction of stage I apoptosis (Susin et al., 2000). If shown to be veritable, the interaction between DECR1 and Neu may prove to be a previously undescribed link between components of lipid metabolism and tumour formation. The role of DECR1 in Neu mediated signal transduction is the subject of exploration in this thesis.

3.2 2,4 dienoyl-CoA-reductase (DECR1) Associates Directly with Neu/ErbB2 Through an Interaction at Y1253 (site E)

To assess DECR1's role in site E mediated signal transduction, the p34 cDNA was cloned. Through a dual RT/plaque lift procedure several cDNAs were isolated. Initial amino acid substitution mutations in the coding region required additional cloning to correct the sequence (see materials and methods section 2.1). Upon confirmation of sequence by BLAST analysis (National Center for Biotechnological Information), protein affinity experiments aimed at resecting DECR1 function by *in vitro* techniques were conducted.

This line of experimentation was further bifurcated in order to address either direct or indirect DECR1/Neu association. To demonstrate an initial protein-protein interaction between site E and DECR1, p34 was *in vitro* translated and assayed through affinity purification using biotinylated peptides corresponding to site E, both phosphorylated and

unphosphorylated (pYE-pep and YE-pep respectively). On account of a residual 'stickiness' of the cloned protein to agarose and sepharose beads, initial experiments had not been able to show a direct association between DECR1 and pYE-pep. To address this problem, a peptide prebinding/biotin blocking technique was employed to lower p34's non-specific association to streptavidin-sepharose (see materials and methods). Data from this experiment indicate that DECR1 associates with peptides corresponding to site E both directly and in a phospho-specific fashion (Figure 3.1a). Further, an NPXpY motif corresponding to another phosphorylation site (site C) within Neu does not complex with p34, demonstrating specificity to only Y1253. However, taking into consideration that the initial p34 'pull-down' was conducted using peptides used in the preceding experiment, further corroboration using a whole-cell lysate system and whole receptor was required.

Since this initial data supported the hypothesis that p34 signals exclusively through Y1253, additional experiments were carried-out to resolve the specificity with which DECR1 interacted with autophosphorylation sites in the whole Neu receptor. Indirect association studies using cellular systems were employed in order to help resolve this question. Immunoprecipitation experiments were conducted using lysates derived from 293T cells co-transfected with DECR1 and the various NeuNT add-back mutants. These data, along with those from similar experiments, using cells co-transfected with p34 and four NeuNT-YE second-site mutants have revealed site-specific binding of DECR1 to the NTYE mutant (Figure 3.1b). Furthermore, the second-site mutant experiments narrowed-down p34 binding specificity to what likely conforms to the PTB-binding consensus motif (NPEY) constituting site E (Figure 3.2). These latter data however, were obtained using

highly stringent conditions suggestive of a strong, direct association between DECR1 consistent with the peptide data. Interestingly, it appears that alteration of protein folding is more important to p34 binding than charge as is evident by the presence of weak binding of both the YE[NDEY] and YE[DPEY] mutants to DECR1. Typically, the requirement of a β-turn in the target peptide supersedes charge requirement for PTB binding (Margolis, 1999). These results coupled to the fact that DECR1 site E interaction is dependent on tyrosine phosphorylation imply that p34 binding may be coordinated through a canonical PTB interaction. Not surprisingly however, amino acid sequence analysis by the Simple Modular Architecture Research Tool (SMART, Letunic et al., 2002; Schultz et al., 1998) failed to identify a PTB domain in p34. Despite this, the notion that association is achieved through a PTB region should not be eliminated, as PTB domains are known to be degenerate and exhibit variable binding specificities. Indeed, a subset of IRS-PTB domains foregoes the phosphotyrosine-binding requirement altogether (Margolis, 1999).

As an alternative to PTB-coordinated binding, DECR1 may be binding via complex formation by interacting with ancillary factors governing PTB specificity. To elucidate whether p34 is indeed binding directly, or through other factors, a direct association assay was devised. Rather than employing a 'far-western' approach, which relies on associations between denatured proteins, an alternate non-denaturing method was developed. DECR1 was grown in 293T cells, and purified by nickel-column purification. The purified protein was run on a vertical slab apparatus, silver stained, and immunoblotted to ascertain purity. Purified protein was spotted on a nitrocellulose membrane, air-dried and probed with biotinylated peptides corresponding to site E, both phosphorylated and dephosphorylated,

site C (containing an NPXpY motif) and the transformation defective YE[DPEY] peptide. Presence of the peptides was then determined by HRP-conjugated streptavidin. What the data show, is that consistent with the observations in the whole cell lysate experiments, p34 binds site E phospho-specifically, and in a sequence-dependent manner (Figure 3.3). Interestingly, residual binding is also observed in pYC-pep and pYE[DPEY]-pep but not in YE-pep, consistent with the notion that tyrosine phosphorylation is a more important determinant than NPXY consensus for DECR1 binding. Similar data were also obtained in the second-site mutant immunoprecipitations discussed above, suggesting that this specificity hierarchy observed is real. Furthermore, the direct blots reveal that the association between p34 and Neu site E is direct. This evidence is further corroborated by the fact that the direct association experiments were carried-out under high stringency conditions (350mM NaCl, 1% Triton X-100) capable of dissociating most cellular complexes (Ausubel, 1998). Unfortunately, what these experiments do not indicate, is whether there is a clear relationship between DECR1 binding and the relative strength of transformation of the NeuNT-YE second-site mutants. At this time, p34-binding data show no direct correlation between transformation and DECR1/ErbB2 receptor association. Despite this, these data do provide an insight into the nature of the p34-Neu interaction.

Considering the evidence, the possibility that DECR1 is interacting though a PTB domain remains viable. Although no SH2 or PTB domain has been identified in p34, two possibilities remain. First, that there exists a previously unidentified phosphotyrosine-binding site, or secondly, DECR1 is in fact interacting through a poorly conserved or partial PTB region not currently identifiable through homology searches. In order to

attempt to resolve this unknown, three preliminary DECR1 deletion mutants were generated. The first mutant (DECR1Δ5') possessed a deletion encompassing the entire 5' region up until the putative Bcl domain (DECR1 domain structure is discussed in section 3.3). The second mutant (DECR1ΔBcl), had its Bcl domain deleted and the final mutant contained a C-terminal truncation consisting of the removal of the region carboxyl-proximal to the putative Bcl domain. All mutants were constructed with an in-frame V5 tag for detection in immunoblotting (Figure 3.4).

3.3 DECR1 Exhibits Sequence Homology to the BH1, BH2 and BH3 Regions of the Bcl Domain of the Bcl-family of Apoptotic Proteins but Its Role as an Apoptotic Factor Remains Unresolved

Although the above results do not provide irrevocable evidence that p34 is a *bona fide* binding partner for site E, these data fortify the interesting possibility that p34 may function in a dual metabolic/signaling role. To further narrow-down the specific role(s) played by p34 in the context of receptor signalling, functional analyses were required. Naturally, the demonstration that p34 binds ErbB2 site E in a sequence specific manner raises intriguing questions as to the biological activity of DECR1 *in vivo*. Interestingly, it has been shown that the activation of the peroxisome proliferator-activator receptor (PPAR) family of transcription factors by hypolipidemic agents such as clofibrate, the J series of prostaglandins, eicosopentaenoic acid (EPA), or thiazolidinediones (TZDs) not only up-regulates transcription of enzymes required in lipid degradation, including DECR1, (Madsen et al., 1998; Vaagenes et al., 1998) but also potentiates apoptosis in various tumour-cell lines

(Clay et al., 1999). Furthermore, recent evidence has also demonstrated that PPARγ activation suppresses ErbB2 and ErbB3 phosphorylation induced by NRG1 and NRG2 (Pignatelli et al., 2001). Indeed, not only does p34 associate with ErbB2 site E directly and in a phospho-specific manner, it also demonstrates an uncanny sequence homology to the Bcl domain of the Bcl-family members of apoptotic regulators (Figure 3.5). This alignment data shows that the Bcl-domain contained in p34 encompasses the BH3, BH2 and BH1 regions, which within Bcl proteins, are sufficient for the oligomerization of various pro- and anti-apoptotic Bcl-family members (reviewed in (Wang, 2001; Zornig et al.)). Figure 3.6 demonstrates the structure of the putative Bcl domain relative to other known regions in the DECR1 amino acid sequence. It can be seen that the Bcl domain bisects the short-chain dehydrogenase domain, suggesting that Bcl-mediated dimerization would likely ablate p34 enzymatic activity by occlusion of the active site of DECR1 (Hiltunen et al., 1993). Furthermore, the putative Bcl domain was discovered using an outlier homologue search-engine suggesting that p34 is more likely a functional homologue whose function evolved in parallel to the Bcl-family of proteins. Although a tenuous link, the possibility that DECR1 plays a direct role in regulating apoptosis through interaction with Bcl-family members could not go untested.

In order to determine if indeed p34 is involved in Bcl-family member oligomerization, immunoprecipitation experiments aimed at resolving whether DECR1 complexed with Bcl members were undertaken. Although, initial immunoprecipitation experiments were promising, subsequent experimentation using both stable and transient cell lines proved inconsistent. However, these results must be discussed as the corroborating

homology evidence, though circumstantial, is too persistent to ignore. Of the five Bcl-family members tested (Bad, Bax, Bcl-2, Bcl-x and Mcl-1) two proteins, Bcl-2 and Bad, showed potential complex formation with p34 (Figure 3.7). Bcl- 2 is the prototypical anti-apoptotic Bcl member, while Bad is a BH3 domain-only antagonist of anti-apoptotic Bcl-family members. Over-expression of Bcl-2 has been shown to confer apoptotic resistance to cells subjected to various stimuli including Myc-inducible apoptosis, and chemotherapeutic agents (reviewed inZornig et al.). Further, compartment specific expression of Bcl-2 has been shown to attenuate either endoplasmic reticulum- (ER) or mitochondria-specific chemotherapeutic agent induced apoptosis (Annis et al., 2001). Conversely, Bad, a BH3 domain-only protein has been shown to suppress anti-apoptotic activity by its dimerization with Bcl-2 and Bcl-$_{XL}$. The role of p34 in apoptosis, if any, may be to modulate the apoptotic regulation through its interaction with these proteins.

Thus, in order to ascertain DECR1's role in apoptosis regulation, Rat1 fibroblast cells stably expressing p34 were derived (Figure 3.8) and treated with the drug staurosporine and assayed for poly-ADP-ribose phosphate (PARP) cleavage (Figure 3.9a). Although technically a success, these experiments failed to elucidate whether p34 plays an active role in programmed cell death. It may be that p34 requires 'activation' by the presence of activated ErbB2. Indeed, DECR1 is tyrosine phosphorylated (Figure 3.11) and it remains a possibility that the kinase activity of Neu is responsible for this state. To this end, various activated ErbB2 add-back mutant/p34 co-stable cell-lines were being derived in order to test this hypothesis. Unfortunately, due to time constraints, and technical issues, the cell-lines were never derived.

As an adjunct, the use of other apoptosis-inducing agents was considered. Staurosporine, though a potent inducer of apoptosis, may in fact act in a pathway parallel to the one that being investigated. Various data suggest that staurosporine, a broad specificity kinase inhibitor, acts in both a caspase dependant and independent manner (Belmokhtar et al., 2001), possibly circumventing Bcl-regulation altogether. In addition, recent data concerning compartment-specific expression of Bcl-2 implies staurosporine exacts its pro-apoptotic effect via the endoplasmic reticulum (Annis et al., 2001), foregoing regulation at the mitochondria altogether. To counteract this experimental flaw, the mitochondrion-specific agent etoposide (Annis et al., 2001; Soucie et al., 2001) was employed in conjunction with transiently transfected Rat1 stable cell-lines. Unfortunately, due to high cell mortality, likely due to the use of cytotoxic transfection reagent and unstable expression of the Neu transgene in the Rat1 stable cell-lines, the experiments could not be carried-out. Instead, experiments using just the DECR1 stable cell-lines were conducted in the hopes that they would provide some insight into apoptotic regulation by DECR1. Despite efforts to narrow down the timeline suitable for allowing a change in PARP-cleavage to be resolved (down from eighteen hours to six), there was no difference in apoptotic activity observed (Figure 3.9b). Presumably, further experimentation along these lines, employing co-stable p34/Neu-addback cell-lines lines, may prove to be fruitful.

3.4 Further Functional Characterization of DECR1 within the Context of Neu Activation

However stimulating, demonstration of phospho-specific binding of DECR1 to Neu, sequence homology, and inconclusive apoptosis data must be further corroborated with biological evidence that DECR1 is involved in signaling downstream of site E. Indeed, drawing a correlation between an 'activation' of p34 and site E association would further fortify the argument that DECR1 plays a role in signalling downstream of Neu. Since p34 must form a homotetramer to be enzymatically active (Kimura et al., 1984), it would seem logical to investigate the multimerization of DECR1 in various NeuNT add-back backgrounds. Unfortunately, all attempts to assess this state by non-denaturing polyacrylamide electrophoresis were unsuccessful. Subsequent data however, using reciprocal-tagging (V5- and Myc-tagged) immunoprecipitation have demonstrated that DECR1-V5 can in fact co-immunoprecipitate DECR1-Myc in the presence of any of the following activated Neu receptors: NeuNT-NYPD, NeuNT-YB, and NeuNT-YE. Further, this co-precipitation is observed in 293T cells lacking activated Neu altogether (Figure 3.10). Although these data do not show a Neu-dependent oligomerization of DECR1, it is reassuring that the V5 antibody does not cross-react with the Myc tag, and that co-precipitation is likely the result of a true oligomerization event (Figure 3.10). Although this result is not especially provocative, it is not entirely surprising considering that the mature p34 is capable of tetramerization on its own in the mitochondria. This result, however, is informative insofar as it demonstrates that neither the V5 nor the Myc

epitope tags interfere with the formation of an intact and (presumably) active DECR1 holoenzyme.

Since this experiment was equally inconclusive at elucidating the role of p34 in signalling, another indirect approach at identifying the role of a putative activation of p34 by Neu was attempted. If, as suggested, ErbB2 is required to activate DECR1, then tyrosine phosphorylation of p34 could be used as a marker for p34 activation. To this end, cells were transfected with p34, and NeuNT, NeuNTNYPD or NeuNTYE, or p34 alone. The cells were treated with etoposide and assayed for p34 phosphorylation. Interestingly, the experiment showed that p34 is tyrosine phosphorylated, suggesting a potential role in signalling (Yaffe, 2002). Unfortunatley, the state of DECR1 phosphorylation is dependant neither on presence of Neu or on treatment by an apoptotic inducer (Figure 3.11). Clearly, until the role of p34 is deduced by methods other than biochemical means, the phenomena revealed in the preceding two sections cannot be properly interpreted.

3.5 DECR1 Co-localizes with Neu/ErbB2

A critical question requiring resolution concerns the localization of p34 in respect to Neu. Clearly, a putative signalling molecule cannot interact with its target protein if it is located in a separate sub-cellular compartment. Naturally, the fact that DECR1 has not only been characterized in the context of fatty-acid catabolism in the mitochondria and (possibly) the peroxisome (Hakkola et al., 1989), but the fact that p34 possess a mitochondrial import peptide in its first 34 amino acids (Hirose et al., 1990) introduces

pessimism to the notion that p34 can in any way function in Neu signalling. However, since *decr1* is a nuclear gene, its polypeptide must be localized to the cytosol prior to mitochondrial import. In addition, current dogma suggests that there is no evidence to suggest that polypeptides cannot form properly folded functional molecules before appropriate localization (Matouschek et al., 2000). Although mitochondrial localization of p34 clearly occurs, this does not preclude the localization of a portion of the species to other compartments. Indeed, computational analysis by the predictive *TargetP* program (Emanuelsson et al., 2000) indicates that although the amino-terminal signalling peptide likely localizes p34 to the mitochondria, it does so only with a reliability class (RC) of three (where RC is likeliness as measured by the size of the difference [diff] between the highest and the second highest output scores, and RC3 is: $0.600 > \text{diff} > 0.400$) indicating potential leakiness in p34 targeting.

To resolve this issue, empirical evidence was required to substantiate the hypothesis that DECR1 interacts with Neu. To assay Neu/p34 co-localization, immunofluorescence was attempted in stable cell-lines derived from Rat1 cells. Unfortunately, since the co-stable cell lines were never derived (see section 3.3), stable cell-lines expressing DECR1-V5 and endogenous Neu were used. Although single-antigen staining using rhodamine-conjugated V5 antibody was successful, the use of fluorescein-conjugated antibodies for the detection of Neu could not be optimised due to technical issues. Equally problematic, co-staining for mitochondria could not be assessed since fluorescein-conjugated anti-V5 could not be seen along side mitochondria stained with MitoTracker Red. Despite this, single-stained p34 stable cell-lines showed a

punctate cytosolic staining pattern (Figure 3.12a) consistent with but not exclusive to membrane localization (R. Perry, *pers. comm.*). In fact, the staining pattern is also reminiscent to mitochondrial staining. Faced with inconclusive data, biochemical means of resolving this problem were attempted; namely through sub-cellular fractionation.

To assay molecular localization, 293T cells were transfected with DECR1, and co-transfected with either NeuNTNYPD or NeuNTYE and fractionated by density gradient centrifugation. The results indicate that indeed DECR1 is not only localized to the mitochondria, but also to the plasma membrane where it can interact freely with Neu (Figure 3.12b). Though it could be argued that there is cross-contamination between fractions, closer inspection of the controls reveals that the mitochondrial fraction contaminates neither the plasma membrane nor the cytosol. Further, it appears that it is only the soluble cytosol that contaminated both the plasma membrane and mitochondrial fractions. Intriguingly, this negligible contamination cannot explain an over-abundance of Neu in the mitochondria. The possibility that Neu over-expression was inducing aberrant targeting was considered, however, previous immuno-histochemisrty studies have shown that Neu can be detected in the mitochondria in both neoplastic and normal mammary and kidney tissue (De Potter et al., 1989; Quatacker et al., 1990). Furthermore, electron microscopy study has revealed that mitochondrial Neu is found in the inner membrane (De Potter et al., 1989) where it should require multiple targeting signals to be localized (Rassow and Pfanner, 2000). Curiously, no other literature about ErbB family members and mitochondrial localization or about signalling from other membrane-bound compartments seems to exist. It remains to be tested, but the localization of Neu and

putative signalling partner p34, may prove an interesting discovery worthy of further investigation.

Figure 3.1. DECR1 binds ErbB2 at Y1253. A 100ng of *in vitro* translated reticulocyte lysate containing DECR1 protein was incubated with 1μg of pre-bound, pre-blocked peptides (phospho-YE, phospho-YC, and de-phosphorylated YE). Affinity precipitates were washed four times in modified PLCγ lysis buffer and run out on an SDS-PAGE gel. Presence of p34 was detected via C-terminal V5-epitope tag and assayed via anti-V5 monoclonal antibody (Invitrogen).

B. 293T cells were transfected with p34 and co-transfected with pcDNA/NeuNT, NeuNT-derived add-back mutants or vector. Cells were allowed to grow for 24-48 hours and were serum starved overnight. Lysates were harvested and used for analysis. **i** 300μg of each lysate was immunoprecipitated at 4^0C for three hours using anti-V5 antibody (Invitrogen) in the presence of 1mM orthovanadate. Immunoprecipitates were washed five times using modified PLCγ lysis buffer supplemented with 1mM orthovanadate. Washed beads were processed by electrophoresis and blotted by standard protein blotting techniques. Blots were probed using anti-Neu antibody (Ab3; Jackson Labs). 10μg of each lysate was run on a gel, blotted and probed for the presence of Neu and C-terminally-tagged DECR1 (anti-V5 antibody; Invitrogen) (**ii**, **iii**). Black arrowheads correspond to protein of interest in each panel.

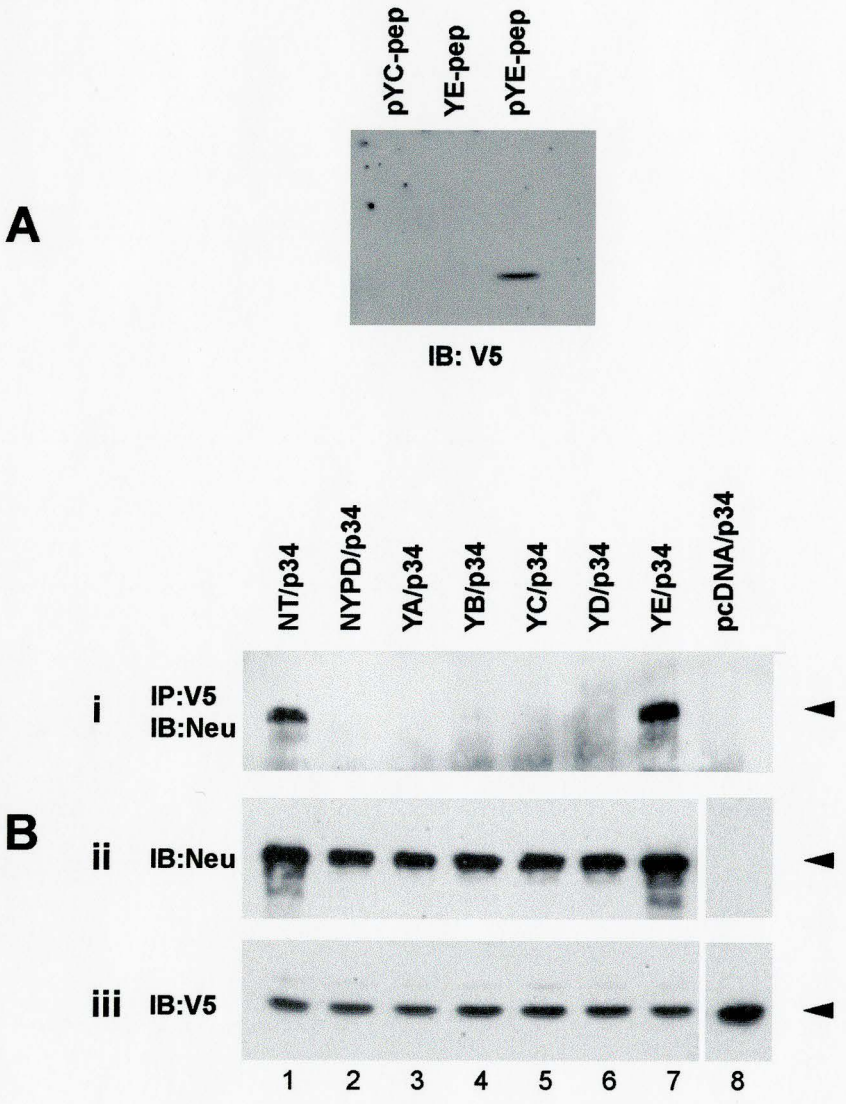

A
pYC-pep
YE-pep
pYE-pep
IB: V5
B
NT/p34
NYPD/p34
YA/p34
YB/p34
YC/p34
YD/p34
YE/p34
pcDNA/p34
i
IP:V5
IB:Neu
ii
IB:Neu
iii
IB:V5
1
2
3
4
5
6
7
8

Figure 3.2. Mutated YE second-site residues impair binding of DECR1 to Y1253 in the intact receptor. 293T cells were transfected with DECR1 and co-transfected with pcDNA/NeuNT-YE, NeuNT-NYPD, or the various transformation defective NeuNT-YE second-site mutants. Cells were allowed to grow 24-48h, and were serum starved overnight. Cells were harvested using modified PLCγ lysis buffer. Lysates were assayed for levels of Neu and p34 (**A**, **B**). 300μg of each lysate was immunoprecipitated at 4^0C for three hours using anti-V5 antibody in the presence of 1mM orthovanadate. Immunoprecipitates were washed five times using modified PLCγ lysis buffer supplemented with 1mM orthovanadate. Washed beads were electrophoresed and blotted by standard protein blotting techniques. Blots were probed using anti-Neu antibody (**C**). p34 displayed no variation in Y1253 affinity when immunoprecipitated under standard conditions (**Ci**). Addition of non-ionic detergent (1% Tween-20) to immunoprecipitation conditions resulted in varied abrogation of p34 binding to YE second-site mutants (**Cii**).

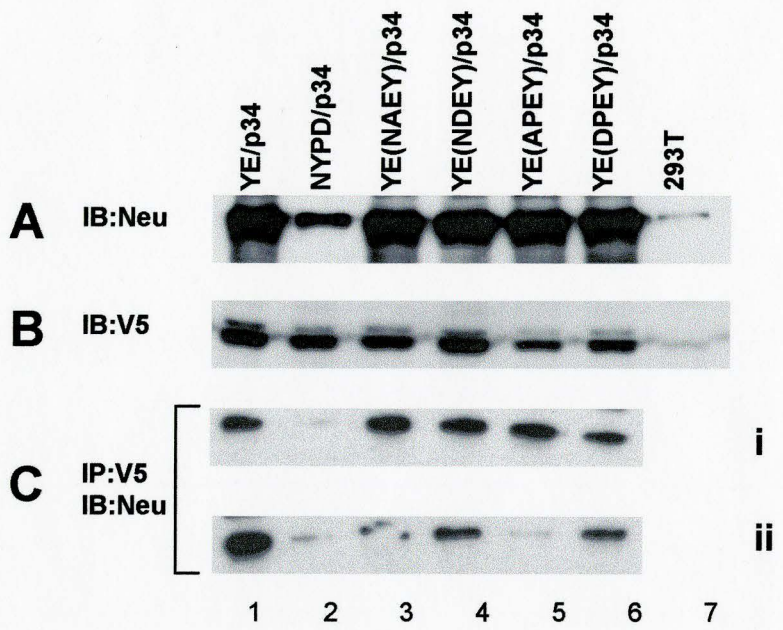

YE/p34
NYPD/p34
YE(NAEY)/p34
YE(NDEY)/p34
YE(APEY)/p34
YE(DPEY)/p34
293T
A
IB:Neu
B
IB:V5
C
IP:V5
IB:Neu
i
ii
1
2
3
4
5
6
7

Figure 3.3. DECR1 interacts with site E directly and in a sequence and phospho-specific manner. 293T cells were transfected with pEF1/DECR1, pooled and lysed in native lysis buffer. Recombinant protein was pre-bound to Ni-NTA-sepharose, washed and eluted. Panel **A** shows immunoblot of purified recombinant p34. Each lane represents 30µl volume of wash, eluate or diluted beads. Lanes 1-4 show wash volumes used for p34 purification. Lanes 5 and 6 show p34 eluted with buffer-250 and buffer-500 respectively. Lane 7 contains post-elution beads. Panel **B** shows silver stain of 1µg whole-cell lysate (lane 1) and 1µg of buffer-250 eluted p34 (lane 2).

In order to demonstrate direct binding, of p34 to Neu site E, 150ng of purified protein containing DECR1 (buffer-250 fraction) was spotted onto nitrocellulose and probed with various phosphorylated peptides. Panel **C** shows 1µg of YE peptide pre- and post dephosphorylation. Peptide was dephosphorylated using CIAP or mock reaction, spotted onto nitrocellulose membrane and probed with α-phosphotyrosine antibody. Panel **D** shows direct binding between immobilized DECR1 and various peptides corresponding to variations on the site E NPXpY motif. DECR1 is seen to interact directly with phosphorylated peptide corresponding to site E, but not to dephosphorylated YE peptide. Weak binding was seen in the mutagenized non-consensus YE[DPEY] peptide , and in YC peptide.

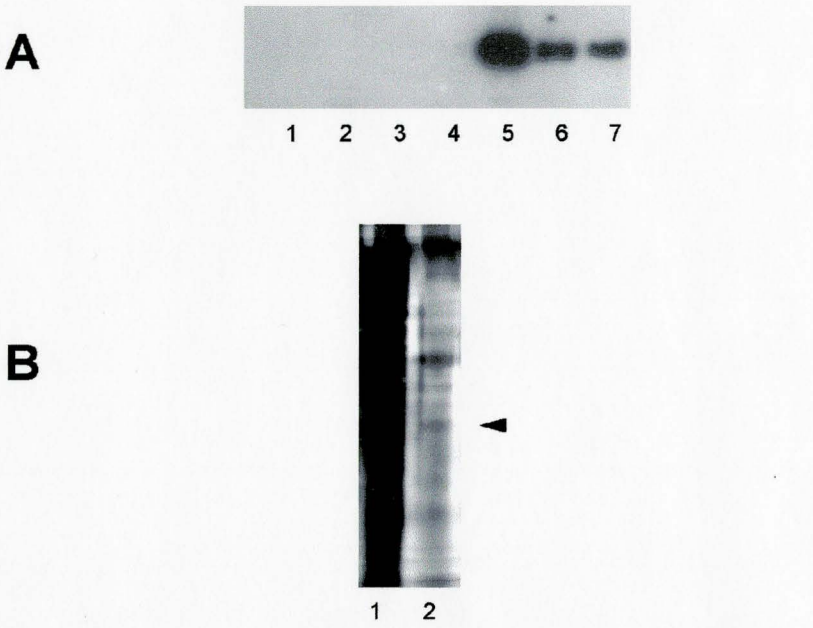

A
1 2 3 4 5 6 7
B
1 2
C
pYE peptide
pYE peptide dephos.
D
probe:
pYE
YE
pYE(DPEY)
pYC

Figure 3.4. DECR1 deletion mutants were generated. Mutants were generated using PCR-directed mutagenesis and cloned into pEF1/V5-His(C). In order to detect proteins by immunoblotting, mutant cDNAs were cloned with an in-frame V5-His tag. Five μg of transfected 293T lysates were electrophoresed and immunoblotted with α-V5 antibody. Lanes 1, 2, and 3 show DECR1Δ5', DECR1ΔBcl and DECR1Δ3' respectively. Mutant proteins corresponded to expected size. Black arrow indicates a molecular weight of 30kD, and grey arrow indicates 24kD.

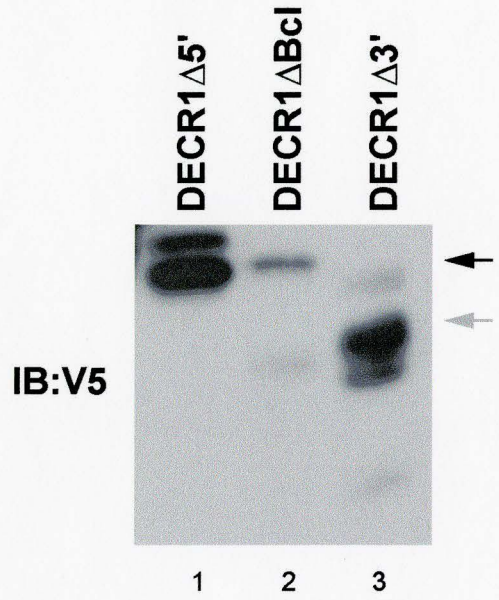

DECR1Δ5'
DECR1ΔBcl
DECR1Δ3'
IB:V5
1
2
3

Figure 3.5 DECR1 demonstrates amino acid sequence homology to the Bcl-domain (BH1, BH2 and BH3) of Bcl-family member proteins. The alignment of the Bcl domains of various Bcl family members against the putative Bcl domain of DECR1 is shown. Coloured bars indicate the degree of conservation amongst aligned residues. Amino acids below the bars correspond to the majority of residues identified as consensus from the alignment. Boxed areas show critical residues characteristic of Bcl domains; the letters in red indicate the nature of amino acids located in those regions. It should be noted that DECR1 satisfies the required domains in all but one case (pos.47). Alignment generated by *Lasergene* (DNASTAR Inc.) using the CLUSTAL method.

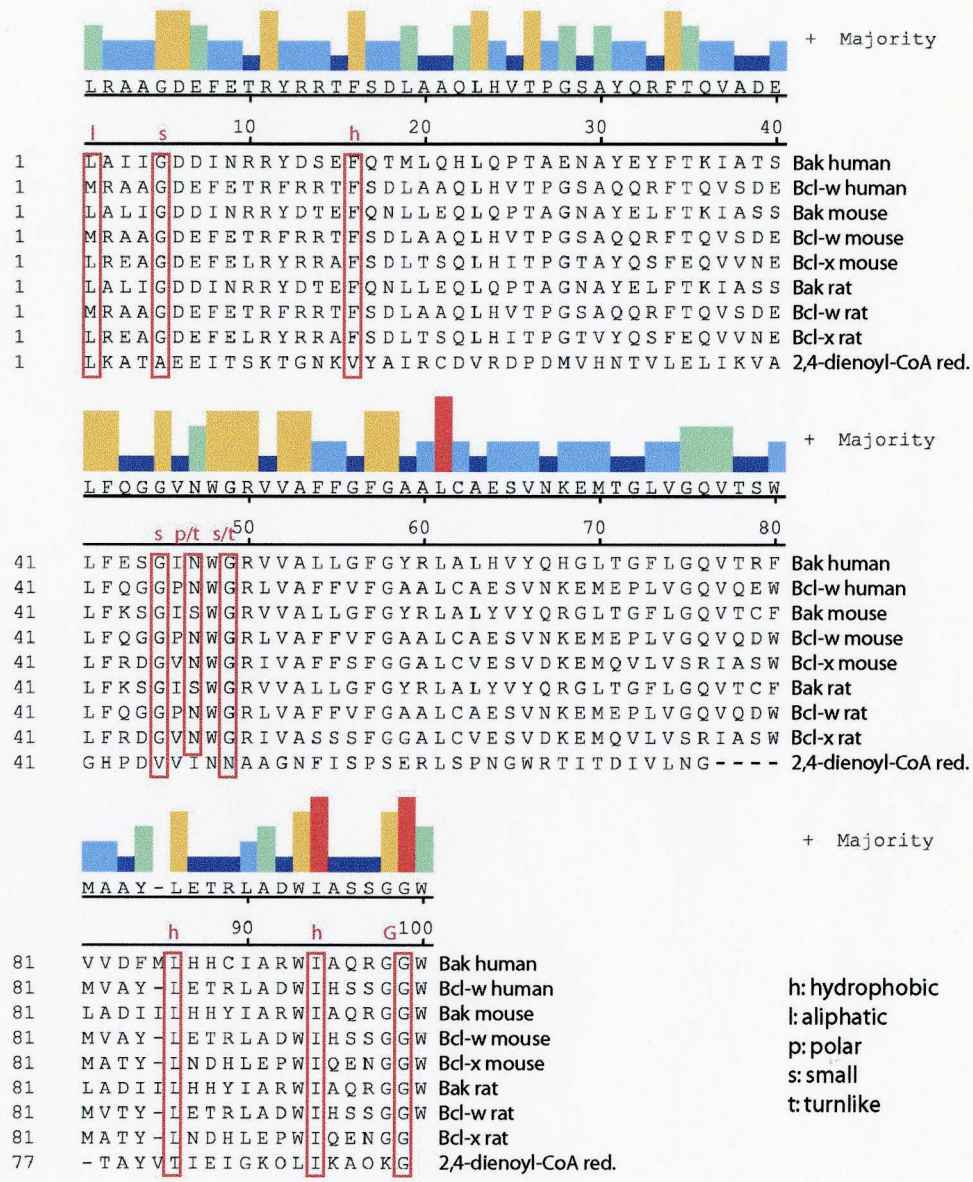

+ Majority
LRAAGDEFETRYRRTFSDLAAQLHVTPGSAYQRFTQVADE
l s 10 h 20 30 40
1 LAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATS Bak human
1 MRAAGDEFETRFRRTFSDLAAQLHVTPGSAQQRFTQVSDE Bcl-w human
1 LALIGDDINRRYDTEFQNLLEQLQPTAGNAYELFTKIASS Bak mouse
1 MRAAGDEFETRFRRTFSDLAAQLHVTPGSAQQRFTQVSDE Bcl-w mouse
1 LREAGDEFELRYRRAFSDLTSQLHITPGTAYQSFEQVVNE Bcl-x mouse
1 LALIGDDINRRYDTEFQNLLEQLQPTAGNAYELFTKIASS Bak rat
1 MRAAGDEFETRFRRTFSDLAAQLHVTPGSAQQRFTQVSDE Bcl-w rat
1 LREAGDEFELRYRRAFSDLTSQLHITPGTVYQSFEQVVNE Bcl-x rat
1 LKATAEEITSKTGNKVYAIRCDVRDPDMVHNTVLELIKVA 2,4-dienoyl-CoA red.
+ Majority
LFQGGVNWGRVVAFFGFGAALCAESVNKEMTGLVGQVTSW
s p/t s/t 50 60 70 80
41 LFESGINWGRVVALLGFGYRLALHVYQHGLTGFLGQVTRF Bak human
41 LFQGGPNWGRLVAFFVFGAALCAESVNKEMEPLVGQVQEW Bcl-w human
41 LFKSGISWGRVVALLGFGYRLALYVYQRGLTGFLGQVTCF Bak mouse
41 LFQGGPNWGRLVAFFVFGAALCAESVNKEMEPLVGQVQDW Bcl-w mouse
41 LFRDGVNWGRIVAFFSFGGALCVESVDKEMQVLVSRIASW Bcl-x mouse
41 LFKSGISWGRVVALLGFGYRLALYVYQRGLTGFLGQVTCF Bak rat
41 LFQGGPNWGRLVAFFVFGAALCAESVNKEMEPLVGQVQDW Bcl-w rat
41 LFRDGVNWGRIVASSSFGGALCVESVDKEMQVLVSRIASW Bcl-x rat
41 GHPDVVINNAAGNFISPSERLSPNGWRTITDIVLNG---- 2,4-dienoyl-CoA red.
+ Majority
MAAY-LETRLADWIASSGGW
h 90 h G 100
81 VVDFMLHHCIARWIAQRGGW Bak human
81 MVAY-LETRLADWIHSSGGW Bcl-w human
81 LADIILHHYIARWIAQRGGW Bak mouse
81 MVAY-LETRLADWIHSSGGW Bcl-w mouse
81 MATY-LNDHLEPWIQENGGW Bcl-x mouse
81 LADIILHHYIARWIAQRGGW Bak rat
81 MVTY-LETRLADWIHSSGGW Bcl-w rat
81 MATY-LNDHLEPWIQENGG Bcl-x rat
77 -TAYVTIEIGKQLIKAQKG 2,4-dienoyl-CoA red.
h: hydrophobic
l: aliphatic
p: polar
s: small
t: turnlike

Figure 3.6 Domain structure of DECR1. The coding region and amino acid sequence for 2,4-dienoyl-CoA reductase precursor (*Rattus norvegicus*) is shown. The short-chain dehydrogenase domain is shown in yellow and the short-chain dehydrogenase/reductase C-terminal domain is shown in green. Putative Bcl domain is shown boxed. Underlined sequence represents transit peptide. All domains were resolved using the Simple Modular Architecture Research Tool (SMART; Schultz, 1998; Lutenic, 2002) except the transit peptide (Hirose, 1990). The putative Bcl domain was identified using the outlier homologue search of the Schnipsel non-redundant database.

```
  1 atggcgctgctggcccgtgcgttctttgctggggtgtcccgcctc
    M  A  L  L  A  R  A  F  F  A  G  V  S  R  L   15
 46 ccctgcgatcccggtcctcagaggtttttcagctttggaacgaaa
    P  C  D  P  G  P  Q  R  F  F  S  F  G  T  K   30
 91 accctgtatcaaagcatcgatgctccacagtctaaattcttccca
    T  L  Y  Q  S  I  D  A  P  Q  S  K  F  F  P   45
136 cccattttaaagcctatgctaccacctaatgcctttcaaggaaaa
    P  I  L  K  P  M  L  P  P  N  A  F  Q  G  K   60
181 gtggctttcatcacgggaggaggcactggccttggcaaggcaatg
    V  A  F  I  T  G  G  G  T  G  L  G  K  A  M   75
226 acaactttcctgtccagcctgggtgcccagtgtgtgatcgcgagc
    T  T  F  L  S  S  L  G  A  Q  C  V  I  A  S  105
271 aggaatattgatgttctgaaagctactgcagaagagattacttct
    R  N  I  D  V  L  K  A  T  A  E  E  I  T  S  120
316 aaaactggaaataaggtctatgcgattcggtgtgacgttcgagat
    K  T  G  N  K  V  Y  A  I  R  C  D  V  R  D  135
361 cctgatatggtacacaacacagtactggagctgatcaaagttgca
    P  D  M  V  H  N  T  V  L  E  L  I  K  V  A  150
406 gggcatcctgatgtggtgataaacaatgcggcagggaacttcatt
    G  H  P  D  V  V  I  N  N  A  A  G  N  F  I  165
451 tctcccagtgagagactgtctcccaatggttggaagaccataact
    S  P  S  E  R  L  S  P  N  G  W  K  T  I  T  180
496 gacatagttctcaatggtacagcctatgtgacgatagaaattgga
    D  I  V  L  N  G  T  A  Y  V  T  I  E  I  G  195
541 aagcagctaattaaagcacagaaaggagctgcctttcttgctatc
    K  Q  L  I  K  A  Q  K  G  A  A  F  L  A  I  210
586 actacgatctatgctgagagcggatcaggctttgtaatgccaagt
    T  T  I  Y  A  E  S  G  S  G  F  V  M  P  S  225
631 tcttcagccaaatcaggcgtggaagccatgaataagtctcttgca
    S  S  A  K  S  G  V  E  A  M  N  K  S  L  A  240
676 gctgaatggggtagatacggaatgcgtttcaacataattcagcca
    A  E  W  G  R  Y  G  M  R  F  N  I  I  Q  P  255
721 gggcctatcaaaaccaaaggtgcctttagccgtttggacccgact
    G  P  I  K  T  K  G  A  F  S  R  L  D  P  T  270
766 ggaaaatttgagaaagatatgatcgagagaatcccctgtggtcgg
    G  K  F  E  K  D  M  I  E  R  I  P  C  G  R  285
811 ctgggaactgtggaggaacttgccaatctagcgactttcctttgc
    L  G  T  V  E  E  L  A  N  L  A  T  F  L  C  300
856 agtgattatgcctcttggatcaatggggcagtcattcgatttgac
    S  D  Y  A  S  W  I  N  G  A  V  I  R  F  D  315
901 ggtggagaggaagtgtttctgtcaggtgaattcaactctctaaag
    G  G  E  E  V  F  L  S  G  E  F  N  S  L  K  330
946 aaggtcaccaaggaggagtgggatgtaatcgaaggcctcatcaga
    K  V  T  K  E  E  W  D  V  I  E  G  L  I  R  345
991 aagacaaaaggctcctaa 1008nt
    K  T  K  G  S  *   355aa
```

Figure 3.7 DECR1 co-immunoprecipitates with Bad and Bcl-2. A. Co-transfected 293T lysates were immunoprecipitated using either anti-V5 antibody (lanes 1,2) or anti-DOKR antibody (lane3). Lysates were precipitated as in Figures 1 and 2. Control lysate specific for protein desired was loaded into lane 4 (upper panel, A431; lower panel, Jurkat T-cells; Transduction Labs). Upper panel shows blot probed for Bad (anti-Bad; Transduction Labs), and lower panel shows blot probed for Bcl-2 (anti-Bcl-2; Transduction Labs).

B. 10μg of each 293T-derived lysate was electrophoresed, blotted and subsequently probed for the presence of Neu (upper panel) and 2,4-DiCAR (lower panel, lanes 1,2) or DOKR (lower panel, lane 3). Cells were transfected as follows: for **A** and **B**; lane1, NeuNTYE/DECR1; lane2, NeuNTNYPD/ECR1, lane3, NeuNTYE/DOKR. Black arrowheads indicate protein of interest, and grey arrowheads indicate IgG light-chain.

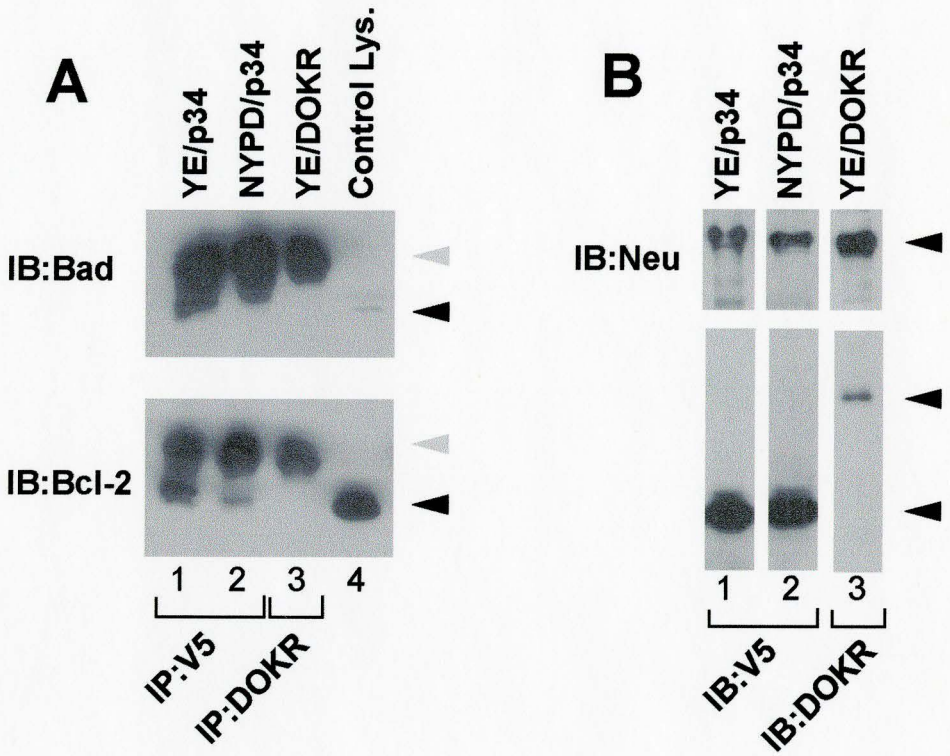

A
YE/p34
NYPD/p34
YE/DOKR
Control Lys.
IB:Bad
IB:Bcl-2
1
2
3
4
IP:V5
IP:DOKR
B
YE/p34
NYPD/p34
YE/DOKR
IB:Neu
1
2
3
IB:V5
IB:DOKR

Figure 3.8. Stable cell lines expressing DECR1 were derived. Rat1 cells were transfected with pEF6/LacZ or pEF6/DECR1 and selected with blasticidin for 15 days. LacZ-expressing control cell-lines are shown in panel **A**, and DECR1 cell lines are shown in panel **B**.

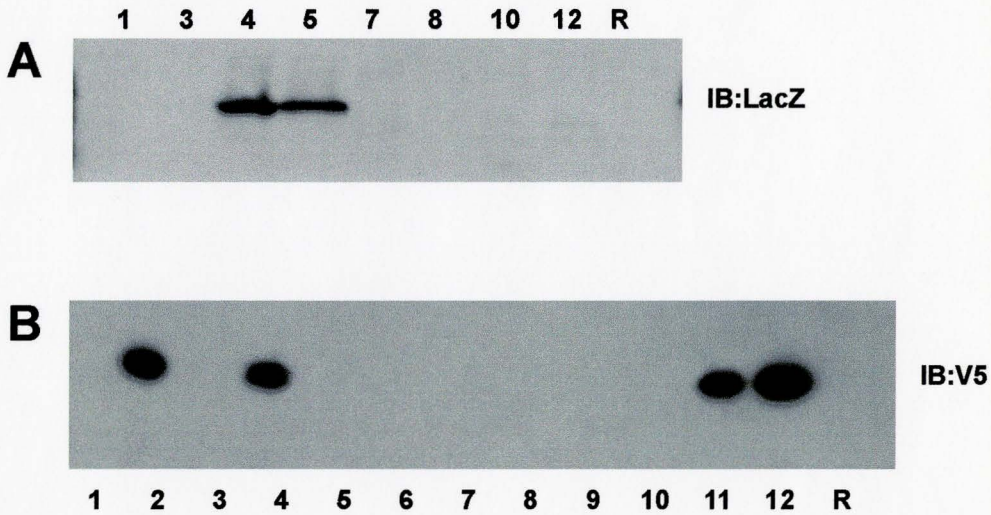
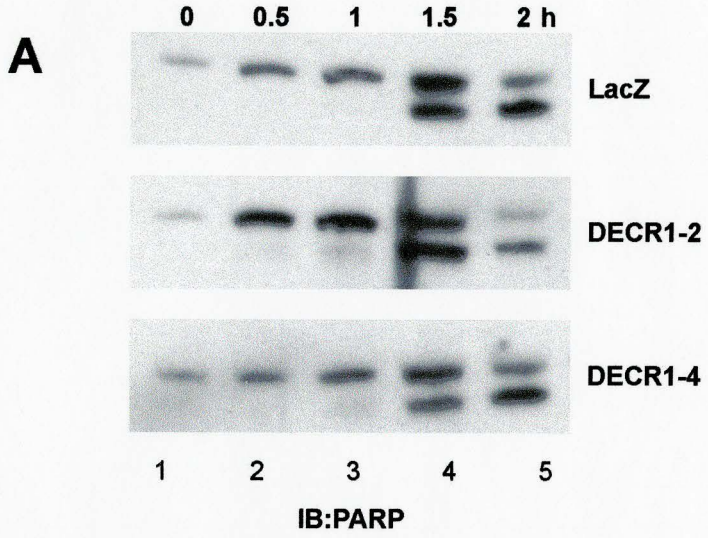

A
1 3 4 5 7 8 10 12 R
IB:LacZ
B
IB:V5
1 2 3 4 5 6 7 8 9 10 11 12 R

Figure 3.9. Rat1 cells over-expressing DECR1 are not differentially sensitised to apoptosis inducing agents. Sub-confluent LacZ control cells, and DECR1 stable cell lines were treated with 10% FBS DMEM supplemented with 1μM staurosporine for the time intervals indicated (**A**). Sub-confluent LacZ control cells, and DECR1 stable cell lines were treated with 0.5% FBS DMEM supplemented with 40μM etoposide for the time intervals indicated (**B**). In both panels, cells were harvested on ice, collected by centrifugation and lysed with boiling SDS-PAGE buffer. DNA was sheared by passing lysates through a 26-gauge needle. 10μg of each lysate was processed by standard SDS-PAGE and blotted for the presence of intact and cleaved PARP (anti-PARP C-2-10 antibody; Biomol).


A
0
0.5
1
1.5
2 h
LacZ
DECR1-2
DECR1-4
1
2
3
4
5
IB:PARP

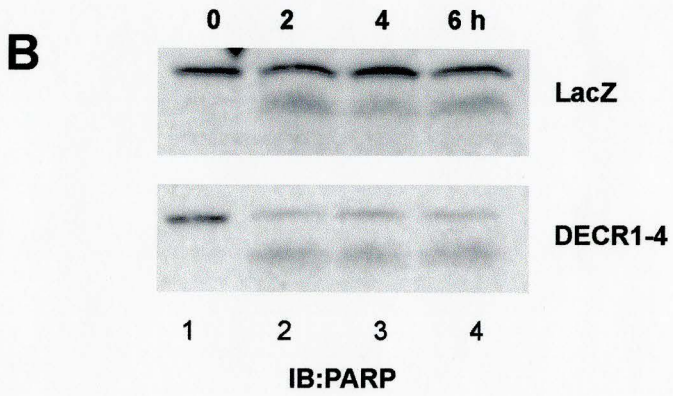

B
0
2
4
6 h
LacZ
DECR1-4
1
2
3
4
IB:PARP

Figure 3.10. Myc-tagged DECR1 co-immunoprecipitates with V5-tagged DECR1 in the presence and absence of NeuNT-derived add-back transient cell lines. 293T cells were transfected with Myc- and V5-tagged p34 and co-transfected with NeuNT-derived add-back mutants or vector. Cells were allowed to grow for 24-48 hours and were serum starved overnight. Lysates were harvested and used for analysis. **D** 300μg of each lysate was immunoprecipitated at 4^0C for three hours using anti-V5 antibody in the presence of 1mM orthovanadate. Immunoprecipitates were washed five times using PLCγ lysis buffer supplemented with 1mM orthovanadate. Washed beads were electrophoresed and blotted by standard protein blotting techniques. Blots were probed using anti-Myc antibody (mouse anti-Myc; Santa Cruz). 10μg of each lysate was electrophoresed, blotted and probed for the presence of Neu and C-terminally-tagged DECR1 (**A**, **B**, **C**). Myc-tagged DECR1 was derived by cloning DECR1 cDNA into pcDNA4/TO-Myc-His vector (Stratagene). Lanes 1-4 that V5 antibody can co-precipitate Myc-tagged DECR1. In the absence of V5-tagged DECR1, this is not observed (lane 5), demonstrating both the specificity of the V5 antibody and that DECR1 forms a multimer.

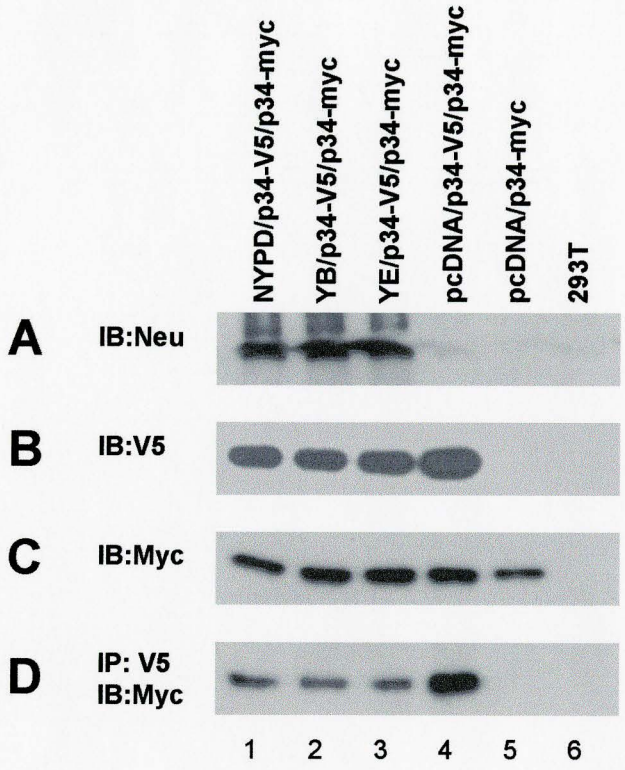

NYPD/p34-V5/p34-myc
YB/p34-V5/p34-myc
YE/p34-V5/p34-myc
pcDNA/p34-V5/p34-myc
pcDNA/p34-myc
293T
A
IB:Neu
B
IB:V5
C
IB:Myc
D
IP: V5
IB:Myc
1
2
3
4
5
6

Figure 3.11. DECR1 is tyrosine phosphorylated in the cell. 293T cells were transfected with p34 with or without NeuNTYE or NeuNTNYPD. Cells were grown overnight and treated with either 0.5% FBS DMEM (-) or 0.5% FBS DMEM with 40μM etoposide for 18 hours (+). Cells were lysed, and used for analysis. 300μg of lysate was immunoprecipitated with anti-V5 antibody and immunoblotted for phosphotyrosine (bottom panels). 10μg of each lysate was used to determine protein levels (all remaining panels). As can be seen, DECR1 is tyrosine phosphorylated, but not in response to either the presence of Neu or to induction of apoptosis by etoposide.

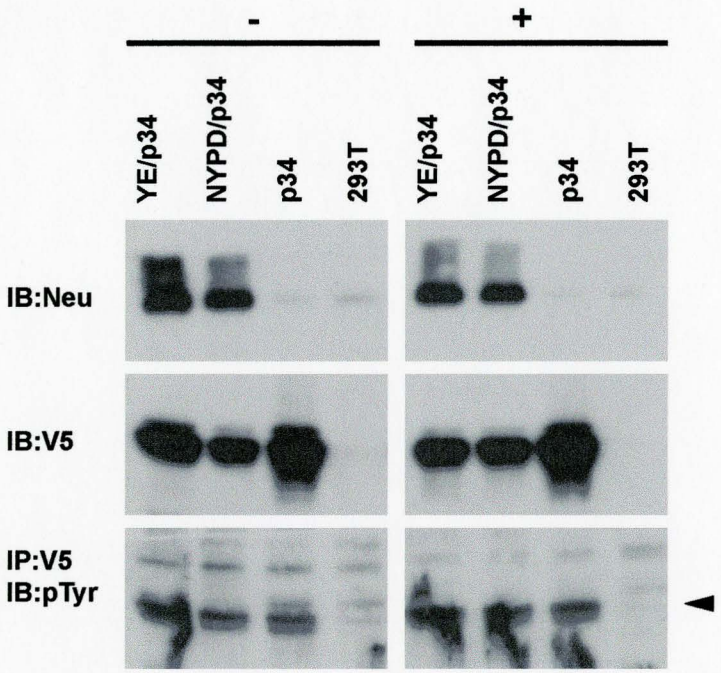
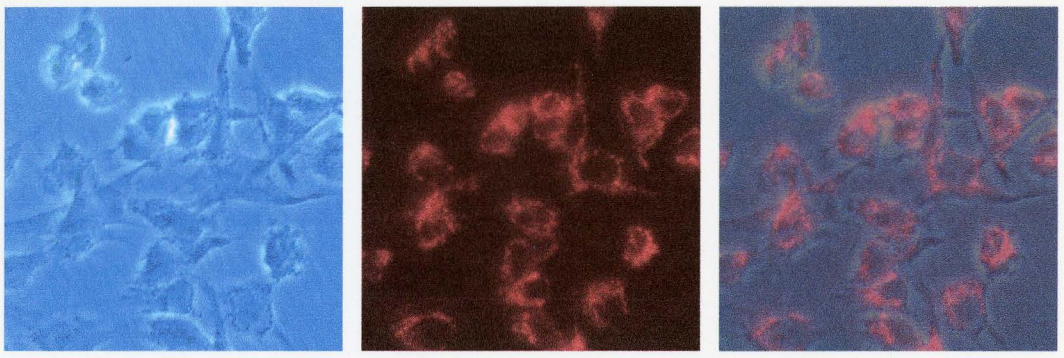

-
+
YE/p34
NYPD/p34
p34
293T
YE/p34
NYPD/p34
p34
293T
IB:Neu
IB:V5
IP:V5
IB:pTyr

Figure 3.12. DECR1 is localized in the same compartments as Neu. A. DECR1-2 stable cell line was fixed and probed for presence of V5 epitope via immunofluorescence. Cells show a punctate extra-nuclear staining reminiscent of focal plasma membrane localization.

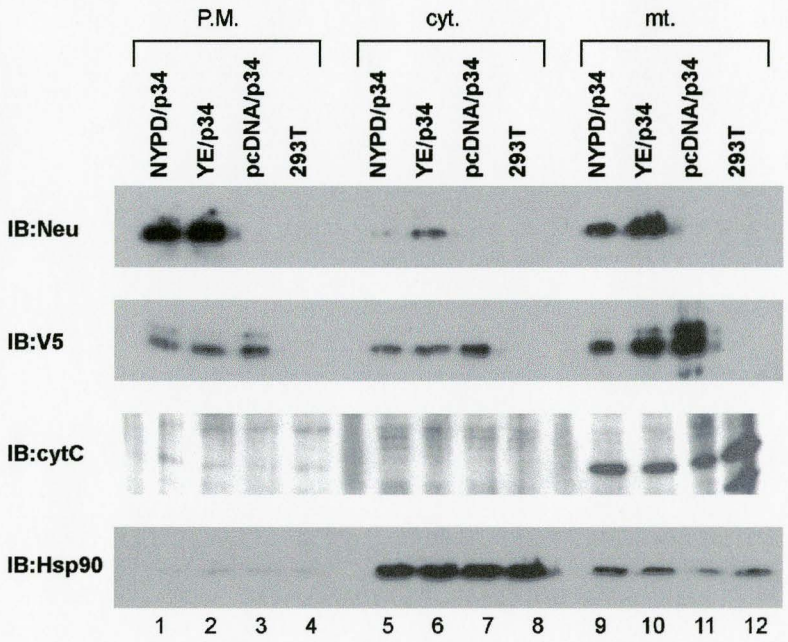
B. 293T cells were transfected with p34 with or without NeuNTYE or NeuNTNYPD. Cells were gown overnight, serum starved for 12 hours, lysed in hypotonic buffer and fractionated. 10μg of each lysate was used for immunoblotting. Cytochrome C (cytC), Hsp90 and Neu were used as a mitochondrial, cytosolic and plasma membrane markers respectively. DECR1 is shown primarily to the mitochondria, but also to the plasma membrane and cytosol. The presence or absence of Neu does not appear to affect DECR1 localization. Paradoxically, Neu also localizes to the mitochondria.

A



visible light rhodamine superimposed

B



CHAPTER 4

DISCUSSION

4.1 Overview

Receptor tyrosine kinases play a vital role in cellular proliferation and disorders that derive thereof. Partial characterization of some of these important molecules, such as Neu/ErbB2 provides insight into not only human disease but also into cellular biology, and development (Kim and Muller, 1999). Indeed the importance of these receptors has been demonstrated in the efforts directed at disabling the species responsible for tumour formation (Herceptin, Genentech). Though the rudimentary workings of most of the ErbB receptors are known, the precise mechanism by which signalling is mediated remains elusive. The characterization of Neu/ErbB2, arguably the most medically relevant ErbB member, by this lab and by others has led to a wealth of knowledge in the understanding of Neu-mediated signalling. Extensive characterization of the effector domain of this molecule through biochemical, cellular and transgenic means has all but unlocked much of the mystery surrounding ErbB2 signalling downstream of receptor activation (Dankort et al., 2001a; Dankort et al., 2001b; Dankort et al., 1997). Important questions still remain. One of these addresses the mode of mitogenic signalling downstream of tyrosine 1253 in Neu. Discovery of a 34kD molecule that associates with Neu site E generated much excitement as to revelation of a *bona fide* component of Neu mediated signal

transduction. The exposition of a mitochondrial pre-protein involved in lipid catabolism, DECR1, raised a number of issues concerning the veracity of the phenomenon unveiled.

This treatise has attempted to address the questions being posed as relating to the problem of Neu signalling through Y1253, via this putative signalling molecule. The verification herein that p34 interacts with Y1253 directly and phospho-specifically, substantiated the premise that DECR1 is indeed a potential candidate for Neu mediated signalling. Further experiments determining the sub-cellular localization, and verifying the co-localization of p34 and ErbB2 further enforced the notion that DECR1 is a component of Neu signalling. However, the inability to determine the mode by which p34 signalling takes place has been a substantive block to the progress of this project and serves to reinforce that scientific reason should remain humble in the face of true knowledge. If the truth of signalling through this molecule is to be revealed, further experiments employing different and perhaps more creative techniques are required.

4.2 DECR1 Associates with Neu/ErbB2 site E via a Putative Phosphotyrosine Binding Domain

Of paramount importance was the confirmation that p34 was a *bona fide* Neu interaction partner. Through indirect immunoprecipitation assays using the whole receptor (Figures 3.1b, 3.2), it was shown that p34 interacts with ErbB2 in a phospho-specific manner, and with sequence specificity not unlike that of a PTB domain. Through a direct association assay, it was shown that DECR1 alone is sufficient for Neu association at site E (Figure 3.3). Specifically, p34 alone possesses the determinant for

both phospho- and sequence specificity, circumventing the necessity for other adapter molecules. Having shown this, these results were not without issue. Critics could argue that DECR1 protein purity was at question in the demonstration of direct binding. Though Figure 3.3b clearly shows co-purification of several other proteins along with recombinant DECR1, it is unlikely that co-factors were isolated along with p34. Four washes with a high salt, high detergent solution (450mM NaCl, 20mM imidazole, and 1% Triton X-100) would likely dissociate any complexes formed (Holzinger et al., 1996; Janknecht et al., 1991). Any residual co-purified proteins would be directly associated to the affinity matrix via their own metal-chelation interactions and not likely influence p34-Neu association. Furthermore, the high stringency conditions used in the co-immunoprecipitation assays would imply a direct association. Indeed, dissociation of other adapter proteins from Neu such as Grb2 can only be achieved with high salt washes, (personal observation,(Dankort et al., 2001b)), suggestive that p34-Neu complex formation is direct.

Interestingly, it is this high stringency issue that poses an alternate problem. The fact that specificity of association can only be achieved using high stringency conditions raises concerns about the validity of the p34-Neu association *in vivo*. Although not trivial, taking into consideration that these experiments were essentially pursued *in vitro* implies that the conditions under which association was achieved were not the same as those in the cell. Molecular aggregation, common in cell-free assays (Mak, 1998), had to be alleviated by addition of non-ionic detergents (Ausubel, 1998). Insofar as this was

concerned, the consistency with which results were re-capitulated using both whole receptor and peptides under essentially native conditions should negate this issue.

The veritable association between Neu and p34 raises interesting questions regarding the nature of the association. Especially interesting is the question of how p34 interacts with ErbB2. Through homology searches it was determined that DECR1 contains no known SH2 or PTB domains. Considering PTB sequence degeneracy, it is not surprising that a consensus motif was not identified. However, the clear requirement for a phosphotyrosine, and specific binding requirement for the canonical NPXpY domain would suggest that DECR1 is a PTB containing protein. Analyses using second site mutants to site E, corroborate the notion that p34 binds through a PTB region. In canonical Src-type PTB domains, the determinant for ligand binding is governed by the phosphotyrosine (Margolis, 1999). Further specificity for binding is co-ordinated by amino acids N-terminal to this tyrosine (Margolis, 1999). Importantly, it is through the formation of a β-turn that PTB specificity achieved (Yaffe, 2002). By looking at the relative binding as determined by co-immunoprecipitation of site E second-site mutants, one can see that these criteria are fulfilled. In Figure 3.2 as in Figure 3.1, it can be seen that p34 does not co-precipitate with NeuNTNYPD, indicating a requirement for tyrosine phosphorylation. Low stringency precipitations show that p34 binds to every second-site mutant demonstrating that the requirement for a phosphotyrosine supersedes additional requirements N-terminal to the tyrosine (Figure 3.2c). Mutations N-proximal to Y1253 reveal the requirement of the β-turn for proper binding when immunoprecipitated at higher stringency. Substitution of alanine for asparagine at the –3 position ablates p34

binding, whereas the substitution of aspartate re-constitutes some binding in this position. Both asparagine and aspartate have a high positional frequency for type I β-turns (0.1085 and 0.0904 respectively) versus that of alanine, which is 0.0284 (Wilmot and Thornton, 1988; Wilmot and Thornton, 1990). Similarly, substitution of proline, a small turn-like amino acid (positional frequency of 0.0349), at the –2 position with alanine also ablates binding. Substitution with aspartate in turn, partially restores binding. It must be noted however, that these data to not agree with prior experiments carried by this lab. The fact that relative to NeuNT; NTYE[NAEY], NTYE[NDEY], NTYE[APEY] and NTYE[DPEY] transform at 98%, 30%, 6% and 4% respectively (Dankort et al., 2001a), is inconsistent with the immunoprecipitation data if it is to correlate with Neu-dependent transformation. The only conclusion that can be reached is that p34 is involved in phenomena other than Neu mediated transformation.

Although it is possible that DECR1 is interacting with Neu via a novel phosphotyrosine-binding domain, the above evidence would suggest that the association of DECR1 to Y1253 of Neu is mediated through a true PTB domain. To further understand this phenomenon, other mutational analyses would have to be carried out in order to identify the specific regions through which p34 is associating. Ideally, structural analyses would have to be conducted to determine the exact nature of the binding domain. That, unfortunately, is beyond the scope of this particular project. However, in anticipation of exploring p34/Neu binding further, DECR1 deletion mutants were produced. Three mutants with non-overlapping mutations were constructed to narrow-

down the regions whereby p34/Neu association takes place (Figure 4). These assays, unfortunately, will have to wait for another time.

4.3 Veracity of DECR1 as a True Neu/ErbB2 Effector Molecule

The relevance of the experiments in this thesis can be called to question when considering the method by which p34 was initially isolated. Naturally, since the identification of DECR1 was carried-out by affinity purification using artificial peptides, suspicion is aroused regarding subsequent experiments conducted using the same peptides (Figures 3.1a, 3.3). The results could be considered *perpetuum mobile*, in effect fulfilling the hypothesis by the nature of the similarity between experiments. However, examination of the data reveals that this is not true.

Experiments aimed at deducing the localization of p34 relative to Neu fell short in the determination of molecular co-localization between Neu and p34, but did demonstrate that both Neu and DECR1 are in the same sub-cellular compartment at the same time (Figure 3.12b), providing ample opportunity for interaction. Indeed, DECR1 and Neu share two compartments in common, namely the mitochondria, and the plasma membrane, providing the possibility for two alternate signalling pathways. These data, namely the presence of Neu in the mitochondria, were at first considered erroneous. However, considering corroborating findings from other sources (De Potter et al., 1989; Quatacker et al., 1990) presenting data that place Neu in the mitochondria in both normal and cancerous cells, intra-mitochondrial signalling provides an interesting avenue for future considerations. Although it is known that Neu is targeted to the inner

mitochondrial membrane (De Potter et al., 1989), the orientation in which it is embedded is not known. For p34/Neu signalling to take place in the mitochondria, presumably Neu must be oriented with the carboxyl-terminus toward the mitochondrial matrix. What the role of p34-Neu interaction could be within the mitochondria is unknown, and considering the conspicuous deficit of literature on the subject, highly speculative.

Though the localization of Neu to the mitochondria is baffling, the multi-compartmental targeting of DECR1 is less mysterious. The targeting of mitochondrial matrix proteins post-localization to other membrane bound compartments is not unprecedented (Soltys and Gupta, 1999). Numerous matrix proteins, typically found in the mitochondria have been found in various extra-mitochondrial compartments such as in secretory vesicles (mtHsp60, cytochromeC, TRAP-1) and the plasma membrane (mtHsp70, mtHsp60) (Cechetto and Gupta, 2000; Cechetto et al., 2000; Singh et al., 1997; Soltys et al., 2001; Soltys and Gupta, 1997). Further, it has been speculated that these proteins are in fact deliberately exported *ex mitochondria*, not merely mislocalized (Soltys and Gupta, 2000). Perhaps DECR1 is one such protein serving dual roles in intra- and extra-mitochondrial compartments. Alternately, p34 may be targeted to the membrane prior to mitochondrial import. Indeed, *TargetP* (Emanuelsson et al., 2000) localized DECR1 to the mitochondria with a relatively low reliability class of three (out of five reliability classes; see section 3.5) suggesting that p34 might be targeted to alternate compartments. One possibility is that p34 piggybacks on Neu. The data shown here (Figure 3.12b), would suggest otherwise however. DECR1 is localized to the membrane regardless of the presence of NeuNTYE, suggesting that another mode for

translocation is employed. Molecular weight studies on p34 could answer if the mitochondrial pre-sequence has been removed in the plasma membrane species indicating if the event was pre- or post mitochondrial. Nevertheless, the localization of DECR1 to domains outside of its canonical location is a real phenomenon that in the context of other atypically localized proteins is itself not unusual.

Taking into consideration the whole-receptor affinity experiments (Figures 3.1b, 3.2) coupled to localization data (Figure 3.12), the association between Neu and DECR1 would appear real. The condition that there does not appear to be any immediate biological relevance, though unfortunate, reinforces the need to investigate this association further. Since the limits within which experiments in this thesis were carried-out were not all encompassing, investigation into the relationship between lipid metabolism, and signal transduction could be a fruitful avenue to be pursued.

4.4 The role of DECR1 in Neu Mediated Signalling

Since the interaction of DECR1 with Neu site E, similar to the case of DOKR (Dankort et al., 2001a), does not correlate to the transformation data observed in site E add-back second-site mutants, the likelihood of p34 acting as a mitogenic activator is unlikely. The pursuit of an alternate biological activity via strong circumstantial data connecting p34 to apoptosis proved inconclusive. However, discounting a possible role for DECR1 in Neu–mediated signalling at this stage would be premature. DECR1 may have a previously undescribed link to apoptotic signalling. The localization of p34 to the mitochondria, in conjunction with the tantalizing findings that place Neu in the same

compartment is intriguing. In recent years, the mitochondrion has increasingly been described as a death signal integrator (Brenner and Kroemer, 2000). Indeed, Neu has for years been implicated in survival signalling through its ability to recruit PI3'K through its dimerization with ErbB3 (Sepp-Lorenzino et al., 1996). PI3'K activation of PKB/AKT through the phosphorylated phosphoinositol second messengers, and the subsequent suppression of apoptosis through PKB dependent inhibitive phosphorylation of Bad and inactivation of the forkhead transcription factors, (responsible for suppression of the transcription of pro-apoptotic Bcl-family members) may provide the key (Alessi et al., 1997; Wang, 2001). The role of p34 may be as an intermediary or signal modifier in this cascade. Indeed, the ability of PPARγ to suppress survival signalling through the PI3'K pathway appears to suggest a direct role between lipid metabolism and proliferative signalling (Pignatelli et al., 2001). Although closer inspection reveals that PPAR induction activates a number of genes implicated in cell proliferation and apoptosis such as *c-myc*, *ras* and *jun* (reviewed in(Vanden Heuvel, 1999)), there nevertheless exists a connexion between the transcriptional activation of *decr1* (Madsen et al., 1998; Vaagenes et al., 1998) by PPARs and PPAR mediated apoptotic regulation. Thus, it is not inconceivable that DECR1 may serve as a bridge between the RTK-mediated signal transduction and lipid metabolism. All efforts at this time have not been able to demonstrate veritable function for DECR1, prompting the re-assessment of methodologies to be employed.

Interestingly, the presence of Neu in the mitochondria calls to question whether Neu has a more direct role in apoptotic signalling. Indeed it also calls to question whether

Neu plays part in more mundane functions of cellular metabolism. However, presence of Neu in mitochondria has been given little attention and its function there lies in the realm of speculation. Since the orientation of Neu, its ability to dimerize or signal within the inner mitochondrial membrane is not known, it is not possible to guess what the role of Neu in this context might be.

4.5 Future Directions

The basis for believing that p34 may be involved in apoptotic signalling stems from a number of observations. The fact that DECR1 contains a putative Bcl domain, the fact that it is localized to the mitochondria, a death integrator organelle (Brenner and Kroemer), as well as the cytoplasm and plasma membrane, and the fact that it interacts with Neu, known to play a part in survival signalling would suggest that p34 could be involved in this pathway. Since the experiments performed herein provided no conclusive data regarding p34 as an apoptotic regulator, further experimentation is required. The repetition of the PARP cleavage assays using DECR1/Neu co-stable cell lines would be an appropriate starting point. Other alternate apoptotic assays, such as TUNEL could be used to quantify cellular apoptosis. Investigations into cell cycle progression may also provide alternate avenues of exploration regarding the function of p34.

Also, further investigations into the nature of p34/Neu co-localization are required. Immunofluorescence studies coupled to confocal microscopy would better show where in the cell Neu and DECR1 interact rather than merely showing which fractions contain both proteins. Experiments to investigate how p34 migrates in response to

apoptotic inducers would also be an interesting avenue to pursue. Presumably, p34 localization could be dependant on the insult incurred by the cell. Perhaps DECR1 moves from one cellular compartment to another in response to various stimuli. GFP-tagged p34 could be used to assess this molecular migration in real-time in living cells. Alternatively, fractionation assays could be used for immunolocalization studies to answer this question. Pending a meaningful outcome, the previously mentioned p34 deletion mutants could be uses to ascertain if partial DECR1 proteins can migrate in the same manner as intact DECR1.

Naturally, the first order of business requiring solution regards the characterization of the role p34 plays in receptor dependant signaling. However, Y1253 has been shown to mediate strong mitogenic signaling, and a mechanism of this signal transduction has still not been determined. Although it has been shown in this thesis that DECR1 binding occurs at site E, a transformation dependent association has not been established. It is most likely that a positive mediator of signalling is yet to be identified. To try to resolve this problem, experiments using the cloning of receptor targets (CORT) assay (Skolnik et al., 1991) technique were attempted to screen potential effectors using mouse cDNA expression libraries. Briefly, pYE-pep, YE-pep and pYC-pep (used as a positive control) peptides were used for positive/negative immuno-screening of bacteriophage expression libraries. The strategy attempts to tease out putative phospho-specific PTB consensus binding partners for site E by direct binding. However, due to significant technical problems, varied attempts at employing the CORT assay failed to yield any positive clones. Alternate strategies using tyrosine-phosphorylated GST-fusion proteins corresponding to sites C and E as well as yeast

dihybrid libraries should be considered. Indeed, two advantages offered by the yeast system, proper protein folding/modification and the ability to detect comparatively sensitive interactions, are highly favourable. Unfortunately, detection of false positives, the inability to control phospho-specificity and high cost are also considerations. At this time however, due to time constraints and the limitation of scope of this thesis, this line of investigation was postponed.

As important as it is to delineate the molecular mechanism by which site E signals, *in vivo* approaches to assess Y1253's biological relevance must also be studied. In particular, the role of site E in mammary tumourigenesis must be evaluated. To assess the relevance of site E in mammary transformation, transgenic mice possessing the NeuNTYE mutation under the control of the tissue-specific mouse mammary tumour virus (MMTV) promoter were developed. Unfortunately, due to a set of unforeseen circumstances, the YE mouse lines had to be terminated on account of inconsistent expression and aberrant genetics. In response, YE transgenic mice were re-derived. To determine the degree to which site E affects tumourigenesis, these mice will be studied for tumour kinetics data and protein expression. Metastasis studies will also be undertaken to determine the rate and frequency at which metastasis occurs in YE transgenics. If cell studies using YE are as indicative of a metastatic phenotype as they are in the case of YB, then it can be expected that rates and frequency of metastasis will be increased in YE transgenics (Dankort et al., 2001b), (Dankort et al., 1997). Knowledge gained from these studies would further broaden our understanding of how RTK mediated signaling contributes to the leading cause of breast

cancer mortality. Not unlike the CORT investigations however, this line of investigation is also being pursued outside the confines of this thesis.

4.6 Summary

Neu has been implicated in various biological phenomena including cancer. Neu is known to be a potent activator of mitogenic signalling, and it is this context that the role of Neu signalling through its most terminal autophosphorylation site, Y1253/site E, was explored.

The discovery that Neu interacts with a mitochondrial lipid-processing enzyme called 2,4-dienoyl CoA reductase (DECR1) in a transformation-dependent fashion was investigated. Experiments described in this thesis showed that DECR1 interacts with Neu at site E in a phospho-specific manner characteristic of a PTB domain-containing protein. Furthermore, despite being a mitochondrial enzyme, DECR1 was shown to co-localize with Neu in the plasma membrane. In addition, it was shown that Neu is also localized to the mitochondria via an unknown mechanism. Sequence analyses revealed that DECR1 contains a putative Bcl domain, suggesting a role in apoptosis. Experiments aimed at resecting DECR1 function in apoptotic regulation were inconclusive.

The data and ideas presented in this thesis demonstrate that DECR1 is a *bona fide* signalling partner for Neu. Furthermore, the startling revelation that Neu is localized to the mitochondria poses more questions that require resolution. Through novel approaches to answering this problem and the question of how DECR1 signalling is achieved, potential new exciting avenues of research could be opened for exploration.

CHAPTER 5

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APPENDIX A

ADDITIONAL RESULTS:

Neu/ErbB2 signals through CrkII at Y1201

A.1 Introduction

The characterization of the effector domain of Neu/ErbB2 receptor tyrosine kinase by this lab and (Matsuda et al., 1990) others has led to a better understanding of the processes involved in mitogenic signalling by ErbB2. This receptor tyrosine kinase (discussed in greater detail in chapter 1 of the main thesis) contains an effector domain consisting of five *bona fide* autophosphorylation sites that serve as docking sites upon receptor activation (Dankort et al., 1997; Siegel and Muller, 1996). Four of these sites, dubbed B, C, D, and E (Y1144, Y1201, Y1226/7, and Y1253 respectively) potentiate mitogenic signalling (Dankort et al., 1997). Recently, characterization of these sites revealed that known adapter molecules mediate these signals. Mutational analyses showed that sites B, D, and C signal through Grb2, Shc and CrkII* respectively leaving only one potentiating site, Y1253, an 'orphan' (Dankort et al., 2001b). The subject of site E is discussed in the main thesis.

Interestingly, what differentiates site C from the other positive mediators of transformation is that site C is refractory to Rap1A inhibition of Ras signalling (Dankort

* The contribution of the author of this thesis is discussed in this appendix.

et al., 2001b). YC mutants have been shown to mediate transformation independent of the Ras mitogenic pathway demonstrating the ability of ErbB2 to mediate a proliferative signal by parallel pathways (Dankort et al., 2001b). Recent experiments have implicated the role of CrkII in site C mediated signalling. CrkII is a 40kD adapter protein containing one SH2 and two SH3 domains (Mayer et al., 1988); Matsuda, 1990 #99; (Matsuda et al., 1993)} that has been shown to bind to EGFR upon receptor stimulation (Birge et al., 1992; Matsuda et al., 1990).

Recent experiments showed peptides corresponding to site C bind the CrkII adapter protein in a phospho-dependent manner (Dankort et al., 2001b). These data are consistent with the notion that site C signals independently of Ras. Indeed, CrkII has been shown to recruit Rap1A, a competitive inhibitor of Ras, to activate the MAP kinase pathway (Gotoh et al., 1995; Vossler et al., 1997) and is known to bind directly to EGFR via its SH2 domain (Birge et al., 1992). More recently, it has also been demonstrate that CrkII activates the MAPK pathway and that it is required for invasion of the ECM in ErbB2 transformed cell lines (Spencer et al., 2000). Although strong evidence suggests that site C funnels through the CrkII (Dankort et al., 2001b), *in vivo* experiments failed to show CrkII-Neu binding in a transformation-dependant fashion (Dankort et al., 2001a; Dankort et al., 2001b).

In order to determine the mode of cellular signaling and oncogenic activation by ErbB2, it was necessary to attempt to answer how ErbB2 signaling activates the mitogenic pathway through tyrosine phosphorylation at site C.

A.2 Materials and method

BL21 strain *E. coli* (Stratagene) were transformed with pGSTag derived GST, GST-PLCγ, GST-Crk plasmids respectively (Dankort et al., 2001b). Overnight cultures were grown and used to inoculate fresh LB-amp media at a 1:20 dilution and were grown at 37°C to an OD of 1.0. 1M IPTG was added to cultures to a final concentration of 0.5 mM and incubated for a further 3 hours. Pelleted bacteria were resuspended in 1/20 volume of PBS and lysed by sonication and/or freeze-thaw. Bacterial lysates were solubilized by the addition of Triton X-100 to a final concentration of 1% v/v and agitated at room temperature for 30min. Lysates were subsequently cleared by two rounds of centrifugation at 13000rpm 4°C and the supernatant was incubated with 1/50 volume of a 1:1 slurry of glutathione-sepharose (CLB4, Pharmacia) for 30min. Beads were washed 5 times in PBS and eluted with two rounds of two volumes each of 40mM Tris-buffered glutathione (pH 8.0) for five minutes. Eluates were pooled and quantified by Bradford assay. Presence of fusion protein was verified by SDS-PAGE and Coomassie stain.

Direct binding assays were performed as follows: Serial dilutions (0.01-1μg) of GST-fusion proteins were spotted onto nitrocellulose membrane and air-dried. Membranes were blocked in 0.3% gelatin in 0.1% TBS-Tween (TBST-G) for one hour. Membranes were then rinsed twice with TBST and incubated with peptides, both phosphorylated and dephosphorylated (pYE, [biotin-FEGTPTAENPE(pY)LGLDVPV]; pYC, [biotin-FAFGGAVENPE(pY)LVPREGT]; used at 1μg/ml), in TBST-G for an hour at room temperature. Blots were washed thrice for 10 minutes with TBST and incubated with a

1:2500 dilution of ^{125}I-streptavidin (IM236, Amersham) in TBST-G for an hour. Blots were washed again, dried and autoradiographed. Peptide dephsophorylation prior to direct blotting was carried out by calf intestinal alkaline phosphatase (CIAP) mediated dephosphorylation (described in section 2.10 in the main thesis).

A.3 Results and discussion

Despite its moderate transforming activity, Y1201 has been shown to signal independently of Ras (Dankort et al., 2001b). This observation indicates that ErbB2 is capable of mediating proliferative signaling via multiple parallel and possibly redundant pathways. In an attempt to use phospho-peptides at identifying potential site E binding targets, CrkII was originally identified serendipitously as a phospho-specific binding partner to a 'negative control' peptide corresponding to site C (biotin-FAFGGAVENPE(pY)LVPREGT, pYC-pep). Immunoprecipitations using these peptides (both phosphorylated and dephosphorylated) were able to precipitate CrkII from Rat1 lysates in a phospho-specific fashion. Further, immunoprecipitation with non-specific, NPXpY containing peptides corresponding to site E (biotin-FEGTPTAENPE(pY)LGLDVPV) showed that CrkII only complexed with site C (Dankort et al., 2001b). Unfortunately, experiments aimed at attempting to precipitate CrkII using the intact Neu receptor proved inconclusive (Dankort and Muller, unpublished). Indeed, it is a possibility that the Src non-tyrosine kinase, known to interact with Neu and CrkII (Luttrell et al., 1994; Matsuda et al., 1990), obscured immunoprecipitation results. Since it has been speculated that Src binds Neu outside of

the canonical effector domain (Kim and Muller, unpublished), it is possible that complex formation between CrkII and Neu and can occur independently of site C. Thus, experiments employing the whole Neu receptor would form immuno-complexes with CrkII despite the ablation of the canonical autophosphorylation sites.

To address the specificity of site C-CrkII interaction, an alternate experimental approach was devised. Experiments carried-out in this thesis demonstrated direct binding between the SH2 domain of the CrkII adapter to phosphorylated versions of the C peptide (Figure A.1) using a direct blot technique (also described in section 3.2 in the main thesis). Non-specific SH2 domain controls using the PLCγ-SH2 domain failed to show association with pYC-pep implicating direct phospho-dependent binding between site C and CrkII. Although the C site contains that the PTB-binding consensus NPXpY motif (Margolis, 1999), it also contains the sequence pYLVP, which conforms to the pYΨXΨ (where Ψ denotes a hydrophobic residue) consensus required for a subset of SH2 interactions. Interestingly, this apparent dual sequence consensus may serve to diversify signalling by pleiotropy through binding multiple signal molecule species in response to different stimuli. Indeed, there is some evidence that the PTB-domain containing multi-adapter protein DOKR associates at site C (Dankort and Muller, unpublished) as well as site E (Dankort et al., 2001b).

In vivo attempts to co-immunoprecipitate NeuNTYC and CrkII have failed to reveal a site-specific interaction, nevertheless, CrkII binding to site C is consistent with Ras independent signaling downstream of Y1201. Studies have shown that the CrkII oncogene v-Crk activates Rap1A dependent activation of MAPK pathway through the binding and

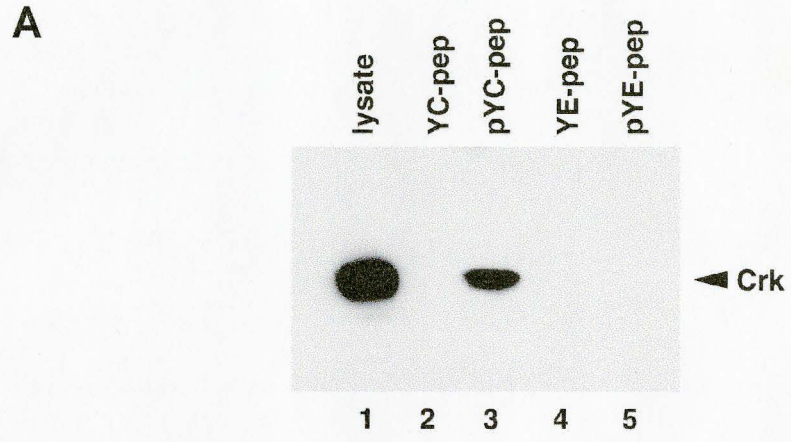
activation of the Rap1A specific guanine exchange factor (GEF) C3G (Knudsen et al., 1994). Binding and activation of C3G/Rap1A complex in turn allows for the co-opting of the Raf-dependent MAPK pathway also required for signaling by Ras (Vossler et al., 1997). Interestingly, proliferative signalling through site C appears to be a parallel pathway to the one through which sites B, D and E signal. In the event of a mutagenic event, this redundancy could serve to retain partial function of the Neu receptor. Though it has been demonstrated here and by others in this lab that CrkII mediated signalling through site C (Dankort et al., 2001b), potentiates proliferative signalling, recent observations also suggest that CrkII can affect cell migration through interaction with p130CAS in an ErbB2-dependent manner (Spencer et al., 2000). Although the mechanism by which this occurs is not understood, cells devoid of Neu cannot undergo CrkII-mediated cell-migration (Spencer et al., 2000). Results from this lab failed to draw a conclusion between increased cellular migration in NeuNTYC providing evidence that CrkII may signal through multiple interaction sites on Neu.

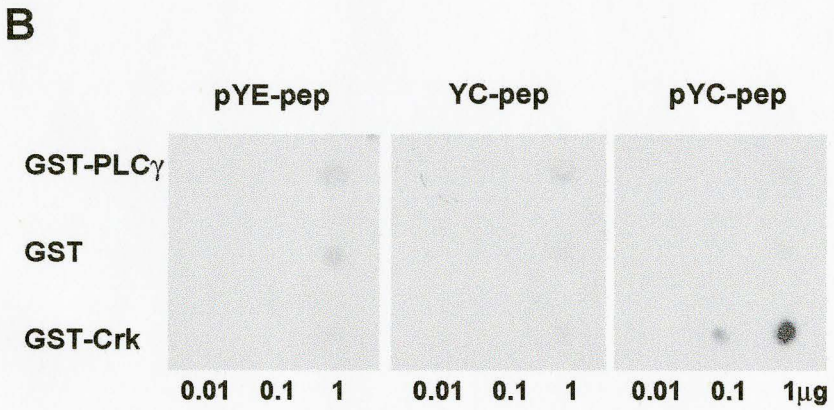
Although a substantive link between site C and CrkII mediated Ras-independent signaling has been demonstrated (Dankort et al., 2001b), further investigations into the specific mechanisms involved in this process are required. Furthermore, identification of other molecules involved in this signaling would indeed offer better understanding of Y1201 mediated proliferative signaling.

Figure A.1. Crk fusion protein interacts directly with phospho-peptides corresponding to tyrosine 1201 (site C). **(A)** Immobilized phosphorylated and unphosphorylated peptides (1μg) were incubated with 500μg Rat1 fibroblast lysates. Associated proteins were electrophoresed on and SDS polyacrylamide gel and were subjected to anti-Crk immunoblot analysis.

B. Membrane-immobilized GST fusion proteins were incubated with phosphorylated and unphosphorylated peptides (1μg/ml). Membranes were subsequently probed with streptavidin-^{125}I and autoradiographed. Serial dilutions of fusion (0.01-1μg) are indicated.

Figure A.1a was contributed by David Dankort (Dankort *et al.*, 2001a).


A
lysate
YC-pep
pYC-pep
YE-pep
pYE-pep
Crk
1 2 3 4 5


B
pYE-pep
YC-pep
pYC-pep
GST-PLCγ
GST
GST-Crk
0.01 0.1 1 0.01 0.1 1 0.01 0.1 1μg

A.3 References

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