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Cell Transformation and Signal Transduction of an Oncogenic Human Insulin-like Growth Factor I Receptor

by

Yixing Jiang

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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11/25/96
Date

Jacalyn H. Pierce
Jacalyn H. Pierce, Ph.D.
Chairman of Examining Committee

12/10/96
Date

Terry A. Krulwich
Terry A. Krulwich, Ph.D.
Executive Officer

Andrew Chan, Ph.D.
Jeanne Hirsh, Ph.D.
Robert Krauss, Ph.D.
Lu-Hai Wang, Ph.D.

Supervisory Committee

The City University of New York

Abstract**Cell Transformation and Signal transduction of an Oncogenic Human Insulin-like Growth Factor I Receptor**

by

Yixing Jiang

Advisor: Dr. Lu-Hai Wang

An oncogenic human insulin-like growth factor I receptor was constructed previously in our laboratory by truncating its EC domain and fusing to retroviral *gag* sequence resulting in a gag-IGFR fusion receptor. In this study, tyrosine residues in the cytoplasmic domain of gag-IGFR were systematically mutated to phenylalanines to investigate their role in the enzymatic and biological function of this fusion receptor. 19 mutants including two deletion mutants were constructed. Tyrosine residues 1131, 1135, and 1136 are important for the receptor PTK activity. Y1221, Y1095 and Y1183 have significant effect on the *in vitro* kinase activity but not on the *in vivo* autophosphorylation. Mutation of Y1136 (F1136) only slightly affects the kinase activity but dramatically reduced the transforming activity. Tyrosines 943 and 950 play important roles in autophosphorylation of the gag-IGFR. Mutation of either of the two tyrosine residues results in significantly reduced phosphorylation of the receptor but not on its PTK activity or transforming ability. Mutation of Y950 from the most oncogenic gag-IGFR produces little effect on the phosphorylation of insulin receptor substrate 1 (IRS-1), association of IRS-1 with PI3 kinase or cell transforming activity. Deletion of 13 amino acids surrounding Y950, however, results in a

significant reduction of IRS1-associated PI3 kinase and transforming activity. Deletion of 19 amino acids (dS2) in this region dramatically reduces transforming activity but not its kinase activity. However, mutation of Y950 from a moderate transforming gag-IGFR results in reduced IRS1-associated PI3 kinase and transforming activity. The most carboxyl tyrosine residue Y1316 is involved in tyrosine phosphorylation of PLC γ , which is, however, not important for cell transforming activity.

Since, dS2 and F1136 retain kinase activity but have greatly reduced transforming ability, their downstream was further analyzed. Both mutants are capable of inducing phosphorylation or activating a series of signaling molecules including phosphorylation of IRS-1, PLC γ and STAT proteins as well as activation of PI3 kinase. Interestingly, despite the loss of mitogenicity, both mutant proteins are able to activate MAP kinase as potently as the wild type gag-IGFR. This implies that pathway other than MAP kinase is essential for the mitogenic activity and is impaired in the mutants. Nevertheless, MAP kinase also plays certain role in cell growth and transformation since a dominant negative mutant of MEK-1 only inhibits the transforming activity of the wild type gag-IGFR by 2 to 3 folds.

To further explore the pathways that are defective in those two mutants, the role of RhoA, Rac1 and Cdc42 were assessed. Activated RhoA and Cdc42 partially rescue the transforming activity of dS2 and F1136 with RhoA more effective for the former, and Cdc42 more effective for the latter, suggesting that the two mutants are defective in different pathways. It appears that RhoA rescues both colony and foci forming activities while Rac1 and Cdc42 only rescue the colony forming but not foci forming activity of the mutants. These results indicate that RhoA and Cdc42 play important roles and have differential

effect in gag-IGFR induced cell transformation. Activation of each pathway alone is not sufficient to support the full transforming activity of gag-IGFR. This is supported by the observation that a dominant negative RhoA significantly but not completely inhibits gag-IGFR-induced cell transformation suggesting that pathways other than RhoA are also involved.

Overall, this study has identified several tyrosine residues of IGFR important for its PTK activity and substrate interaction. The transforming potential of the gag-IGFR correlates well with its ability to phosphorylate cellular substrates in general and to activate PI3 kinase via IRS1 in particular. MAP kinase, RhoA, and Rac1/Cdc42-mediated pathways play certain role in the transforming activity of gag-IGFR. Activation of each pathway alone is insufficient for the gag-IGFR-induced cell growth and transformation. It appears that RhoA is involved in regulating contact inhibition and anchorage independent growth while Rac1 and Cdc42 are more involved in regulating the latter activity.

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Format of thesis

This thesis was prepared in accordance with guidelines of City University of New York. Chapter 3 contains results published as Jiang et al., Journal of Biological Chemistry (1996) 271: 160-167. Chapter 3, 4 and 5 each has a brief introduction and discussion. A general introduction is put at the beginning and a general discussion at the end of the thesis. "Materials and Methods" and "References" have been consolidated and put in the respective section. Figures and tables are placed at the end of each chapter.

Table of Contents

Abstract.....	IV
Acknowledgments.....	VII
Format of Thesis.....	IX
Table of Contents.....	X
List of Table.....	XI
List of Figures.....	XII
Chapter 1. Introduction.....	1
Chapter 2. Material and Methods.....	28
Chapter 3. Effect of Tyrosine Mutations on the Kinase Activity and Transforming Potential of an Oncogenic Human Insulin-like growth factor I Receptor.....	36
Chapter 4. Effects of Juxtamembrane Region Truncation and Tyrosine 1136 Mutation on the Transforming Potential of an Oncogenic Human Insulin-like Growth Factor I Receptor in Chick Cells and Mammalian Cells.....	61
Chapter 5. The role of RhoA and Cdc42 in an Oncogenic Human Insulin-like Growth Factor I Receptor Mediated Cell Transformation.....	91
Chapter 6. General Discussion and Future Perspectives.....	110
References.....	114

Lists of Tables

Table 3-1 Biological Properties of Mutant Viruses.....	60
Table 5-1 The Role of Dominant negative or Activated RhoA and Cdc42 in the Colony Forming Ability of gag-IGFRs.....	108
Table 5-2 Activated Rac1V12 promotes colony formation of dS2 and F1136.....	109

List of Figures

Fig. 3-1 Schematic representation of UIGFR and NM1 chimeric receptors.....	52
Fig. 3-2 anchorage-independent growth of transfected CEFs.....	53
Fig. 3-3 <i>In vitro</i> kinase activity and intracellular phosphorylation of mutant gag-IGFRs.....	54
Fig. 3-4 Phosphorylation of mutant F943 and F950 proteins.....	55
Fig. 3-5 Phosphorylation of cellular proteins by mutant gag-IGFRs.....	56
Fig. 3-6 Phosphorylation of annexin II in mutant gag-IGFR transformed cells.....	57
Fig. 3-7 Mutation gag-IGFR- and IRS-1-associated PI3 kinase activity and phosphorylation of IRS-1.....	58
Fig. 3-8 Tyrosine phosphorylation of PLC γ	59
Fig. 4-1 Colony forming ability of mutant gag-IGFRs in CEF.....	75
Fig. 4-2 Colony forming ability of mutant receptors in 3T3 cells.....	76
Fig. 4-3 <i>In vitro</i> kinase activity and intracellular phosphorylation of mutant receptors.....	77
Fig. 4-4 Growth rate of 3T3 cells expressing gag-IGFRs.....	78
Fig. 4-5 Tyrosine phosphorylation of annexin II in CEF.....	79
Fig. 4-6 Tyrosine phosphorylation of PLC γ in 3T3 cells expressing gag-IGFRs.....	80
Fig. 4-7 Tyrosine phosphorylation of catenins.....	81

Fig. 4-8 Activation of STAT proteins by gag-IGFR.....	82
Fig. 4-9 Tyrosine phosphorylation of STATs in CEF.....	83
Fig. 4-10 Tyrosine phosphorylation of STAT3 and STAT5 in 3T3 cells expressing gag-IGFRs.....	84
Fig. 4-11 Association of PI3 kinase with IRS-1 and gag-IGFR and in mutant expression cell lines.....	85
Fig. 4-12 Activation of MAP kinase in 3T3 cells expressing gag-IGFRs.....	86
Fig. 4-13 Dominant negative MEK-1 partially inhibits transforming activity of NM1 protein.....	87
Fig. 4-14 Dominant negative MEK-1 inhibits focus forming activity of NM1 protein.....	88
Fig. 4-15 Inhibition of MAP kinase activity by dominant negative MEK-1.....	89
Fig. 4-16 Activation of JNK in 3T3 cells expressing dS2, F1136 and NM1 proteins.....	90
Fig. 5-1 Dominant negative RhoA inhibited transforming activity of NM1 protein.....	99
Fig. 5-2 JNK and MAP kinase activities in 3T3 cells co-expressing dominant negative RhoA and NM1 protein.....	100
Fig. 5-3 Activated RhoA partially rescued anchorage independent growth activity of dS2 and F1136 proteins.....	101
Fig. 5-4 Activated RhoA promotes focus activity of dS2 and F1136.....	102

Fig. 5-5 Activated Cdc42 partially rescues the colony forming activity of dS2 and F1136.....	103
Fig. 5-6 Activated Cdc42 is unable to promote focus forming activity of dS2 and F1136.....	104
Fig. 5-7 Activated Rac1 enhances colony forming activity of dS2 and F1136.....	105
Fig. 5-8 Activated Rac1 is not able to enhance focus forming activity of dS2 and F1136.....	106
Fig. 5-9 Activated RhoA but not Rac1 and Cdc42 promotes the focus forming activity of an oncogenic RAS.....	107

Chapter 1

I Receptor Protein Tyrosine Kinases

II Signal Transduction Through Receptor Tyrosine Kinases

III IR and IGFR

INTRODUCTION

Receptor Protein Tyrosine Kinases (RPTKs)

RPTKs are cell surface receptors with an intrinsic protein kinase activity in their cytoplasmic domains, and are capable of specifically responding to the stimulation of their ligands, such as growth factors, differentiation factors and hormones. They share several common structural features, including a glycosylated extracellular domain containing the ligand binding site, a single hydrophobic transmembrane domain anchoring the receptor molecules to the plasma membrane and a cytoplasmic domain comprising a catalytic tyrosine kinase domain (Yarden and Ullrich, 1988; Williams, 1989; Cantley et al., 1991). Unlike non-receptor protein tyrosine kinases, RPTKs lack SRC homology 2 (SH2) domains and SRC homology 3 (SH3) domains (Cantley et al., 1991). Binding of ligand to the extracellular domain of a RPTK leads to dimerization of the receptor (Schlessinger, 1988) and subsequent cross-phosphorylation on their tyrosine residues (Yarden and Schlessinger, 1987). This inter-molecular autophosphorylation event is crucial for RPTKs to initiate their signaling pathways.

Based on the similarities of their sequences and structural motifs, RPTKs are classified into eight families (Heldin, 1995). These include epidermal growth factor receptor (EGFR), insulin receptor (IR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor (HGFR), vascular endothelial growth factor receptor (VEGFR), neurotrophin receptor and EPH receptor families. Each family has its own structural characteristics. For instance, EGFR family members possess two cysteine-rich repeats in the extracellular (EC) domain. IR family members including insulin-like growth factor receptor (IGFR) are heterotetrameric complexes consisting of two external α subunits and two transmembrane β subunits linked by disulfide bonds. The cysteine-rich sequences are also present in the extracellular domain of IGFR. PDGFR family members have five immunoglobulin-like motifs in the extracellular domain, while FGFR family members have three (Ullrich and Schlessinger, 1990; Heldin, 1995).

The kinase activities of RPTKs are tightly regulated by their ligands under physiological conditions. The oncogenic variants of RPTKs usually have constitutively elevated kinase activity mainly caused by mutations in the receptor. First, deletion of large portions of the EC domain of a RPTK often results in constitutive activation of the receptor. V-Ros, an oncogenic counterpart of c-Ros which is a RPTK, contains only seven amino acids in the EC domain fused in-frame with an N-terminal viral gag protein. The resulting gag-Ros protein has a high level of kinase activity and oncogenic potential (Neckameyer et al., 1986; Neckameyer and Wang, 1985). Based on this knowledge, a chimeric protein gag-IGFR, which is artificially constructed by fusing IGFR, truncated of most of its EC

domain, with the *gag* sequence of avian sarcoma virus UR2. This gag-IGFR fusion receptor exhibits cell transforming potential and a constitutive kinase activity (Liu et al., 1992). Second, a single amino acid substitution for certain RPTKs can cause oncogenic activation. For example, a single amino acid change from valine to glutamate in the transmembrane region of NEU, a RPTK encoded by a carcinogen-induced oncogene *neu*, leads to a constitutively dimerized and activated receptor with transforming activity (Ben-Levy et al., 1992). Finally, mutations in the cytoplasmic domain can also convert a normal RPTK to an oncogenic receptor (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990). For instance, v-FMS has a truncation of 37 amino acids at carboxyl terminus of c-FMS (Coussens et al., 1986; Furman et al., 1986). Virtually, mutations and truncations can occur in any part of a RPTK. The outcome of the above described mutations is facilitation of ligand-independent dimerization and inter-molecular autophosphorylation.

Signal Transduction Through Receptor Protein Tyrosine Kinases

Growth factors function by binding to the extracellular domains of their cognate receptors, resulting in receptor oligomerization, autophosphorylation, and initiation of signaling cascades. The signal transduction ultimately leads to gene activation, DNA synthesis, and cell proliferation or differentiation.

1. The SH2 domain and SH3 domain containing proteins

The ligand-induced autophosphorylation on tyrosines not only enhances the kinase activity of receptors but also creates binding sites for specific signaling molecules. Phosphorylated tyrosine residues on RPTKs provide binding sites for SH2-domain-containing proteins. The SH2 domain contains approximately 100 amino acids and

interacts specifically with phosphorylated tyrosine residues (Heldin, 1991; Cohen et al., 1995). The SH2 domain mediates receptor-substrate interactions and also determines the specificity of the interaction. Using a degenerate phosphopeptide library, it has been shown that SH2 domains derived from different signaling molecules interact with different phosphotyrosine peptides (Songyang et al., 1994). The SH3 domain has a distinct sequence of about 50 amino acids. This domain is also involved in protein-protein interactions. Unlike the SH2 domain, the SH3 domain interacts with proline-rich sequences (Ren et al., 1993; Cicherri et al., 1992; Koch et al., 1991).

SH2 domain-containing proteins are generally divided into two groups (Pawson and Gish, 1992). One group has enzymatic activity, e.g. phospholipase C gamma (PLC γ), GTPase activating protein (GAP), protein tyrosine phosphatase 1C (PTP1C) and SRC family kinases. The other group has no enzymatic activity. This group, also known as adaptor or docking proteins, includes growth factor receptor binding protein 2 (GRB2), SHC, p85 subunit of phosphatidylinositol-3 kinase (PI3K), CRK, and NCK. With the exception of SHC, these adaptor proteins contain SH3 domains. Recently, a new domain named phosphotyrosine binding domain (PTB) has been defined (Kavanaugh et al., 1994). SHC has both SH2 and PTB domains (Kavanaugh et al., 1994; Blaikie et al., 1994). The interactions of SHC with certain RPTKs including IR and IGFR occur through its PTB domain but not its SH2 domain (Gustafson et al., 1995). Another PTB domain-containing docking protein is insulin receptor substrate 1 (IRS-1) (Gustafson et al., 1995; Wolf et al., 1995). In addition to the PTB domain, IRS-1 has a pleckstrin homology (PH) domain responsible for its association with plasma membrane (Voliovitch et al., 1995).

A group of transcriptional factors involved in cytokine receptor signal transduction, known as signal transducers and activators of transcription (STATs), also contain SH2 and SH3 domains (Fu, 1992; Shuai et al., 1992). The SH2 and SH3 domains are located at the carboxyl terminus of STAT proteins. The SH2 domains are highly conserved among the STAT proteins and are involved not only in binding phospho-tyrosine residues on the cytokine receptors but also in the formation of homodimers or heterodimers between STATs. Unlike SH2 domains, the SH3 domains of STAT proteins are much less conserved among the STAT proteins. The function of the SH3 domains of the STAT proteins remains unclear (Ihle et al., 1994 ; Ihle et al., 1995).

2. The RAS pathway

The RAS pathway has been extensively studied and well established. Molecules involved in the RAS pathway are GRB2, son of sevenless (SOS), RAS, RAF, MEK, and MAP kinase.

Beginning with the binding of ligand to a RPTK, which triggers the signal transduction cascade of the receptor, subsequent inter-molecular autophosphorylation of the receptor facilitates the recruitment of SH2 domain-containing molecules. GRB2, an SH2 domain-containing docking protein, is one of the signaling molecules immediately recruited to the activated receptor. GRB2 was first cloned from an expression library by using a tyrosine phosphorylated EGFR cytoplasmic polypeptide fragment as a probe. Upon EGF stimulation, GRB2 binds to the phosphorylated tyrosine residue on EGFR through its SH2 domain (Lowenstein et al., 1992). From amino acid sequence comparison, GRB2 is highly homologous to the *C. elegans* SEM-5 protein. Based on the genetic study in *C.*

C. elegans, SEM-5, LET-23 (EGFR homologue), and LET-60 (RAS homologue) lie along the same signal transduction pathway. According to these results, microinjection experiments have been designed to evaluate the role of GRB2 in the RAS activation. Microinjection of both GRB2 and H-RAS proteins into rat fibroblasts resulted in the stimulation of DNA synthesis. However, injection of each protein alone failed to induce a mitogenic effect (Lowenstein et al., 1992). These experiments suggest that GRB2 plays an important role in the RPTK-mediated RAS activation pathway.

Besides the SH2 domain, GRB2 has two SH3 domains. The SH2 domain of GRB2 binds to the phosphorylated tyrosine residues on a RPTK upon ligand stimulation, while its SH3 domains bind to another important signaling molecule, SOS (Rozakis-Adcock et al., 1993; Li et al., 1993). In *Drosophila*, the *sos* gene was first identified on the basis of a dominant allele that enhances signaling by a mutant Sevenless protein which is homologous to the Ros RPTK (Rogge et al., 1991). Using a genetic screening strategy, it has been found that *sos* gene encodes a protein which is homologous to CDC25. CDC25 is a guanine nucleotide exchange factor of RAS proteins in budding yeast (Broek et al., 1987; Robinson et al., 1987; Jone et al., 1991). Later, the human homologue of SOS (hSOS) was cloned and found to function as a guanine nucleotide exchange factor for RAS (Chardin et al., 1993). Association of GRB2 with the proline-rich region of SOS couples receptor tyrosine kinases to the activation of RAS (Egan et al., 1993; Oliver et al., 1993). SOS converts the inactive RAS-GDP form to the active RAS-GTP form. Thus, SOS is a positive regulator of RAS.

GRB2 and SOS constitutively form a complex in fibroblasts and their association is

not affected by growth factor stimulation (Gale et al., 1993). The main function of GRB2 is to recruit SOS to the plasma membrane (Rozakis-Adcock et al., 1993). Addition of an artificial membrane targeting signal to SOS leads to the constitutive activation of RAS. This observation indicates that this plasma membrane translocation is crucial for the activation of RAS (Aroheim et al. 1994). More recent studies indicate that recruitment of SOS by GRB2 may not be the only mechanism for the activation of SOS. SOS has PH and Dbl domains at its N-terminus, and a GRB2 binding site at its C-terminus (McCollam et al., 1995). Mutant SOS with an intact GRB2 binding domain but lacking the N-terminal domain is not sufficient to enhance the GTP loading of p21^{RAS}. Taken together, the role of GRB2/SOS complex is to connect RPTKs with the RAS pathway.

For the receptors that do not bind GRB2, it is believed that SHC is required to recruit the GRB2/SOS complex to the receptor. SHC is capable of inducing transformation of fibroblasts and neuronal differentiation of PC12 cells when overexpressed (Pellicci et al., 1992; Schlessinger, 1993). The fact that dominant negative RAS can inhibit SHC-induced PC12 cell differentiation indicates that SHC is upstream of RAS in the RPTK signal transduction pathway (Rozakis-Adcock et al., 1992; Pronk et al., 1994). SHC binds phosphotyrosines through its newly defined phosphotyrosine-binding (PTB) domain located at the amino terminus of SHC (Kavavanugh and Williams, 1994; Blaikie et al., 1994). While the sequence of the PTB domain differs from the SH2 domain, the PTB domain is an alternative mechanism for binding to PTKs in mediating signal transduction pathways.

The critical mediator in the RAS pathway is p21^{RAS}. Numerous reports have demonstrated that RAS is associated with tumor formation. For example, high frequency

mutations on *c-K-ras* is found in human colorectal adenocarcinoma (Ando et al., 1991). In carcinogen induced hamster lung cancer, *c-K-RAS* is overexpressed (Ning et al., 1991). Overexpression of $p21^{RAS}$ has also been found in benign tumors such as ameloblastoma, ameloblastic fibromas and odontogenic myxomas (Sandros et al., 1991). An oncogenic form, *v-RAS*, can cooperate with nuclear oncoproteins, such as *Myc*, or a viral nuclear protein, such as adenovirus E1A, to transform primary rodent cells (Lowy, 1993).

RAS is a small GTP binding protein with intrinsic GTPase activity (Marshall, 1991). *RAS* can bind either GDP or GTP. *RAS*-GDP is an inactive form while *RAS*-GTP is an active form. *RAS* has at least two regulators. One of them is GTPase activating protein (GAP), which is considered a negative regulator (Lowy, 1993). Another regulator, as mentioned above, is a guanine nucleotide releasing factor, SOS, a positive regulator, which converts the *RAS*-GDP form to the *RAS*-GTP form (Shou et al., 1992). From previous studies, it is clear that *RAS* acts as a downstream mediator of protein tyrosine kinases. Early studies demonstrated that microinjection of neutralizing anti-*RAS* antibody can inhibit NGF induced differentiation in PC12 cells (Haggag et al., 1986). Moreover, overexpression of *RAS* may also induce differentiation in some types of cells such as PC12 cells (Barbacid, 1987). Dominant negative *RAS* mutant can inhibit the proliferation of mouse fibroblasts (Feig and Cooper, 1988). *RAS* has also been shown to be required for cell transformation by certain activated protein tyrosine kinases including SRC and EGFR (Smiths et al. 1986). These evidences indicate that *RAS* functions downstream of RPTKs.

Activation of tyrosine kinases induces the activation of RAF kinase, a 74kD serine/threonine kinase (Morrison et al., 1989). RAF functions downstream of *RAS*.

Microinjection of neutralizing antibody to RAS does not block v-RAF-induced cell transformation (Smith et al., 1986). Expression of oncogenic RAS is sufficient to induce the hyperphosphorylation of RAF in NIH3T3 cells (Morrison et al., 1988). Furthermore, transformation of NIH3T3 cells by oncogenic RAS is partially dependent on the function of RAF (Kolch et al., 1991). By what mechanism does RAS activate RAF? Accumulating evidence suggests that RAS physically associates with RAF and brings it to the plasma membrane where it can be activated by RAF activators. The evidence that RAS directly associates with RAF is supported by numerous studies. It has been shown that RAS-GTP forms a triple-complex with RAF and mitogen-activated protein kinase kinase (MAP kinase kinase, MEK) which can phosphorylate MAP kinase (ERK) (Moodie et al., 1993). It has also been found that both normal and oncogenic RAS directly bind to the amino-terminal regulatory domain of RAF (Zhang et al., 1993 ; Warne et al., 1993 ; Vojtek et al., 1993). Other studies have shown that RAS mediates the activation of RAF simply by recruiting RAF to the plasma membrane. Targeting RAF to the plasma membrane by adding CAAX signal at its carboxyl terminus results in a constitutively active RAF, and this activation is RAS independent (Leevers et al., 1994; Stokoe et al., 1994). From these experiments, it was speculated that some proteins located on the plasma membrane may be the RAF activators and RAS only functions to bring RAF to the plasma membrane. A family of proteins called 14-3-3 has been found to be a candidate of RAF activator (Fantl et al., 1994; Irie et al., 1994; Freed et al., 1994). Expression of 14-3-3 proteins in *Xenopus* oocytes enhances RAF-1 activity and promotes RAF-1-dependent oocyte maturation. A dominant negative mutant of RAF inhibits the effects of 14-3-3 protein. Recent studies

indicate that 14-3-3 is a phosphoserine binding protein (Muslin et al., 1996). Using serine phosphorylated peptides derived from RAF, the binding motif of 14-3-3 has been identified (Muslin et al., 1996). These phospho-peptides can competitively disrupt the association of RAF with 14-3-3 protein and inhibit the maturation of *Xenopus* oocytes. Since 14-3-3 protein is not an enzyme, the mechanism by which it activates RAF still remains unknown. One study has demonstrated that PKC α might be responsible for the direct phosphorylation of RAF (Kolch et al., 1993). However, the relationship between 14-3-3 and PKC α has not been defined.

RAS mediated activation of RAF is not the only mechanism. RAF can also be activated in a RAS-independent manner. Using germline mosaics in *Drosophila*, it was demonstrated that D-RAF can be activated by torso receptor in the absence of RAS, SOS or GRB2 (Hou et al., 1995). These studies provide *in vivo* evidence that RAF can be activated by RPTKs in a RAS-independent pathway.

RAF activation results in the activation of MEK or MAP kinase kinase. MEK is a tyrosine and serine/threonine dual specific kinase and phosphorylates MAP kinases or ERKs (extracellular signal-regulated kinases) at both threonine and tyrosine residues. MEK functions downstream of RAF. This is supported by the observations that MEK is constitutively active in NIH3T3 cells expressing v-RAF (Kyriakis et al., 1992), and that mammalian and bacterial expressed RAF can activate MEK *in vitro* (Dent et al., 1992). As mentioned above, RAS-GTP, RAF and MEK are in one complex (Moodie, et al., 1993), which suggests that MEK activation may be through direct interaction with RAF. Using the yeast two hybrid system, it has been found that MEK interacts with the carboxyl catalytic

domain of RAF (Van Aelst et al., 1993). The proline-rich sequences in MEK1 and MEK2 are required for RAF binding and regulation of MEK function (Catling et al., 1995). Unlike most kinase-substrate interactions, which are transient, RAF stably binds to its substrate MEK (Crew and Erikson, 1993). It has been proposed that RAF acts as a bridge to allow formation of RAS/RAF/MEK complex. Four MEKKs have been identified thus far. They are MEKK1, MEKK2, MKK3 and MKK4. RAF is considered to be one of the MEKKs. However, not all four MEKs are activated by RAF. RAF activates MEK1 and MEK2 which subsequently activate MAP kinase (Derijard et al., 1995). Both MKK3 and MKK4 are responsible for activating p38 while MKK4 also activates JNK (JUN N-terminal kinase) (Derijard et al., 1995). Both ERKs and JNK are members of the MAP kinase family and therefore are considered JNK subgroup and ERK subgroup MAP kinases (Minden et al., 1994b).

As discussed above, growth factors such as EGF, NGF, and insulin rapidly induce phosphorylation of ERKs, mainly ERK1 and ERK2 (Boulton et al., 1992; Thomas, 1992). ERKs are serine/threonine kinases (Crews et al., 1992), and their activities correlate with their own tyrosine and threonine phosphorylation (Boulton et al., 1991). Activated ERKs translocate to the nucleus (Chen et al., 1992) where they phosphorylate certain transcription factors such as c-Jun or another serine/threonine kinase called ribosomal S6 kinase (RSK) (Blenis, 1991; Pulverer et al., 1991).

The signaling pathway from growth factor receptors to ERKs or MAP kinases is fairly well understood. ERKs are rapidly phosphorylated and activated following stimulation by growth factors. However, the mechanism of a parallel thr/ser kinase pathway leading to

the activation of JNK and p38 is less clear. EGF or NGF induced JNK activation are RAS-dependent, whereas TNF induced JNK activation is RAS-independent (Minden et al., 1994a). These appear to have distinct mechanism in the activation of JNK.

The RAS pathway is involved in signaling cascades mediated by various receptor tyrosine kinases, such as EGF receptor, PDGF receptor, insulin receptor, IGF-I receptor, FGF receptor, and NGF receptor (Gale et al., 1993; Morrison et al., 1989; Thomas et al., 1992; Li et al., 1993). Besides RPTKs, other molecules can also activate RAS. For example, TPA-activated PKC requires RAS for induction of mitogenesis, indicating that RAS is downstream of PKC. However, other observations suggest that RAS can also activate PKC (Majerus et al., 1986; Morris et al. 1989). Furthermore, cAMP inhibits RAS-dependent RAF activation, indicating that other pathways such as G protein-coupled receptors, may also affect RAS pathway (Wu et al., 1993; Cook and McCormick, 1993). These observations demonstrate that the RAS pathway interacts with other pathways, and does not function independently.

Activation of the RAS pathway may lead to different consequences in different cell types. In most fibroblasts, RAS activation leads to a mitogenic effect. In PC12 cells, RAS or RAF activation results in neurite outgrowth and cell differentiation. What is the specificity of the signal transduction? Studies using PC12 cells as a model system provide some clues about this question. Overexpression of insulin receptor or EGF receptor in PC12 cells leads to sustained activation of MAP kinase and cell differentiation, while stimulation of endogenous insulin receptor and EGF receptor results in transient activation of MAP kinases and ultimately causes cell growth rather than differentiation (Dikic et al., 1994;

Traverse et al., 1993; Traverse et al., 1994). The results in PC12 cells also demonstrate that sustained activation of MAP kinases results in their nuclear translocation while transient activation of MAP kinases does not result in a detectable signal in the nucleus as measured by immunofluorescence. They also suggest that the nuclear accumulation of MAP kinases leading to phosphorylation and activation of transcription factors may have a scheme of gene activation different from that resulting from transient activation of MAP kinases. Based on these experimental results, it has been proposed that the differential response of PC12 cells to the activation signal is dependant on the duration of MAP kinase activation (Marshall, 1995).

2. The Alternate Pathways

PLC γ and PI3 kinase can be activated by and associate with several receptor tyrosine kinases including PDGFR and EGFR (Meishenhelder et al., 1989; Bjorge et al., 1990; Coughlin et al., 1989). These two pathways are considered alternatives to the RAS pathway.

(a) PLC γ pathway

PLC γ is an enzyme involved in lipid metabolism. Activated PLC γ breaks down phosphatidylinositol-4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP₃) (Stahi et al., 1988; Majerus et al., 1986). DAG activates protein kinase C (PKC) while IP₃ increases intracellular calcium levels (Berridge and Irvine, 1989).

Early studies have shown that PLC γ is a substrate of the PDGFR, EGFR, and c-Kit (Meisenhelder et al., 1989; Kim et al., 1991; Kinashi et al., 1995). Following EGF or PDGF treatment of A431 human epidermal carcinoid cells or quiescent 3T3 mouse fibroblasts,

respectively, PLC γ was phosphorylated within 30 seconds. Later, it was shown that tyrosine phosphorylation of PLC γ correlates with its increased catalytic activity (Nishibe et al., 1990). PLC γ has an SH2 domain that binds to the phosphorylated tyrosine residues on RPTKs (Anderson et al., 1990). This association is dependent on the receptors' kinase activity (Margolis et al., 1990a). The binding site for PLC γ on the PDGFR has been mapped to tyrosine-1021 (Valius et al., 1993). Using the phosphopeptide corresponding to tyrosine-1021 and the SH2 domain of PLC γ , the complex has been analyzed by nuclear magnetic resonance (Pascal et al., 1994).

The effects of the second messengers, IP3 and DAG, generated by PLC γ are well understood. IP3 increases intracellular Ca²⁺ levels and DAG activates PKC. However, the biological role of PLC γ in regulating cell growth, differentiation, and cell transformation is still controversial. Early studies indicated that overexpression of PLC γ increased tyrosine phosphorylation of PLC γ and intracellular IP3 but did not increase intracellular calcium signaling and PDGF-dependent DNA synthesis (Margolis et al., 1990b). Based on this result, it was speculated that enzymes other than PLC γ might limit PDGF-induced DNA synthesis and calcium signaling. Deletion of the kinase insert or substitution of tyrosine 825 with phenylalanine in PDGFR abrogates its ability to transmit mitogenic signals, but is still capable of interacting and phosphorylating PLC γ at tyrosine residue in response to PDGF stimulation (Morrison et al., 1990). This result suggests that PLC γ is not sufficient for the mitogenic effect of PDGFR. This is also supported by a recent finding that a mutant C-KIT, which lacks the PLC γ binding site, can stimulate cell growth (Kinashi et al., 1995). Another piece of evidence in FGFR indicated that a point mutation in FGFR

eliminated phosphatidylinositol hydrolysis without affecting mitogenesis (Mohammadi et al., 1992; Petera et al., 1992). Nevertheless, PLC γ could be the downstream mediator of PDGFR's mitogenic signal (Valius and Kazlauskas, 1993). Mutations of five major phosphorylation sites in PDGFR generates a mitogenic defective receptor. By "add-back" mutation strategy, the receptor's mitogenic signaling was restored when the PLC γ binding site was reconstructed. In PC12 cells, a good model for studying cell differentiation, activation of TRK (nerve growth factor receptor) with NGF can induce PC12 cell differentiation marked by neurite outgrowth. Mutation of tyrosine-785, which abolishes the association of PLC γ with the receptor, does not affect neurite outgrowth (Loeb et al., 1994; Peng et al., 1995). Taken together, PLC γ seems not to be essential in regulating cell differentiation while its role in mitogenic response is still controversial.

(b) PI3 kinase pathway

PI3 kinase is another enzyme involved in lipid metabolism. PI3K consists of two subunits, a p85 regulatory subunit and a p110 catalytic subunit. P110 phosphorylates the inositol ring of phosphatidylinositol-4 phosphate (PI-4-P), PI-4,5P₂ or PI at the 3' position (Whitman et al., 1988). In early studies, it was first observed that an 85 kD protein became tyrosine phosphorylated and PI kinase activity was increased in 3T3 cells treated with PDGF (Kaplan et al., 1987). Activation and association of PI3 kinase with RPTKs occurs upon activation of PDGFR and a number of other receptors including EGFR, CSF-I receptor, insulin receptor and IGFI receptor (Varticovski et al., 1989; Skolnik et al., 1991; Sun et al., 1991). However, tyrosine phosphorylation of p85 was only observed with PDGFR. Since p85 can associate with RPTKs, p85 was cloned by both expression library

screening and affinity purification using peptides derived from RPTKs as probes. For example, by using a carboxyl-terminal tail of EGFR as a probe, p85 was cloned from a bacterial expression library (Skolnik et al., 1991). Also, using PDGFR as an affinity reagent, p85 was purified from cell lysates, and consequently its cDNA was cloned (Escobedo et al., 1991). Upon sequencing of p85, it was found that this regulatory subunit has both SH2 and SH3 domains. It is clear that upon ligand stimulation, the p85 subunit of PI3 kinase binds to a phosphorylated tyrosine on a RPTK through its SH2 domain resulting in the activation of p110 catalytic activity, and eventually increases phosphoinositides in cells.

What is the role of PI3 kinase and its second messengers in the regulation of cell growth and transformation? PI3 kinase has been considered a mitogenic mediator for a long time. In previous studies, it was speculated that PI3 kinase was important for PDGFR-mediated mitogenic signaling (Coughlin et al., 1989; Valius and Kazlauskas, 1993). Deletion of the PDGFR kinase insert resulted in a receptor that was still capable of causing PDGF-induced autophosphorylation but abrogated its association with PI3 kinase, and this mutant receptor was defective in PDGF-induced PI3 kinase activation and promoted cell proliferation less effectively than the wild type receptor (Coughlin et al., 1989). This experiment implicated the role of PI3 kinase in the transmission of mitogenic signals. This hypothesis was further supported by mutating the PI3 kinase binding site in PDGFR ("add-back" mutation) (Valius and Kazlauskas, 1993). When PI3 kinase binding site was reconstructed in a mitogenic deficient PDGFR mutant, the mitogenicity was restored.

It is apparent that RPTK is upstream of PI3 kinase and directly or indirectly (ie, through IRS-1 in case of insulin receptor) associates with PI3 kinase. Recent studies indicate that PI3 kinase can be either upstream or downstream of RAS. A recent study showed that PI3 kinase was a direct target of RAS (Rodriguez et al., 1994). It has been shown that RAS interacts with the catalytic subunit of PI3 kinase in a GTP-dependent manner, and dominant negative RAS N17 inhibits growth factor induced PI3 kinase activity in PC12 cells. In contrast to the above findings, another study found that PI3 kinase was upstream of RAS (Hu et al., 1995). In this study, expression of a constitutively active p110 subunit of PI3 kinase in NIH 3T3 cells induced transcription from the *fos* promotor. Moreover, co-expression of a dominant negative RAS with the active p110 subunit blocked this action, suggesting that RAS is downstream of PI3 kinase.

Efforts to look for the downstream effectors of PI3 kinase lead to the discovery of other kinases. One of them is ribosomal S6 (pp70^{S6K}) kinase, a serine /threonine kinase that is activated by PI3 kinase (Chung et al., 1994). Activation of pp70^{S6K} kinase by serine phosphorylation results in 40S ribosomal protein S6 phosphorylation which is important for the G1/S transition in a variety of cell lines. In one study, it has been found that PI3 kinase mediates signaling from PDGF or IR to pp70^{S6K} kinase. Activation of pp70^{S6K} kinase by PI3 kinase is independent of RAS (Ming et al., 1994). In cells expressing PDGFR lacking the PI3 kinase binding site in the kinase insert region, PDGF activates RAS but not pp70^{S6K} kinase. PI3 kinase inhibitors, wortmannin and demethoxyviridin, can inhibit the activation of pp70^{S6K} kinase, which again suggests that PI3 kinase is the activator of pp70^{S6K} kinase (Petritsch et al., 1995).

Another target of PI3 kinase known thus far is a serine/threonine protein kinase encoded by the AKT proto-oncogene (Franke et al., 1995; Burgering and Coffey, 1995). c-AKT is also called protein kinase B (PKB) because it shares sequence homology with protein kinase A and C. AKT can be activated by PDGF, EGF, and insulin. The activation of AKT can be inhibited by the PI3 kinase inhibitor wortmannin, and by co-expression of dominant negative mutant of PI3 kinase. Furthermore, gag-PKB, an oncogenic protein that is homologous to v-AKT, displays increased ligand-independent kinase activity. This activity is sufficient to activate pp70^{S6K} kinase, which indicates that pp70^{S6K} is downstream of AKT (Franke et al., 1995; Burgering and Coffey, 1995). Taken together, activation of PI3 kinase results in AKT activation followed by activation of pp70^{S6K}.

Besides its mitogenic property, PI3 kinase also has some other newly characterized functions. First, it has been recently demonstrated that PI3 kinase is required for NGF-initiated prevention of apoptosis (Yao and Cooper, 1995). NGF induces cell differentiation and is essential for the survival of neurons. The ability of NGF to prevent apoptosis is inhibited by two specific inhibitors of PI3 kinase, wortmannin and LY294002. PDGF prevents apoptosis in PC12 cells expressing wild type PDGFR but not mutant PDGFR that is defective in activating PI3 kinase. Second, PI3 kinase mediates the translocation of glucose transporters in response to insulin stimulation (Quon et al., 1995). And finally, PI3 kinase induces membrane ruffling, which suggests that it may participate in cytoskeletal signaling (Ridley and Hall, 1992a; Ridley et al., 1992b; Ridley, 1994).

PI3 kinase and PLC γ share one common substrate, PIP2. The phosphorylation of PIP2 inositol ring at the 3' position by PI3 kinase generates an uncleaveable form for the

PLC γ . It is likely that the PI3 kinase and PLC γ pathways may regulate each other (Serunian et al., 1989). As previously mentioned, the activation of PLC γ leads to the activation of PKC. PKC subsequently activates RAS (Mucay et al., 1985). Furthermore, it was shown that PI3 kinase could be either upstream or downstream of RAS (Rodriguez et al., 1994; Hu et al., 1995). Therefore, the interaction among signaling molecules of different pathways increase the complexity of the signal transduction network.

3. The JAK/STAT pathway

Besides the RAS, PI3K, and PLC γ pathways, another signaling pathway, the JAK/STAT pathway, also known as the “direct pathway”, has recently been established. As described above, STAT proteins have SH2 and SH3 domains (Fu, 1992; Shuai et al., 1992). The SH2 domains of STATs have two functions. One function is directing STATs to bind to the tyrosine residue on the receptor. The other function is regulating the formation of STAT homodimers and heterodimers (Ihle et al., 1995). There are six STATs (STAT1, 2, 3, 4, 5 and 6) that have been identified thus far (Schindler and Darnell, 1995). STATs are involved in the cytokine and interferon signaling pathways (Lerner et al., 1993; Silvennoinen et al., 1993; Sadowski et al., 1993). Upon various cytokine stimulation, STATs are tyrosine-phosphorylated and translocated to the nucleus where they bind to specific DNA sequences and transactivate cellular gene expression. Tyrosine phosphorylation is important for the transactivation activity of STATs. However, a recent study showed that serine phosphorylation may also be required for maximal activation of STATs (Wen et al., 1995). The protein tyrosine kinases that phosphorylate STAT proteins

are Janus kinases (JAK). JAKs physically associate with the membrane-proximal region of the ligand-bound cytokine receptors, which leads to tyrosine phosphorylation and activation of JAKs. The activated JAKs subsequently phosphorylate the cytokine receptors and STATs. Different cytokines activate different JAKs and STATs, generating diverse signaling pathways in response to different cytokines. For instance, IFN- α/β activates JAK1 and TYK2, while IFN- γ activates JAK1 and JAK2. Both IFN- α/β and IFN- γ activate STAT1 (Ihle et al., 1994).

Recently, more and more evidence indicate that STAT proteins are involved in RPTK signaling as well. The first STAT protein found to be involved in RPTK signaling is STAT1 (Fu and Zhang, 1993). It has been shown that STAT1 is tyrosine phosphorylated and physically associated with EGFR in response to EGF stimulation. The activated STAT1 has increased DNA binding activity and is translocated from cytosol to nucleus. STAT3 can also be activated by EGF (Zhong et al, 1994). Besides EGF, PDGF can activate STAT1 and STAT3 as well (Silvennoinen et al., 1993). The most recent evidence indicates that STAT5 interacts with insulin receptor in the yeast two hybrid system and is tyrosine phosphorylated and activated in response to insulin stimulation (Chen et al., 1996 submitted). Unlike the IFN and other cytokine mediated activation of STATs, the activation of STAT1 and STAT3 in response to EGF or PDGF is independent of the presence of individual JAKs. STAT1 and STAT3 may be directly phosphorylated by the receptor tyrosine kinases (Vignais et al., 1996; Meraz et al., 1996).

It has been indicated that STATs also participate in non-receptor signaling. STAT3 phosphorylation and activation have been observed in v-SRC transformed cells (Yu et al.,

1995; Cao et al., 1996). JAK3, STAT5, and STAT6 are activated in v-ABL transformed pre-B cells (Danial et al., 1995). Phosphorylation and activation of STAT1 correlate the transforming activity of EYK RPTK (Zhong et al., 1996). STAT proteins are important components in IFN signal transduction, evidence implicated from genetic studies. Mice lacking STAT1 protein manifest a deficiency in IFN- γ signaling and are susceptible to infections (Meraz e et al., 1996). However, the significance of STAT proteins in receptor and non-receptor tyrosine kinase signaling pathways is less well understood.

4. RhoA/Rac1/Cdc42 pathway

Cell transformation is a multi-factorial event involving enhancement of cell growth, changes in cell shape, and reduced secretion of extracellular matrix components. Different oncogene-induced cell transformations have distinct cell morphologies. For example, chicken embryo fibroblasts (CEF) transformed by human oncogenic IGFR exhibit refractile, elongated, fusiform morphology, which are strikingly different from normal CEF (Liu et al., 1992). In contrast, CEF transformed by v-Src have a rounded cell shape and tend to detach from culture dishes. These dramatic changes in cell shape are caused by the reorganization of cytoskeletal structure and the alteration of membrane properties. This process is likely to be regulated by Rho family proteins. The Rho family, a group of small GTP/GTPase proteins related to p21^{RAS}, includes Rho (RhoA, B, and C), Rac1, and Cdc42. Like RAS protein, they have CAAX farnesylation or geranylation motifs at their carboxyl terminus (Mizuno et al., 1991; Kinsella et al., 1991). Some early investigations found that Rho proteins could induce rapid changes in cell morphology (Paterson et al., 1990). Microinjection of constitutively activated Rho protein into 3T3 cells induced a

contracted cell body, finger-like process, and actin filament reorganization. This observation indicates that the function of RhoA is to control some aspect of cytoskeletal organization. Studies have shown that RhoA, Rac1, and Cdc42 are important in regulating the formation of stress fibers, lamellipodia (membrane ruffling) and filopodia (Ridley, 1994; Nobes and Hall, 1995; Symons, 1996).

Unlike the RAS pathway, the signaling pathway initiated by Rac/RhoA/Cdc42 is less understood. Rac1 and Cdc42 participate in a signaling pathway parallel to the RAS-RAF-MAP kinase pathway, resulting in activation of JNK/SAPK and p38. As mentioned above JNK is JUN N-terminal kinase or stress-activated protein kinase (SAPK). Both JNK and p38 are considered to be distinct MAP kinases, and p38 is also called MPK2. The kinase which activates JNK and p38 is a tyrosine and serine/threonine dual specificity kinase called JNKK, and it functions as a mediator between MEKK and JNK (Lin et al., 1995). JNKK activates JNKs but does not activate the ERKs and is unresponsive to RAF-1. Rac and Cdc42 are upstream mediators in JNK activation, while RhoA is not involved in this pathway (Coso et al., 1995; Minden et al., 1995). Using the yeast two hybrid system, a serine/threonine protein kinase called protein kinase N (PKN) was found to bind to GTP-RhoA and can be activated by binding to GTP-RhoA *in vitro* and *in vivo* (Watanabe et al., 1996). This suggests that RhoA activates PKN, and activated PKN may subsequently phosphorylate cytoskeletal components or initiate a kinase cascade.

The precise signal transduction pathways mediated by these small G proteins are elusive. Their upstream activators and downstream effectors are less well studied. How these signaling pathways lead to the reorganization of cytoskeletal structure still remains

unclear. Nevertheless, the role of these small G proteins in regulating cell growth and transformation has been demonstrated. One study suggests that RhoA, Rac1, and Cdc42 transmit a mitogenic signal which is important for cell cycle progression through G₁ (Olson et al., 1995). Microinjection of RhoA, Rac1, and Cdc42 into Swiss 3T3 cells stimulates cell cycle progression through G₁ and consequently increases DNA synthesis. Injection of dominant negative forms of RhoA, Rac1, and Cdc42 blocks serum-induced DNA synthesis. These data have demonstrated that RhoA, Rac1, and Cdc42 are essential for cell growth. Other evidences indicate that RhoA and Rac1 may be important in the cell transformation process. Using various mutants of RhoA and Rac1, it has been shown that these two proteins are important in mediating RAS-induced cell transformation (Khosravi-Far et al., 1995).

IR and IGFR

Both IR and IGFR are RPTKs. They are heterotetramers consisting of two extracellular ligand-binding α subunits and two transmembrane catalytic β subunits (Ullrich et al., 1986; Ebina et al., 1985). IGFR and IR are very closely related receptors and share 80% amino acid sequence identity in their kinase domain (Ullrich et al., 1986). However, the physiological functions of IGFR and IR differ somewhat. Cells treated with insulin increase glucose uptake and lipid and glycogen syntheses, and only upregulate DNA synthesis after 12 hour of stimulation (Randszso et al., 1990), while IGF-1 appears to be more potent in stimulating DNA synthesis and regulating cell growth.

The signal transduction of insulin receptor has been studied more extensively than that of IGFR. Seven major phosphorylation sites in IR have been mapped. They are

tyrosines 953, 960, 1146, 1150, 1151, 1316, and 1322. Phosphorylation of the tyrosines at positions 1146, 1150, and 1151 are involved in controlling the kinase activity (Smith et al., 1994). IRS-1, an adaptor protein and the major substrate for insulin receptor, lacks SH2 and SH3 domains (Sun et al., 1991). Instead, IRS-1 has a PTB domain and a PH domain at its N-terminus (Kavanaugh et al., 1994; Voliovitch et al., 1995). The tyrosine phosphorylation of IRS-1 by insulin receptor is dependent on the presence of the PH domain, indicating that membrane association plays a role in IRS-1 function (Voliovitch et al., 1995). Mutational analysis of the IR juxtamembrane region indicates that tyrosine 960 is the binding site for IRS-1 (Kaburagi et al., 1993). Substitution of tyr960 with phenylalanine impairs the phosphorylation of IRS1. It has been found that the PTB domain of IRS-1 interacts with the NPEY motif in the juxtamembrane region of the receptor (Gustafson et al, 1995). A recent study demonstrated that overexpression of IRS-1 can rescue insulin action in CHO cells expressing mutant IR lacking the NPEY motif (Chen et al., 1995).

IRS-1 has multiple tyrosine phosphorylation sites and plays an essential role in IR signal transduction. It has been shown that IRS-1 mediates insulin-induced xenopus oocyte maturation and is necessary for insulin-mediated mitogenic signal transduction (Chuang et al., 1993; Rose et al., 1994). Overexpression of IRS-1 induces NIH 3T3 cell transformation and activation of MAP kinases (Ito et al., 1996).

Phosphorylated IRS-1 associates with different downstream signaling molecules including the GRB2/SOS complex, PI3 kinase, SHPTP SYP, and NCK (Baker et al., 1992; Giorgetti et al., 1993; Skolnik et al., 1993; Xiao et al., 1994; Lee et al., 1993). Association

of IRS-1 with GRB2/SOS links IR signaling to the RAS pathway (Skolnik et al., 1993). Interaction of IRS-1 and PI3 kinase leads to the activation of PI3 kinase. It was first noticed that activation of PI3 kinase by insulin requires an unidentified endogenous substrate (Baker et al., 1992). In CHO cells, overexpression of IRS-1 results in increased tyrosine phosphorylation of IRS-1 and PI3K activity. PI3 kinase plays an essential role in insulin-stimulated glucose transporter translocation (Quon et al., 1995). Dominant negative p85 subunit of PI3 kinase and wortmanin inhibit the translocation of glucose transporter. SYP is a phosphatase and its biological role in insulin action is not known, although it has been shown to associate with IRS-1. Microinjection of SYP antibody or SYP GST-SH2 can inhibit DNA synthesis, suggesting that SYP might be a positive regulator of mitogenic signal transduction (Xiao et al., 1994).

IRS-1 is not the only molecule that couples IR signaling to the RAS pathway. SHC also interacts with the NPEY motif of IR via its PTB domain (Gustafson et al., 1995). Phosphorylated SHC proteins then recruit GRB2/SOS complex, resulting in RAS activation.

Accumulating studies suggest that IRS-1 is not the only important element for regulating IR signal transduction. IRS-1 knock-out mice have been generated recently (Araki et al., 1994; Tamemoto et al., 1994). These mice showed smaller body size, and some degree of insulin resistance. The fact that PI3 kinase activity is still detected in response to insulin in these mice suggests that there may be an alternative signaling molecule, named IRS2, which can compensate for the function of IRS-1. It was subsequently confirmed that IRS-2 is the protein called 4PS which is the major substrate of IL-4 receptor associated kinase (Patti et al., 1995). In IRS-1 deficient mice,

phosphorylation of both 4PS and the associated PI3 kinase are enhanced following insulin treatment, suggesting that 4PS is an alternative substrate for IRS-1. From sequence alignment, it has been noted that IRS-2 also contains PTB and PH domains. However, its binding to IR is not dependent on the presence of the NPEY motif (Sawka Verhelle et al., 1996).

IRS-1 is not only involved in IR signaling but is also involved in other signal transduction pathways. IRS-1 was shown to be a major substrate of IL-4 receptor associated kinase, and the IL-4 receptor contains an IRS-1 binding motif (Keegan et al., 1994). IRS-1 is also associated with integrins and thus couples extracellular matrix receptor signaling to the RAS pathway (Vuori and Rouslahti, 1994). This finding indicates a mechanism for the synergistic action of growth factor and extracellular matrix receptors.

IGFR and IR are closely related receptors and thus share similar signaling pathways. Like IR, IGFR also has a cluster of three tyrosines (tyr1131,1135,1136) in its kinase domain. These three tyrosines are phosphorylated upon IGF-I stimulation. Replacement of the three tyrosines with phenylalanines abolishes the kinase activity both *in vitro* and *in vivo* (Gronbory et al., 1993). It has been shown that IRS-1 is a common component in IR and IGFR signal transductions (Myers et al., 1993). From sequence alignment, it has been proposed that tyr950 in the juxtamembrane domain of IGFR is the binding site for IRS-1 (Keegan et al., 1994). All this evidence and some earlier studies suggest that IGFR also utilizes the RAS pathway. More recent studies using the IGFR knockout cells (R⁻cells) demonstrated that IGFR is required for optimal progression through all phases of the cell cycle. Introduction of oncogenic RAS into R⁻ cells fails to support cell

growth in serum free media or to induce colonies in soft agar. It has been postulated that IGFR signaling has two pathways; one is the RAS pathway and the other is a RAS-independent pathway which is required in addition to the RAS pathway for cell growth and transformation (Sell et al., 1994). Furthermore, transfection of EGFR into R⁻ cells cannot induce EGF-depend cell transformation; however, co-transfection of IGFR and EGFR into R⁻ cells can restore EGF-induced cell transformation, suggesting that IGFR is required for EGFR mediated transformation (Copola et al., 1994).

Our laboratory has previously demonstrated that fusion of a viral gag to N-terminus truncated IR or IGFR results in the activation of the transforming potential of these receptors (Wang et al., 1987; Liu et al., 1992). Deletion of the entire extracellular domain of IGFR generates a constitutively activated and oncogenic gag-IGFR (Liu et al., 1993). Deletions of the C-terminus of IGFR have differential modulatory effects on the receptor's kinase and transforming activities. Some mutants retain the ability to form colonies in soft agar but are unable to form tumors in chickens (Liu et al., 1993). More and more evidence indicate that IGFR may be involved in the development of various tumors. In order to understand the cell transformation process induced by IGFR, I used the mutagenesis approach to dissect gag-IGFR-mediated signal transduction in the regulation of cell growth and transformation. In this study, I engineered various mutations in an oncogenic human IGFR (gag-IGFR) to disrupt receptor-substrate interactions and used these mutants as tools to dissect the pathways and identify molecules that are important for cell transformation.

Chapter 2

Materials and methods

Cells and viruses. The preparation of CEF and colony formation assay of virus-infected CEF were done according to published procedures (Liu et al., 1992). CEF were maintained in F10 medium supplemented with 5% bovine calf serum and 1% chicken serum (GIBCO Laboratories). UR2 and its associated helper virus UR2 AV have been described previously (Liu et al., 1992). Mutant viruses stocks were obtained by collecting medium about 10 days after transfection.

Construction of recombinant plasmids. Mutant IGFRs were constructed by using site-directed mutagenesis (Promega altered sites in vitro mutagenesis system). The parental plasmid used in the mutagenesis is pNM1 which is an N-terminus truncated IGF1 receptor fused to Avian Sarcoma virus UR2 *gag* sequence (Fig. 3-1) and cloned into a UR2 retroviral vector (Liu et al., 1993a). The *Sma*I-*Bam*HI DNA fragment of pNM1 was first freed from the UR2 vector and subcloned into a phagemid, pALTER-1, which contains a tetracycline resistant gene and a mutated ampicillin resistant gene. Recombinant bacteria phages were harvested from an overnight culture and precipitated with 3% polyethylene glycol (PEG8000) and 0.4M NaCl. Single stranded DNA template was purified from the phage pallet using phenol chloroform extraction followed by ethanol precipitation. Two oligonucleotids, a mutagenic oligonucleotide and an ampicillin repair oligonucleotide, were used for annealing to the DNA template. Double stranded DNA synthesized using bacterial

T4 polymerase was used to transform a repair minus strain of *E. coli*. and ampicillin resistant colonies were selected. Each clone was sequenced to confirm the intended mutation. The mutant NM1 sequence was then subcloned back to the UR2 vector containing two LTR.

The Y950 mutation and the 13 a.a. deletion were generated by ligating two DNA fragments. A 5' BglII site-containing oligonucleotide and a 3' mutagenic oligonucleotide containing the Y950 to F950 mutation and a HgaI site were used with either pNM1 or pUIGFR as a template for PCR to generate the 5' fragment. The 3' DNA fragment used for both UF950 and F950 was synthesized using a 5' mutagenic oligonucleotide containing a HgaI site and a 3' SphI site-containing oligonucleotide. The two fragments were digested with HgaI and ligated. The ligated product was used in a second round PCR with the BglII and SphI oligonucleotides to amplify the ligated product. The final fragment was cloned back into pUIGFR or pNM1 using the BglII and SphI sites. The Deletion mutant was constructed in a similar manner but using different mutagenic oligonucleotides containing a HgaI site and the sequence flanking the 13aa deletion from Tyrosine 943 to Valine 956.

Antibodies. Rabbit antiserum anti-IB was raised against the IGFR β -subunit (Liu et al., 1992). Recombinant alkaline phosphatase conjugated anti-phosphotyrosine antibody, RC20, was purchased from Transduction Laboratories. Rabbit polyclonal anti-PLC γ antibody was purchased from Upstate Biotechnology INC. Anti-murine annexin II was purchased from Zymed Laboratories. A polyclonal anti-chicken Annexin II serum was a gift from Dr. Tony Hunter. Goat anti-rabbit and goat anti-mouse IgG conjugated with alkaline phosphatase were purchased from Boehringer Mannheim.

To prepare the polyclonal rabbit anti-IRS1 antibody, the 3' sequence coding for rat C-terminal 271 amino acid of IRS1 was cloned by polymerase chain reaction and inserted into a GST bacterial expression vector; this was done by C.S.Zong in our lab. GST-IRS1 fusion protein was purified with glutathione agarose beads followed by gel electrophoresis. 250 µg of purified protein was emulsified with an equal volume of complete Freund's adjuvant (SIGMA) and used to immunize a New Zealand white rabbit (2.5 Kg, female). For boost injections, 150 µg of protein emulsified with an equal volume of incomplete Freund's adjuvant every two weeks. Bleeding was performed weekly starting 10 days after the second boost.

Plasmids used: Dominant negative MEK-1 plasmid, pBABE/LIDA was obtained from Dr. Michael Weber (Catling et al., 1995). Activated RhoA, Rac1 and Cdc42 constructs were obtained from Dr. Toru Miki. Dominant negative RhoA plasmids was from Dr. Channing Der.

DNA transfection. About 1×10^6 CEF were plated on 6-cm dishes; 18 hours later, the culture medium was removed and 1 ml fresh medium was added. 20 µg of recombinant DNA plasmid, 2 µg of SacI digested pUR2AV helper virus DNA and 30 µg of polybrene were added into the medium. The culture was incubated at 37°C for 6 hours with occasional mixing. At the end of the incubation, the cells were shocked with 30% dimethyl sulfoxide in regular medium at room temperature for 3 min, followed by washing three times with fresh medium and then maintained at 37°C. The cells were transferred to 10-cm dishes at confluence and overlaid with soft agar medium next day to enhance the growth of transformed cells.

Biological assays. Cell transformation was monitored by observation of morphological change in the monolayer cell cultures and anchorage-independent growth (colony formation) in the agar medium. Transfected CEF were overlaid with soft agar to select for transformed cells (Liu et al., 1990). Colony assay was set up three days after transfection as described (Hanafusa, 1969). Briefly, 10^6 cells were plated out in each 10 cm dish containing MEM agar medium and the cultures were maintained in 41°C for two weeks for colonies to grow.

NIH3T3 cells transfected with different plasmids were selected with appropriate antibiotics and the pool of drug resistance colonies was used for colony formation assay. 10^5 cells were plated in each 6 cm dish containing DEM agar medium and the cultures were maintained in 37°C for two weeks for colonies to grow.

Focus forming assay was set up as described (LI et al., 1996). 1.5×10^5 NIH3T3 cells were transfected in 10 cm dish with different plasmids by calcium phosphate method as described (LI et al., 1996). The transfected 3T3 cells were maintained in DMEM supplemented with 5% of calf serum for three weeks to allow focus formation. The dishes were fixed, stained with Giemsa dye and photographed.

Metabolic labeling. Cells were plated in 6 cm dishes and pre-starved for 2 hours in the methionine free medium. Medium was removed and 1ml fresh methionine free medium and $80 \mu\text{Ci}$ of ^{35}S -methionine were added. The cells were incubated at 37°C for 4 hours. Cells were lysed in RIPA buffer containing 50 mM Tris-HCl pH7.5, 150 mM NaCl, 1% triton X100, 1% deoxycholate, 1 mM sodium vanadate, 1% trasylol and 1 mM PMSF. Immunoprecipitation was done in RIPA buffer containing αIB at 500 folds dilution in 4°C

for 2 hours. Antigen-antibody complex was captured on the protein A beads and the precipitates were washed three times in the RIPA buffer. The immunoprecipitates were boiled in the SDS-PAGE sample buffer and analyzed in 10% SDS-PAGE gel. Gel was dried and autoradiography was performed to visualize the labelled proteins.

In vitro kinase assay Cells were lysed in RIPA buffer and the lysate was immunoprecipitated with α IB followed by protein A-agarose beads (Repligen) binding to the immunocomplex. The immunoprecipitates were washed three times with RIPA buffer and twice with kinase buffer (50 mM Tris-HCl pH 8.0, and 10 mM $MnCl_2$) and finally resuspended in 50 μ l kinase buffer. The kinase reaction was carried out at room temperature for 10 min in the presence of 10 μ Ci γ - ^{32} P-ATP. After reaction, the beads were washed three times with RIPA buffer to remove free γ - ^{32} P-ATP. The samples were boiled in the SDS-PAGE sample buffer for 5 min and analyzed in 10% SDS-PAGE gel. After gel electrophoresis, the gel was fixed in 10% acidic acid and 30 % methanol and dried. The kinase activity was detected by autoradiography.

Western blot Western analysis was done according to the previously described method (Liu et al., 1992) with following modifications: after separation of proteins by SDS-PAGE, the gel was immediately placed onto an electrotransferring apparatus and transferred to a nitrocellulose filter in transfer solution composed of 25 mM Tris base, 192 mM glycine and 20% methanol. Transfer procedure was done for 1 hour at 100 V at room temperature. The filter was blocked for 1 hour at room temperature with 3% BSA in 10 mM Tris-HCl pH7.5, 50mM NaCl and 1% Tween-20 (TNT) and then subjected to binding of antibody in TNT at either 4 °C for 6 hours or at room temperature for 2 hours. After

binding, the filter was washed in TNT three times for 20 min each at room temperature. The washed filter was incubated with secondary antibodies (either goat anti-rabbit or rabbit anti-mouse immunoglobulin) conjugated with alkaline phosphatase. The filter was then washed extensively to remove nonspecifically bound antibodies and was developed by adding alkaline phosphatase substrates, BCIP and NBT.

PI 3-kinase Assay. The PI 3-kinase assay was done according to the published procedure with slight modifications (Liu et al., 1993b). CEF were lysed in Nonidet P-40 (NP-40) buffer (20 mM Tris-HCl pH7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4 , 1 mM phenylmethanesulfonyl fluoride, 100 mM NaF, 50 mM sodium PP_i). The cell lysates were centrifuged at 15,000 g for 10 min at 4 °C to remove cell debris. The supernatant was incubated with αIB for 2 hours at 4 °C. Then 15 μl of protein A-agarose beads was added, and the mixture was further incubated for 1 hour at 4 °C. The immunoprecipitates were washed as described above, and the washed beads were resuspended in 25 μl TGN buffer (20 mM Tris-HCl pH7.5, 100 mM NaCl and 0.5 mM EGTA). Ten micrograms of the substrate, phosphatidylinositol (PI) (20 $\mu\text{g}/\mu\text{l}$ in dimethyl sulfoxide; purchased from Avanti Polar Lipids, INC) was added to the resuspended beads and mixed. The mixture was incubated for 10 min at room temperature to form micelles of PI. $\gamma\text{-}^{32}\text{P}\text{-ATP}$ (10 μCi per assay) and MgCl_2 (final concentration, 20 mM) were premixed and added to the immunoprecipitates suspension. The mixture was incubated at room temperature for 10 min. Formation of PI 3-phosphate (PIP) was linear at this time period (L-H Wang, unpublished data). The reaction was stopped by addition of stop solution (Chloroform:Methanol:12N HCl=100:200:2) and lipids extracted with chloroform. The

extracted PIP was washed three times with a mixture of methanol and 1 N HCl (1:1). The washed PIP was dried, resuspended in the 15 μ l chloroform and analyzed in a thin-layer chromatography Silica Gel 60 plate (Merk) as described previously (Liu et al., 1993b).

MAP kinase assay Cells were lysed in RIPA buffer and the lysate was immunoprecipitated with rabbit polyclonal antibody specific to p42-MAPK, TR10 (a gift from Dr. Michael J. Weber). The immunocomplex were captured by protein A-agarose beads. The immunoprecipitates were washed three times with RIPA and twice with MAP kinase buffer (10 mM Hepes, PH 7.4 and 10 mM MgAc) and resuspended in 20 μ l MAP kinase buffer. 20 μ l substrate (myelin basic protein, 2 mg/ml) and 20 μ l 3X hot mix (5 μ Ci γ -³²P-ATP, 150 μ M ATP, 30 mM MgAc and 30 mM Hepes) were added to this bead-antibody-antigen complex and incubated at 30 °C for 30 minutes. The reaction was stopped by adding 60 μ l 2X SDS-PAGE loading buffer and boiled for five minutes. The supernatant was taken and analyzed in 15% SDS-PAGE gel. After electrophoresis, gel was fixed and dried. The phosphorylation of MBP was visualized by autoradiography.

Cytoskeletal Protein Fractionation Monolayer cells were incubated with 0.5 ml CSK buffer (10 mM PIPES pH6.8, 100 mM KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, 0.3 M sucrose, 1 mM PMSF, 1% trasylol, 1 mM Na₃VO₄, 10 μ M Na₂MoO₄ and 1% Triton X-100) on ice for 3 min and the supernatant was collected. Then the cells were incubated again with 0.5 ml CSK buffer for another minute, supernatant was collected and combined with the previous one. The remaining structure was extracted with Western lysis buffer. The collected supernatant was ethanol precipitated and dissolved in Western lysis buffer. Both factions were analyzed in SDS-PAGE gel and blotted with appropriate antibodies.

JNK kinase assay Monolayer NIH 3T3 cells were starved in serum free medium over night and treated with 600 μM Na_3VO_4 for half hour. Cells were lysed in NP-40 buffer and 250 μg total cellular protein was incubated with either immobilized GST alone or GST-JUN fusion protein over night at 4°C. The beads were washed three times with NP-40 buffer and twice with kinase buffer (50 mM Tris-HCl pH 8.0, 5 mM MnCl_2 , 5 mM MgCl_2). The agarose beads were resuspended in 30 μl kinase buffer and kinase assay was started by adding 1 μl $\gamma\text{-}^{32}\text{P}\text{-ATP}$ and incubated for 10 minutes at room temperature.

Chapter 3

Effect of Tyrosine Mutations on the Kinase Activity and Transforming Potential of an Oncogenic Human Insulin-like Growth Factor I Receptor

(Jiang et al., 1996. J. Biol. Chem. 271: 160-167)

Abstract

The tyrosine residues in the cytoplasmic domain of an oncogenic human insulin-like growth factor I receptor (IGFR), a gag-IGFR fusion protein constructed previously, were systematically mutated to phenylalanines to investigate the role of those residues in the enzymatic and biological functions of the fusion receptor. My results indicate that tyrosine residues 1131, 1135, 1136, and 1221 are important for the receptor PTK activity. Single mutation of Y1221 greatly reduced the *in vitro* kinase activity, but not the *in vivo* autophosphorylation. Triple mutation of Y1131/1135/1136, but not the double mutation of Y1135/1136, abolishes both *in vitro* and *in vivo* kinase activity. In addition, Y1095 and 1183 also have a significant effect on the *in vitro* kinase activity. Single mutation of Y1136 only slightly affects the kinase activity, but has a dramatic effect on overall substrate phosphorylation, in particular, annexin II, which is strongly phosphorylated by the oncogenic gag-IGFR but not by the F1136 mutant. Those mutants with reduced total substrate phosphorylation also have weak or abolished transforming ability. The F1136 mutant has dramatically reduced transforming activity despite that its PTK activity is near the wild type level. Tyrosines 943 and 950 play important roles in phosphorylation of the

gag-IGFR. Single mutation of either of the two tyrosines results in significantly reduced phosphorylation of the receptor but not on its PTK activity or transforming ability. Mutation of Y950 from the most oncogenic gag-IGFR produced little effect on the phosphorylation of insulin receptor substrate 1 (IRS-1), association of IRS-1 with PI3 kinase or cell transforming activity. Deletion of 13 amino acids surrounding the Y950 did have a significant reduction of IRS1-associated PI3 kinase and transforming activity. However, mutation of Y950 from a moderate transforming gag-IGFR resulted in reduced IRS-1-associated PI3 kinase and transforming activity. My data also suggest that the most carboxyl tyrosine residue Y1316 is involved in tyrosine phosphorylation of PLC γ , which is, however, not important for cell transforming activity.

Overall, this study has identified several tyrosine residues of IGFR important for its PTK activity and substrate interaction. The transforming potential of the gag-IGFR correlates well with its ability to phosphorylate cellular substrates in general and to activate PI3 kinase via IRS1 in particular.

Introduction

Previous studies of our laboratory on IGFR has shown that the cell transforming activity of native IGFR was significantly enhanced in an N-terminus truncated receptor, containing 36 aa of the extracellular domain, the entire transmembrane (TM) and cytoplasmic domains of IGFR β subunit fused to the avian sarcoma virus UR2 *gag* sequence (Liu et al., 1992). The *gag*-IGFR encoding virus called UIGFR has an enhanced transforming potential over that of native IGFR in cultured CEF but is not tumorigenic *in vivo*. The *gag*-IGFR is a dimerized transmembrane protein (Liu et al., 1992). Further examination of this extracellular 36 aa sequences revealed that it has a negative modulating effects on IGFR transforming and tumorigenic potential (Liu et al., 1993). Deletion of the entire 36 aa resulted in a strong transforming and tumorigenic variant called NM1 (Liu et al., 1993). Functional role of the carboxyl terminus of IGFR was examined by deleting C-terminus of the UIGFR encoded fusion receptor (Liu et al., 1993). Deletion of 27 aa from the C-terminus of this receptor, including a potential PI3 kinase binding motif YXXM (aa 1316-1319) produced little effect on the function of the *gag*-IGFR. Further deletion of 20 aa abolished the receptor's kinase and transforming activities. Surprisingly, deletion of 67 aa restored both kinase activity and transforming ability, and deletion of 88 aa abolished all the activities. Overall, previous studies on the *gag*-IGFR indicate a strong correlation between the receptor kinase activity and its transforming ability.

In this study, I further explored the mechanism of cell transformation induced by this oncogenic *gag*-IGFR. Since receptor-substrate interactions play important roles in RPTK

signal transduction and phosphorylated tyrosines on the receptor provide binding sites for the substrates, tyrosine residues in the cytoplasmic domain of NM1 encoded gag-IGFR were systematically replaced by phenylalanines. In addition, since IRS-1 is a substrate of IGFR, a more extensive study of tyrosine-950 and its neighboring sequence, the presumed docking site for IRS-1, was undertaken. Although substantial studies on mutation of tyrosine residues in insulin receptor have been carried out (see Discussion), the role of tyrosine residues in IGFR has largely been inferred from insulin receptor thus far. My study provides a direct systematic examination of the role of tyrosine residues in an oncogenic form of IGFR.

Results

Construction and expression of Mutant IGFR In order to explore the mechanism of cell transformation induced by gag-IGFR, mutants that possess kinase activity but lack transformation ability will be very useful for identifying the essential downstream components for cell transformation by comparing their substrates with those of the parental NM1 protein. Since the phosphorylated tyrosine residues are potentially critical sites for the receptor to interact with its downstream substrates, my initial mutagenesis study focused on the tyrosine residues in the cytoplasmic domain of NM1 encoded gag-IGFR. There are 15 tyrosine residues in the cytoplasmic domain of the IGFR β subunit and each tyrosine except Y1316 was substituted with phenylalanine. Early study of our laboratory has already shown that a mutant, CM2, with deletion of carboxyl 27 aa including Y1316 had little effect on the PTK activity and transforming function of the UIGFR encoded gag-IGFR (Liu et al., 1993). In addition, mutants with combinatory mutations of certain tyrosine residues were also constructed. All the mutants were named according to amino acid positions (Ullrich et al., 1986) of the mutation (Fig. 3-1). (F950, d950 and UF950 were constructed and their biological and biochemical properties were analyzed by Joseph Chan in the lab.)

Transforming activity of the tyrosine-phenylalanine mutants NM1 or its mutants DNA was cotransfected with two micrograms of SacI digested UR2AV DNA into CEF as described in Chapter 2. Cells were overlaid with soft agar the next day to select for the transformed cells. Colony formation assay was set up three days after transfection

and maintained at 42°C for two weeks, after which plates were scored for number of colonies (Table3-1). The morphological appearance of monolayer cultures was also monitored and recorded (data not shown). Normal CEF and weak- or non-transforming virus-infected cells had spindle shaped morphology while transformed cells showed elongated fusiform morphology and had increased refractility. Most of the mutants have a similar transforming ability as the parental NM1. However, mutants F1121, F1136, double tyrosine mutant F1135/36, and triple tyrosine mutant F3 (Fig. 3-1) have either reduced or completely lost the transforming ability (Table3-1, Fig. 3-2). Interestingly, F1136, which contains the third tyrosine mutation in the three tyrosine cluster, has a significantly reduced cell transforming and growth promoting activity (7 folds reduction of colonies in comparison with NM1) despite having a near wild type level of PTK activity (see below). The effect of Y950, the presumed IRS1 interacting sites was addressed using point and deletion mutants. Mutation of Y950 from the highly oncogenic gag-IGFR encoded by NM1 produced little effect, however, when a similar mutation was made in UIGFR (UF95) the transforming activity was greatly reduced. Deletion of a 13-aa stretch including the Y950 from NM1, unlike the point mutation, did result in a significant reduction of colony forming ability (Fig. 3-2).

Tyrosine kinase activity and intracellular phosphorylation of the mutant receptors The kinase activity of various mutant gag-IGFR receptors were assessed by comparing their abilities to autophosphorylate *in vitro* and intracellularly (Fig. 3-3A). Those activities were compared in parallel with the levels of protein expression. For those transformation defective mutants, the protein expression levels in long term culture were

relatively low, therefore, transiently transfected cells were used for comparison (Fig. 3-3C). With a few exceptions, most of the mutant proteins exhibited similar levels of *in vitro* kinase activity and intracellular autophosphorylation as that of NM1. F1095 and F1183 proteins appeared to have 4 to 5 fold reduction in the *in vitro* kinase activity, however, their intracellular phosphorylation remained the same as NM1 protein (Fig. 3-3A). Interestingly, F1136, which had a significantly reduced cell transforming and growth promoting abilities, retained the *in vitro* and *in vivo* autophosphorylation kinase activities similar to those of the NM1 protein. However, its ability to phosphorylate cellular substrates in general is significantly reduced (see below). Surprisingly, the extent of intracellular autophosphorylation of the mutant F1221 and double mutant F1135/36 proteins, both of which had dramatically reduced transforming activity, were similar to that of NM1 in the transient transfection assay. However, the *in vitro* kinase activity of F1221 is greatly reduced. The triple mutant F3, which had lost completely the transforming activity, was devoid of *in vitro* kinase activity and intracellular autophosphorylation (Fig. 3-3C). F943 and F950 mutant proteins displayed faster mobility of receptor bands particularly in the antiphosphotyrosine blot suggesting that these two sites play an important role in the phosphorylation of the receptor (Fig. 3-4), however UF950 of UIGFR containing the corresponding Y950 mutation did not exhibit this characteristic mobility shift.

Phosphorylation of Cellular Proteins To evaluate tyrosine phosphorylation of cellular substrates by the mutant proteins, total cell lysates of different mutant transfected CEFs were analyzed by immunoblotting with RC20, a recombinant antiphosphotyrosine antibody conjugated with alkaline phosphatase (Fig. 3-5). The overall tyrosine

phosphorylation of cellular proteins were remarkably increased over normal CEF in cells transfected by all the mutants except the weak and non-transforming variants, namely F1221, F1136, F1135/36 and F3. There was no obvious difference when compared among the strong transforming mutants and NM1. The F950, UF950 and d950 did not appear to have a difference in the pattern and extent of substrate phosphorylation, despite the fact that both UF950 and d950 have a reduced transforming activity from their respective parental viruses UIGFR and NM1. Despite the F1136 protein being as efficiently autophosphorylated as that of the NM1 protein (Fig. 3-3A), it has a significantly reduced ability to phosphorylate cellular proteins in general. By comparing the substrate pattern of F1136 protein with those of strong transforming mutants and NM1, the mutant F1136 protein failed to induce tyrosine phosphorylation of a 36KD protein, in particular, which was strongly phosphorylated in NM1- and other strong transforming mutant-infected cells. In other weak transforming or non-transforming mutants-infected CEF, this 36kD protein was not phosphorylated either. Annexin II, a 36kD phospholipid binding protein, was reported to be tyrosine phosphorylated in *src* transformed cells (Kube et al., 1991). A monoclonal antibody against annexin II was used to identify this 36kD protein in the mutant and NM1 transformed cells. The protein band detected by the monoclonal Ab superimposed with the tyrosine phosphorylated 36kD protein detected with the anti-P-tyr Ab (Fig. 3-6A). To examine subcellular localization of the 36KD protein, NM1 transformed cell lysates were fractionated into soluble and cytoskeletal (CSK) fractions. The proteins from the two fractions were resolved in SDS-PAGE gel, blotted and reacted with RC20 or the anti-annexin II Ab (Fig. 3-6B). The result showed that the 36kD protein and annexin II were co-

localized in the CSK fraction. To further confirm that annexin II is phosphorylated in NM1 transformed cells, NM1 infected cells were lysed in RIPA buffer containing 0.1% SDS and immunoprecipitated with a polyclonal anti-annexin II serum. Annexin II was strongly phosphorylated in NM1-infected cells but not in normal CEF (Fig. 3-6C). However, we can not conclude that annexin II is the sole 36kD protein observed since it remains possible that there are more than one proteins co-migrating at this position. It is concluded that the overall tyrosine phosphorylation of transforming viruses infected cells is remarkably higher than that of non- or weak-transforming viruses infected cells and annexin II is tyrosine phosphorylated in the transformed cells but not in the normal or weakly-transformed cells.

IRS-1 phosphorylation and PI3 kinase activity IRS-1 is a major phosphorylation substrate of IR and IGFR and is believed to be important in the recruitment of other signalling molecules such as PI3 kinase, Grb2/Sos complexes and Syp. To determine the ability of those mutant proteins to phosphorylate IRS1, the infected cell lysates were immunoprecipitated with anti-IRS-1 serum and Western blotted with antiphosphotyrosine Ab. The phosphorylation of IRS-1 was not significantly changed in most mutants-infected cells in repeated experiments (Fig. 3-7). Surprisingly, UF950 and F950 proteins, in which the putative IRS-1 docking site has been mutated (Keegan et al., 1994), phosphorylate IRS-1 as efficiently as their parental UIGFR and NM1 proteins. Even the deletion mutant d950 protein retains the ability to phosphorylate IRS-1 though at a slightly lower level. Association of IRS-1 with PI3 kinase results in activation of its activity (Baker et al., 1992; Myers et al., 1993). To examine the activation of PI3 kinase, its activity was assayed in the receptor or IRS-1 immunoprecipitates of cell extracts prepared from mutant receptor

plasmids transfected CEF in comparison with those from normal CEF and NM1 transfectants (Fig. 3-7). For most mutants, receptor- and IRS-1-associated PI3 kinase activity were not affected. The F950 mutant retained a wild type level of receptor- and IRS-1-associated PI3 kinase activity but UF950 and d950 proteins exhibited a decrease in the associated PI3 kinase activity when compared to their respective parental proteins. The basal level of receptor associated PI3 kinase is largely due to the cross reactivity of anti-IB for chicken endogenous IGFR. IRS-1-associated PI3 kinase activity was approximately four to five folds higher than the receptor-associated PI3 kinase activity. This could be due to the possibility that the IRS-1-PI3 kinase complex is more stable than the receptor-IRS-1-PI3 kinase triple complex or alternatively, the receptor-IRS-1 interaction is much more transient. Overall, except for the kinase inactive mutants, UF950 and d950, no significant difference in either gag-IGFR- or IRS-1-associated PI3 kinase activity was observed.

Phosphorylation of PLC γ An anti-PLC γ antibody was used to examine PLC γ phosphorylation in the cells infected with various mutants including a carboxyl deletion mutant, CM2, which lacks the carboxyl 27 amino acids including Y1316 in the YAHM sequence which is considered a potential motif for interacting with SH2-containing proteins. CM2 protein is active in PTK and cell transforming activities (Liu et al., 1993). All transforming mutants are capable of inducing PLC γ tyrosine phosphorylation while CM2 is unable to (Fig.3- 8). This result suggests that tyrosine 1316 together with its neighboring sequence may be involved in the association and/or phosphorylation of PLC γ . However, phosphorylation of PLC γ appears not to be essential for CEF transformation. A 70kD and a 180kD tyrosine phosphorylated proteins were reproducibly co-immunoprecipitated with

PLCy from NM1-infected but not from UR2-infected cells. The identities of those proteins are unknown.

DISCUSSION

My results show that mutation of tyrosine residues of the gag-IGFR fusion receptor can affect its kinase activity and transforming ability. The cluster of the three tyrosines (Y1131/1135/1136) plays an important role in regulating the PTK activity of the receptor. Those three tyrosines correspond to the tyrosine residues 1158, 1162 and 1163 of insulin receptor shown to be located at the gate of the kinase catalytic center (Hibbard et al., 1994; Wei et al., 1994). It was reported earlier that the autophosphorylation sites of the insulin receptor are the tyrosines 965, 972, 1158, 1162, 1163, 1328 and 1334 with tyrosines 1158, 1162 and 1163 being the main regulators of the enzymatic activity for insulin receptor (Kohanski, 1993; Ellis et al., 1986; Zhang et al., 1991). Mutation at each site reduces insulin-stimulated autophosphorylation by 45 to 60% of that of the wild type receptor. Double mutation reduces autophosphorylation by 70%, and replacement of all three tyrosines with phenylalanines almost abolishes the kinase activity (Smith et al., 1994). My study indicates that single mutation at position 1135 or 1136 produces little effect on the kinase activity of the chimeric receptor. However, mutation at position 1136 drastically reduced cell transforming and growth promoting activities without affecting significantly the receptor kinase autophosphorylation activity *in vitro* and intracellularly. The F1136 mutation does significantly affect its ability to phosphorylate cellular substrates in general and the 36kD annexin II protein in particular. This property correlates well with its reduced biological function. These observations suggest that mutation of this tyrosine may change the receptor conformation or that phosphorylation of this residue is important for

substrate recognition. Interestingly, mutation of the Y1136 corresponding tyrosines in the two closely related RPTKs, IR and ros, also results in significantly reduced transforming activity without affect their PTK activity (C. Zong, J. Chan and L.-H. Wang, 1996 J. Biol. Chem. in press). However, no global decrease in substrate phosphorylation like F1136 was observed in the IR and Ros mutants, although reduction in interaction with specific substrates were observed. Double mutation of Y1135 and Y1136 did not produce the drastic effect as that of Y1162 and Y1163 in native IR. It is possible that the NM1 encoded gag-IGFR is constitutively and highly activated such that it is less sensitive to changes in those sites. Mutations at position 943 and 950 result in faster mobility of the receptor proteins indicating that these two sites play important roles in the IGFR phosphorylation. The down shift in the autophosphorylation products *in vitro* and missing of the upper band in the anti-P-tyr blot of those mutants proteins suggest that Y950 and Y943 are the prominent autophosphorylation sites. This is in agreement with an earlier study showing that the juxtamembrane tyrosines of IR are the preferred autophosphorylation sites in an *in vitro* assay at low ATP concentrations (Kohanski, 1993). The two corresponding tyrosines in IR are tyrosine 965 and 972 which have been shown to be *in vivo* autophosphorylation sites (Ellis et al., 1986). The Y972 mutation of a gag-IR also resulted in a faster mobility of the protein in anti-P-tyr immunoblot (J. Chan and L.-H. Wang unpublished). Surprisingly, when the reported major phosphorylation sites, tyrosines 1135 and 1136, were substituted with phenylalanines, no significant change in the receptor protein mobility was observed. It is possible that tyrosine 943 and 950 mutations may indirectly affect serine/threonine phosphorylation as well and resulting in a more

pronounced effect. My observation suggests that, similar to IR, IGFR also contains several potential tyrosine autophosphorylation sites and mutation of any of them, excepting Y943 and Y950, does not significantly affect the overall phosphorylation of the receptor.

IRS-1 is a key component in the IR signal transduction. It has been shown that tyrosine-972 is critical for the binding of IRS-1 to IR. Point mutation of this tyrosine results in impaired phosphorylation of IRS-1 upon insulin stimulation (Kaburagi et al., 1993). From sequence alignment, tyrosine-950 in IGFR could be the interacting site for IRS-1. However, no clear experimental evidence has been provided (Keegan et al., 1994). Mutation of tyrosine-950 in the highly activated and oncogenic NM1 encoded gag-IGFR does not appear to affect its ability to induce IRS-1 phosphorylation or to promote association of PI3 kinase with IRS-1, but when the mutation is generated in the less active and moderate transforming gag-IGFR, encoded by UIGFR, there is a significant decrease in both cell transforming ability and IRS-1-associated PI3 kinase activity while tyrosine phosphorylation of IRS-1 remains unchanged. Deletion of 13 aa flanking the tyrosine-950 (943-956) of NM1 gag-IGFR, however, impacts on all three activities, namely causing a decrease in transformation, IRS-1 phosphorylation and PI3 kinase activity. While my result with UIGFR is consistent with the notion that Y950 of IGFR is involved in interaction of IGFR with IRS-1, the data with NM1 are not in complete agreement. There are three possible explanations for these paradoxical results. First, tyrosine 950 may not be the only site for the IRS-1 interaction with IGFR, and IRS-1 may have another redundant binding site. Redundancy in substrate binding sites has been shown in other RPTKs such as PDGFR (Marshall, 1995). In PDGFR, SHC has four binding sites which are tyrosine 579, 740, 751 and 771.

The second explanation is that IRS-1 is phosphorylated by other tyrosine kinases activated by IGFR. Interactions of RPTKs with non-receptor tyrosine kinase has been reported. SRC can be activated by PDGFR and the binding sites for Src in PDGFR have been identified as tyrosines 579 and 581 (Marshall, 1995). Furthermore, IGFR is activated in v-SRC transformed cells (Peterson et al., 1994). The highly activated receptor, such as NM1 gag-IGFR in particular, may have a higher potential of cross activating other PTKs. The third possibility is that this mutation can be compensated for by either overexpression or hyperactivation of the gag-IGFR. Recent studies suggested that IRS-1 interacts with IR through a phosphotyrosine binding (PTB) domain (Kavanaugh and Williams, 1994; Gustafson et al., 1995) and a pleckstrin homology (PH) domain (Voliovitch et al., 1995). Phosphorylation of IRS-1 can be abolished if the PH domain is deleted, however, it can be restored by overexpression of IR (M. White private communication). It has also been shown that overexpression of full length IR containing F972 can rescue the phosphorylation of IRS-1 despite the mutated interaction site (M.White, private communication). The constitutively activated NM1 gag-IGFR may compensate for the Y950 mutation with its high level expression and catalytic activity. My result with d950 suggests that the sequence surrounding the site is involved in the interaction with IRS-1 and Y950, however, is not critical in the highly activated receptor. IRS-1 is apparently phosphorylated to a similar extent in all mutants infected cells. However, it remains possible that different sites are phosphorylated which may account for the reduced PI3 kinase activity in d950 and UF950.

Annexin II is a tetrameric Ca^{2+} dependent phospholipid binding protein and is

associated with the cytoskeleton (Ma et al., 1994). However, other report showed that annexin II was mainly found in the membrane associated fraction (Drust and Creutz, 1991). Annexin II was first shown to be a substrate of *v-src* in its transformed cells (Kube et al., 1991). Tyrosine phosphorylation of annexin II can also be induced by PDGF receptor after treatment of PDGF (Bramibilla et al., 1991). Annexin II in addition to being a substrate for protein tyrosine kinases can also be a substrate for protein kinase C (Johnstone et al., 1992). Overexpression of annexin II was reported in human pancreatic carcinoma cells and primary pancreatic cancers, as well as in multidrug resistant small lung cancer (Cole et al., 1992; Tressler et al., 1993). Study of the RAW large lymphoma cells showed that depletion of cell surface annexin II could inhibit RAW cells from adhering to the liver microvessel endothelial cells suggesting that annexin II might be involved in metastasis (Vishwanatha et al., 1993). Moreover, annexin II was shown to be the receptor of tenascin-C indicating that annexin II may mediate cellular response to soluble tenascin-C in the extracellular matrix (Chung and Erickson, 1994). Furthermore, annexin II is one of the *fos* target genes (Braselman et al., 1992). However, the exact function of annexin II remains unknown. We have identified that annexin II is a substrate for the gag-IGFR and its phosphorylation correlates with the extent of cell transformation by the mutant gag-IGFRs raising the possibility that it may play a role in the cell transformation.

Fig. 3-1. Schematic representation of UIGFR and NM1 chimeric receptors. The tyrosine to phenylalanine mutants are indicated by their amino acid positions (Ullrich et al., 1986). The deletion in mutant d950, encompasses Tyrosine 943 to Valine 956. The CM2 mutant was reported previously (Liu et al., 1993).

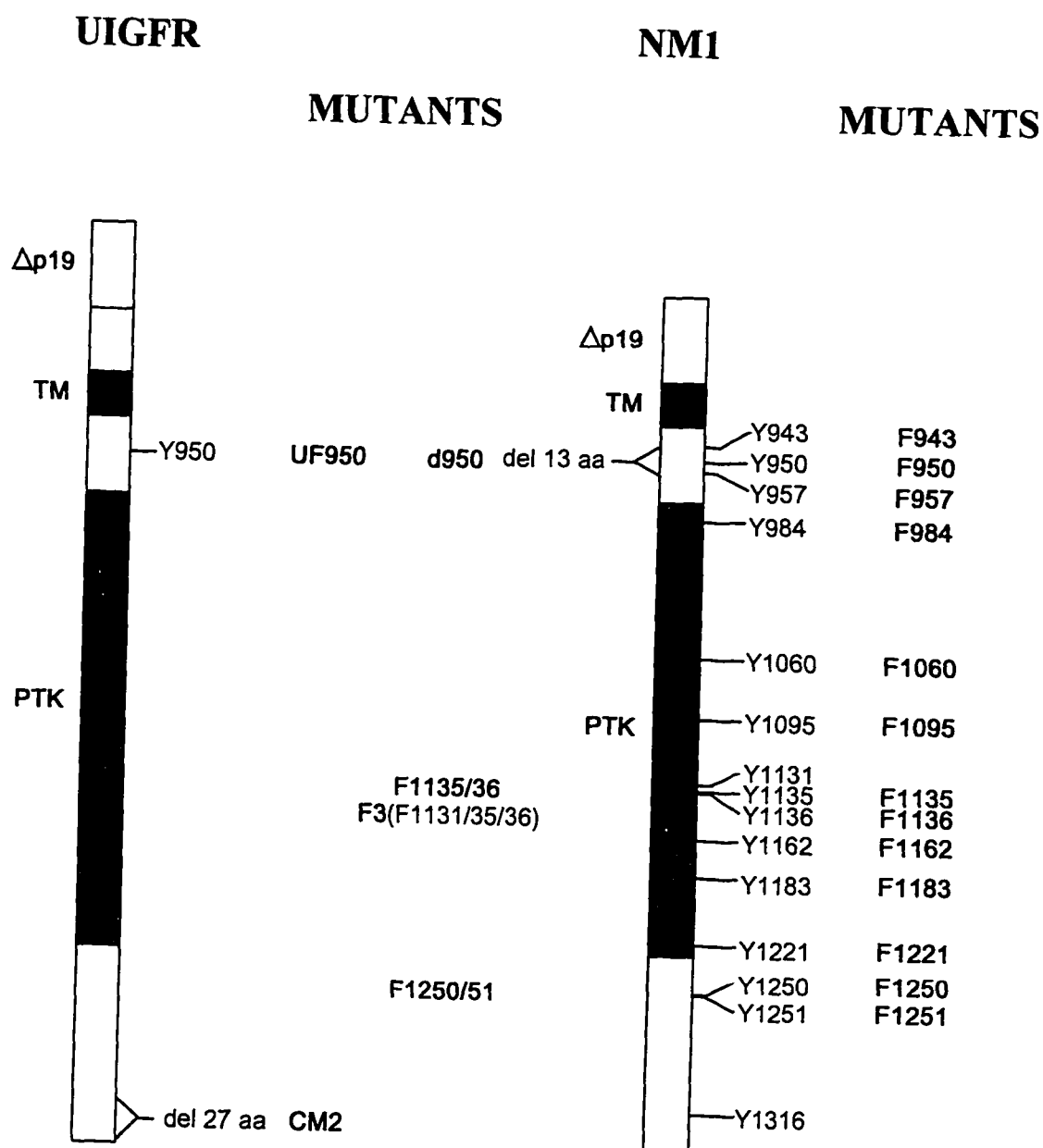


Fig. 3-2. Anchorage-independent growth of transfected CEFs. Colony assays were set up three days after transfection and the cultures were maintained at 41°C. The pictures were taken ten days after incubation.

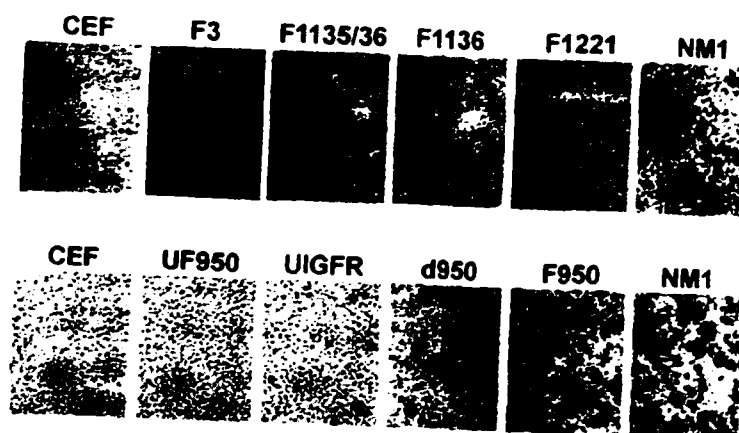


Fig. 3-3. In vitro kinase activity and intracellular phosphorylation of mutant gag-IGFRs. Confluent virus infected cells were extracted with RIPA buffer and equivalent protein amounts of total lysates were used in each immunoprecipitation using an anti-IGFR antiserum, anti-IB. Half the immunoprecipitated proteins were subjected to an *in vitro* kinase assay while the other half was Western blotted with anti-IB to monitor the amount of IGFR. A parallel culture was treated with 200 μ M Na_3VO_4 for 4 hours then lysed and immunoprecipitated similarly. The intracellular phosphorylation of the receptor was determined by blotting with an anti-phosphotyrosine antibody, RC20, coupled with alkaline phosphatase (panel A, B and E). Alternatively, cells were labelled with ^{35}S -Met before lysis and the relative amounts of gag-IGFR protein were determined by immunoprecipitation, gel analysis and autoradiography (panel D). The assays in the panel C were done with transiently transfected cells and the protein amount of gag-IGFR was monitored by Western blot. The slower mobility of UIGFR and UF950 proteins is due to the presence of additional 36 aa of the extracellular sequence in comparison with that of NM1 (Fig. 3-1) and glycosylation of the UIGFR proteins.

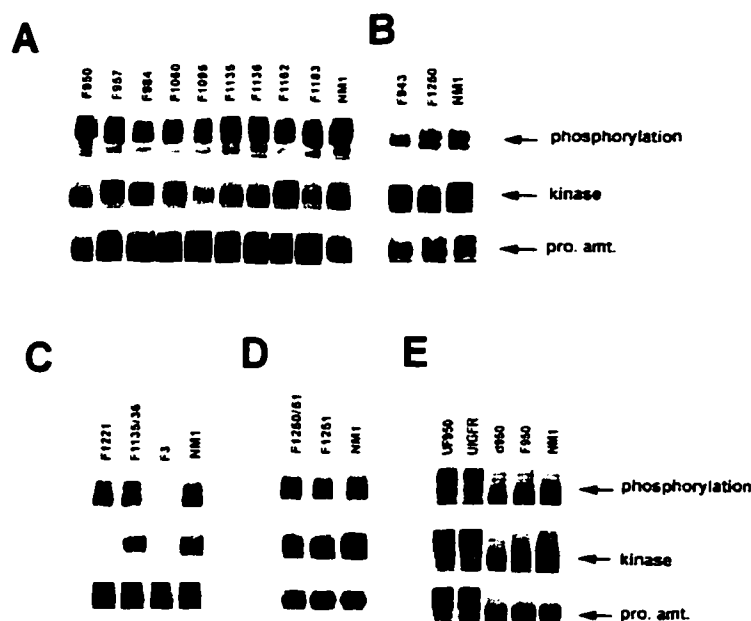


Fig. 3-4. Phosphorylation of mutants F943 and F950 proteins. Mutant gag-IGFR transfected CEFs were treated with Na_3VO_4 as in Fig. 3-3 and lysed. 200 μg each of total cellular proteins was immunoprecipitated with anti-IB and subjected to Western blotting with either anti-IB or anti-P-tyr (RC20). (A) F943 and NM1 proteins were detected by using a goat-anti-rabbit secondary antibody conjugated with alkaline phosphatase and followed by color reaction. (B) F950 and NM1 proteins were detected by ^{125}I -protein A labelling. The extent of tyrosine phosphorylation was detected by alkaline phosphatase coupled anti-P-tyr Ab, RC20, in both (A) and (B).

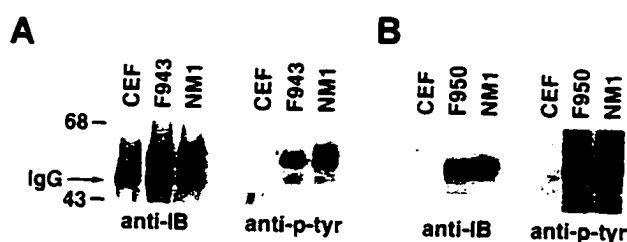


Fig. 3-5. Phosphorylation of cellular proteins by mutant gag-IGFRs. Na_3VO_4 pre-treated cells were lysed in the Western lysis buffer and 10 μg each of total cellular proteins was resolved in the 10% SDS-PAGE gels. The filters were blotted and reacted with anti-P-tyr (RC20).

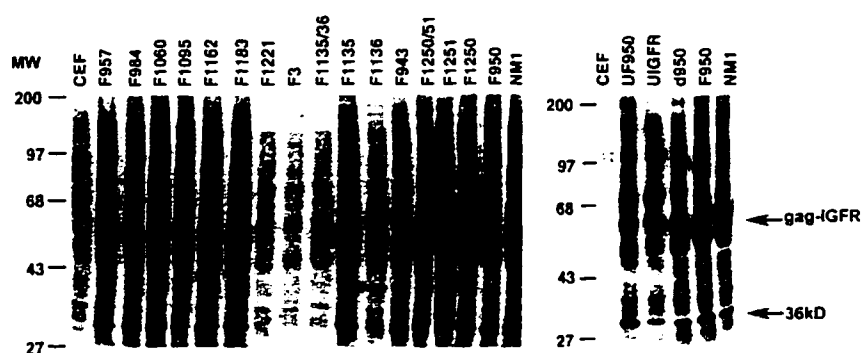


Fig. 3-6 Phosphorylation of annexin II in mutant gag-IGFR transformed cells. (A) Na_3VO_4 pretreated cells were lysed in the Western lysis buffer and 10 ug each of total cell lysate was analyzed in the SDS-PAGE gel. The filter was first blotted with anti-annexin II to detect the annexin II protein (lower panel). The same filter was stripped off the antibody and further reacted with an anti-P-tyr monoclonal antibody (PT22) followed by ^{125}I -protein A labelling. **(B)** Na_3VO_4 treated NM1 transformed cells were fractionated into soluble (Sol.) and cytoskeletal (CSK) fractions as described in the material and methods. The soluble fraction was precipitated with 70% ethanol and redissolved in Western lysis buffer. Both fractions were analyzed in the gel followed by Western blotting with either RC20 or anti-annexin II. **(C)** NM1 transformed cells were treated with Na_3VO_4 . In lanes 1 and 2, cells were lysed with RIPA buffer containing 0.2% SDS and the lysate was immunoprecipitated with the anti-annexin II. In lanes 3 and 4, cells were lysed in the Western lysis buffer. The anti-annexin II precipitates and direct Western buffer extracts were analyzed in the same gel and Western blotted. The filter was reacted with RC20.

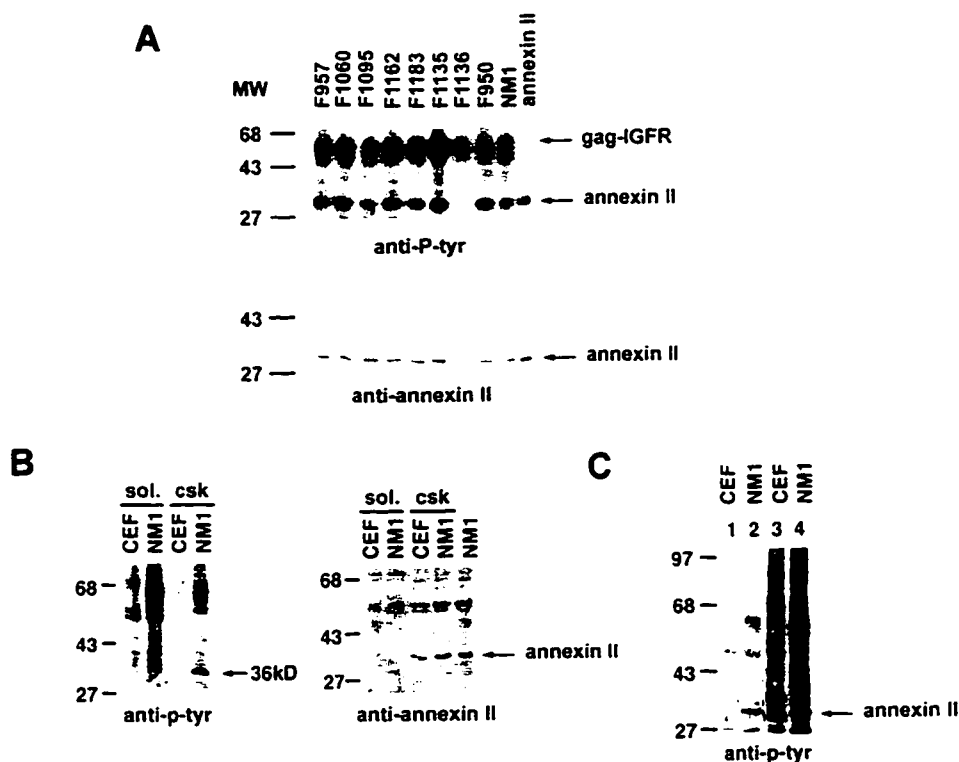


Fig.3-7. Mutant gag-IGFR- and IRS-1-associated PI3 kinase activity and phosphorylation of IRS-1. Cells pre-treated or untreated with Na_3VO_4 were lysed in RIPA or NP-40 buffer and used for detecting IRS-1 tyrosine phosphorylation or PI3 kinase assay, respectively, after immunoprecipitation with either anti-IRS-1 or anti-IB. For PI3 kinase assay, 300 μg each of lysate was used, 1 mg was used for IRS1 tyrosine phosphorylation and 50 μg was used for monitoring the gag-IGFR protein. The reduced IRS-1-associated PI3 kinase activity of F1162 was due to experimental fluctuation and repeated experiments showed no difference from NM1.

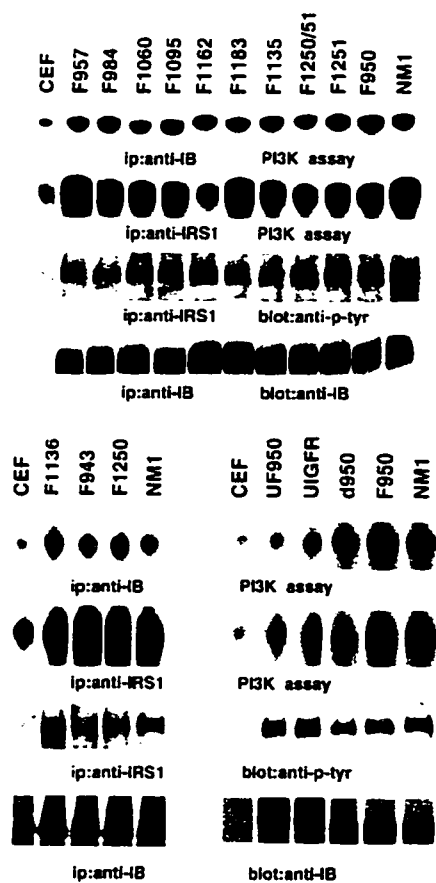


Fig. 3-8 Tyrosine phosphorylation of PLC γ . Various mutant gag-IGFR transformed cells were treated with Na_3VO_4 and lysed with RIPA. 1 mg each of total cellular proteins was used in each immunoprecipitation with anti-PLC γ . The immunoprecipitates were analyzed in the 10% SDS-PAGE gel and followed by immunoblotting with RC20.

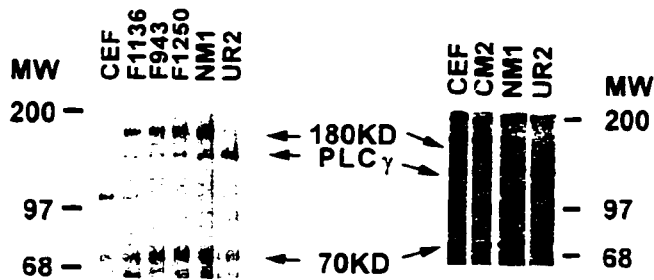
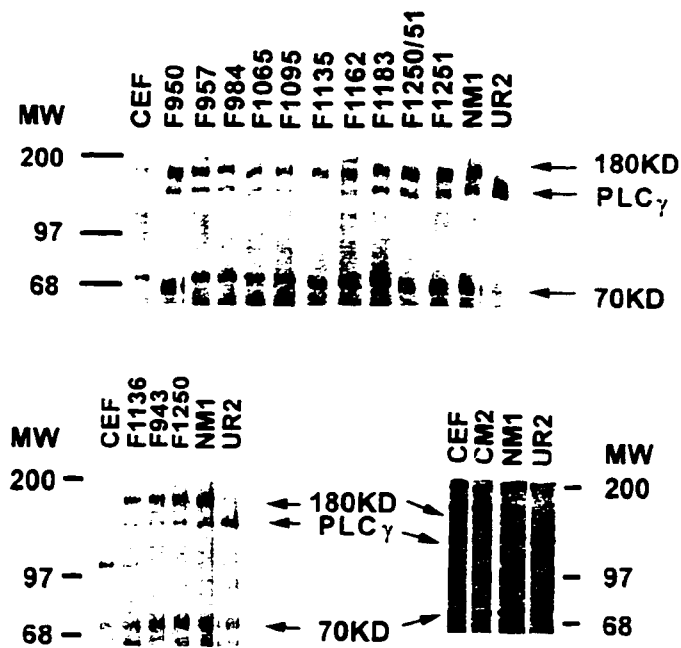


Table 3-1 Biological Properties of Mutant Viruses

Mutants	Morphology	Colonies/million cells
None(CEF)	Spindle	0
F943	Fusiform	1428
F957	Fusiform	1548
F984	Fusiform	1492
F1060	Fusiform	1500
F1095	Fusiform	1628
F1135	Fusiform	1476
F1135/1136	Spindle	20
F1136	Spindle	186
F3	Spindle	0
F1162	Fusiform	1220
F1183	Fusiform	1276
F1221	Spindle	32
F1250	Fusiform	1284
F1251	Fusiform	1320
F1250/1251	Fusiform	1236
NM1	Fusiform	1204

Chapter 4

Effects of Juxtamembrane Region Deletion and Tyrosine 1136 Mutation on the Transformation Potential of the Oncogenic gag-IGFR

Abstract

Site-specific mutagenesis was undertaken to dissect the signaling and transforming functions of the oncogenic gag-IGFR. Two mutants are particularly interesting, they are a juxtamembrane domain deletion mutant dS2 and a phenylalanine substitution mutant of Y1136, the third tyrosine of the triple tyrosine cluster in the PTK domain of IGFR.

Both mutants retain active PTK activity, but have dramatically reduced mitogenic and transforming abilities. Both mutant proteins are capable of inducing phosphorylation of IRS-1, PLC γ and STAT proteins as well as promoting association of PI3 kinase with IRS-1. Despite the loss of mitogenicity, both mutant proteins are able to activate MAP kinase as potently as the wild type gag-IGFR. This indicates that activation of MAP kinase is not sufficient for the gag-IGFR-mediated mitogenicity and that certain signaling pathway(s) other than MAP kinase is essential for the activity and this pathway(s) is impaired in these mutants. The role of MAP kinase was further investigated by using a dominant negative (dn) mutant of MEK-1, which is upstream of MAP kinase. The dnMEK-1 inhibits the transforming activity of the wild type gag-IGFR by 2 to 3 fold. Therefore, MAP kinase plays some roles in gag-IGFR induced cell growth and transformation but its activation alone is insufficient for those functions.

Introduction

Substitution of tyrosine 1136 with phenylalanine (F1136) in the gag-IGFR results in dramatic decrease in the transforming potential of this oncogenic receptor (Chapter 3). Tyrosine 950 is the binding site for IRS-1, deletion of 13 amino acids surrounding this tyrosine in NM1 gag-IGFR generates a mutant that has moderately decreased IRS-1-associated PI3 kinase activity and transforming activity. Since the juxtamembrane region of IGFR contains not only tyrosine residues but also serine/ threonine residues, it is likely to be involved in various receptor-substrate interactions. To illustrate the role of this region in the IGFR-mediated cell transformation, the fusion receptor containing a larger deletion (19aa, gly938-tyr957) of this region was constructed (dS2). Both F1136 and dS2 proteins retain their kinase activity but have very little transforming activity. These two mutants provide us useful tools to dissect the signaling pathways which may be important in cell transformation. In this study, I analyzed the downstream signaling molecules of dS2 and F1136 together with NM1. My study identifies certain signaling pathways that are important in gag-IGFR-induced cell transformation.

Results

Construction and expression of F1136 and dS2 proteins In order to explore the mechanism of cell transformation by gag-IGFR, mutants that have kinase activity but lack transforming ability are very useful for identifying essential downstream components

required for cell transformation by comparing their substrates with those of the parental NM1 protein. In Chapter 3, I used CEF to study the role of tyrosine residues in the cytoplasmic domain of gag-IGFR in its biological function. Disadvantages in CEF system: 1) gene expression requires co-transfection of a helper virus and replication of viruses could lead to further mutation, 2) unfeasibility to establish permanent lines and to sequentially introduce different genes, 3) lack of suitable immunoreagents for various signaling molecules. To overcome those difficulties, I introduced the mutants into NIH 3T3 cells (the high efficiency transfection was done by Dr. Weiqun Li in NIH). For this purpose, I constructed the mutant receptors and the parental NM1 protein, gag-IGFR, in both UR2 retroviral vector and mammalian expression vector, pMx which is a moloney MmLV LTR-based expression vector (Liu et al., 1991).

F1136 contains the third tyrosine mutation of the cluster of three tyrosines in PTK domain. It was constructed by site-directed mutagenesis as described above (Chapter 2). I have shown that the F1136 retains *in vitro* kinase activity and is tyrosine phosphorylated *in vivo*. However, F1136 has about 7 fold reduced transforming activity when compared to NM1. dS2 contains a deletion of 19 amino acids from gly938 to tyr957. It was constructed by PCR method described previously (Chapter 2). These two mutants and NM1 were cloned in chicken retroviral UR2 vector (pF1136, pdS2 and pNM1) as well as in the mammalian expression vector pMx containing a Neo selection marker (pMxF1136, pMxdS2 and pMxNM1) (Liu et al., 1991). pF1136, pdS2 and pNM1 were co-transfected with the helper virus UR2AV DNA into CEF while pMxF1136, pMxdS2 and pMxNM1 were individually transfected into NIH3T3 cells. The 3T3 cells were selected for Neo-resistance

colonies and the pool of drug resistant colonies were used as massive cultures for the biological and biochemical analysis.

Transforming activity of F1136 and dS2 proteins in CEF and NIH 3T3 cells

CEF transfected with pF1136, pdS2 and pNM1 were overlaid with soft agar the day after transfection to select for transformed cells. The colony forming assay was set up three days after transfection and maintained in 42°C for two weeks and the appearance of colonies was monitored. The transforming ability of F1136 and dS2 is dramatically reduced when compared with that of NM1 in CEF (Fig. 4-1). However, F1136 and dS2 still have wild type levels of *in vitro* kinase activity and *in vivo* autophosphorylation (see below).

3T3 cells expressing F1136, dS2 and NM1 proteins were assayed for their transforming activity. Colony forming assay was performed and the cultures were maintain in 37°C for 2 weeks. In agreement with the result obtained in CEF, F1136 and dS2 proteins exhibit greatly reduced transforming ability in 3T3 cells in comparison with NM1 protien (Fig 4-2A). 3T3 cells transformed by NM1 protein are more reflectile, elongated and some of them have a rounded morphology (Fig. 4-2B). 3T3 cells expressing F1136 protein show some degree of elongation and reflectility, however, dS2 cells are essentially indistinguishable from the normal 3T3 cells (Fig. 4-2B). This result indicates that the biological properties of dS2, F1146 and NM1 proteins correlate well in both chicken and mammalian cells.

***In vitro* kinase and intracellular phosphorylation of dS2 and F1136 proteins**

in CEF Upon activation of a RPTK, its kinase activity and level of tyrosine phosphorylation

are increased. Autophosphorylation of a RPTK is essential for initiating the signaling cascades. To assess the ability of autophosphorylation, kinase activity and intracellular tyrosine phosphorylation of dS2 and F1136 proteins were compared with those of the NM1 protein (Fig. 4-3). Both mutant proteins have a level of *in vitro* kinase activity similar to NM1 protein. dS2 protein displayed a smaller molecular size as expected from its deletion. Both mutant proteins are tyrosine phosphorylated *in vivo* at the same level as NM1 protein. However, dS2 has a noticeably reduced protein expression level. The lower expression level of dS2 protein was also observed in the established 3T3 cell line (Fig. 4-2C). This could be due to the deletion in the juxtamembrane region, which renders the protein less stable. Another mutant, dS1, containing 22 aa deletion in the same region could not be expressed successfully, the stability of the protein is likely the reason contributing to the lower expression level of the dS2 protein.

The mitogenic property of dS2 and F1136 proteins IGFR has been shown to be required for the optimal cell growth (Sell et al., 1994). In several cell types, stimulation with IGF-I results in increase of DNA synthesis and cell proliferation. Increased mitogenicity, morphological alteration and anchorage independent growth are among the most prominent events occurring upon cell transformation. These events are not necessary linked. dS2 and F1136 proteins have diminished ability in inducing morphological change and anchorage independent growth. The mitogenic activity of these two mutant receptors was examined in 3T3 cells expressing dS2, F1136 and NM1 proteins (Fig. 4-4). In DMEM medium containing 0.5 % calf serum, NM1-expressing cells grow much faster than normal 3T3 cells. However, the growth rate of dS2- and F1136-

expressing cells is much lower than that of NM1-expressing cells and is indistinguishable from that of normal 3T3 cells. Therefore, dS2 and F1136 proteins are defective in both mitogenicity and transforming activity related to morphological alteration and anchorage independent growth.

Cellular proteins phosphorylated in the mutant- and NM1-expressing cells

To determine which pathways are defective and what signaling components are missing in dS2- and F1136-expressing cells, antibodies against specific signaling molecules were used to detect their tyrosine phosphorylation or activity.

We have previously reported that annexin II is phosphorylated by NM1 protein, gag-IGFR, but not by its transformation defective variants in CEF (Jiang et al., 1996). In current study, phosphorylation of annexin II was examined in dS2-, F1136- and NM1-infected CEF (Fig. 4-5). Increased tyrosine phosphorylation of 36kD annexin II was detected in NM1-infected but not in dS2-, F1136-infected cells in comparison with normal CEF. However, enhanced tyrosine phosphorylation of the 36kD protein was not observed in NM1-transformed 3T3 cells. This indicates that cell type specific substrate phosphorylation in the process of cell transformation. Annexin II might be important for the transformation of chick cells but not for mammalian cells.

Tyrosine phosphorylation of PLC γ in NM1- and mutant-expressing 3T3 cells was analyzed (Fig 4-6). Phosphorylation of PLC γ was about 2 to 3 folds higher in dS2-, F1136- and NM1-expressing cells than that of normal 3T3 cells. This result indicates that phosphorylation and presumably activation of PLC γ is not sufficient to promote gag-IGFR induced mitogenicity and transformation. Two proteins, a 180kD and a 70kD, were co-

immunoprecipitated with PLC γ and the former was strongly tyrosine phosphorylated in dS2, F1136 and NM1 cells. The association of PLC γ with two proteins of similar molecular weight was also observed in CEF (Chapter 3). The identities of these two proteins remain unknown.

Cell transformation involves loss of contact inhibition and cytoskeletal rearrangement. Here, I examined some of the cytoskeletal protein phosphorylation. Cadherins are cell surface adhesion molecules. Catenins (α , β , and γ) associate with the cytoplasmic domain of cadherin and are thought to bridge the interaction with actin. Antibodies against α , β , and γ catenins were used to examine their phosphorylation (Fig. 4-7). Only α -catenin showed slightly increased phosphorylation in F1136 and NM1 cells. dS2 cells and normal 3T3 cells had similar level phosphorylation of α -catenin (Fig. 4-7C). Phosphorylation level of β - and γ -catenins did not increase in these three cell lines over that of the control cells (Fig. 4-7A and B).

It has been reported that STAT proteins are phosphorylated and become activated by receptor and non-receptor PTKs including EGFR, PDGFR and SRC (Fu and Zhang, 1993; Zhong et al., 1994; Cao et al., 1996; Danial et al., 1996). Phosphorylation of STAT proteins was assessed in NM1-expressing 3T3 cells and CEF (Fig. 4-8A and B). Phosphorylation of STAT3 was increased in NM1 transformed CEF (Fig 4-8B). Phosphorylation of STAT1 was not detected in CEF (Fig. 4-8B). In NM1-expressing 3T3 cells, increased phosphorylation of STAT3 and STAT5 but not STAT1 was observed (Fig. 4-8A). Phosphorylation of STAT3 and STAT5 correlated with their increased DNA binding activity (Fig. 4-8C) (the gel shift assay was done by Dr. Sadowski). Increased

phosphorylation of STAT3 was also detected in dS2- and F1136-expressing CEF and 3T3 cells (Fig. 4-9B and Fig. 4-10A). However, phosphorylation of STAT5 was only increased moderately in 3T3 cells (Fig. 4-10B). These results suggest that STAT3 is most likely activated by gag-IGFR.

Association of PI3 kinase with receptor and IRS-1 in dS2-, F1136- and NM1-expressing cells IRS-1 is a key substrate for both IGFR and IR. Phosphorylation of IRS-1 recruits p85 of PI3 kinase and GRB2/SOS complex. The ability of the mutant receptors in phosphorylating IRS-1 and activating PI3 kinase was assayed. The particular 3T3 cells we are working on have a high background level of IRS-1 phosphorylation (Fig.4-11B). This is also reflected in the IRS-1-associated PI3 kinase activity (Fig. 4-11A, left panel). The IRS-1-associated PI3 kinase only increased about two fold in dS2, F1136 and NM1 cell lines comparing with that of the control 3T3 cells. The basal level of IRS-1-associated PI3 kinase activity in 3T3 cells used here is significantly higher than we observed in the other 3T3 cells used previously (Xiong et al., 1996). By contrast, the receptor-associated PI3 kinase activity was more markedly increased in the gag-IGFR expressing cells (Fig. 4-11A, right panel).

Activation of MAP kinase in dS2-, F1136- and NM1-expressing cells As mentioned above, phosphorylation of IRS-1 leads to the activation of RAS-RAF-MAP kinase pathway. It has been known that the RAS pathway is important for the mitogenic response. Since dS2 and F1136 proteins are not only defective in transforming activity but also in mitogenicity, it is interesting to know whether or not dS2 and F1136 proteins are capable of activating MAP kinase. MAP kinase activation requires both tyrosine and

serine/threonine phosphorylation, which results in a mobility up-shift of the MAP kinase protein band in SDS gel. Activated MAP kinase can phosphorylate an exogenous substrate, myelin basic protein (MBP). Therefore, MAP kinase protein mobility shift and phosphorylation of MBP are two readouts for measuring the activity of MAP kinase. MAP kinase activity in dS2, F1136 and NM1 cells was measured by mobility shift and phosphorylation of MBP (Fig. 4-12A and B). Surprisingly, MAP kinase activity is as pronouncedly increased in dS2 and F1136 cells as in NM1 cells (Fig. 4-12A and B). These results indicate that activation of MAP kinase is not sufficient for oncogenic gag-IGFR induced cell growth and transformation and that other pathways are required.

Dominant negative (dn) MEK-1 partially inhibits gag-IGFR (NM1 protein) induced cell transformation pMxNM1 was co-transfected with either control plasmid or dnMEK-1 plasmid, pBABE/LIDA into 3T3 cells. Cells were selected for resistance to neomycin and puromycin and the pool of colonies were used to study their biological and biochemical properties. NM1 cells showed strong transforming activity in the colony forming assay. However, the dnMEK-1/NM1 co-expressing cells exhibited 2 to 3 fold reduction of total colony number and had fewer larger colonies (Fig. 4-13 top panel). Similar result was obtained in the focus forming assay (Fig. 4-14). Similar expression level of gag-IGFR in those culture was detected (Fig. 4-13 left side of bottom panel) and as expected, the double transfectants overexpressed dnMEK-1 (Fig. 4-13 right side of bottom panel).

Since MEK-1 is upstream of MAP kinase, the activity of MAP kinase was assayed by its mobility shift and ability to phosphorylate MBP (Fig 4-15 top and bottom panel). As

expected, MAP kinase activity was reduced in the dnMEK-1/ NM1 co-expressing cells.

JNK activity in dS2, F1136 and NM1 cells Cell transformation involves rearrangement of cytoskeleton protein. Small G proteins Rho, Rac1 and Cdc42 regulate stress fiber formation, membrane ruffling and formation of filopodia. Cdc42 and Rac1 have been shown to lead to JNK activation (Coso et al., 1995; Minden et al., 1995), a serine/threonine protein kinase similar to MAP kinase. To inquire about the role of JNK in cell transformation, JNK activity was measured using GST-JUN as a substrate (Fig.4-16). 3T3 cells appear to have a high basal level of JNK activity. JNK activity in dS2, F1136 and NM1 cells exhibits about 2 to 3 fold increase, indicating that defectiveness of the mutant receptors is not due to the failure to induce JNK activation.

Discussion

In our previous study, we reported that mutation at tyrosine1136 reduced the transforming potential of NM1 protein, gag-IGFR, in CEF. My current results indicate that mutant F1136 has the similar biological properties in NIH3T3 cells. Furthermore, the juxtamembrane deletion mutant, dS2, also has a dramatic reduction in transforming activity while it retains a wild type level kinase activity.

It was reported that the cluster of three tyrosines in the kinase domain of IR is important for the receptor's kinase activity (Kohanski, 1993; Ellis et al., 1968; Zhang et al., 1991). Our previous study demonstrated that the corresponding cluster of three tyrosines in IGFR also plays an important role in regulating the kinase activity of IGFR. However, replacement of any of three tyrosines to phenylalanines seems not to affect the receptor's kinase activity. Mutation of the third tyrosine, F1136, however, dramatically reduced the transforming and mitogenic activities. This result is consistent with the study done in a IGFR knock-out cell line (R⁻ cell) (Li et al., 1994). Overexpression of the full length IGFR with Y1136 mutation in R⁻ cells could not promote the optimal growth and colony formation of these cells. However, this mutant receptor is able to phosphorylate IRS-1 and SHC. My results also indicate that F1136 is still capable of phosphorylating IRS-1 (Fig. 4-11) and SHC (data not shown).

IRS-1 is an important substrate for IGFR and IR signaling. Tyrosine 960 of IR is the interacting site for IRS-1. Similarly, tyrosine 950 in the juxtamembrane domain of IGFR

is the binding site for IRS-1 (Keegan et al., 1994). IRS-1 is thought to regulate the mitogenic response of IR (Chuang et al., 1993; Rose et al., 1994). My results about dS2 mutant indicate that deletion of the juxtamembrane region sequence reduces receptor's transforming and mitogenic capability without significantly affecting its kinase activity. However, dS2 protein is still able to induce phosphorylation of IRS-1 and promotes the association of IRS-1 with PI3 kinase even though it lacks the IRS-1 binding site. There are three possible explanations for this observation. First, tyrosine 950 may not be the only site for IRS-1 interaction and IRS-1 may have other alternative binding sites on the receptor. Multiple binding sites of a substrate on a RPTK have been reported earlier. For example, SHC has four binding sites on PDGFR (Marshall, 1995). Second, IRS-1 may be phosphorylated by other tyrosine kinases activated by the mutant IGFR. It was shown that SRC can phosphorylate and activate IGFR (Peterson et al., 1994). Thirdly, under the high concentration of the overexpressed receptor, the requirement for the site specific interaction may be less stringent. The PH domain of IRS-1 was shown to be important for the phosphorylation of IRS-1 by IR and the function of the PH domain is thought to direct IRS-1 to the plasma membrane (Voliovitch et al., 1995). Phosphorylation of IRS-1 can be impaired when the PH domain is deleted, however, it can be restored by overexpression of IR protein.

Activation of PI3 kinase by IGF-I or insulin is achieved through interaction between the p85 regulatory subunit of PI3 kinase and IRS-1 (Backer et al., 1992). However, recent study suggest that PI3 kinase can also directly interact with IR at tyrosine 1322 near the carboxyl-terminus of the receptor (Van Horn et al., 1994). There is a corresponding

tyrosine at position 1316 in IGFR and, thus, activation of PI3 kinase could occur via interaction with either IRS-1 or the carboxyl tyrosine.

STATs were initially shown to be involved in the interferons and cytokines mediated signal transduction. During recent years, several RPTKs have been shown to activate STATs (Fu et al., 1993; Silvennoinen et al., 1993). Using yeast two hybrid system, STAT5 was found to directly interact with IR and was rapidly phosphorylated *in vivo* upon insulin stimulation under physiological condition (Chen et al., 1996 submitted). However, the role of STATs in regulating cell transformation remains unknown. My results indicate that gag-IGFR is able to induce tyrosine phosphorylation of STAT3 and STAT5 raising a possibility that they could be the direct substrate of IGFR. Since dS2 and F1136 proteins can also induce phosphorylation of STAT3 and STAT5 and these two mutant receptors have reduced transforming activity, activation of STAT3 and STAT5 is not sufficient for gag-IGFR-mediated cell transformation.

Tyrosine phosphorylation of IRS-1 results in recruitment of GRB2/SOS complex leading to the activation of RAS-RAF-MEK-MAP kinases pathway. Alternatively, activated receptor can recruit SHC which then interacts with GRB2/SOS complex resulting in RAS pathway activation. The RAS pathway has been considered important for mitogenic signaling (Blenis, 1991). Activated MAP kinase translocates to the nucleus and phosphorylate transcription factors such as c-JUN resulting in gene activation and cell proliferation. Expression of oncogenic RAS alone in R⁻ cells was not able to support optimal cell growth in serum free medium suggesting that activation of RAS alone is not sufficient to gain full mitogenicity (Sell et al., 1994). This result also indicates that IGFR

has RAS-dependent and RAS-independent pathways. It was speculated that the RAS-independent pathway may be important for cell growth and transformation since oncogenic RAS could not transform R⁻ cells in the absence of serum. My study shows that MAP kinase is fully activated in dS2 and F1136 cells, however, their growth rate is much lower than that of NM1 cells and is similar to that of 3T3 cells under the low serum concentration. This observation indicates that activation of MAP kinase alone is not sufficient to promote the gag-IGFR-induced mitogenicity and cell transformation and that other RAS-independent pathway(s) is required. I speculate that the RAS-independent pathway could be mediated by a group of small G proteins such as RhoA and Rac1/Cdc42.

Fig. 4-1 Colony forming ability of mutant gag-IGFRs in CEF. CEF was co-transfected with individual mutant plasmids and helper virus UR2AV DNA. Three days after transfection, one million cells were plated per 10 cm dish in MEM medium containing soft and the cultures were maintained in 42°C for two weeks (Chapter 2). Pictures were taken ten days after the colony assay was set up.

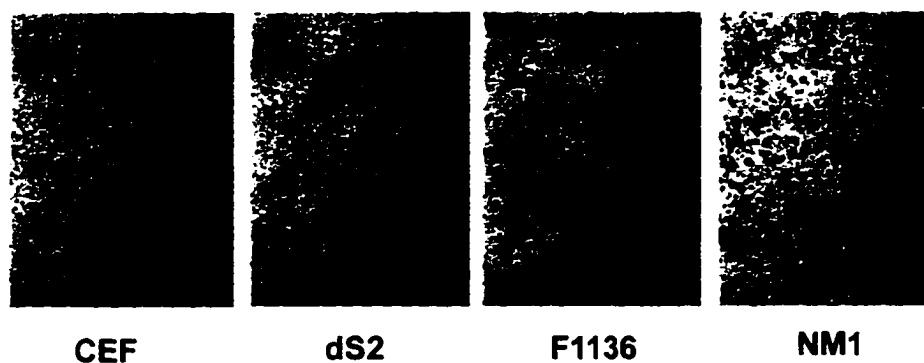


Fig.4-2 Colony forming ability of mutant receptors in 3T3 cells. 3T3 cells were transfected with pMxdS2, pMxF1136 and pMxNM1 and selected with neomycin. The pool of colonies were used as mass cultures for the biological and biochemical analysis. (A) 10^5 cells were plated in each 6 cm dishes containing DMEM agar medium and the cultures were maintained in 37°C for two weeks. The pictures were taken ten days after the colony assay was set up. (B) Monolayer cultures of 3T3, dS2-, F1136- and NM1-expressing cells. (C) Cells were lysed in RIPA. 400 μ g each of total cellular proteins was immunoprecipitated with anti-IGFR and the immunoprecipitate was analyzed by Western blotting with anti-IGFR to detect the expression of gag-IGFR.

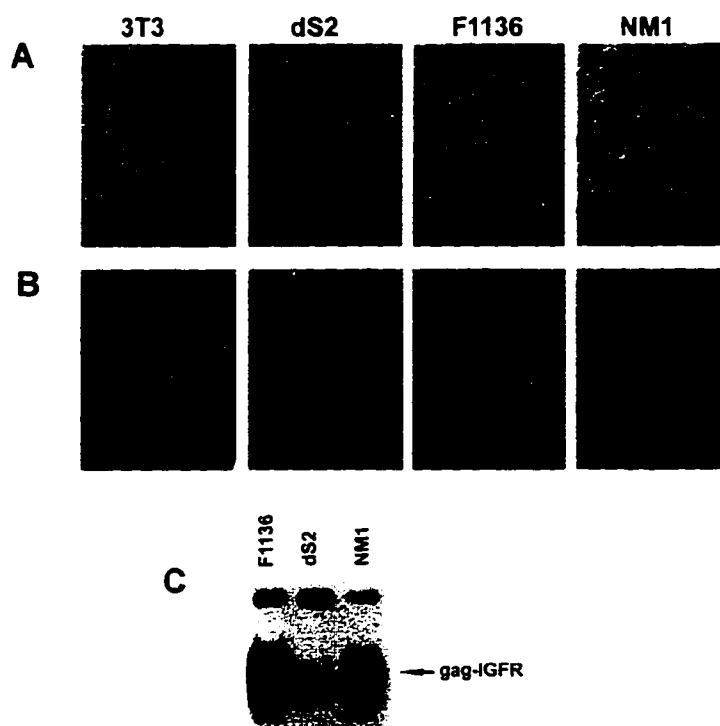


Fig. 4-3 *In vitro* kinase activity and intracellular phosphorylation of mutant receptors. The parallel CEF cultures used in the colony assay in Fig. 4-1 were treated with 200 μ M Na_3VO_4 for 3 hours and lysed in RIPA buffer. Equivalent amount of cell lysates was immunoprecipitated with anti-IGFR. Each immunoprecipitate was divided into three aliquots and subjected to *in vitro* kinase assay, Western blotting with anti-P-tyr to detect tyrosine phosphorylation or blotting with anti-IGFR to detect the protein amount.

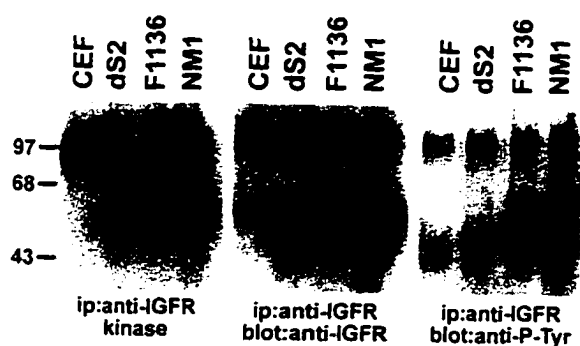


Fig. 4- 4 Growth rate of 3T3 cells expressing gag-IGFRs. 1.5×10^4 cells were plated per 3 cm dish in duplicates and the cultures were maintained in DMEM containing 0.5% calf serum. Cells were counted every other day.

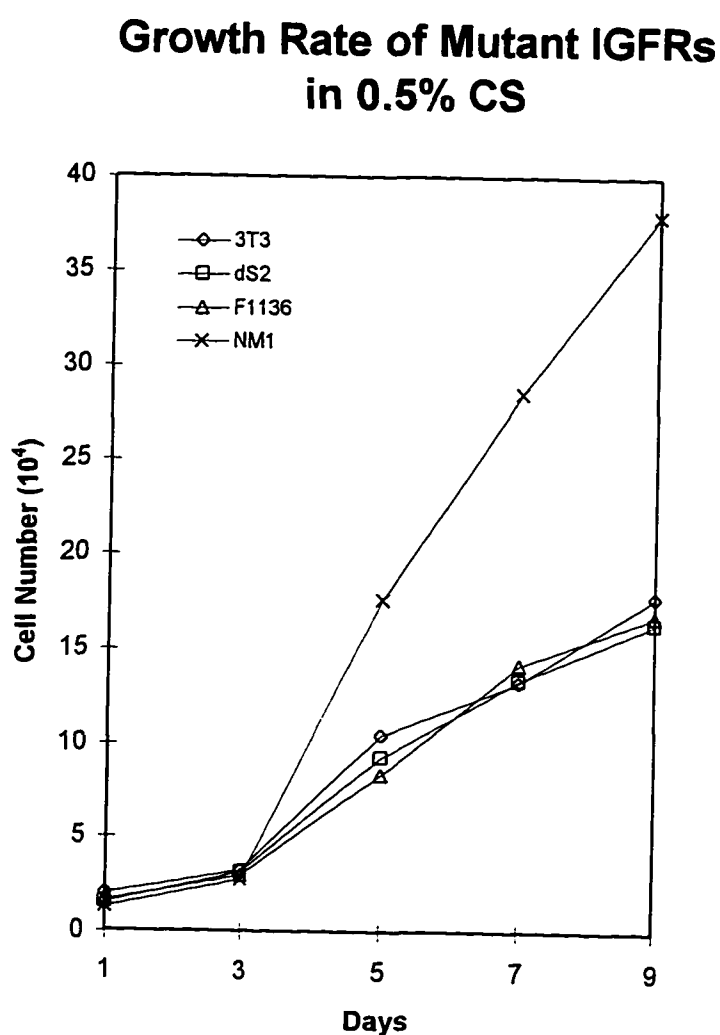


Fig. 4-5 Tyrosine phosphorylation of annexin II in CEF. CEF infected with various mutant viruses were treated with 200 μ M Na_3VO_4 for 3 hours and lysed in RIPA buffer. 500 μ g each of total cellular proteins was immunoprecipitated with anti-annexin II and the immunoprecipitates were analyzed by Western blotting with anti-P-tyr to detect tyrosine phosphorylation.

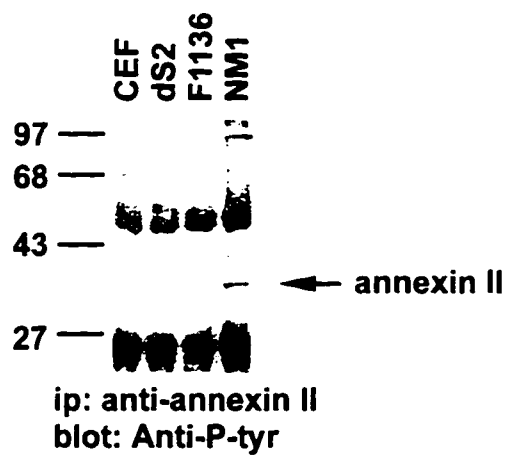


Fig. 4-6 Tyrosine phosphorylation of PLC γ in 3T3 cells expressing gag-IGFRs. dS2-, F1136- and NM1-expressing 3T3 cells were treated with 200 μ M Na_3VO_4 for 3 hours and lysed in RIPA buffer. 1 mg each of total cellular proteins was immunoprecipitated with anti-PLC γ and subjected to Western blotting with either anti-P-tyr to detect tyrosine phosphorylation (top panel) or anti-PLC γ to detect protein (Bottom panel).

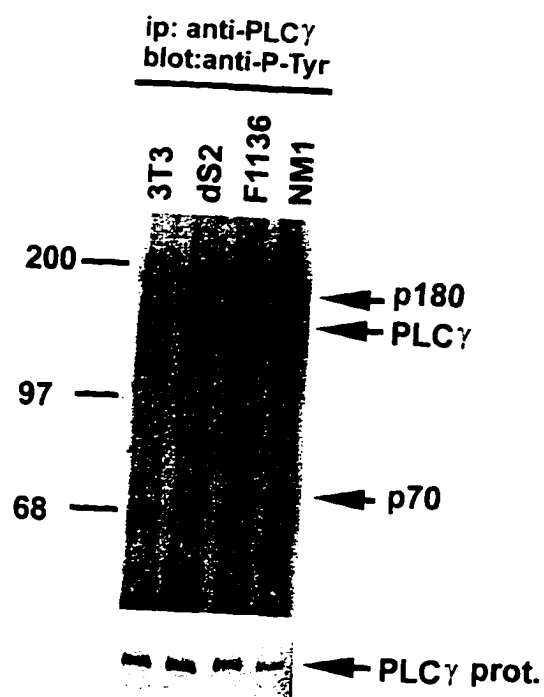


Fig. 4-7 Tyrosine phosphorylation of catenins. 3T3 cells expressing dS2, F1136 and NM1 proteins were treated with 600 μ M Na_3VO_4 for half an hour and lysed in RIPA. 1 mg each of total cellular proteins was immunoprecipitated with anti- β -catenin (A), or anti- γ -catenin (B), or anti- α -catenin (C). Half of each immunoprecipitate was analyzed by Western blotting with anti-P-tyr to detect tyrosine phosphorylation and the other half was analyzed by Western blotting with correspondent anti-catenin antibodies to detect protein.

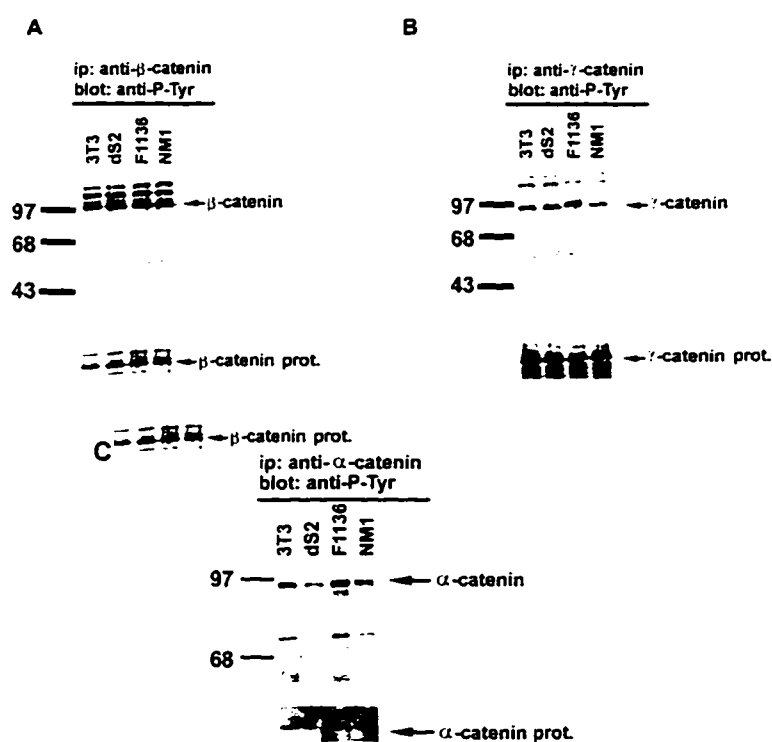


Fig. 4-8 Activation of STAT proteins by gag-IGFR. (A) NM1-expressing 3T3 cells were treated with 600 μM Na_3VO_4 for half an hour and lysed in RIPA buffer. 1.2 mg each of total cellular proteins was immunoprecipitated individually with anti-STAT1, anti-STAT3 or anti-STAT5. Two third of each immunoprecipitate was subjected to Western blotting with anti-P-Tyr to detect tyrosine phosphorylation and one third of the immunoprecipitate was subjected to Western blotting with correspondent antibodies to detect STAT proteins. (B) NM1 transformed CEF were treated with 200 μM Na_3VO_4 for 3 hours and lysed in RIPA. Phosphorylation and protein of STAT1 and STAT3 were detect as described in (A). (C) NM1-expressing 3T3 cells were treated with 600 μM Na_3VO_4 for half an hour and subjected to gel shift assay as described (Sadowski et al., 1993)

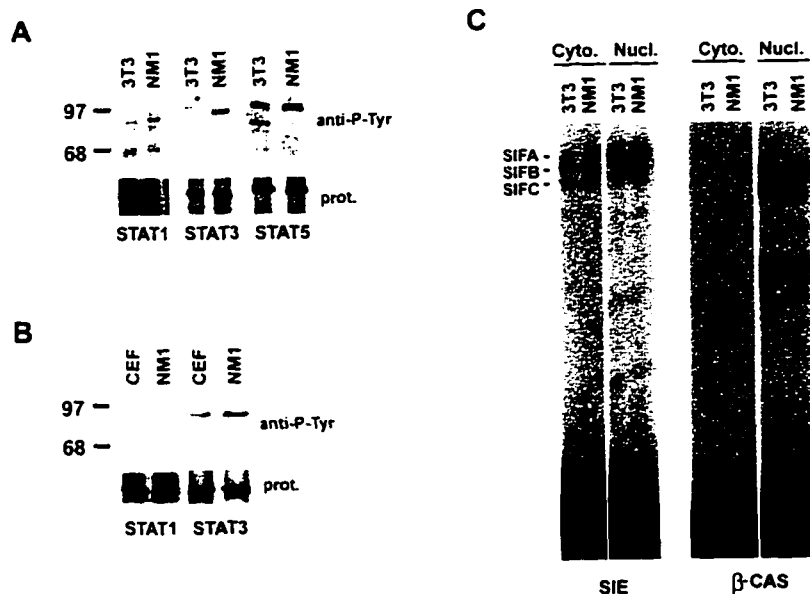


Fig. 4-9 Tyrosine phosphorylation of STATs in CEF. CEF infected with various mutant viruses were treated with 200 μ M Na_3VO_4 for three hours and lysed in RIPA. 1 mg each of total cellular protein was immunoprecipitated with either anti-STAT1 (A) or anti-STAT3 (B). The immunoprecipitates were subjected to Western blotting with anti-P-tyr or anti-STATs to detect phosphorylation or protein respectively.

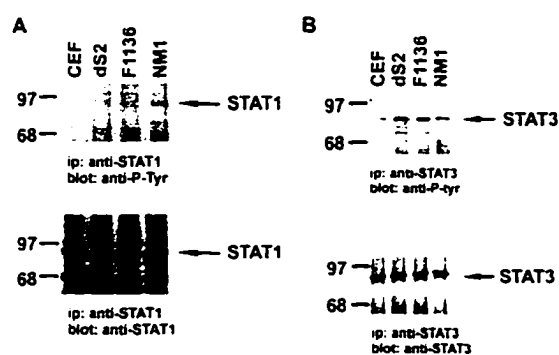


Fig. 4-10 Tyrosine phosphorylation of STAT3 and STAT5 in 3T3 cells expressing gag-IGFRs. dS2-, F1136- and NMi-expressing 3T3 cells were treated with 600 μ M Na_3VO_4 for half an hour and lysed with RIPA. 1 mg each of total cellular protein was immunoprecipitated with either anti-STAT3 (A) or anti-STAT5 (B) and the immunoprecipitates were subjected to Western blotting with anti-P-tyr or anti-STATs to detect phosphorylation or protein respectively.

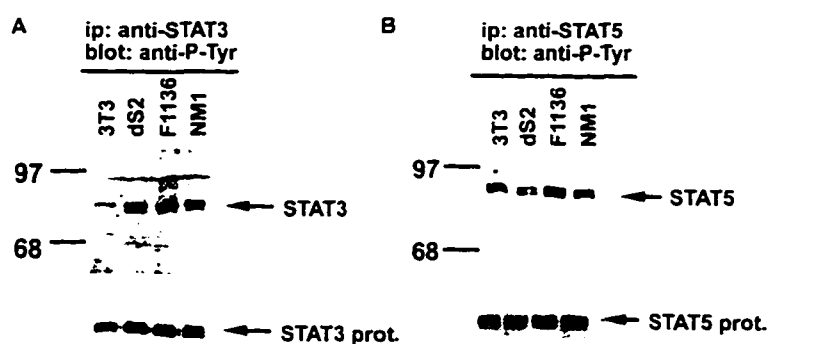


Fig. 4-11 Association of PI3 kinase with IRS-1 and gag-IGFR and in the mutant receptor expression 3T3 cells. Cells were starved in serum free medium over night and lysed in NP-40 buffer. (A) 300 μ g each of total cellular proteins was immunoprecipitated with either anti-IGFR (right panel) or anti-IRS-1 (left panel) and the immunoprecipitates were subjected to PI3 kinase assay. (B) 1 mg each of total cellular proteins was immunoprecipitated with anti-IRS-1 and the immunoprecipitates were subjected to Western blot with anti-P-Tyr to detect phosphorylation or with anti-IRS-1 to detect protein.

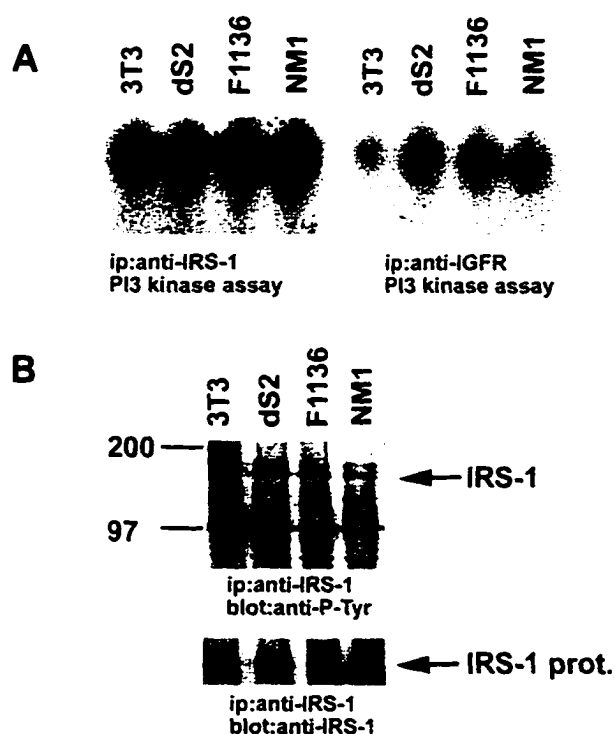


Fig. 4-12 Activation of MAP kinase in 3T3 cells expressing gag-IGFRs. Cells were starved in serum free medium overnight and treated with 600 μM Na_3VO_4 for half an hour. The treated cells were lysed in RIPA. (A) 20 μg each of total cellular proteins was loaded in each lane in a lowcross-linking gel for Western blotting with anti-ERK2 to detect the mobility shift of MAP kinase. (B) 200 μg of total cellular proteins was immunoprecipitated with anti-ERK2 and subjected to an *in vitro* kinase assay using MBP as an exogenous substrate (Chapter 2).

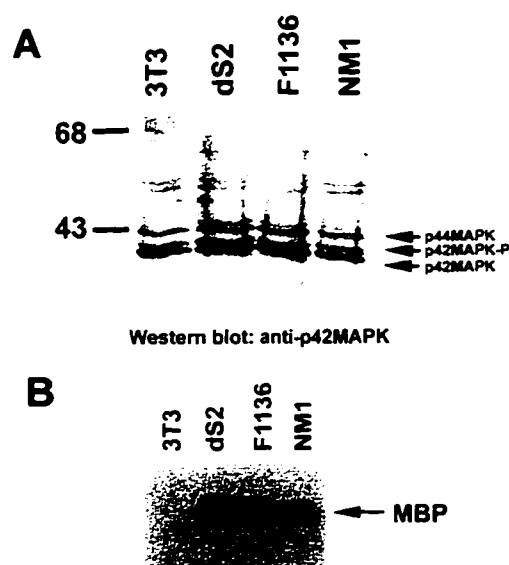


Fig. 4-13 Dominant negative MEK-1 partially inhibits transforming activity of NM1 protein. 3T3 cells were co-transfected with pMxNM1 and control vector or dominant negative MEK-1 plasmid, pBABE/LIDA. Transfected cells were double selected with neomycin and puromycin. The pool of colonies were used for the biological and biochemical analysis. Colony assay was set up as described in Chapter 2 (top panel). The colonies were stained with DMEM containing 50% INT (Sigma) dye (middle panel). The overexpression of dominant negative MEK-1 was determined by Western blotting (bottom panel). 500 ug each of total cellular proteins was immunoprecipitated with anti-MEK-1 and the immunoprecipitates were analyzed by Western blotting with anti-MEK-1 (right side of bottom panel). 200ug each of the same lysate was immunoprecipitated with anti-IGFR and the immunoprecipitates were analyzed by Western blotting with anti-IGFR to detect the expression of gag-IGFR(left side of bottom panel).

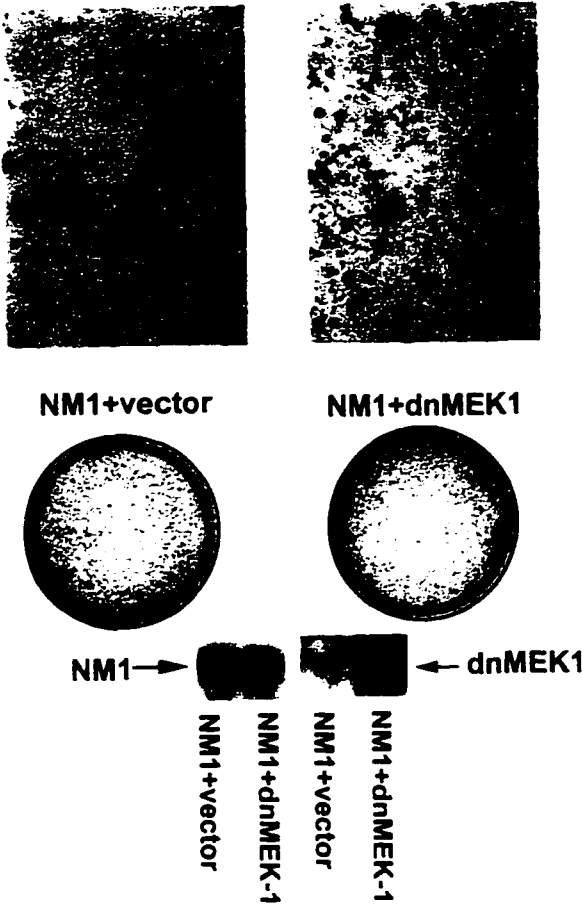


Fig 4-14 Dominant negative MEK-1 inhibits focus forming activity of NM1 protein. 3T3 cells were co-transfected with pMxNM1 and control vector (pBABE) or dominant negative MEK-1 plasmid, pBABE/LIDA, and the focus assay was set up as described in Chapter 2.

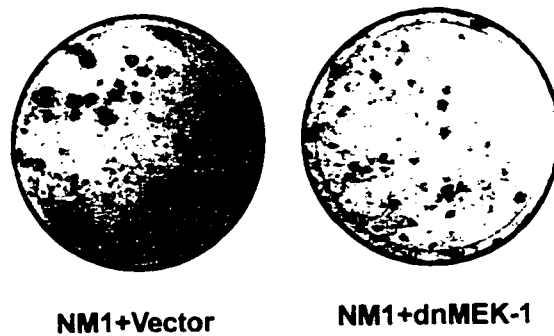


Fig. 4-15 Inhibition of MAP kinase activity by dominant negative MEK-1. The cultures described in Fig. 4-13 were starved in serum free medium overnight and treated with 600 μ M Na₃VO₄ for half an hour. The treated cells were lysed in RIPA and subjected to Western blotting with anti-ERK-2 or MAP kinase assay as described in Chapter 2.

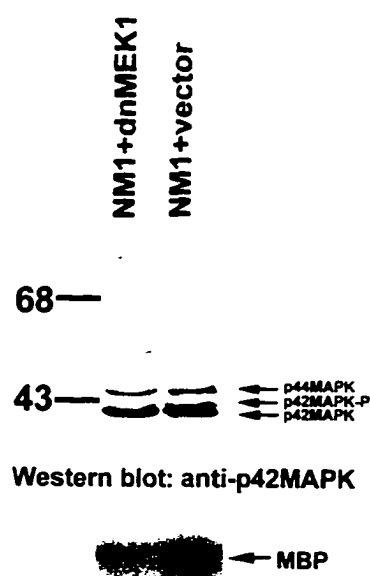
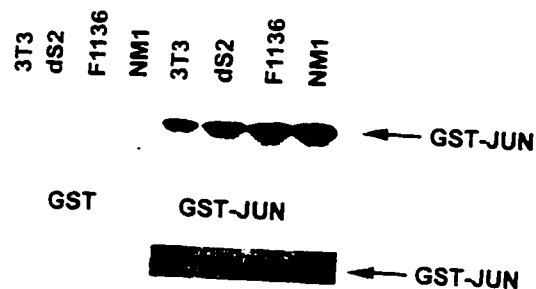


Fig. 4-16 Activation of JNK in 3T3 cells expressing dS2, F1136 and NM1 proteins. Cells were pretreated with 600 μM Na_3VO_4 for half an hour and lysed in NP-40 buffer. 200 μg each of total cellular proteins was incubated with immobilized GST or GST-JUN fusion protein over night at 4°C. The beads were washed and subjected to *in vitro* kinase assay (Chapter 2).



Chapter 5

The Role of RhoA, Rac1, and Cdc42 in Oncogenic Human Insulin-like Growth Factor I Receptor-Mediated Cell Transformation

Abstract

Activated RhoA, Rac1, and Cdc42 partially rescue the transforming activity of dS2 and F1136 proteins. RhoA is more effective in dS2 and Cdc42 is more effective in F1136 mediated transformation. Dominant negative RhoA dramatically inhibits the transforming activity of NM1 protein. Apparently, the two mutants are defective in different signaling pathways. Results also indicate that RhoA, Rac1, and Cdc42 play important roles in gag-IGFR-induced cell transformation; however, activation of each alone is not sufficient.

Together with previous data, I conclude that MAP kinase, RhoA and Cdc42/Rac1 pathways cooperate in gag-IGFR-induced cell transformation and cell proliferation.

Introduction

Cell transformation is a multifactorial process. It involves enhancement of cell proliferation, loss of contact inhibition, reduced secretion of extracellular matrix components and reorganization of cytoskeletal structures. Some of these processes are believed to be mediated by small G proteins such as RhoA, Rac1, and Cdc42 (Chapter 1)

I have constructed two mutants derived from the NM1-encoded gag-IGFR oncogenic receptor; one has a 19 aa deletion within the juxtamembrane domain (dS2) and

the other has a tyrosine to phenylalanine substitution at position 1136 (F1136). Both mutant receptors retain kinase activity but have greatly reduced transforming potential. Both mutants are capable of activating MAP kinase implying that the RAS/MAP kinase pathway is not affected by the mutations. To examine the pathways that are defective in these two mutants, I co-expressed constitutively activated RhoA, Rac1 or Cdc42 with either dS2 or F1136 proteins in 3T3 cells to determine whether these activated small G proteins could rescue the transforming ability of these mutants. These small G proteins have been shown to be important for RAS-mediated transformation and are known to signal the activation of ser/thr kinase pathways parallel to the MAP kinase pathway (Kosravi-Far et al., 1995; Coso et al., 1995; Minden et al., 1995). Conversely, I also co-expressed dominant negative mutant RhoA to determine whether it could inhibit the transforming activity of the NM1 encoded gag-IGFR oncogenic receptor.

Results

Dominant negative RhoA inhibits the transforming activity of an oncogenic gag-IGFR (NM1 protein) To investigate the role of the small G protein, RhoA, in the transforming process of NM1 protein, pMxNM1 with pZipNeo or pZipRhoA19N (a dominant negative mutant of RhoA obtained from Dr. Channing Der) were co-transfected into NIH3T3 cells which were then selected for G418 resistant clonal populations (transfection and selection was done by Dr. Weiqun Li). The transfected cells were assayed for focus formation. The pool of drug resistant colonies were used for further

analysis including colony assays and biochemical characterization of the cells. The expression level of NM1 protein in cells transfected with either pMxNM1 alone or pMxNM1 and pZipRhoA19N was comparable (Fig. 5-1B).

The transforming properties of NM1 cells and NM1/dnRhoA co-expressing cells were compared by colony and focus forming assays. Cells co-transfected with pMxNM1 and pZipRhoA19N developed much fewer and smaller colonies than the NM1 cells (Fig. 5). Dominant negative RhoA reduces the colony forming ability of NM1 protein by about 3 fold (Table 5-1). This result indicates that RhoA is involved in gag-IGFR mediated cell transformation.

Since Rac1 and Cdc42 but not RhoA are upstream activators of JNK and MAP kinase, JNK and MAP kinase activities, as reflected by *in vitro* kinase assay and gel mobility shift, were not affected by dominant negative RhoA (Fig. 5-2).

Constitutively active RhoA (RhoAQ63) rescues the transforming activities of dS2 and F1136 proteins Both dS2 and F1136 proteins retain wild type level kinase activities but have greatly diminished transforming ability. To determine whether the G protein-mediated pathways are defective in dS2- and F1136-expressing cells, either pMxdS2 or pMxF1136 was co-transfected with pCEV29RhoAQ63 (obtained from Dr. Toru Miki) into 3T3 cells. The transfected cells were assayed directly by focus forming assay or selected with neomycin and the pool of drug resistance colonies were used for colony formation assay and biochemical analysis (transfection was done by Dr. Wei quen Li). The results are shown in Table 5-1 and Fig. 5-4. Cells transfected with pMxdS2 or pMxF1136 developed very few small colonies and foci (Fig. 5-3 and Fig. 5-4). pCEV29RhoAQ63

transfectants displayed considerably more robust colony and focus forming abilities (Fig. 5-3 and Fig. 5-4). The dS2/RhoAQ63 and F1136/RhoAQ63 co-expressing cells developed more and larger sized colonies as well as numerous foci (Fig. 5-3, Fig 5-4). The size of some colonies appeared in dS2/RhoAQ63 cells were as large as NM1-transformed 3T3 cells (Fig. 5-3). However, RhoAQ63 rescued the colony forming activity of dS2 protein more effectively than F1136 (14 fold increase versus 0.4 fold increase, table 5-1), suggesting that these two mutants may be defective in different signaling pathways. However, RhoAQ63 cannot restore the transforming activity of dS2 and F1136 to that of NM1 protein. RhoAQ63 can rescue both colony and focus forming activities of dS2 and F1136 proteins suggesting that RhoA may be involved in promoting anchorage independent growth and loss of contact inhibition. This is consistent with the observation that RhoAQ63 alone is able to induce a basal level of foci and colonies (Table 5-1, Fig. 5-4).

Constitutively activated Cdc42 (Cdc42V12) enhances the colony but not focus forming activity of dS2 and F1136 proteins Cdc42 and RhoA are involved in distinct signaling pathways. Cdc42 activates the JNK/p38 pathway while RhoA activates a PKN pathway (Coso et al., 1995; Watanabe et al., 1996). To evaluate the role of Cdc42 in regulating gag-IGFR mediated cell transformation, the same co-transfection strategy as described above was used to establish Cdc42V12-, dS2/Cdc42V12- and F1136/Cdc42V12-expressing cells. The transforming properties of these cells were analyzed by focus and colony formation assays. Unlike RhoAQ63, Cdc42V12 alone did not induce focus formation in a monolayer culture and it was unable to rescue the focus

forming activity of dS2 and F1136 proteins (Fig. 5-6). Cdc42V12 enhanced the colony forming activity of both dS2 and F1136 proteins (Fig. 5-5 and Table 5-1). Cdc42V12 promoted the colony forming ability of F1136 protein better than RhoAQ63, whereas RhoAQ63 rescued dS2 more effectively than that of F1136. This observation implies that these two mutants are defective in different signaling pathways as measured by their colony forming activities. These results also suggest that the pathway initiated by Cdc42 is more likely to be involved in regulating anchorage independent growth rather than promoting the loss of contact inhibition. Similar to RhoAQ63, Cdc42V12 is unable to restore the colony forming activities of dS2 and F1136 proteins to that of NM1 protein. This suggests that Cdc42 pathway only plays a partial role in mediating gag-IGFR-induced cell transformation.

Constitutively activated Rac1 (Rac1V12) rescues the colony forming but not focus forming activity of dS2 and F1136 proteins Rac1 and Cdc42 overlap the same signaling pathway leading to the activation of the ser/thr kinases JNK and p38 (Coso et al., 1995; Minden et al., 1995). Since Cdc42 promoted colony but not focus forming activity of dS2 and F1136 proteins, the ability of Rac1 to rescue the transforming activity of these two mutants was also assessed. Expression of Rac1V12 alone resulted in a basal level of colonies and very few foci (Fig. 5-7, Fig. 5-8, table 5-2). Similar to Cdc42V12, co-expression of Rac1V12 with either dS2 or F1136 proteins significantly enhanced the colony forming activity of both mutants (Fig. 5-7). However, Rac1V12 did not enhance focus forming activity of either mutant (Fig. 5-8). Like Cdc42, Rac1 may be more important in promoting anchorage independent growth but less important in

promoting the loss of contact inhibition.

Constitutively activated RhoA, but not Rac1 and Cdc42, cooperates with oncogenic RAS (RASL12) to promote focus formation To further confirm that Rac1 and Cdc42 do not promote the loss of contact inhibition in monolayer culture. RASL12/RhoAQ63, RASL12/Rac1 and RASL12/Cdc42 co-expressing cells were assayed for their focus forming activity. RhoAQ63 cooperates with RAS to promote foci formation while Rac1V12 and Cdc42V12 do not (Fig. 5-9).

Discussion

F1136 and dS2 affect the mitogenic, focus and colony forming activities of the oncogenic gag-IGFR. While both mutants are equally impaired in their mitogenicity, F1136 protein seems to retain higher focus and colony promoting activities than dS2 protein. These mutants provide us tools to dissect distinct signaling pathways involved in the oncogenic IGFR-induced cell transformation. Results presented in Chapter 4 and here suggest that dS2 and F1136 do not affect the RAS/MAP kinase pathway, but impair other small G protein-mediated signaling pathways.

Two evidence in this study indicate that the RhoA pathway plays an important role in gag-IGFR induced cell transformation. One is that dominant negative RhoA inhibits NM1-induced cell transformation activity by 3 to 4 fold. The other is that activated RhoA rescues the transforming ability of dS2 and F1136 proteins. However, the inhibition and rescue are partial, which suggests that the RhoA pathway alone is not sufficient to fully support oncogenic IGFR-mediated cell transformation. The observation that activated

RhoA enhances both colony and focus forming activity of dS2 and F1136 proteins suggests that RhoA may be involved in regulating anchorage independent growth as well as loss of contact inhibition. The small G proteins RhoA, Rac1 and Cdc42 were shown to be involved in the rearrangement of the cytoskeleton including the formation of stress fibers, membrane ruffling, and filopodia (Ridely et al., 1994; Nobes and Hall., 1995; Symons, 1995). Recently, activated RhoA was shown to be important in RAS-mediated cell transformation (Khosravi-Far et al., 1995). Activated RhoA can cooperate with activated RAF to transform 3T3 cells while RhoA itself does not cause transformation. This is different from what we observed. However, we have reproducibly observed the basal level of activated RhoA-induced foci and colonies under our conditions. The different amount of RhoAQ63 DNA used in our experiments may explain the discrepancy from the published reports.

Since Cdc42 and Rac1 overlap the signaling pathway to activate the downstream ser/thr kinases, it is likely that Cdc42 and Rac1 have similar effect in gag-IGFR induced cell transformation. My results show that both activated Cdc42 and Rac1 rescue the colony but not the focus forming activity of dS2 and F1136 proteins. Moreover, activated RhoA cooperates with an oncogenic RAS to promote focus formation in monolayer culture while activated Cdc42 and Rac1 do not. These results suggest that Cdc42 and Rac1 are involved in promoting anchorage independent growth but not the loss of contact inhibition while RhoA is involved in both functions.

My data also show that JNK and MAP kinase activities were not affected by expression of dominant negative RhoA in NM1 cells. This is consistent with the

established pathway showing that Cdc42 and Rac1, but not RhoA, are the upstream activators of MAP kinase and JNK (Minden et al., 1995; Olson et al., 1995).

JNK belongs to a family of protein kinases called stress-activated protein kinases which are strongly activated by UV light, heat shock, protein synthesis inhibitors and TNF- α (Adler et al., 1995; Sluss et al., 1995). In most cells, growth factors that stimulate RAS can only activate JNK weakly and oncogenic RAS only partially activates JNK. Thus, the role of JNK in cell transformation is obscure. In our previous study, we found that JNK was activated in dS2 and F1136 cells to the same degree as that in NM1 cells. In the current study, my data show that Cdc42 and Rac1 only partially rescue the transforming activity of dS2 and F1136 mutants. These results suggest that although JNK activation could still play some role in cell transformation, its activation alone is clearly not sufficient. Alternatively, Rac1/Cdc42-mediated activation of the JNK pathway may not be relevant to cell transformation.

Taken together, the results described here and in Chapter 4, I conclude that the RAS pathway, RhoA, Cdc42, and Rac1 pathways all contribute to the process of cell transformation mediated by gag-IGFR. Activation of each pathway alone is not sufficient to support the full potential of gag-IGFR-induced cell transformation, and each pathway appears to have a distinct effect on the cell transforming process.

Fig. 5-1 Dominant negative RhoA inhibits transforming activity of NM1 protein. 3T3 cells were co-transfected with pMxNM1 and control vector (pZipNeo) or dominant negative RhoA plasmid (pZipRhoA19N). The transfected cells were selected with neomycin and the pool of colonies were used for the biological assay. (A) Colony assay was set up as described in Chapter 2. (B) Western blot analysis of NM1 protein in NM1 expressing and NM1/dnMEK-1 co-expressing cells. Cells were lysed in RIPA and 15 μ g each of total cellular proteins was used for direct Western blotting with anti-IGFR.

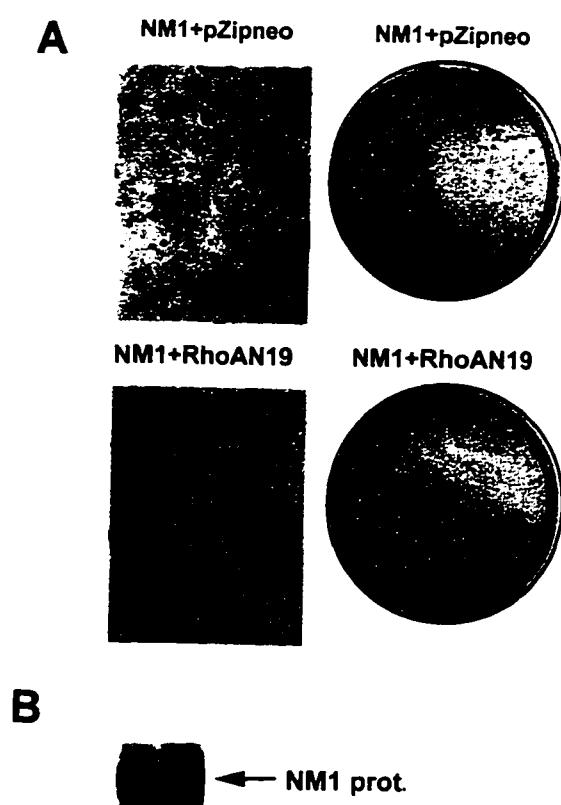


Fig. 5-2 JNK and MAP kinase activities in 3T3 cells co-expressing dominant negative RhoA and NM1 protein. The same cultures described in Fig. 5-1 were treated with 600 μ M Na_3VO_4 for half an hour. (A) Cells were lysed in RIPA and 20 μ g each of total cellular proteins was analyzed in a 10% lowcross-linking gel followed by Western blot analysis with anti-ERK2. (B) Cells were lysed in NP-40 buffer and 200 μ g each of total cellular proteins was incubated with immobilized GST or GST-Jun fusion protein over night in 4°C. The GST agarose beads were then washed and subjected to *in vitro* kinase assay (Chapter 2).

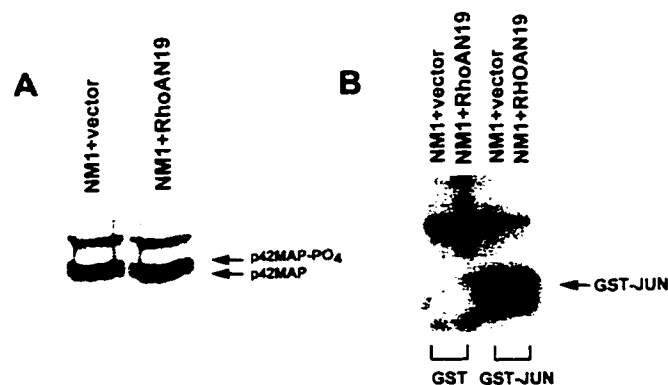


Fig. 5-3 Activated RhoA partially rescues anchorage independent growth activity of dS2 and F1136 proteins. 3T3 cells were co-transfected with activated RhoA plasmid (pCEV29RhoAQ63) and pMxdS2 or pMxF1136, or individually transfected with pCEV29RhoAQ63, pMxdS2 , pMxF1136 and pMxNM1. The transfected cells were selected with neomycin and the pool of drug resistant colonies were used as mass cultures for biological analysis. (A) Colony forming assay was set up as described in Chapter 2. (B) Cells were lysed in RIPA and 15 μ g each of total cellular proteins was analyzed by direct Western blotting with anti-IGFR to detect the expression of gag-IGFRs.

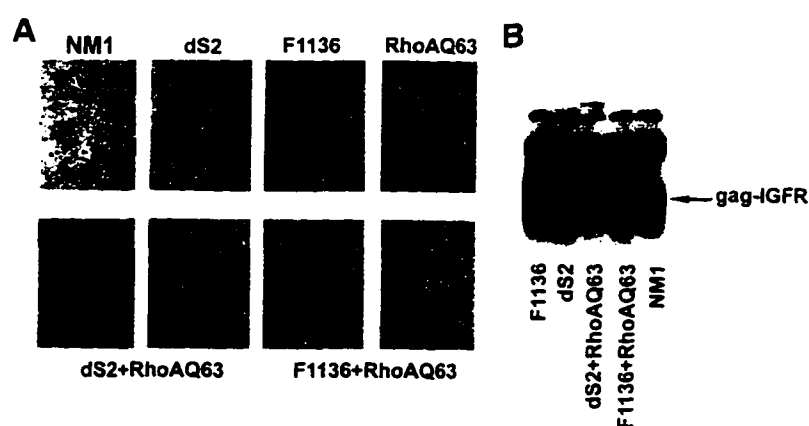


Fig. 5-4 Activated RhoA promote focus forming activity of dS2 and F1136. 3T3 cells were individually transfected with pCEV29RhoAQ63, pMxdS2 and pMxF1136, or co-transfected with pCEV29RhoAQ63 and pMxdS2 or pMxF1136. The transfected cells were analyzed by focus formation assay as described in Chapter 2.

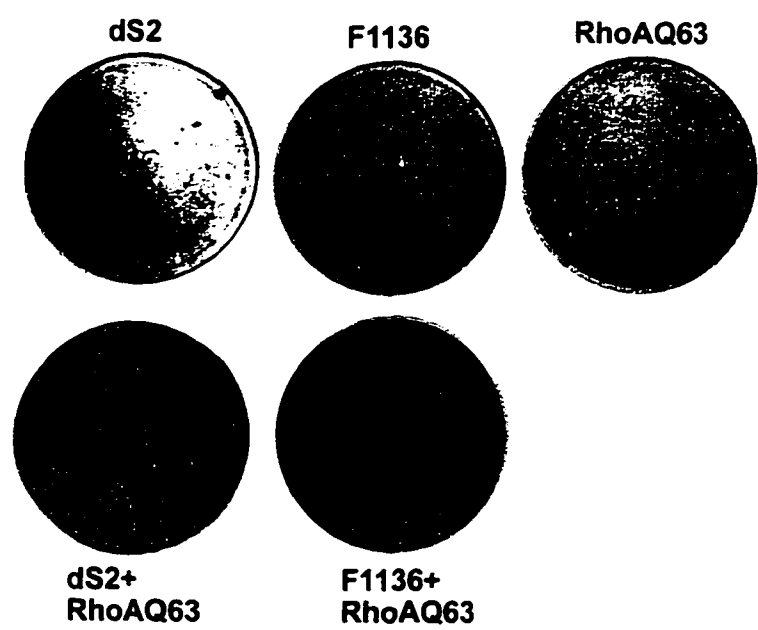


Fig. 5-5 Activated Cdc42 partially rescues the colony forming activity of dS2 and F1136. 3T3 cells were individually transfected with pCEV29Cdc42V12, pMxdS2 and pMxF1136, or co-transfected with activated Cdc42 plasmid (pCEV29Cdc42V12) and pMxdS2 or pMxF1136. Transfected cells were selected with neomycin and the mass cultures were used for biological analysis. (A) Colony assay was done as described in Chapter 2. (B) Cells were lysed in RIPA and 15µg each of total cellular proteins was analyzed by direct Western blotting with anti-IGFR to detect the expression of gag-IGFRs.

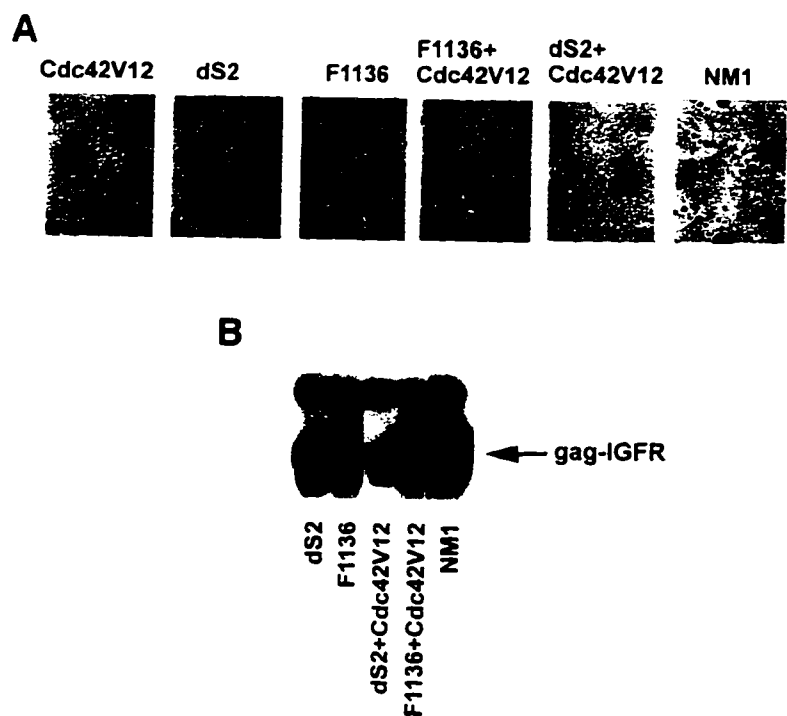


Fig. 5-6. Activated Cdc42 is unable to promote focus forming activity of dS2 and F1136. 3T3 cells were transfected as described in Fig. 5-4 and analyzed by focus forming assay (Chapter 2).

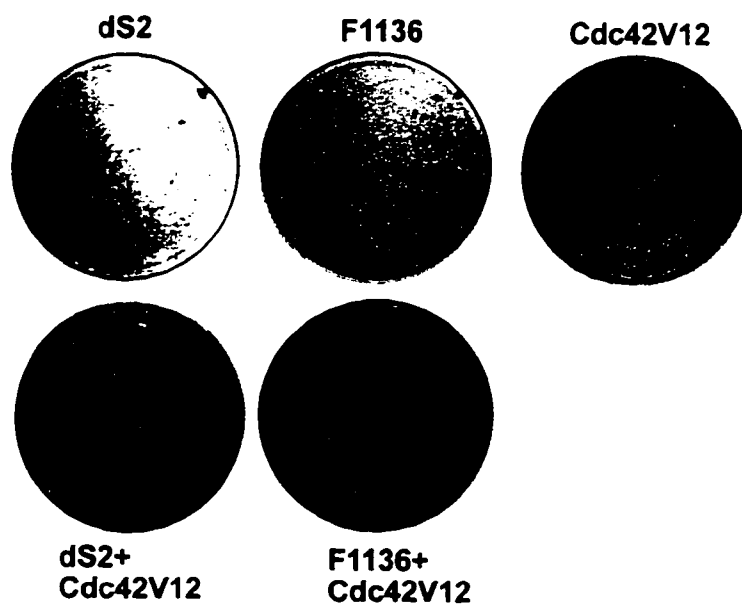


Fig. 5-7 Activated Rac1 enhances colony forming activity of dS2 and F1136. 3T3 cells were individually transfected with pCEVRac1V12, pMxdS2 and pMxF1136, or co-transfected with pCEVRac1V12 and pMxdS2 or pMxF1136. (A) The transfected cells selected with neomycin and the pool of colonies were used for colony forming assay as decried in Chapter 2. (B) Cells were lysed in RIPA and 15 µg each of total cellular proteins was analyzed by direct Western blotting with anti-IGFR to detect the expression of gag-IGFR.

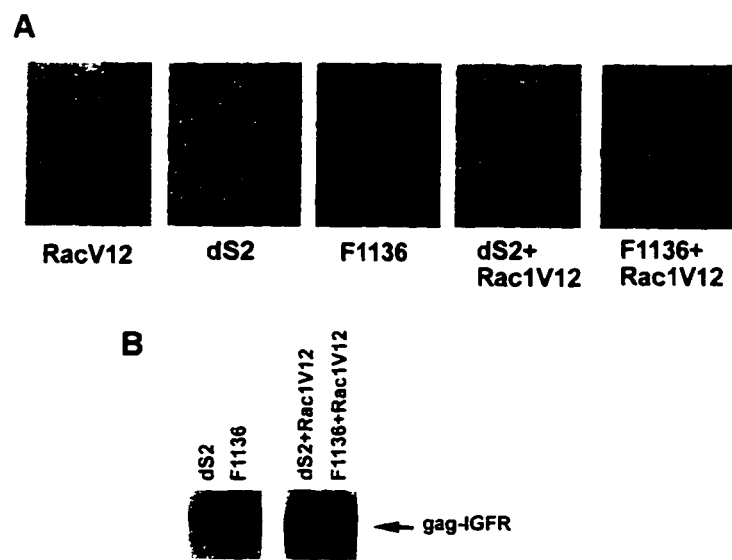


Fig. 5-8 Activated Rac1 is not able to enhance focus forming activity of dS2 and F1136.
3T3 cells were transfected as described in the legend of previous figures and analyzed by focus forming assay (Chapter 2).

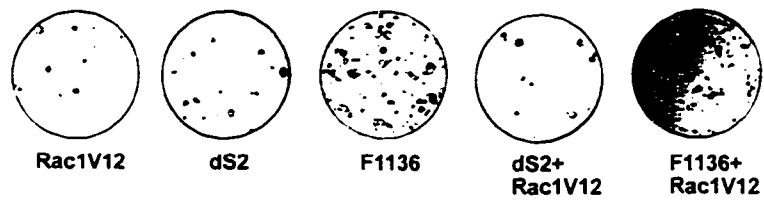


Fig. 5-9. Activated RhoA but not Rac1 and Cdc42 promotes the focus forming activity of an oncogenic RAS. 3T3 cells were individually transfected with pZipRhoAQ63, pRASL12, pCEV29dc42V12 and pCEV29Rac1V12, or co-transfected with the following pair combinations: pRASL12+pCEV29RhoAQ63, pRASL12+pCEV29Rac1V12 and pRASL12+pCEV29Cdc42V12. The transfected cells were subjected to focus forming assay as described in Chapter 2.

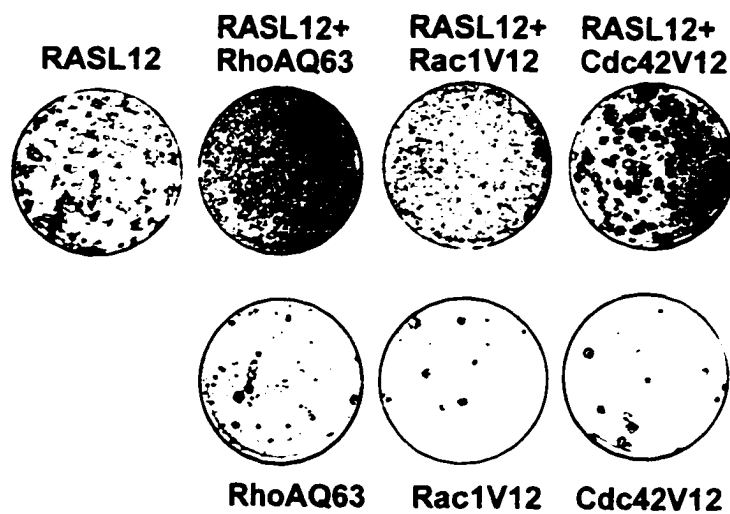


Table 5-1. The role of dominant negative or activated RhoA and Cdc42 in the colony forming ability of gag-IGFRs

plasmids	colony number/ 10 ⁶ cells	number of colonies changed	percentage increase/inhibition
pMxNM1+pZipNeo	1684		
pMxNM1+pZipRhoA19N	512	-1172	330
pMxdS2+control plasmid	56		
pMxF1136+control plasmid	292		
pCEV29RhoAQ63	104		
pCEV29Cdc42V12	176		
pMxdS2+pCEV29RhoAQ63	964	+804	1400
pMxF1136+pCEV29RhoAQ63	506	+226	40
pMxdS2+pCEV29Cdc42V12	360	+179	230
pMxF1136+pCEV29Cdc42V12	1092	+624	210

Colonies with diameter larger than 0.1mm were scored.

Table. 5-2 Activated Rac1V12 Promotes Colony Formation of dS2 and F1136

Transfected plasmids	Colonies/10 ⁵ cells	number of colonies increased	percentage increase
pMxdS2+control plasmid	132		
pMxF1136+control plasmid	400		
pCEV29Rac1V12	408		
pMxdS2+pCEV29Rac1V12	892	+352	260
pMxF1136+pCEV29Rac1V12	1836	+1028	257

Colonies with diameter larger than 0.1mm were scored.

Chapter 6

General Discussion and Future Perspectives

1. Tyrosine residues are important in regulating gag-IGFR kinase activity and transforming potential.

Ligand binding, receptor dimerization and autophosphorylation are the initial steps in RPTK signal transduction. Kinase activity is essential for a receptor to transmit the signals. Tyrosine residues play important roles in regulating kinase activity and directing receptor-substrate interactions. In insulin receptor, it has been shown that the cluster of three tyrosines in the kinase domain is important in regulating the kinase activity of the receptor (Chanter 1 and Chapter 3 discussion). Another closely related RPTK, Ros, also has these three corresponding tyrosines. Mutation of each tyrosine alone produces little effect on the receptor's kinase activity while mutation of two or replacement of all three tyrosines with phenylalanines have dramatic negative effect on the Ros kinase activity as well as transforming activity (C.S. Zong, J. Chan and L.H. Wang 1996, in press). The corresponding tyrosines in IGFR are tyrosine 1131, 1135 and 1136. My study indicates that these three tyrosines are important for the gag-IGFR PTK activity. Single mutation of 1136 or double mutations of 1135/1136 have little effect on the *in vitro* kinase activity or *in vivo* autophosphorylation. Triple mutation of these three tyrosines completely abolishes the enzyme activity *in vitro* and *in vivo*. Besides these three tyrosines, my study further demonstrates that tyrosine 1221, 1095, and 1183 are also involved in regulating *in vitro* kinase activity, but do not appear not to affect *in vivo* autophosphorylation.

Although replacement of tyrosines 1136, 1135/1136, and 1221 have little effect on *in vivo* autophosphorylation of the mutant receptors, these mutations dramatically reduce the ability of gag-IGFR to phosphorylate cellular proteins and transform cells. In particular, these mutants are unable to phosphorylate annexin II, whose role in cell transformation is not known. It appears that annexin II is a substrate specifically in CEF but not in 3T3 cells, indicating that there is cell type specific substrate phosphorylation during the process of cell transformation. The ability of gag-IGFR to phosphorylate overall cellular substrates correlates well with its transforming ability.

2. Activation of MAP kinase is not sufficient for gag-IGFR mediated mitogenicity and cell transformation.

Two weakly transforming mutants (dS2 and F1136) are particularly useful since they retain kinase activity almost at the wild type level. Both F1136 and dS2 have reduced mitogenic activity in CEF and NIH3T3 cells. Activation of MAP kinase has been well established to be important for mitogenic stimulation by growth factors (Blenis, 1991). Despite the loss of mitogenicity, dS2 and F1136 can still lead to activation of MAP kinase as potently as NM1 encoded oncogenic gag-IGFR. Dominant negative MEK-1 inhibits NM1-induced focus and colony forming ability in 3T3 cells by 2 to 3 folds. These results suggest that activation of MAP kinase is necessary but not sufficient to promote gag-IGFR-mediated mitogenicity and cell transformation. This is consistent with the results obtained in the IGFR knock-out (R^-) cells. Introducing oncogenic RAS into the R^- cells could not support optimal cell growth and colony formation in soft agar in the absence of serum indicating that activation of the RAS-RAF-MAP kinase pathway alone is not

sufficient to fully promotes mitogenicity and cell transformation (Sell et al., 1994).

3. Small G proteins, RhoA, Rac1, and Cdc42, play important roles in cell transformation mediated by gag-IGFR.

Small G proteins have been implicated in the change in cell morphology, cytoskeletal protein reorganization, as well as mitogenic response (Chapter 1). Their roles in cell transformation have also been recognized recently (Khosravi-Far et al., 1995). However, the signaling pathways initiated by these G proteins are not well understood. My study demonstrates that activated RhoA, Rac1, and Cdc42 can rescue the colony forming ability of dS2 and F1136 with RhoA rescuing dS2 better and CDC42 rescuing F1136 better. Conversely, dominant negative RhoA can inhibit the transforming activity of NM1 protein. However, the rescue and inhibition are partial indicating that each pathway alone is not sufficient to fully promote cell transformation induced by gag-IGFR. Interestingly, Rac1 and Cdc42 seem to be more involved in enhancing anchorage independent growth than in regulating the loss of contact inhibition, while RhoA is involved in both functions. It appears that the MAP kinase, RhoA and Cdc42 pathways may cooperate to achieve the full potential of gag-IGFR induced cell proliferation and transformation.

4. Future perspectives.

These established cell lines are very useful in studying the mechanism by which RhoA, Rac1, and Cdc42 rescue the transforming activity of dS2 and F1136. First, PI3 kinase has been implicated to be downstream of G proteins and to be involved in the cytoskeletal reorganization (Chapter 1). It is worthwhile to examine if the association of

certain cytoskeletal proteins, such as integrin, with IRS-1 and PI3 kinase is affected in these cells. Second, the downstream molecules of PI3 kinase including AKT and S6 kinase can be examined to assess their roles in the cell transformation process. Third, the activity of PKN can be measured in these cell lines to address the role of RhoA-mediated signaling. It has been proposed that RhoA causes cytoskeletal rearrangements through activation of PKN (Chapter 1) and that it is responsible for initiating a cytoskeletal signaling cascade. Finally, I observed that activated Rac1 and Cdc42 only rescue colony but not focus forming ability of dS2 and F1136 proteins. Furthermore, Rac1 and Cdc42 did not synergize with oncogenic RAS to enhance focus formation. To address whether or not Rac1 and Cdc42 are involved only in regulating anchorage independent growth but not in contact inhibition, dominant negative Rac1 and Cdc42 will be tested to see if they can interfere with the focus and colony forming activity of NM1 protein.

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