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Signal Transduction and Cell Transformation of Receptor-Like Protein Tyrosine Kinase Ros

By

Cong Zong

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

1998

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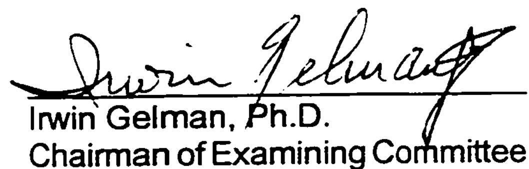

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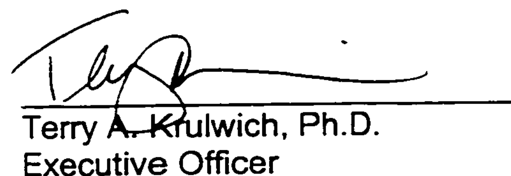

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Abstract

Signal Transduction and Cell Transformation of Receptor-Like Protein Tyrosine Kinase Ros

by

Cong Zong

Advisor: Professor Lu-Hai Wang

The transforming gene of avian sarcoma virus UR2, v-*ros*, encodes a receptor-like protein tyrosine kinase (PTK). To identify the pathways and signaling molecules important for Ros-mediated cell transformation, two approaches were taken.

The first approach employed loss-of-function mutants to correlate their functional impairment with specific defects in signal transduction. Among the mutants of $P68^{gag\text{-}ros}$, F419 and DI are particularly interesting since they retain wild type PTK and mitogenic activities, but have dramatically reduced oncogenicity. Both mutants are able to activate the Ras/MAP kinase pathway. However, F419 protein is unable to induce tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) and to promote its association with phosphatidylinositol 3 (PI3) kinase. In contrast, the DI protein is normal in those respects suggesting that phosphorylation of IRS-1 and activation of PI3 kinase may be important but not sufficient for Ros-induced transformation. Both mutant proteins display reduced ability to induce tyrosine

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phosphorylation of a series of cytoskeleton and cell-cell interacting proteins. Thus, the F419 and DI mutations define the v-Ros sequences important for cytoskeleton signaling, and the impairment of which correlates with reduced cell growth in soft agar. Analysis of other mutants suggested that Y414, Y418 and Y419 play an important role in modulating the Ros PTK activity, whereas Y564 is important for v-Ros autophosphorylation, and phosphorylation of phospholipase Cγ, whose activation, however, appears not to be essential for v-Ros-induced transformation.

In the second approach, dominant negative (dn) mutants were used to assess the role of Stat signaling in Ros-induced cell transformation. Stat3 was specifically activated by Ros in NIH 3T3 cells. Therefore, dnStat3 mutants were introduced into NIH 3T3 cells expressing a EGFR-Ros chimera which had been shown to induce transformation in response to EGF. Co-expressing the dnStat3 mutant with EGFR-Ros resulted in a dramatic inhibition of this receptor PTK-mediated colony forming activity, but having only a mild effect on mitogenicity of the cells in monolayer. The inhibition of Ros-induced anchorage independent growth of cells correlated with inhibition of the DNA binding and transcriptional activities of Stat3. Therefore, activation of Stat3 plays an important role in the establishment and maintenance of Ros PTK-induced anchorage independent growth.

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Finally, I would like to express my deepest gratitude to my parents and my sister for their unconditional love and encouragement, for their confidence and pride in all my accomplishments. Without their constant support this thesis would not have been possible.

I would like to dedicate this thesis to my parents.

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Format of Thesis

This thesis was prepared in accordance with guidelines of the City University of New York. Chapter 3 contains results published as Zong et al., Journal of Biological Chemistry (1997) 272: 1500-1506. Chapters 3 and 4 each has a brief introduction and discussion. A general introduction is placed at the beginning and a general discussion at the end of the thesis. “Materials and Methods” and “References” have been consolidated and placed in the respective sections. Figures and tables are placed at the end of each chapter.

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Chapter 1

Introduction

Signal transduction through receptor protein tyrosine kinases.

Receptor protein tyrosine kinases (RPTKs) are a family of cell surface molecules that function to transmit extracellular stimuli intracellularly. Binding of the cognate ligand to a RPTK results in its oligomerization, autophosphorylation, activation of kinase activity, and creation of phosphotyrosine site(s) for substrate interaction. A series of signals are transmitted through cascades of effector molecules that lead to metabolic changes, DNA synthesis, gene expression, cell growth, or cell differentiation.

The interaction between an activated RPTK and its substrates is mostly mediated by a family of Src-homology domain 2 (SH2) or phosphotyrosine binding (PTB) domain-containing proteins (Blaikie et al., 1994; Kavanaugh and Williams, 1994; Koch et al., 1991; Pawson and Grish 1992). Receptor PTKs in general do not contain the SH2 or SH3 sequences; instead, most of their downstream signaling proteins do. Tyrosine phosphorylation of certain sites on a RPTK is essential for recruitment of specific substrates. The immediate effector molecules of RPTK are a family of proteins containing the SH2, SH3 sequences, the PTB domain, or a combination of these sequences. Many of those proteins do not have any known enzymatic activity and appear to serve merely as intermediate molecules that further recruit downstream signaling components. Therefore, they have been

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referred to as adaptor or docking proteins. A number of pathways have been well characterized to mediate the RPTK signaling (Fig.1-1).

1. The Ras/MAP kinase pathway.

The SH2/SH3- or PTB-containing adaptor proteins interact with phosphotyrosine sites on the RPTKs. Multiple tyrosine sites on a RPTK may interact with different substrates, eliciting signaling pathways for distinct biological functions (Marshall, 1995). The best characterized pathway is the Ras/mitogen activated protein (MAP) kinase pathway (Fig.1-1) which has been well established and shown to be involved in mitogenic activation (Cantley et al., 1991; Blenis, 1993; Davis, 1993). Two adaptor proteins Grb2 and Shc (Lowenstein et al., 1992), both containing SH2 domains, are involved in the Ras/MAP kinase pathway. Upon ligand stimulation, activated RPTKs including epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors have been shown to associate with Grb2 and Shc through interaction between specific phosphotyrosine residues on the receptors and the SH2 domains of these adaptors (Avidsson et al., 1994; Betzer et al., 1994 and 1995). Additionally, Shc contains an N-terminal PTB domain, which is also capable of binding to phosphotyrosine and becomes tyrosine phosphorylated by the RPTK (Betzer et al., 1995). Grb2 is capable of binding to the receptor directly or to tyrosine phosphorylated Shc via its SH2 domain (Rozakis-Adcock et al., 1992). The SH3 domain of Grb2 binds to the proline-rich region of Sos (Son of Sevenless), a guanine nucleotide exchange factor and positive modulator of Ras, and thereby recruits Sos to the plasma membrane to stimulate the exchange of GTP for GDP in the Ras complex. GTP-bound Ras activates the downstream serine/threonine

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kinase Raf via its recruitment to the plasma membrane. Activated Raf leads to the sequential activation of MEK (MAP kinase kinase) and MAP kinase. The latter translocates to the nucleus to phosphorylate and activate transcription factors such as SRF, c-Jun (AP1), and Elk all of which control gene expression (Blenis, 1993; Whitemarsh et al., 1995). To explore the function of those effectors, various approaches have been employed, they include the use of constitutively activated variants, dominant negative mutants, and blocking antibodies of those signaling molecules. Moreover, genetic studies of similar pathways in Drosophila have been very instrumental in establishing the order of the effectors in a given pathway (Simon et al., 1991). Those genetic approaches coupled with biochemical analyses of protein-protein interactions have proven to be very powerful in dissecting the signal transduction pathways.

2. Other signaling pathways.

The pathways involving phospholipase Cγ (PLCγ), insulin receptor substrate 1 (IRS-1), and phosphatidylinositol 3 kinase (PI3 kinase) are less well understood in comparison with the Ras/Map kinase pathway. PLCγ is a SH2/SH3-containing enzyme (Stahl et al., 1988). Its activation and subsequent cleavage of its substrate, phosphatidylinositol-4,5-bisphosphate (PIP2), results in the generation of secondary messengers 1,4,5-inositol triphosphate (IP3) and diacylglycerol (DAG), which effect the release of Ca^{2+} from the membrane bound fraction and activation of PKC, respectively. IRS-1 has been shown to serve as the major adaptor for insulin receptor (IR). IRS-1 contains a PTB domain and a pleckstrin homology (PH)

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domain in its N-terminal region, those domains have been implicated in the interaction with specific phosphotyrosine motifs and lipids respectively (Wolf et al., 1995). IRS-1 has neither SH2/SH3 sequences nor any known enzymatic activity. There are at least two ways that the activated IR can be linked to the Ras pathway: one is through the interaction of IR with Shc followed by binding of Grb2 (Myers et al., 1994; Skolnik et al., 1993), and the other is via interaction of IR with IRS-1 which has been shown also to bind Grb2 (Baltensperger et al., 1993; Skolnik et al., 1993; Myers et al., 1994). In addition, tyrosine phosphorylated IRS-1 is capable of binding to and activating PI3 kinase (Myers et al., 1992). The IRS-1 recognition site on the insulin receptor has been identified as the NPEY motif in the juxtamembrane region of IR, in which the N, P and Y residues of the motif are important for the interaction (Gustafson et al., 1995). Mutation of the IRS-1 recognition motif NPEY was shown to diminish insulin induced tyrosine phosphorylation of IRS-1 and activation of PI3 kinase (White et al., 1988). Studies of IRS-1 null cell lines and gene knock-out animals have shown that IRS-1 plays an important role in the signaling of IR (Araki et al., 1994; Tamemoto et al., 1994).

Activation of PI3 kinase has been implicated in diverse functions including mitogenesis (Cheatham et al., 1994; Kaplan et al., 1987), GLUT4 translocation/glucose transport (Cheatham et al., 1994; Quon et al., 1995), prevention of apoptosis (Yao and Cooper, 1995), membrane ruffling (Ridley and Hall, 1992 ; Ridley et al.,1992 and 1994) and activation of p70 S6 kinase which is involved in protein synthesis (Cheatham et al., 1994). Recently, it has been shown that Akt/PKB, a PKA related serine/threonine protein kinase, can be activated by PI-

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3,4,P_2 which is one of the product of PI3 kinase, suggesting that Akt is a downstream effector of PI3 kinase (Franke et al., 1995). Three types of experiments have strongly suggested that PI3 kinase is necessary and sufficient for growth factor-dependent activation of Akt. First, PDGF receptor mutants that are deficient in activating PI3 kinase failed to mediate activation of Akt/PKB (Burgering and Coffer, 1995; Franke et al., 1995). Second, a dominant inhibitory mutant of PI3K prevents activation of Akt (Burgering and Coffer, 1995) and constitutively activated PI3 kinase increased Akt/PKB activity independent of growth factor stimulation (Franke et al., 1997; Klippel et al., 1996; Marte et al., 1996). Third, the PI3 kinase inhibitor wortmannin blocks activation of Akt/PKB by growth factor (Burgering and Coffer, 1995; Franke et al, 1995 and 1997). Recently, accumulating evidences suggest that Akt/PKB is involved in PI3K-dependent pathway, which is implicated in the regulation of cell survival (D'Mello et al., 1997; Kulik et al., 1997). This pathway, which is independent of MAPK and $P70^{S6kinase}$, is able to prevent apoptosis induced by a variety of cellular challenges (Kauffmann-Zeh et al., 1997). The observation that PI3 kinase and Akt/PKB are involved in cell survival could explain why so many onco-proteins, growth factors and survival factors have evolved mechanisms for activation of PI3 kinase.

3. The JAK-STAT pathway.

Signal transducers and activators of transcription (STAT) were originally discovered as transcription factors involved in interferon-induced gene expression (Darnell, 1997; Ihle, 1996). The STATs were subsequently shown also to be involved in mediating signal transduction of a large number of cytokines important

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for differentiation and maturation of immune cells. The structure of STAT proteins includes the SH2 domain , SH3 domain, DNA binding domain, and transcription activation domain. Binding of a type I or type II cytokine to its cognate receptor results in its dimerization and the recruitment and activation of specific tyrosine kinase(s) of the Janus kinase (JAK) family. Tyrosine phosphorylation of the cytoplasmic domain of the cytokine receptor by a JAK generates sites for the binding of STAT proteins via their SH2 domain. Subsequent tyrosine phosphorylation of the carboxyl domain of STATs catalyzes their dimerization which is followed by translocation to the nucleus and functioning as an activated transcription factor. Therefore, STAT signaling is distinguished from the other types of signaling in that it transmits signals directly from the membrane to the nucleus. To date, 7 STATs (1 through 6 including 5a and 5b) and 4 JAKs (1 through 3 and Tyk2) have been identified in mammalian cells (Darnell, 1997; Ihle, 1996). Differential expression and activation of specific cytokine receptors, JAKs and STATs in different types of cells constitutes the complexity and specificity of the JAK-STAT-mediated signaling and gene regulation in those cells. Recent studies indicate that STATs could also function as effectors for a variety of growth factor receptors including epidermal growth factor receptor (EGFR) (Fu and Zhang, 1993; Sadowski et al., 1993; Silennoinen et al., 1993; Zong et al., 1994), platelet-derived growth factor receptor (Patel et al., 1996; Silvennoinen et al., 1993; Vignais et al., 1996), colony stimulating factor I receptor (Barahamand-Pour et al., 1995; Patel et al., 1996; Silvennoinen et al., 1993; Vignais et al., 1996;) and insulin receptor (Chen et al., 1997; Coffer et al., 1997; Sawka-Verhelle et al., 1997). In the case of growth

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factor receptor-induced tyrosine phosphorylation of STATs, accumulating evidences suggest that JAKs may not be required (Leaman et al., 1996; Meraz et al., 1996; Vignais et al., 1996). These observations suggest that STATs may also play a role in RPTK-mediated functions including cell growth and differentiation. Moreover, STATs have also been implicated in either promoting or preventing growth arrest and apoptosis (Bromberg et al., 1996; Fukada et al., 1996; Muli et al., 1996; Takeda et al., 1997). For example, Stat1 is required for EGF-induced expression of cyclin inhibitor $p21^{cip1}$ and growth arrest of A431 cells (Chin et al., 1996), whereas Stat3 functions in preventing apoptosis of pre-B cells (Fukada et al., 1996).

Cytoskeleton Regulation

The cytoskeleton plays important roles in physiological functions of cells including maintenance of cell shape and mediation of cell motility. Cell shape and motility are controlled by the actin cytoskeleton which consists of actin filaments and actin-binding proteins. It has been demonstrated that the Rho family proteins, which are members of the Ras GTPase superfamily, signal to regulate cell motility, cell shape, and formation of actin stress fibers. These small G-proteins may serve to link signaling between a membrane receptor and the cytoskeleton (Fig.1-1).

The Rho family of GTP-binding/GTPase proteins consist of three subgroups and at least eight members in mammalian cells. They are the Rho subfamily (RhoA, RhoB, RhoC and RhoG), the Rac subfamily (Rac1, Rac2, TC10), and the Cdc42 subfamily (Bourne et al., 1990). The involvement of Rho family members in regulating actin stress fiber formation and other membrane properties has been

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established in fibroblasts (Ridley and Hall , 1992; Ridley et al., 1992). Rho controls the formation of stress fibers and focal adhesions. Rac induces the assembly of actin filaments at membrane to form membrane ruffles and lamellipodia, and Cdc42 also causes cells to form focal complexes that are smaller than focal adhesions but contain the same cytoskeletal and signaling components (Nobes and Hall, 1995; Ridely, 1994; Symons, 1996). Little is known about the molecular basis underlying the differences in cell morphological changes induced by activated Rac, Rho or Cdc42. However, several properties are shared such as an apparent increase in polymerized actin, clustering of integrins, and assembly of large protein complexes containing vinculin, talin, focal-adhesion kinase (FAK) and paxillin. The Rho-family proteins are regulated by GAPs (GTPase-activating proteins), GEFs (guanine nucleotide exchange factors) and GDI (GDP dissociation inhibitors). In addition to the effect on cell morphology and motility, Rho family protein signaling may also play a role in cell transformation. The *Dbl* oncogene product and a growing number of related proteins with so called Dbl-homology domains form a family of GEFs for Rho-family proteins and are themselves able to induce cell transformation (Khosravi-Far et al., 1995). Studies of various mutants of Ras, Rac1, and Rho have shown that the latter two are important in mediating Ras-induced cell transformation (Khosravi-Far et al., 1995; White et al ., 1995). Little is known about how Rho is activated in vivo, although the mechanism is probably similar to Ras activation which is mediated by GEFs recruited to membrane receptors. Several proteins have been found to contain a Rho/GAP-homology domain (Machesky and Hall, 1996) and function as negative regulators of Rho by modulating its GTPase activity; for

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example, one such regulator is p190^{RhoGAP}. Similar to the Ras-activated MAP kinase pathway, Rac1 and Cdc42 were shown to initiate a parallel pathway involving a cascade of serine/threonine kinases including p21-associated kinase (PAK) and MEK kinase (MEKK). This parallel pathway signals to activate c-Jun kinase (JNK) and p38, a stress factor activated serine/threonine kinase (Coso et al., 1995; Minden et al., 1994; Olson et al., 1996; Symons, 1996). The downstream targets of Rho are very complex and may involve several pathways: Rho is able to activate multiple protein kinases including PKN and ROKα, which regulate phospholipases and phospholipid kinases (Leung et al., 1996; Machesky and Hall, 1996). In addition, Rho binds to a number of proteins whose functions are still unknown (Machesky and Hall, 1996). Some data suggest that a series of transcription factors including SRF (serum response factor) can be activated by the Rac-Rho pathways (Hill et al., 1995).

Signaling involving cytoskeleton has recently been gaining increasing attention. More studies have been devoted to understand the transmission of signals resulting from interaction of integrins and their extracellular matrix (ECM) ligands, particularly at adhesion plaques, a process which is called "outside-in" signaling. Conversely, the alteration of the biochemical and physical status of focal adhesion proteins as a result of growth factor stimulation or oncogenic transformation is a process called "inside-out" signaling (Gumbiner and McCrea, 1993; Ridley 1994; Sastry and Horwitz, 1993) (Fig.1-2). The focal adhesion kinase p125^{FAK} co-localizes with the integrin receptor at sites of cell attachment to ECM proteins and is activated upon engagement of integrin receptor with its ligand or

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upon Src transformation (Burridge et al., 1992; Cobb et al., 1994; Guan et al., 1992; Hanks et al., 1992; Hildebrand et al., 1993). Since integrin receptor lacks catalytic activity, it association with FAK and activation of its PTK activity most likely play an important role for the integrin-mediated signal transduction (Hanks and Polte, 1997). In addition to activation of FAK, binding of ECM protein to integrins can lead to changes in the status of tyrosine phosphorylation of a number of different signaling proteins including p130^{cas}, Shc, and Cbl, as well as that of structural proteins such as paxillin and tensin. Integrin stimulation can also promote an increase in intracellular calcium level (Sjaatad et al., 1994), protein kinase C activity (Lewis et al., 1996; Vuori et al., 1993) and PI3 kinase activity (Chen et al., 1996; King et al., 1997). Accumulating evidences suggest that one downstream event of integrin-mediated signaling is activation of the MAP kinase pathway (Chen et al., 1994; Miyamoto et al., 1995; Morino et al., 1995; Schlaepfer et al., 1994; Zhu and Assoian, 1995). Therefore, maximal stimulation of the MAP kinase results from inputs of multiple signaling pathways. Integrin-mediated signaling to MAP kinase is dependent on the integrity of the actin cytoskeleton and involves activation of both Rho and Ras families of small GTP-binding/GTPase proteins (Clark and Hynes, 1996; Renshaw et al., 1996). However, the link between integrin signaling and the activation of the GTP-binding proteins has not been clearly defined yet. Some studies showed that antibody-mediated clustering of $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins in suspended cells can stimulate Shc tyrosine phosphorylation, Grb2 binding to Shc and MAP kinase activation without detectable tyrosine

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phosphorylation of FAK (Wary et al., 1996). Overexpression of a Shc protein mutated at the Grb2 binding site can block integrin-stimulated MAP kinase activation (Wary et al., 1996). These observations underscore the importance of Grb2-Shc interaction in the integrin- mediated signaling.

Cadherin and its associated catenins constitute another aspect of cytoskeleton-mediated signaling (Fig.1-2). The cadherins are a superfamily of transmembrane glycoproteins, whose extracellular domains mediate Ca^{2+}-dependent cell-cell adhesion via homophilic interaction with adjacent cell surface cadherin molecules. The cytoplasmic region of cadherin interacts with catenins: β catenin and γ catenin (Plakoglobin) and $p120^{ctn}$ which appear to modulate adhesion and/or bridge cadherins to the actin cytoskeleton. $p120^{ctn}$, β-catenin, and Plakoglobin are structurally similar, each containing a central armadillo repeat domain (Arm domain) originally described in the *Drosophila* β-catenin homolog *Armadillo*. The major PTK targets in the cadherin complex are the Arm catenins (p120, β-catenin and Plakoglobin) which are tyrosine phosphorylated in response to transformation by Src (Behrens et al., 1993; Hamaguchi et al., 1993; Matsuyoshi et al., 1992; Reynolds et al, 1989 and 1994; Tsukita et al., 1991) and in response to stimulation by EGF, TGFα, PDGF, CSF-1 and HGF growth factors (Downing and Reynolds, 1991; Fujii et al., 1996; Hoschuetzky et al., 1994; Kanner et al., 1991; Shibamoto et al., 1994 and 1995; Shibata et al., 1996). It is believed that tyrosine phosphorylation of the cadherin complex plays an important role in modulating cell adhesion, but the mechanism remains unclear (Daniel and Reynolds, 1997). While

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tyrosine phosphorylation of β-catenin and p120^{ctn} correlates with Src-induced transformation, the precise role of these molecules remains to be elucidated. Besides bridging the interaction between cadherin and actin, recent finding about catenins suggests that they may also function as signaling effectors (Gumbiner and McCrea, 1993).

The *ros* oncogene

Oncogenes were first described as retrovirus-encoded genes that induced tumors in birds and rodents. The genes were then shown to be dominant mutated forms of normal host genes (proto-oncogenes) that had been transduced by retroviruses. Most proto-oncogenes encode proteins that are involved in the signal transduction pathways initiated by growth factors.

Avian sarcoma virus (ASV) UR2 was originally derived from a chicken pancreas myofibroma. (Balduzzi et al., 1981). The tumor was observed by R. E. Luginbuhl in a 36 week-old White Rock chicken in 1963. In 1970, a tumor suspension made from the frozen materials was inoculated into the wing webs of chickens to produce secondary tumors by H. R. Morgan at the University of Rochester. Cell free extracts from the tumors were shown to transform chicken embryo fibroblasts (CEF). A more detailed characterization and purification of this virus was carried out later (Balduzzi et al., 1981). Initial analysis of the UR2 genome by nucleic acid hybridization and oligonucleotide fingerprinting revealed that it contains a transforming gene that is different from those of other known avian

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sarcoma viruses (Wang et al., 1982). The transforming gene carried in avian sarcoma virus UR2 named v-*ros* was cloned, sequenced, and shown to encode a receptor-like protein tyrosine kinase (Neckameyer and Wang, 1985; Wang et al., 1982).

Proto-oncogene c-*ros* is the cellular counterpart of the v-*ros* and has been cloned in avian and mammalian species (Birchmeier et al., 1990; Chen et al., 1991; Matsushime et al., 1990). C-Ros, the protein product, of proto-oncogene c-*ros* shares a high degree of sequence and structural homology with the Drosophila sevenless protein (Chen et al., 1991). C-*ros* is expressed specifically in epithelial cells in a number of organs including the collecting ducts of kidney, the villi and underneath crypts of intestine (Chen et al., 1994), and the tubules of epididymis (Sonnenberg-Riethmacher et al., 1996). Recently, a study from c-*ros* knock-out mice suggested that c-Ros controls regionalization and differentiation of the epithelial cells in epididymis (Sonnenberg-Riethmacher et al., 1996). Targeted mutations of c-*ros* in mice resulted in male infertility whereas the fertility of female animals was not affected (Sonnenberg-Riethmacher et al., 1996). The function of c-Ros in kidney and intestine, the two major organs of its expression, remains unknown. The c-*ros* gene codes for a receptor PTK with an extended extracellular (EC) domain (Birchmeier et al., 1990; Chen et al., 1991; Matsushime et al., 1990). The v-*ros* gene differs from c-*ros* in that all but 21 nucleotides of the EC domain is truncated and the remaining c-*ros* gene is fused in frame at its 5' end to the viral gag sequence. As a result, v-*ros* codes for a gag-Ros transmembrane fusion protein of 68 kilodaltons called $P68^{gag\text{-}ros}$. In addition, there are minor

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alterations in the transmembrane (TM) domain and the carboxyl region of v-Ros when compared with c-Ros (Chen et al., 1991; Matsushime et al 1990; Neckameyer and Wang, 1985; Zong et al., 1993). The kinase domain of Ros shares a high degree of homology with those of insulin receptor (IR) and insulin-like growth factor I receptor (IGFR) (Ebina et al., 1985; Neckameyer and Wang 1985; Ullrich et al., 1985 and 1986).

Mammalian cell lines expressing an inducible Ros PTK would be very useful for studying the mechanism of its signal transduction and cell transformation since the ligand for c-Ros is presently unknown. A previous student Qinghua Xiong has established NIH 3T3 cell lines expressing an EGF inducible EGFR-Ros chimeric receptor, called ER2, which contains the EC domain derived from EGFR and the TM and cytoplasmic domains from c-Ros. The chimeric receptor can be activated by EGF and is capable of phosphorylating and activating an array of cellular signaling proteins (Xiong et al., 1996). ER2 is able to promote cell growth in a monolayer culture and colony formation in soft agar in response to EGF stimulation (Xiong et al., 1996).

The purpose of this study was to explore the mechanism of signal transduction and cell transformation by v-Ros and to identify the signaling molecules that are critical for Ros-mediated mitogenic activity and oncogenicity. For this purpose, the kinase active and transformation negative (K^+T^-) or kinase active and transformation attenuated($K^+T^{\pm}$) mutants would be very useful. By comparing the effect of mutant receptor PTKs on the downstream signaling components with that of the parental receptor, it is hoped that can identify the molecule(s) essential for

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cell growth and transformation. Moreover, the kinase and mitogenicity positive, but transformation defective mutants ($K^+M^+T^\pm$) are particularly useful since they would allow me to dissect the signaling pathways leading to distinct biological functions. Signaling molecules suspected to be important for cell transformation can be confirmed by introducing dominant negative mutants of these molecules into Ros-transformed cells to assess their roles in Ros-mediated cell growth and transformation. Using loss-of-function mutants of the Ros oncogene and dominant negative mutants of certain signaling molecules, I have been able to identify a number of signaling pathways important for Ros-induced cell transformation.

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Fig. 1-1. **Signal transduction through receptor protein kinases.**

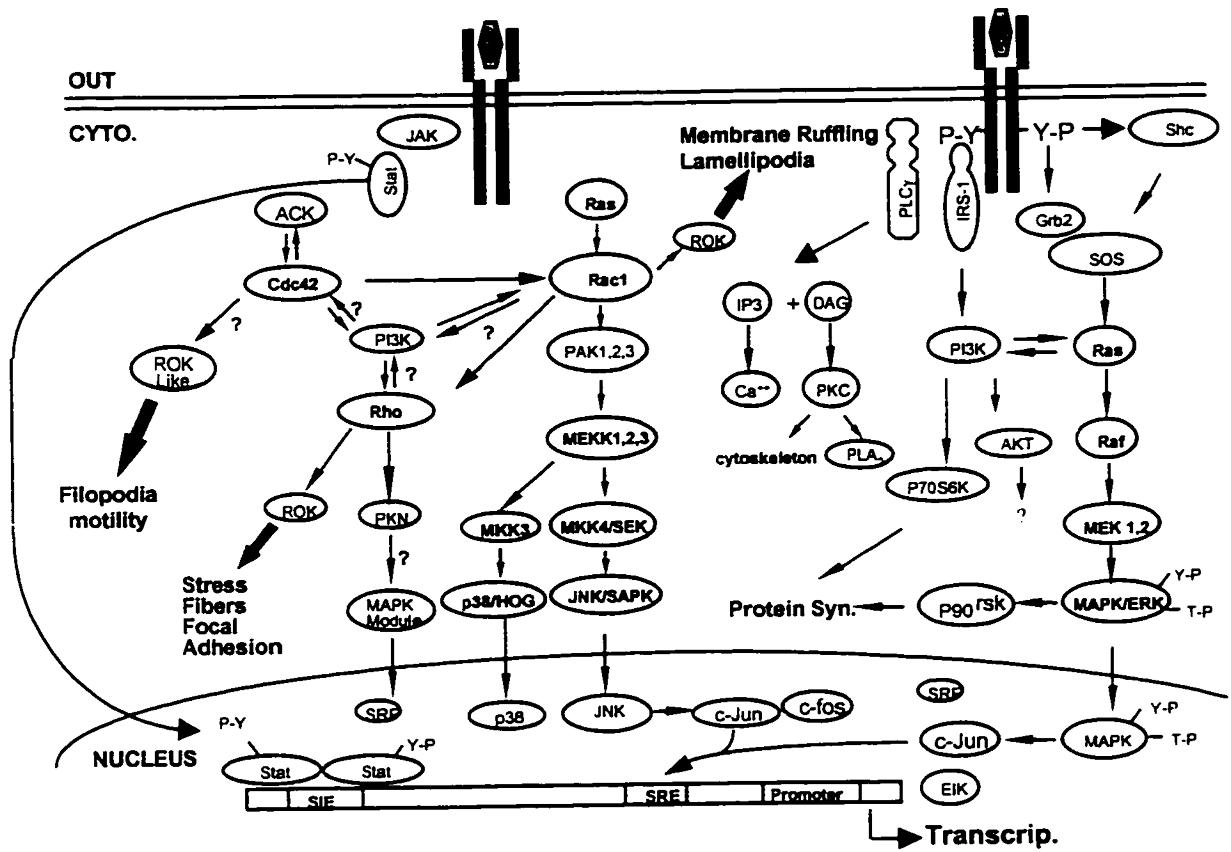

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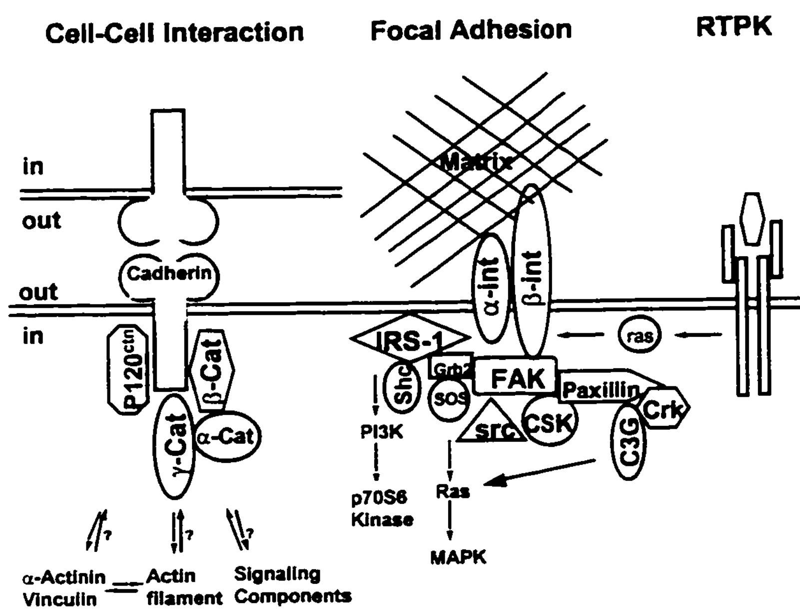
Fig.1-2. **Cytoskeleton regulation.**



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Chapter 2

Materials and Methods

Cells and viruses. Chicken embryo fibroblasts (CEF) were prepared from 11 days old embryos and maintained according to the published procedure (Hanafusa et al., 1969). CEF were maintained in F10 medium supplemented with 5% bovine calf serum and 1% chicken serum (GIBCO Laboratories). Molecularly cloned avian sarcoma virus UR2 and its helper virus UR2AV have been described (Neckameyer and Wang, 1985; Zong et al., 1993). Mutant virus stocks were obtained by collecting medium about 10 days after transfection with the respective viral DNA plasmids. NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% or 10% bovine calf serum. NY68 is a mutant of subgroup A Rous sarcoma virus containing the temperature sensitive (ts) *src* (Kawai et al.; 1971). UR2 ts 251 is a subgroup A avian sarcoma virus containing the ts *ros* (Chen et al., 1994).

DNA transfection. CEF were transferred 1 day before DNA transfection at a density of about 10^6 cells per 60-mm dish. 20 μg of UR2 or its mutant recombinant DNA plasmids, and 2 μg of SacI-digested pUR2AV helper viral DNA (to linearized the permuted genomic DNA in the plasmid) were mixed and added to cells in 1 ml medium containing 30 μg polybrene. The culture was incubated at 37^0 for 5 to 6 hours with occasional mixing. Then cells were shocked with medium containing 30% dimethyl sulfoxide, washed and returned to incubation. NIH 3T3 cells transfection were carried out by standard calcium phosphate method(Chen et al.,

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1988; Graham et al.,1973). Cells were seeded at 5 X10^5 /100-mm-diameter dish in DMEM plus 10% CS at 18-24 hours prior to transfection. Total DNA for transfection was about 10 to 20 μg of appropriate plasmids per dish plus 20 μg calf thymus DNA. Transfection was terminated 15 hours later by removing the medium, washing twice with fresh medium and then maintained in the same medium. Stable clones were selected in growth medium containing 400 μg/ml G418 (Geneticin, Life Technologies), 200 μg/ml Hygromycin B (Sigma) or 2 μg/ml puromycin (Sigma) depending on the plasmid used 48 h after transfection.

Biological assay. Cell transformation was monitored by morphological change and anchorage-independent growth as described previously (Liu et al., 1993; Jiang et al., 1996; Zong et al., 1993 and 1994). For colony formation in methyl cellulose, medium containing 1.3% pure methyl cellulose was used for the top layer on the same platform of bottom layer agar. If EGF was included in the assay, soft agar medium was containing 10 to 50 ng/ml of EGF used for top agar overlay and DMEM medium containing EGF was added on top every 5 days. The control dishes were overlaid with regular soft agar medium and subsequently fed with regular DMEM containing 5% CS.

Antibodies. Anti-Ros and anti-IRS-1 antibodies (Abs) were prepared in our laboratory and described previously (Jiang et al., 1996; Jong and Wang 1987). Anti-MAP kinase polyclonal Ab TR10 was a gift from M. Weber, Anti-annexin II polyclonal Ab was a gift from T. Hunter. Monoclonal Abs for cortactin, tensin and CAS were gifts from T. Parsons and A. Bouton. Anti-p190 RhoGAP polyclonal Ab

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was a gift from S. Parsons. Anti-Grb2 polyclonal Ab was a gift from B. Mayer. Antibodies for FAK, β- and γ-catenin and an alkaline phosphatase-coupled anti-phosphotyrosine (P-Tyr) antibody RC20 were purchased from Transduction Lab. Antibodies for PLCγ and PI3 kinase were purchased from Upstate Biotechology Inc. Anti-β1-integrin and anti-N-cadherin were purchased from Sigma and Zymed Laboratories Inc. respectively. Anti-Stat1 (E-23), anti-Stat3 (C-20), anti-Stat5b (C-17) and anti-Flag (D-8) antibodies were purchased from Santa Cruz Biotechnology.

Construction of pBUR2. The plasmid pUR2H1 contains the full length UR2 genome cloned at the HindIII site of pBR322 (Neckameyer and Wang 1985; Zong et al., 1993). The UR2 genome in this plasmid is permuted with respect to the HindIII site at the 3' region of the UR2 genome. To facilitate DNA transfection and expression of viral genes, a nonpermuted UR2 plasmid was reconstructed. This was done by isolating the 2.3 kb PstI to NruI fragment containing the entire gag-Ros coding sequence from pUR2H1 and using it to replace the gag-IGFR sequence in a nonpermuted viral plasmid pBUIGFR-II constructed previously (Liu et al., 1993). The resulting plasmid was named pBUR2 which served as the parental plasmid for mutant construction.

Construction of mutants. The mutants were engineered by a M13-mediated mutagenesis method (Taylor et al., 1985), the Promega altered sites in vitro mutagenesis system (Promega) described previously (Jiang et al., 1996) or polymerase chain reaction (PCR) using specific mutant oligonucleleotides. In the first two methods, an appropriate UR2 DNA fragment containing the desired site of mutation was released from pUR2H1 and transferred to the mutagenizing plasmid

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(pAlter-1, Promega). After mutagenesis and sequence confirmation of the mutation, the DNA fragment was transferred to the 2-LTR plasmid pBUR2 to yield the final construct. The site of mutagenesis was sequenced again to confirm the mutation. DI and F2 were constructed by the M13 method. F414, F3, F418 and F419 were constructed by the PCR method using pBUR2 as the templet directly. The F2 construct described above was used as the template for F3 construction. Since the region of the cluster of triple tyrosines is very close to a downstream StuI site (Neckameyer and Wang, 1985), mutant oligonucleotides extending from the StuI site to the site of mutation were used as the 3' primers for PCR with a 5' oligonucleotide containing BglII site. The PCR amplified mutant DNA fragment was subcloned into pBluescript and sequenced to confirm the mutation. The 0.25 Kb BglII to StuI fragment was then retrieved from pBluscript and transferred to pBUR2 to obtain the final mutant construct. The rest of mutants except F4 were constructed by the altered sites mutagenesis system. F4 was constructed by swapping the DNA fragment containing F419 mutation with the corresponding region of the F564 DNA to obtain the double mutation.

Plasmids Used (in Chapter 4). pECE-ER1 and pECE-ER2 are two EGFR-Ros chimeric receptor constructs that differ only in their origins of the transmembrane (TM) domain, ER1 contains the TM domain from EGFR whereas ER2 derives its TM domain from Ros as described previously (Xiong et al., 1996). phEFER2 and pMV12ER2 were generated by removing the EGFR-Ros insert from the pECE-ER2 construct (Xiong et al., 1996) and cloned into the human elongation factor promoter-based neomycin plasmid phEF (unpublished) and Moloney leukemia virus LTR

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driven hygromycin resistant plasmid pMV12 (Cacace et al., 1993) respectively. Dominant negative (dn) Stat3-1 contains $E^{434}E^{435}$ to AA mutation (Horvath et al., 1995) and dnStat3-2 contains the same EE to AA and $V^{461}V^{462}V^{463}$ to AAA mutation (Bromberg et al., 1998) in the DNA binding domain of Stat3. Both were cloned in a pRcCMV based expressing vector (Bromberg et al., 1998; Horvath et al., 1995) and were kindly given to us by Drs. James Darnell and Curt Horvath. Two reporter plasmids were used, pLucTKS3 (Turkson et al., 1998), which contains multimerized Stat3-specific binding sites derived from the human C-reaction protein (CPR) gene inserted upstream of the TK minimal promoter, was kindly given to us by Dr. Richard Jove; Ly6ELuc (Wen and Darnell, 1995), which contains three GAS sites from the promoter of the Ly6E gene, was kindly given to us by Dr. Curt Horvath. CMV-β-gal plasmid was purchased from Invitrogen.

Protein analysis. For immunoprecipitation, cells were extracted with RIPA (50 mM Tris hydrochloride pH7.5, 150 mM NaCl, 5 mM EDTA, 1 mM sodium vanadate, 1% Triton X-100, 1% sodium deoxycholate, 1 % Trasylol and 1 mM phenylmethylsulfonyl fluoride), and anti-serum was added to the cleared cell extract for at least 2 hours at 4°C. This was followed by protein A-Sepharose (RepliGen) binding for 1 hour. The immune complex was washed with RIPA three times before it was boiled in the sample buffer for SDS-polyacrylamide gel electrophoresis. For direct Western blotting, cells were extracted with protein extraction buffer (10 mM Tris hydrochloride pH7.5, 5 mM EDTA, 1 mM sodium vanadate, 1 mM sodium molybdate, 1 % Trasylol, 1 mM phenylmethylsulfonyl fluoride and 1% SDS), and

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boiled for 5 minutes before mixing with the sample buffer and boiled again prior to gel electrophoresis. Western immunobloting was done according to the procedure previously described (Jong et al., 1987). After separation of proteins by SDS-PAGE, the gel was 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. The filter was blocked for 1 hour at room temperature with 3 % BSA in 10 mM Tris-HCl pH7.5, 50 mM NaCl and 1 % Tween-20 (TBS-T) and then subjected to binding of antibody for overnight at 4 ^{0}C. After binding, the filter was washed in TBS-T three times for 20 min each time at room temperature. The washed filter was reacted 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. After Western blotting, the proteins were detected by color development as follows. The filter was immersed briefly in color-development solution (100 mM Tris-HCl pH9.5, 100 mM NaCl and 5 mM $MgCl_2$) and then incubated in the same solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphophate (Promega) for an appropriate period of time at room temperature. The reaction was stopped by rinsing the filter in water.

Alternatively, proteins were detected by the enhanced chemiluminescence (ECL) method as follows. The filter was blocked with 5% non-fat milk in TBS-Tween and was probed with the appropriate mAb or pAb followed by incubating with a horseradish peroxidase (HRP)-conjugated secondary antibody. After washing with TBS-Tween, the Western blot was developed by ECL method (Amersgam).

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In vitro kinase assay. Cells were extracted with RIPA and cell extracts were used for immunoprecipitation as described above. The immune complex for kinase assay was washed 3 times with RIPA followed by 2 times with kinase buffer (50 mM Tris hydrochloride pH8.0, 1 mM $MnCl_2$). Kinase reaction was initiated by adding (γ-^{32}P)-ATP (10µCi) to 50µl of the kinase buffer and reaction mixture was incubated at room temperature for 10 min. The immune complex after kinase reaction was further washed with RIPA buffer before SDS-PAGE. After electrophophrosis, gel was fixed in 10 % acidic acid and 30 % methanol and dried. The kinase activity was detected by autoradiography.

PI3 kinase assay. Cells were lysed in Nonidet P-40 (NP-40) buffer (20mM Tris-HCl pH7.5, 5 mM EDTA, 150 mM NaCl, 1%NP-40, 1 mM Na_3VO_4, 1 mM PMSF, 100 mM NaF, 50 mM sodium PPi). The immunoprecipitation was done as described above. The immune complex was washed with the following solutions: NP-40 3X, PBS 1X, LT(0.5 M LiCl and 20 mM Tris-HCl pH7.5) 1X, H_2O 1X, and TEN (20 mM Tris-HCl, 0.5 EDTA and 100 mM NaCl) 1X. The washed beads were resuspended in 25 µl of TGN buffer (20 mM Tris-HCl pH7.5, 0.5 mM EGTA and 100 mM NaCl). 10 µg of the substrate PI (20µg/µl in dimethyl sulfoxide; Avanti Polar Lipids, Inc) was then added to the resuspended immunoprcipitates and mixed. The mixture was incubated at room temperature for additional 10 min. Premixed (γ-^{32}P)-ATP (10µCi per assay NEN) and $MgCl_2$ (final concentration, 20 mM) solution was then added, and the mixture was incubated at room temperature for 10 min. The reaction was stopped by adding 150µl of stop solution (Chloroform:Methanol:12N

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HCl=100:200:2) and extracted with equal volume of chloroform. The extracted PIP was washed three time with a mixture of methanol and 1N HCl (1:1). The washed PIP was then dried, resuspended in 15 µl chloroform and analyzed by thin-layer chromatography in a Silica Gel 60 plate (Merk).

MAP kinase assay. Cells were lysed in RIPA buffer and the lysate was immunoprecipitated with rabbit polyclonal antibody specific to p42 MAP kinase TR10 (a gift from Dr. Michael J. Weber). The immunocomplex was captured by protein A-agoras beads. The immunoprecipitates were washed three times with RIPA and twice with MAP kinase buffer (10 mM Hepes pH7.5 and 10 mM MgAc) and resuspended in 20 µl MAP kinase buffer. 20 µl substrate (myelin basic protein, 20 mg/ml) and 20 µl 3X hot mix (5µCi γ-^{32}P-ATP, 150 µM ATP, 30 mM MgAc and 30 mM Hepes pH 7.5) were added and the reaction mixture was incubated at 30^{0}C for 30 minutes. The reaction was stopped by adding 60 µl 2X SDS-PAGE gel sample buffer. After electrophoresis, gel was fixed and dried. The phosphorylation of MBP was visualized by autoradiography.

Electrophoretic Mobility Shift Assay (EMSA) Cultures of stable transfectants or cells 48 h after transfection were starved for 20 h in serum free DMEM and then stimulated with EGF for 15 min. Whole cell lysates were prepared from the control and EGF treated cells. Briefly, after washing twice with PBS, cells were harvested in 1ml PBS and pelleted by centrifugation. Cells were resuspended in twice the pellet volume of high salt buffer (20 mM Hepes pH7.9, 20 mM NaF, 1 mM NaPPi, 1 mM Na_3VO_4, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 400 mM NaCl, 20% Glycerol,

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0.1% Triton-X100, 1 mM phenylmethylsulfonyl fluroride, 1% Trasylol and 1 μg/ml Leupeptin) and were rocked for 30 min at 4^0C. The lysates were centrifuged at 15,000g for 20 min at 4^0C, and protein concentrations of the clarified lysates were determined by Bradford Assay. Cytosol and nuclear fractions were prepared as according to the published procedure (Sadowski et al., 1993a; 1993b). The M67 SIE (Wagner et al., 1990) oligodeoxynucleotide containing Stat1 and Stat3 binding sites was labeled with ^{32}P-α-dGTP by Klenow end filling and purified by electrophoresis in a 12% nondenaturing gel with a bis to acrylamide ratio of 1:19. The β-CAS oligodeoxynucleotide probe contains the Stat5 binding sequence derived from the rat β-casein gene promoter (Mui et al., 1995). DNA binding was performed by adding 15 to 20 μg of protein lysate to 18μl reaction mixture containing 65 mM NaCl, 10 mM Hepes pH7.9, 1 mM DTT, 2% Ficoll 400, 4% Glycerol and 1 μg Poly(dI-dC) (Pharmacia Biotech), the mixture was preincubated on ice for 15 min and then approximately 30,000 cpm (1-1.5 ng) of the oligodeoxynucleotide was added to the reaction mixture which was further incubated for 30 min at room temperature. The samples were then analyzed in a 20 cm X 20 cm 5 % polyacrylamide gel with a bis to acrylamide ratio of 1:39 containing 2.5% Glycerol and 0.5X TBE (45 mM Tris-base, 1 mM EDTA). The electrophoresis was carried out at 175 volts until the faster migrating bromophenol blue dye was about 4 cm from the bottom edge. The gel was dried and subjected to autoradiography using Kodak XAR5 film for 1 to 3 days with intensifying screens at -70^0C.

Trascriptional Assay Transcriptional assays were performed by transiently

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transfecting NIH3T3 cells with ER2 or NM1 and with either control or dn Stat3 together with the reporter plasmid and the pCMV-β-gal plasmid which served as a control for transfection efficiency. Twenty four hour after transient transfection, the cells were starved for serum overnight, followed by stimulation with EGF (100ng/ml) for 6 h, the cells were then lysed and the extract assayed for luciferase activity with a luminometer according to the protocol provided by the manufacturer (Promega). Part of the lysate was used for measuring β-galactosidase activity detected by colorimetric assay at A_{420} according to the protocol in the hand book of Molecular Cloning Manual (Sambrook, Fritsch and Maniatis). The luciferase activity was normalized to the β-gal activity serving as an internal control for transfection efficiency.

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Chapter 3

Mutations of Ros Differentially Effecting Signal Transduction Pathways Leading to Cell Growth Versus Transformation

(Zong et al., 1997. J. Biol. Chem. 272: 1500-1506)

Abstract

The signaling function of the oncogenic protein tyrosine kinase (PTK) v-Ros were studied by systematically mutating all of the tyrosine residues in its cytoplasmic domain. The carboxyl Y564 mutation produces the most pronounced effect on v-Ros autophosphorylation and interaction with phospholipase (PLCγ). A cluster of 3 tyrosine residues, Tyr-414, Tyr-418 and Tyr-419, within the PTK domain of v-Ros plays an important role in modulating its kinase activity. The mutant F419 and the mutant DI, deleting 6-amino acids near the catalytic loop, retain wild type protein tyrosine kinase and mitogenic activities, but have dramatically reduced oncogenicity. Both mutant proteins are able to phosphorylate or activate components in the Ras/MAP kinase signaling pathway. However, F419 protein is unable to phosphorylate insulin receptor substrate 1 (IRS-1) or promote association of IRS-1 with phosphatidylinositol 3- kinase. This tyrosine residue in the context of NDYY motif may define a novel recognition site for IRS-1. Both F419 and DI mutants display impaired ability to induce tyrosine phosphorylation of a series of cytoskeletal and cell-cell interacting proteins. Thus the F419 and DI mutations define v-Ros sequences important for cytoskeleton signaling, the impairment of

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which correlates with the reduced cell transforming ability.

Introduction

Comparing the PTK domain of Ros with those of the Src family and other receptor PTKs, two sequence features were noticed (Chen et al., 1991). One is the presence of a cluster of 3 tyrosine residues consisting of a single tyrosine followed by twin tyrosines four residues downstream, of which the middle tyrosine corresponds to the major autophosphorylation site Y416 of v-Src (Takeya et al., 1982). The other feature is a 6 aa insertion 3 aa downstream of the predicted PTK catalytic loop based on the crystal structure of the kinase domain of insulin receptor (IR) (Hubbard et al., 1994). These characteristic features of the v-Ros PTK domain are also shared by its closely related RPTKs, IR and insulin-like growth factor I receptor (IGFR) (Ebina et al., 1985; Ullrich et al., 1985). Double mutation of the twin tyrosines or triple mutation of the tyrosine cluster resulted in greatly reduced PTK activity of IR and IGFR (Li et al., 1994; Jiang et al., 1996; Yonezawa et al., 1991; Zhang et al., 1991). The importance of those tyrosine residues was further suggested by solution of the crystal structure of the PTK domain of IR (Hubbard et al., 1996). In that study, it was suggested that the middle tyrosine residue Y1162 of IR plays a major role in the gating of the catalytic site of IR by controlling the accessibility of ATP and substrate binding sites. Based on the sequence homology with IR, it is likely that the triple tyrosine residues of Ros also play an important role in regulating its catalytic activity.

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If specific tyrosine residues of an oncogenic RPTK are involved in interacting with different intermediate substrates leading to distinctive signal transduction pathways, it may, therefore, be possible to differentially impair specific pathways responsible for different biological effects by making mutation at those tyrosine residues. To explore the functional role of specific tyrosine residues of the oncogenic v-Ros, particularly with respect to their roles in mitogenic versus transforming activity, I have systematically mutated all of the tyrosine residues in the cytoplasmic domain of v-Ros. In addition, I have also removed the 6-aa insertion in the v-Ros catalytic domain. These mutants allowed me to identify the tyrosine residues of v-Ros that are important for regulating its PTK activity and interaction with specific substrates. They have also allowed me to differentiate signaling pathways leading to mitogenicity versus morphological transformation and anchorage independent growth.

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Results

Construction of v-Ros site specific mutants All the tyrosine residues in the cytoplasmic domain of $P68^{gag\text{-}ros}$ were converted individually, or in combination to phenylalanines (Fig.3-1). The viruses encoding the mutant v-Ros proteins were named according to the positions of the mutated tyrosine residues (Neckameyer and Wang, 1985) with the exception of double and triple mutants F2, F3 and F4. In addition, a unique 6-aa insertion located 3 aa downstream of the predicted catalytic loop was deleted to generate the mutant DI.

Biological properties of the v-Ros mutants Two or three parallel clones of each mutant v-Ros expression plasmid were individually co-transfected with UR2AV helper virus DNA into chicken embryo fibroblasts (CEF) to assess their biological activity. Most mutants have both mitogenic and transforming activity indistinguishable from that of parental UR2 (Fig.3-1). However, the mutant F2 containing the Y418 and Y419 double mutation has only a residual transforming activity (Fig.3-2A). The triple mutant F3 has undetectable mitogenic and transforming activity. Mutants F419 and DI have dramatically reduced transforming ability as reflected in morphological alteration of the transfected cells (not shown) and their ability to form colonies in soft agar (Fig.3-2B). However, both mutants displayed the wild type mitogenic activity when the infected cells were maintained as monolayer culture in either 5% or 0.5% serum-containing medium (Fig.3-2 C, D).

Double mutant F4 displayed a further reduced transforming capability in

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comparison with F419 (data not shown) despite the fact that a single mutation of Y564 produced no detectable difference from UR2. This result indicates that the effect of Y564 mutation which impairs the interaction of v-Ros with PLCγ(see below) can only be detected in the background of a week transforming protein such as F419.

Tumorigenicity of F419 ,DI and the parental UR2 was compared. The result (Table 3-1) shows that both mutants have dramatically reduced tumorigenicity. In fact, none of the chicks succumbed to the tumors induced by the mutants in some of the animals during the one month observation period (Table 1) and even in an extended period of two months in a separate experiment (data not shown). This result is consistent with the impaired ability of the two mutants in promoting anchorage independent growth, but dose not correspond to their mitogenic activity in monolayer cultures.

PTK activity of the mutant v-Ros proteins The kinase activity of mutant v-Ros proteins was analyzed by *in vitro* auto- and trans-phosphorylation, *in vivo* tyrosine phosphorylation of the Ros proteins, as well as their ability to phosphorylate cellular proteins (Fig.3-3, 4). Mutation of both Tyr-418 and Tyr-419 (F2) resulted in greatly reduced kinase activity (Fig.3-3A), particularly *in vivo* autophosphorylation (Fig.3-3B) and ability to phosphorylate cellular substrates (Fig.3-4). Only a residual *in vitro* kinase activity and no detectable *in vivo* kinase activity was detected for the F3 protein containing the triple mutation of Y414F,Y418F and Y419F (Fig.3-3A, B). These results suggest that these residues, particularly the Tyr-418 and Tyr-419, play a major role in modulating the PTK activity of v-Ros. Surprisingly, despite lack

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of detectable activity in the *in vitro* autophosphorylation (Fig.3-3A) and phosphorylation of the exogenously added substrate, enolase (Fig.3-3D), the DI protein appears to have wild type kinase activity intracellularly as reflected in its *in vivo* autophosphorylation (Fig.3-3B) and phosphorylation of cellular proteins (Fig.3-4). The apparent paradox of the *in vitro* and *in vivo* kinase activity of the DI protein will be discussed. As expected, the DI protein exhibited a slightly faster mobility in the SDS-gel when detected with anti-Ros Ab (Fig.3-3C and bottom panel of D). The F564 protein containing the carboxyl-terminal tyrosine to phenylalanine mutation appears to be underphosphorylated and to have a faster mobility when detected in Western blotting with anti-Tyr(P) antibody (Fig.3-3B,3-4). However, F564 protein has no detectable decrease in the *in vitro* kinase activity, although its phosphorylated products also appear to be down-shifted in gel mobility in comparison with those of the parental v-Ros (Fig.3-3A). The mobility downshifting of *in vivo* phosphorylated protein was also apparent for the F4 protein containing Y419F and Y564F double mutation (Fig.3-3A). Again, no detectable difference of *in vitro* and *in vivo* kinase activity was observed for the F4 protein. The expression level of the various mutant v-Ros proteins in transfected cells was comparable, with the exceptions of F2 and F3, where the expression level was 4-5-fold lower. More protein lysates from F2- and F3-infected cells were needed in order to normalize the Ros protein in the experiments shown in Fig.3-3 and3-4. Except for the mutants described above, no effect on the *in vitro* or *in vivo* PTK activity was observed for the rest of mutations.

Phosphorylation and activation of specific signaling proteins To identify the

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tyrosine site(s) required for interaction of v-Ros with specific substrates, the mutant proteins were compared for their ability to phosphorylate or activate various signaling molecules. Fig.3-5 shows that with the exception of the kinase defective mutants, F2 and F3, all the mutants are capable of inducing tyrosine phosphorylation of Shc and 5C2, an 88 kD cellular protein previously identified to be a prominent substrate of v-Ros (Zong and Wang, 1994). In addition to F2 and F3, the F564 protein was also unable to cause tyrosine phosphorylation of PLCγ despite its wild type kinase activity. As expected this is also true for F4. Therefore, Tyr-564 may serve as PLCγ recognition site directly or be critical for the conformation of a PLCγ site elsewhere in the protein. My observation that F564 has wild type transforming activity level indicates that phosphorylation of PLCγ is not essential for this activity. However, since mutation of Tyr-564 in the background of F419 further reduces its transforming ability, it is likely that phosphorylation of PLCγ plays some role in enhancing transformation by v-Ros.

Activation of MAP kinase To further explore the biochemical basis for the reduced transforming activity of F419 and DI, I examined the ability of those mutants to activate MAP kinase, a downstream effector of the Ras signaling pathway. Consistent with their ability to induce tyrosine phosphorylation of Shc, both the F419 and DI proteins are able to promote association of Grb2 with three distinct tyrosine phosphorylated proteins with gel mobilities corresponding to those of Shc proteins (46, 52 and 66 kD)(Fig.3-6A). The mutants also activate MAP kinase as efficiently as the wild type v-Ros (Fig.3-6B). This is consistent with the observed mitogenic activity of F419 and DI mutants.

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IRS-1:Phosphorylation and association with PI3 kinase I next examined the signaling molecules IRS-1 and PI3 kinase, which were previously shown to be phosphorylated and activated by v-Ros (Zong and Wang, 1994). My result shows that mutation of Tyr-419 specifically decreases the ability of v-Ros to cause tyrosine phosphorylation of IRS-1 (Fig.3-7B). No such effect was observed for any of the other mutants, with the exception of the kinase inactive ones (Fig.3-7A). Consistent with the reduced phosphorylation of IRS-1, F419 protein also failed to promote association of PI3 kinase with IRS-1 as reflected in the in vitro PI3 kinase assay (Fig.3-8). This observation was confirmed by reciprocal immunoprecipitation and Western blotting using anti-p85 and anti-IRS-1 antibodies to detect their physical interaction. Association of IRS-1 with the 85 kD subunit of PI3 kinase was observed in DI- and UR2-, but not in F419-infected cells.(data not shown). These results indicate that phosphorylation of IRS-1 and activation of PI3 kinase may be important but not sufficient for promoting cell transformation and are not essential for promoting the growth of cells in monolayer culture.

Effect of v-Ros mutation on cytoskeleton-associated proteins Reorganization of cytoskeletal structure is intimately related to morphological transformation. The effect of cytoskeleton alteration on cell to cell and cell to matrix interactions could play an important role in the growth of cells in agar. I compared v-Ros and its mutant proteins for their ability to cause tyrosine phosphorylation and interaction of a series of cytoskeletal proteins involved in the formation of focal adhesion plaques and cell-cell interaction. No difference was observed among F419,DI and UR2 PTKs in causing tyrosine phosphorylation of FAK, cortactin, paxillin, CAS (Sastry

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and Horwitz,1993), a Crk-associated protein, and annexin II (Cooper and Hunter,1981), a cytoskeleton-associated Ca^{++}-dependent phospholipid binding protein (Fig.3-9). Greatly increased tyrosine phosphorylation of annexin II, cortactin and paxillin was observed in the wild type and mutants infected cells. In contrast, the increase in tyrosine phosphorylation of FAK and CAS is only about two fold above the control CEF. A tyrosine phosphorylated 190 kD protein was found to be associated with β1 integrin in UR2-, but much less in F419- and DI-infected cells. However, no significant tyrosine phosphorylation of integrin was detected (Fig.3-10). In addition, tensin was more abundantly phosphorylated in UR2- than in the mutants-infected cells. Similarly, the $p190^{Rho/GAP}$ was more highly phosphorylated in UR2- than in the mutants-infected cells, particularly in comparison with the F419 cells.

For the proteins involved in cell-cell interaction, we observed significantly more abundant tyrosine phosphorylation of β- and γ-catenin, as well as more association between cadherin and β-catenin, in UR2-, than in F419- and DI-infected cells (Fig.3-11). Association of β-catenin with cadherin was confirmed by Western blotting of the anti-cadherin immunoprecipitats with an anti-β-catenin antibody (data not shown). Neither α-catenin nor cadherin was significantly tyrosine phosphorylated by any of our v-Ros protein. These results indicate that F419 and DI proteins are either incapable or less effective in promoting tyrosine phosphorylation and interaction between various proteins involved in the formation of focal adhesion plaques and cell-cell interaction.

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Discussion

This study identifies several sequences in v-Ros that play important role in regulating PTK activity and cell transforming functions. The kinase positive and transformation negative($K^{+}T^{-}$) or attenuated ($K^{+}T^{\pm}$) mutants are useful in that they may allow identification of the signaling components essential for cell growth and transformation. I have previously generated a mutant called TM1 deleting 3 aa in the TM domain of v-Ros (Fig.3-1, Zong and Wang, 1994). This mutation has no effect on the kinase activity, but impairs both the mitogenic and transforming activities of the v-Ros. The kinase positive, mitogenicity positive but transformation defective mutants ($k^{+}M^{+}T^{\pm}$), represented by F419 and DI in this study, are thus particularly useful since they are selectively impaired in signaling pathways leading to distinct biological properties. The results with F419 and DI indicate that activation of Ras/MAP kinase pathway is not sufficient for cell transformation. Those data also suggest that the cytoskeleton protein-mediated signaling may be more closely related to morphological transformation and anchorage independent growth of cells. Numerous site specific deletion mutants within the N-terminal region of v-Src have been reported to affect its ability to induce morphological transformation and promote colony formation in soft agar (Wang and Parsons,1989). However, it is not clear how those mutants affect the growth of cells in monolayer culture. The F419 and DI mutants resemble a recently reported tyrosine 807 mutant of v-*fms*, which retains mitogenic but not morphological transforming activity (Trouliaris et al., 1995).

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Our data show that all the tyrosine residues in the cytoplasmic domain of gag-Ros, except Tyr-419 and Tyr-564 are not individually required for Ros's biochemical and biological properties. Our finding of the effect of the triple tyrosine mutation cluster on v-Ros PTK activity is consistent with those of other RPTKs including IR (Ellis et al., 1986), IGFR (Gronborg et al.,1993; Jiang et al., 1996) which also contain such a tyrosine cluster. 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). However, mutation of Tyr-418 of v-Ros, which corresponds to Tyr-1162 of IR, suggested to be the "gate-keeper" of its catalytic site (Hubbard et al., 1994), did not yield any detectable biochemical or biological effect on v-Ros. Instead, mutation of the third tyrosine Tyr-419 in the cluster resulted in impairment of the v-Ros transforming ability and substrate specificity. This result is consistent with our previous observation on the mutation of the corresponding tyrosine residue Tyr-1136 of an oncogenic gag-IGFR fusion PTK except that the mutation in that case resulted in dramatic decrease of both mitogenic and transforming activity (Jiang et al., 1996). Deletion of the 6-aa insertion near the catalytic loop of v-Ros resulted in the loss of *in vitro* kinase activity, but produces little effect on tyrosine phosphorylation of the mutant DI protein . Moreover, the DI protein is able to induce tyrosine phosphorylation of the array of cellular substrates with a pattern indistinguishable from that of the wild-type v-Ros. The simplest explanation for this observation is that the deletion results in an enzyme whose conformation is

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relatively unstable and is easier to be inactivated during cellular protein extraction and *in vitro* processing. However, the possibility that the mutant DI protein is phosphorylated by other endogenous PTK(s) and becomes activated *in vivo* can not be ruled out. If so, the active DI protein is apparently inactivated again during the protein extraction and processing since neither auto- nor trans-phosphorylation activity could be detected *in vitro*.

The IRS-1 and Shc recognition site on IR has been identified as the NPEY motif in the juxtamembrane region of IR, in which the N, P and Y residues are important for the interaction (Gustafson et al., 1995). The v-Ros is capable of inducing tyrosine phosphorylation of IRS-1 and Shc (Zong and Wang, 1994). However, there is no corresponding NPXY sequence in v-Ros. The Y419F mutation specifically decreases the tyrosine phosphorylation of IRS-1, but not of Shc. Therefore, the NDYY sequence of v-Ros likely defines an alternative recognition site for IRS-1. Shc must interact with v-Ros at another site. Alternatively, presence of either of the twin tyrosines in NDYY may be sufficient for Shc recognition.

The Tyr-564 is the only residue that upon single mutation results in a pronounced reduction of intracellular autophosphorylation of v-Ros. Tryptic mapping of the *in vitro* autophosphorylated v-Ros proteins of UR2, F2(Y418F/Y419F), and F564 revealed that several tryptic spots were missing in F564, but not in F2, protein in comparison with those of UR2 v-Ros protein (data not shown). These observations suggest that Tyr-564 is the major phosphorylation site of v-Ros *in vitro* and *in vivo*. Alternatively, initial phosphorylation of Y564 is required for phosphotylation of other site(s) of the v-Ros. Mutation of Y564 also indicates that

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it is important for recognition of PLCγ. The interaction site for PLCγ mapps to the carboxyl tyrosine residues of a number of RPTKs including EGFR (Rotin et al., 1992), PDGFR (Kim et al., 1991), IGFR (Jiang et al., 1996) and Met (Ponzetto et al., 1994). Activation or overexpression of PLCγ has been implicated in stimulating DNA synthesis and promoting cell transformation by EGF and PDGF receptors (Valius and Kazlauskas, 1993). Some other reports, however, concluded that PLCγ was not important for PDGF-induced DNA synthesis (Hill et al., 1990). My results shows that PLCγ plays only a minor role in v-Ros-mediated transformation of CEF.

Activation of PI3 kinase has been implicated in diverse functions. The precise mechanisms of PI3 kinase signaling leading to those diverse functions are not completely clear. The result with F419 and DI suggests that PI3 kinase could play a significant role in v-Ros-induced cell transformation, but its activation is insufficient for morphological transformation and anchorage-independent growth and is not important for growth in monolayer culture.

Our data suggest that signaling involving cytoskeletal proteins and cell-cell interaction may play an important role in morphological transformation and anchorage independent growth. The Rho family of GTP-binding/GTPase proteins including, Rho, Rac and CDC42 are key players in regulating the cytoskeletal structure and membrane properties and also important in mediating Ras-induced cell transformation (Nobesa and Hall,1995; Ridley and Hall,1992). In this regard, it is intriguing that $p190^{RhoGAP}$, a regulator of Rho, is underphosphorylated in the F419- and DI-infected, in comparison with the UR2-infected cells (Fig.3-10). EGF-dependent actin cytoskeleton disassembly is modulated by expression of c-Src and

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correlates with increased tyrosine phosphorylation of $p190^{RhoGAP}$ (Chang et al., 1995). This phenomenon could be explained by increased activity of RhoGAP resulting in diminished abundance of RhoGTP needed to promote the formation of actin stress fibers.

The observation of the increased tyrosine phosphorylation of tensin and a β1 integrin-associated 190kD protein, as well as cadherin-catenin complex involved in cell-cell interaction in UR2-, but not in F419- or DI-infected cells is also intriguing. It raises a possibility that those proteins are involved in mediating morphological transformation and anchorage-independent growth of UR2. Further work is required to elucidate the identity of the 190 kD protein which can not be recognized by the antibody against $p190^{Rho/GAP}$ (data not shown).

The cytoplasmic region of cadherin interacts with α-, β- and γ- catenins which may serve as the bridge for interaction with actin, as well as signaling effectors (Gumbiner et al., 1994). In v-Src transformed cells, β-catenin is tyrosine phosphorylated and although cadherins are expressed on cell surface, they are functionally inactive (Volberg et al., 1991). The homology between β-catenin and a segment polarity gene in *Drosophila* called armadillo raises the possibility that β-catenin has a similar role in developmental signaling (McCrea et al., 1991). Thus catenins may play a dual role in cell-cell interaction and in signaling. The increased tyrosine phosphorylation of catenins and their enhanced association with cadherin in v-Ros transformed cells may not only affect the function of cadherin, but also modulate the cytoplasmic pool of the catenin involved in signaling.

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Fig.3-1. **Mutants of the UR2 v-Ros.** The UR2 encoded p68$^{gag\text{-}ros}$ is shown with different structural domains indicated. The numbers correspond to the amino acid positions (Neckameyer et al., 1985). TM1 deletes 3-aa insertion in the TM domain of UR2 P68 and has been described (Zong et al., 1994). The kinase activity and relative mitogenic and cell transforming activities of each mutant are indicated. The symbol ± means a decrease of activity to different extents as detailed in the text. The DI PTK has very little *in vitro* kinase activity, but displays a wild type level activity *in vivo*.

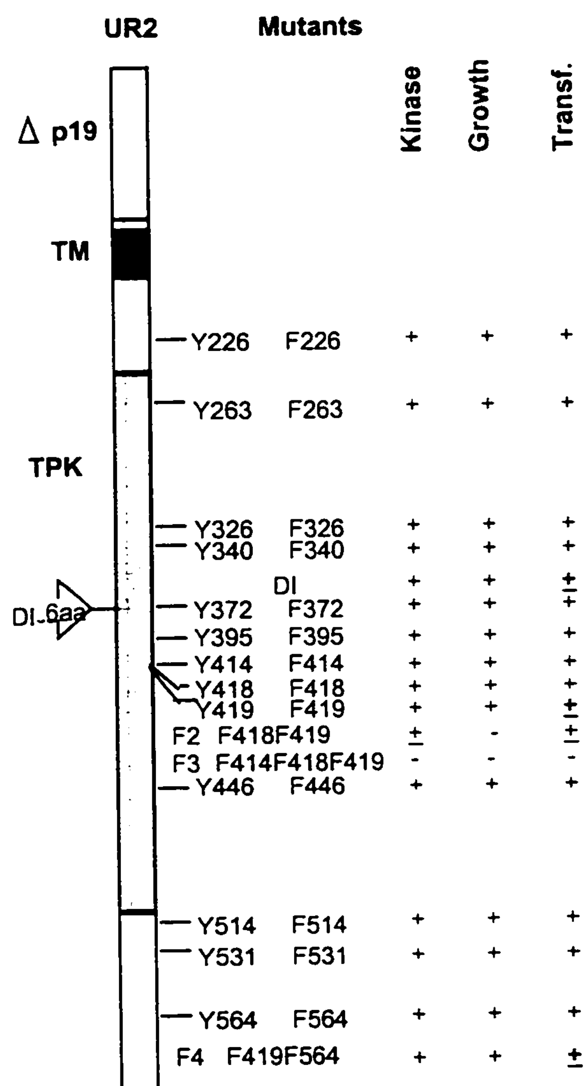

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Fig.3-2. **Biological activities of v-Ros mutants.** (A) and (B). Colony formation of UR2- and mutants-infected cells: CEF and mutants infected cells were seeded in soft agar at 10^5 cells per 60 mm dish. The pictures were taken 2 weeks later. (C) and (D). Growth rate of normal and various viruses infected cells. Cells were grown in medium containing either 0.5% (C), or 5% (D) serum. Cell numbers from duplicate dishes were counted every other day. Day 1 represents one day after initial seeding of cells.

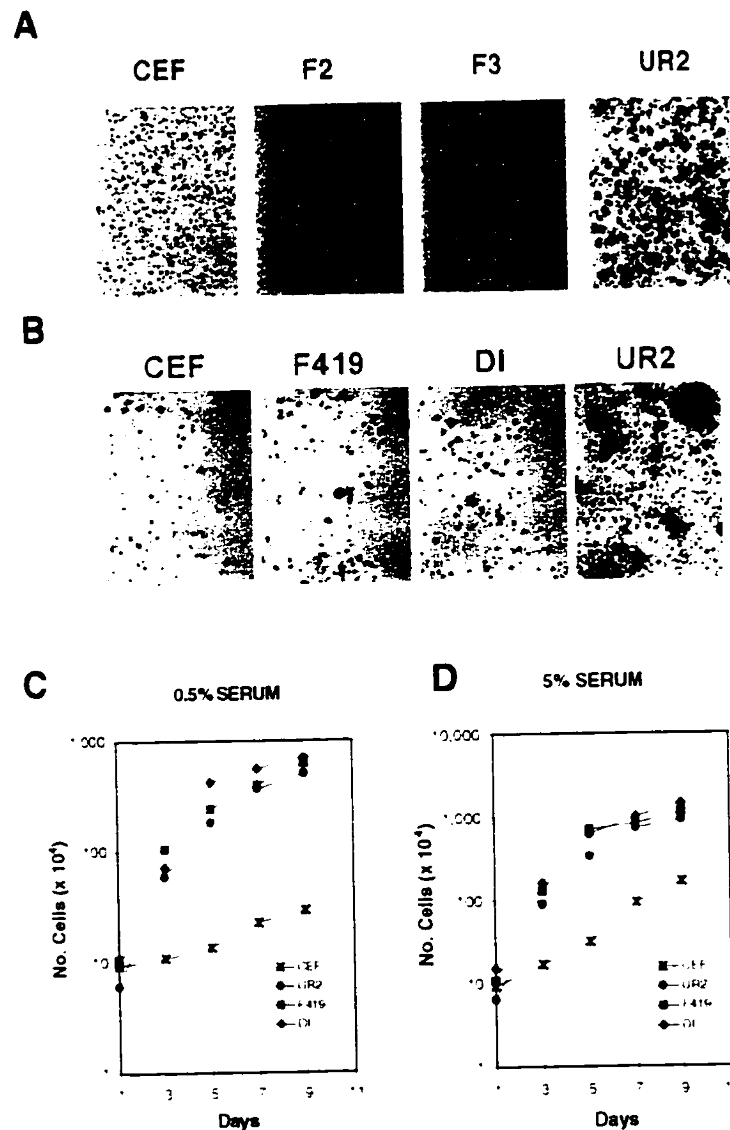

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Fig.3-3. **PTK activities of mutant v-Ros proteins.** Equal amount of cell lysates from control or infected CEF were immunoprecipitated with anti-Ros and subjected to in vitro kinase assay (A), Western blotting with anti-P-Tyr (B), or anti-Ros (C). Four to five fold more lysates were used for F2 and F3 in order to normalize the amount of Ros protein. (D) Upper panel, same as (A) except 2μg of acid treated enolase (Jong and Wang 1987) was included in the kinase reaction. Lower panel, Ros protein detection.

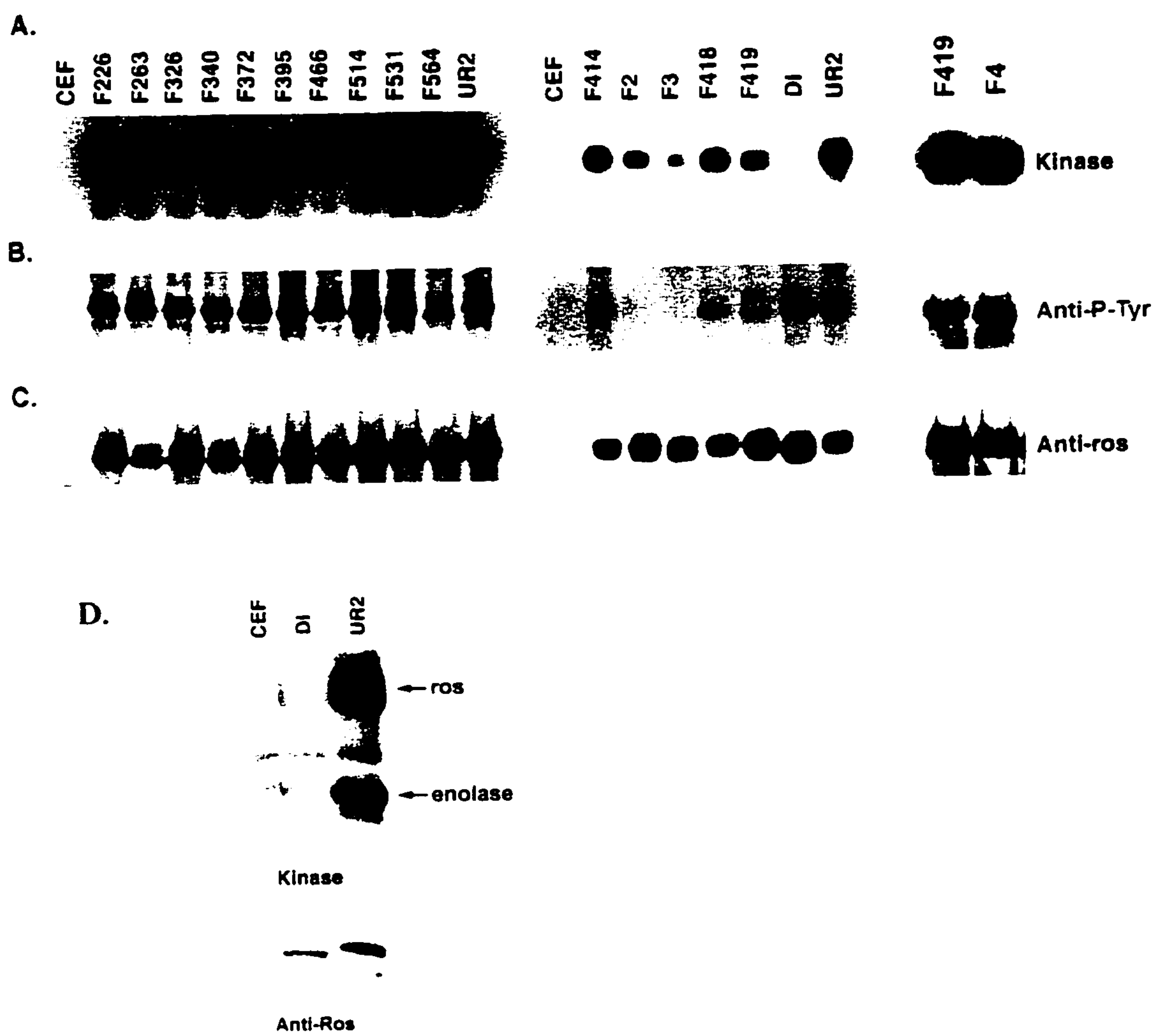

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Fig.3-4. **Tyrosine phosphorylation of cellular proteins.**
20 μg each of total cell lysates were separated by SDS-PAGE, followed by Western blotting with anti-P-Tyr antibody (RC20). The arrow indicate the Ros protein bands.

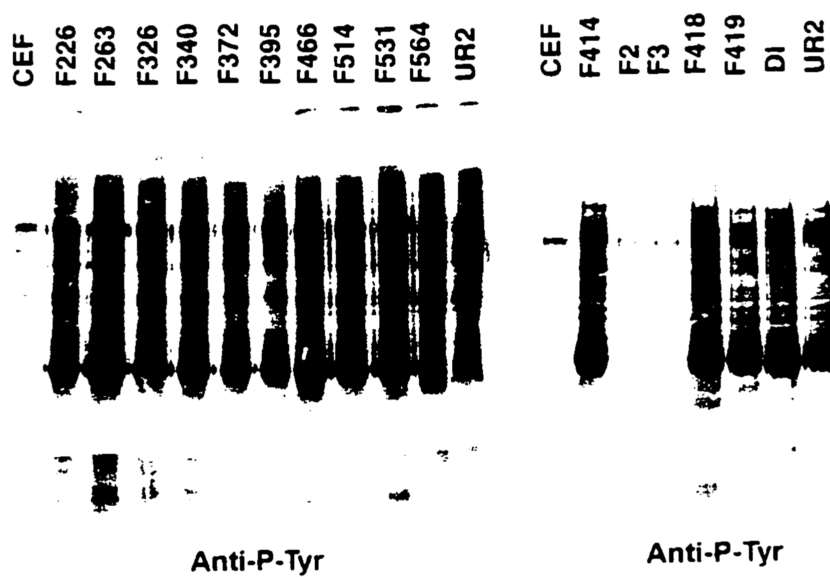

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Fig.3-5. **Tyrosine phosphorylation of PLCγ, 5C2 and Shc.** 500 μg each of cell lysates were immunoprecipitated with anti-PLCγ (A), 5C2 (B) or anti-Shc (C) Ab, and subjected to Western blotting with anti-P-Tyr Ab(RC20).

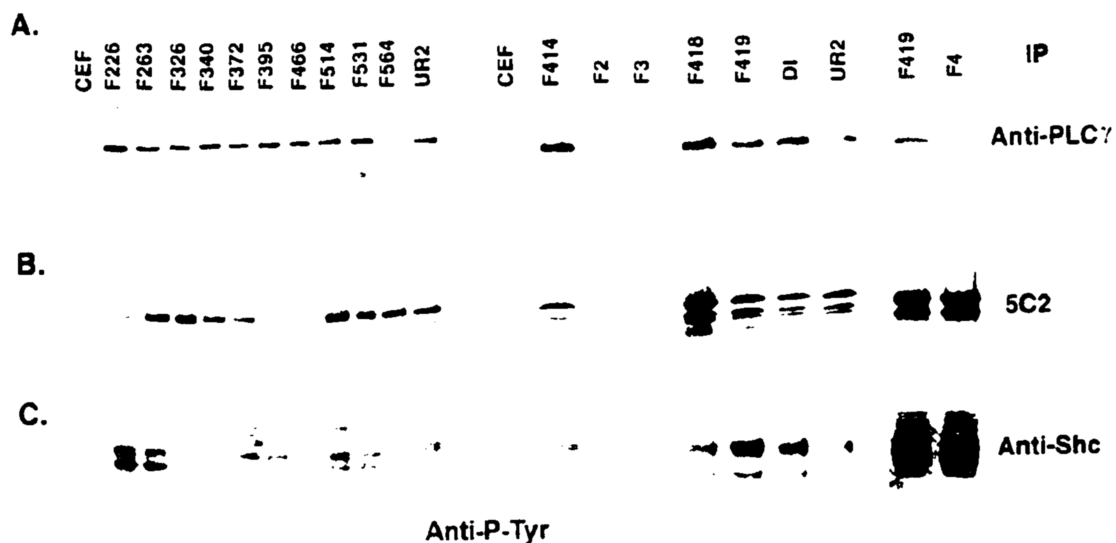

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Fig.3-6. **Association of Shc with Grb2 and activation of MAP kinase.**
(A). Grb2-associated Shc. 500 μg of cell lysates were immunoprecipitated with anti-Grb2, followed by Western blotting with anti-P-Tyr (RC20) (upper panel) or anti-Grb2 (lower panel).The arrows indicate Shc protein bands. (B) Activation of MAP kinase. 400 μg of cell lysates were immunoprecipitated with anti-MAP kinase TR10 and half of the immunoprecipitates were used for MAP kinase assay as described in Materials and Methods. Labeled myelin basic protein (MAP) was separated by SDS-PAGE and visualized by autoradiography (upper panel). The other halves of the immunoprecipitates were subjected to Western blotting with anti-MAP kinase (lower panel).

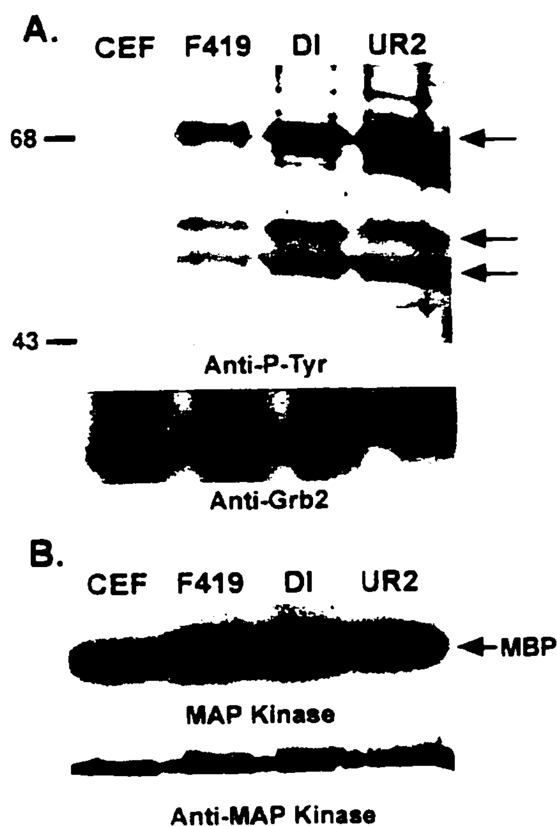

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Fig.3-7. **Tyrosine phosphorylation of IRS-1.** 800 μg of cell lysates were immunoprecipitated with anti-IRS-1 serum and subjected to Western blotting with anti-P-Tyr (RC20) (A). Panel B shows the repeated experiments with DI and F419 mutants. The bottom panel shows the IRS-1 protein from the equivalent immunoprecipitates of Exp1 and was analyzed by Western blotting with anti-IRS-1.

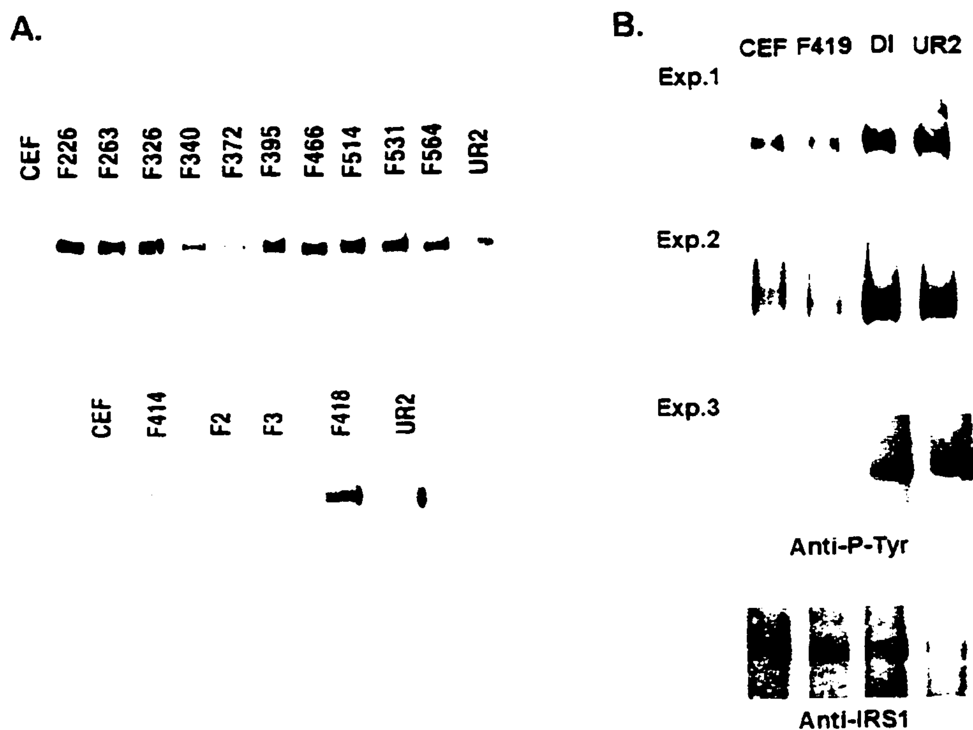

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Fig.3-8. **IRS-1-associated PI3 kinase activity.** 500 μg of cell lysates were immunoprecipitated with anti-IRS-1 serum, followed by PI3 kinase assay. Upper panel shows the autoradiography of one experiment. The histogram represents the quantitative analysis of the signals from three independent experiments.

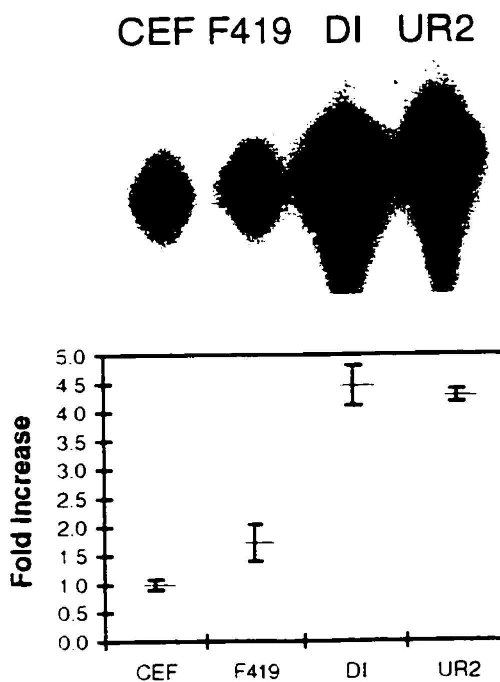

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Fig.3-9. **Tyrosine phosphorylation of cytoskeleton-associated proteins.** 500 μg of cell lysates were immunoprecipitated with respective antibodies indicated and duplicate immunoprecipitates were subjected to Western blotting with either anti-P-Tyr (RC20) (left panel) or the respective antibodies shown at the bottom of each right panel.

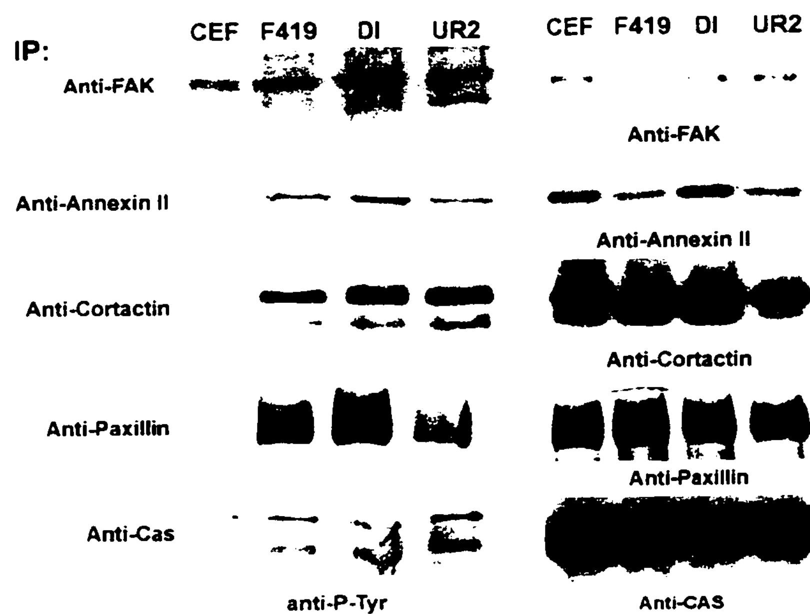

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Fig.3-10. **Tyrosine phosphorylation of integrin, tensin and p190 Rho/GAP.** 500 μg to 1 mg of total cell lysates were immunoprecipitated with the respective Abs indicated at the bottom of each panel and duplicate immunoprecipitates were analyzed by Western blotting with either anti-P-Tyr RC20 (upper 2 panels), or with the original Ab used for IP (bottom panels). The arrows indicate an integrin-associated phosphorylated protein. The bottom panels were derived from Exp.1.

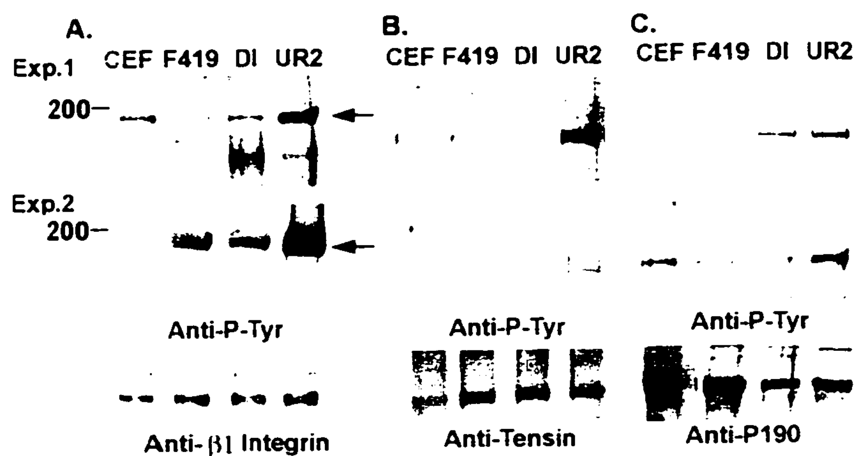

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Fig.3-11. **Tyrosine phosphorylation and association of cadherin and catenins.** 500 to 600 μg of total cell lysates were immunoprecipitated with the indicated Abs and duplicate samples were analyzed similarly as described in Fig.3-10. The arrow indicates β-catenin.

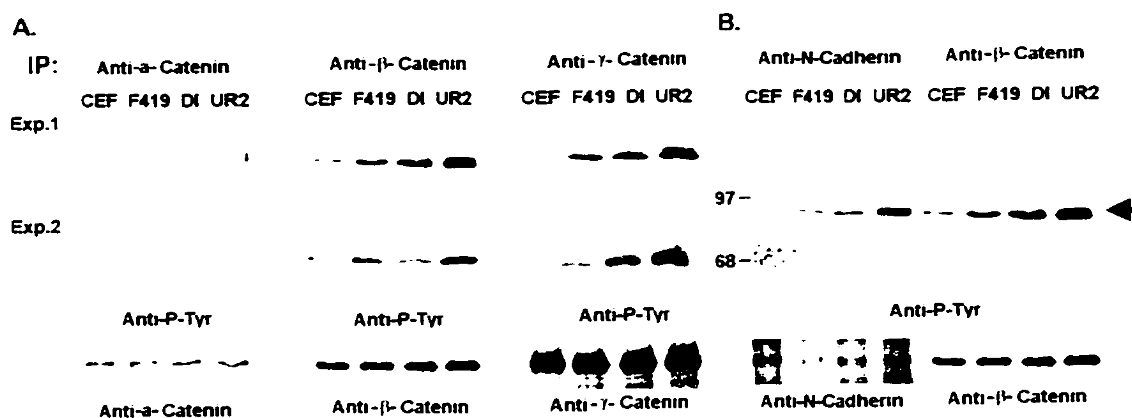

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Table 3-1[1]

	Tumor Incidence	Onset[2] (days)	Average Size[3] (mm^2)	Death[4] Incidence
Control	0/4	-	-	-
UR2	6/6	7-10	239.2	6/6
F419	6/6	17-21	66.5	0/6
DI	4/6	21-30	37.7	0/6

1. Cell-free overnight media from fully infected cultures producing similar amount of Ros proteins were used for injecting 1-day-old chicks. Each chick was injected with 0.1 ml virus stock containing about 10^5 infectious units per wing web. Control chicks were similarity injected with regular medium.

2. Days after injection when tumors were first visible.

3. The size of tumors were measured by their surface area 1 week after their detection. The average size was obtained by dividing the sum of total tumor area by the number of tumors measured.

4. The number of chicks succumbed to tumors 1 month after virus injection when the experiment was terminated. All the chicks inoculated with UR2 died within 4 weeks after injection.

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Chapter 4

Stat3 Plays An Important Role in Oncogenic Ros- and Insulin-like Growth Factor I Receptor-Induced Anchorage Independent Growth

(Zong et al, 1998, in press)

Abstract

Recent studies have suggested that Signal Transducers and Activators of Transcription (STAT) may play some role in the regulation of cell growth and transformation by normal and oncogenic protein tyrosine kinases (PTKs). However there has been little direct evidence of the involvement of STATs in PTK-induced oncogenesis. Our laboratory previously described an inducible EGFR-Ros chimeric receptor PTK called ER2 and a constitutively activated insulin-like growth factor I receptor (IGFR) called NM1, both of which are able to cause anchorage independent growth of NIH 3T3 cells. Characterization of ER2 and NM1 expressing NIH 3T3 cells revealed that these receptor PTKs cause Stat3 activation as measured by tyrosine phosphorylation and DNA binding activity of Stat3. Co-expressing either of the two dominant negative Stat3 mutants with ER2 or NM1 in transiently transfected cells or in stable expressing lines resulted in a dramatic inhibition of these receptor PTK-promoted colony forming activity, but the mutants had only a mild effect on mitogenicity of ER2 or NM1 expressing cells in monolayer. My results indicate that Stat3 is not only important for initial phase of transformation as demonstrated by inhibition of the EGF-inducible colony formation of the ER2

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cells, but also is required for the maintenance of transformation as the mutant is able to revert the transformed state of the NM1 expressing cells. As expected, the DNA binding and transcriptional activities of the endogenous Stat3 were greatly inhibited in the ER2 and NM1 cells co-expressing the Stat3 mutants. These results show that Ros and NM1 gag-IGFR RPTKs are able to activate Stat3 and that activated function of Stat3 is required for the establishment and maintenance of these oncogenic receptor PTKs-induced anchorage independent growth. (The experiments involving NM1 in this chapter were performed by Liyu Zeng of our laboratory).

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Introduction

In addition to be implicated in signaling for cell growth, differentiation and apoptosis as described above (Chapter1), recently, STATs were found to be activated in certain tumors and oncogene transformed cell lines (Cao et al., 1996; Carlesso et al, 1996; Chaturvedi et al., 1997; Danial et al., 1995; Garcia et al., 1997; Gouilleux-Gruart et al., 1997; Ilaria and van Etten 1996; Shuai et al., 1996; Watson and Miller 1995; Yu et al., 1995; Zong et al., 1996). Stat3 is most prominently activated in *src* transformed NIH 3T3 cells (Cao et al., 1996; Yu et al., 1995), whereas Stats 1, 3 and 5 are activated in *src* transformed myeloid cells(Chaturvedi et al., 1997). Stat3 is also constitutively activated in v-*sis* and v-*fps* transformed fibroblasts (Garcia et al., 1997). The oncogenic v-*abl* and Eyk PTKs induce activation of Stat1,5 and 6 in pre-B cells (Danial et al., 1995) and Stat1 and 3 in chicken embryo fibroblasts (Zong et al., 1996) respectively. Furthermore, specific members of STATs were found to be activated in various human tumors and cell lines derived from them (Garcia et al., 1997; Gouilleux-Gruart et al., 1997; Shuai et al., 1996; Yu et al., 1995; Weber-Nordt et al., 1996; Nielsen et al., 1997). These observations raised yet another possible functional role for STATs, namely in oncogenesis. However, direct evidence for the involvement of STATs in oncogenes-induced cell transformation has only very recently begun to emerge. Using a dominant negative mutant of Stat1, Zong et al suggested that it plays an important role in EyK induced transformation of NIH 3T3 cells (Zong et al., 1996). This

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observation together with previously reported activation of specific STATs by v-*src* and v-*abl* further strengthens the possibility that STATs plays some important role in oncogenic PTK- induced cell transformation.

Our laboratory has been interested in exploring the mechanism of cell transformation by oncogenic RPTKs Ros, insulin receptor (IR) and insulin-like growth factor I receptor (IGFR). V-*ros* is an oncogenic gag-Ros fusion receptor gene spontaneously transduced by an avian leukosis virus (Neckameyer and Wang, 1985). A previous member of our laboratory had constructed an EGFR-Ros chimeric receptor and shown that it was capable of inducing EGF-dependent transformation of NIH 3T3 cells (Xiong et al., 1996). Another member of our laboratory had engineered a constitutively oncogenic gag-IGFR fusion receptor called NM1 and demonstrated its transforming ability in chicken embryo fibroblasts (CEF) (Liu et al., 1993) and NIH 3T3 cells (Li et al., 1998). Using a temperature sensitive v-*ros* mutant, ligand inducible EGFR-Ros chimeras and the constitutively activated NM1 gag-IGFR, we have previously observed cell type-specific activation of STAT proteins in CEF and NIH 3T3 cells by Ros and IGFR (Zong, C., Jiang, Y. and Wang, L.-H. unpublished). To further explore the role of Stat3 in Ros and NM1-induced cell transformation, I have employed dominant negative (dn) mutants of Stat3 to investigate its role in the establishment and maintenance of cell transformation by these oncogenic RPTKs. My results show that both Ros and NM1 activate predominantly Stat3 in NIH 3T3 cells and that Stat3 is required for Ros- and NM1-induced anchorage independent growth, whereas Stat3 is less critical for the ability of these RPTKs to stimulate cell growth in monolayer. During the course of

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this study, two reports were published demonstrating that Stat3 plays an essential role in v-*src*-induced transformation of NIH 3T3 cells. (Bromberg et al., 1998; Turkson et al., 1998). Overall, these studies provide initial direct evidence for the role of STATs in cell transformation and oncogenesis.

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Results

Tyrosine Phosphorylation and Activation of STAT proteins in ER2 Expressing cells To determine whether STAT proteins are activated by the oncogenic RPTKs Ros and gag-IGFR (NM1), we expressed temperature sensitive (ts) or a ligand inducible Ros and the constitutively activated NM1 gag-IGFR in CEF or NIH 3T3 cells and analyzed for tyrosine phosphorylation and DNA binding activity of STAT proteins. CEF infected with NY68 encoding the ts *src* was included for comparison. In CEF, increase of Stat1 and Stat3 tyrosine phosphorylation correlated with Src activation (Fig.4-1A). This is in contrast to the specific Stat3 activation by Src in NIH 3T3 cells reported previously (Yu et al., 1995). STAT protein amounts in all cases were indistinguishable. There was very little detectable tyrosine phosphorylation of Stat1 and a constant basal level of phosphorylation of Stat3 in control CEF (Fig.4-1A). A significantly higher than basal level of tyrosine phosphorylation was observed for Stat1 and particularly Stat3 in Src and Ros expressing CEF even at nonpermissive temperature. This could be due to leakiness or presence of revertant viruses in the NY68 and UR2ts251 stocks. Higher basal level observed for Stat3 than for Stat1 suggests that Stat3 is more effectively phosphorylated by Src and Ros. Such basal level tyrosine phosphorylation of Stat1 and Stat3 was not observed in NIH 3T3 cells transfected with Ros expressing vector ER2 (Fig.4-1B). In NIH 3T3 cells, activation of the ER1 and ER2 EGFR-Ros chimera by EGF led to significant increase in tyrosine phosphorylation of Stat3 (Fig. 4-1B) This increased tyrosine activation was correlated with increased DNA binding

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ability of Stat3 (Fig.4-1C). EMSA analysis revealed increase in Stat3 homodimer (SIFA) and Stat3/Stat1 heterodimer (SIFB) binding to the SIE probe upon Ros activation in both cytosolic and nuclear fractions although the later was more prominent (Fig.4-1C). There was a basal level of Stat1 tyrosine phosphorylation which was largely insensitive to EGF stimulation (Fig.4-1B). In contrast, tyrosine phosphorylation of Stat5b was greatly increased upon EGF treatment in control and ER2 expressing cells (Fig.4-1B) implying its activation via endogenous EGFR. This result indicates that activation of Ros does not lead to further activation of Stat5 beyond the level of activation in the control cells. In addition, stat3 was found also to be specifically activated in NM1 gag-IGFR expressing NIH 3T3 cells (Jiang and Wang, unpulished). It is concluded that Ros and NM1 are able to specifically activate Stat3 in NIH 3T3 cells.

Dominant Negative Stat3 (dnStat3) mutants Inhibit Ros- and NM1-Induced Cell Transformation Since in NIH 3T3 cells both Ros and NM1 PTKs are able to activate Stat3 signaling, its potential role in Ros- and NM1-induced cell growth and transformation was investigated. For this purpose, I employed the approach of dominant negative mutants to block the endogenous Stat3 function. The Stat3 mutants used here are defective in DNA binding and have been shown to be able to block the wild type Stat3 activity presumably by forming a nonproductive heterodimer (Horvath et al., 1995). Dominant negative Stat3 mutants were introduced into the ER2 or NM1 expressing cells. Stable clones co-expressing the oncogenic PTK and a mutant Stat3 were selected using the appropriate drugs. Fig.4-2 compares the colony forming abilities of clones expressing ER2 alone or co-

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expressing ER2 and the dn mutants. DnStat3 expressing cells formed significantly fewer and smaller colonies compared to the control vector transfected parental ER2 cells. The control and dnStat expressing lines expressed equivalent amount of ER2 protein, whereas the dnStat lines express about 2 fold excess of the dnStat3 protein (Fig.4-2D). In addition, the ER2 chimeric receptor in all those clones are equally responsive to EGF stimulation (Fig. 4-2C). Upon long term passages of those stable lines, I observed that some of the clones displayed a reduced expression level of dnStat3 with concurrent loss of inhibition on colony formation (data not shown). Furthermore, the inhibition of Ros-induced colony formation by dnStat3 was also assessed by transient transfection method (Fig.4-3). Three independent experiments were carried out by transiently transfecting the dnStat3 mutant into ER2 expressing cells which were assayed for colony formation 48 h after transfection. An average of 50% inhibition was observed (Fig.4-3A). ER2 and dnStat3 protein expression shown in Fig 4-3B represents one of the three experiments. Again, we observed that introduction of dnStat3 resulted in inhibition of the Ros-induced colony forming ability of 3T3 cells. The inhibition by dnStat3 was not due to apoptosis or decreased cell viability as no reduction in drug-resistant colonies were observed when the transfected cells were subjected to drug selection (data not shown).

Similar experiments were carried out with NM1 by transfecting dnStat3 mutants or control empty vector into the stable NM1 expressing 3T3 cells. Mass stable cultures expressing only the NM1 or co-expressing NM1 and either of the dnStat3 mutants were prepared. Result similar to that of Ros was obtained. The

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dnStat3 expressing cells formed fewer and smaller colonies (Fig.4-4A, B, C), although the size rather than the number of colonies appeared to be more significantly affected. Again, the control and dnStat3 expressing lines express about equal amount of NM1 protein (Fig.4-4D). The dnStat3-1 expressing cells had about 2 fold excess of total Stat3 protein and the dnStat3-2 transfectants expressed the FLAG-tagged dnStat3-2 protein as expected (Fig.4-4D). Furthermore, transient transfection of 3T3 cells with 5 μg of the NM1 plasmid together with control or dnStat mutant plasmid in a ratio of 1 to 3 showed that dnStat3-1 and dnStat3-2 inhibited the NM1 colony forming activity by 52 and 62% respectively.

To assess if inhibition of the anchorage independent growth by dnStat3 mutants was due to its general inhibition on mitogenicity, I then performed assays of cell growth in monolayer culture. Three independent dnStat3-1 expressing ER2 lines and two control parental ER2 lines were compared for their growth rate in 5% calf serum plus 50 ng/ml EGF. The dnStat3 expressing cells appeared to have a slower rate of growth during the initial phase resulting in a 30 to 40 % fewer cells at the end point. (Fig.4-5A). Similar result was obtained with mass cultures expressing NM1 alone or co-expressing NM1 and the dnStat3-1 mutant (Fig.4-5B). Here I observed a 20 to 30 % reduction of the numbers of cells during the later time points. It is concluded that the dnStat3 mutant is able to block the Ros-induced establishment of cell transformation as reflected by anchorage independent growth, and it is able to revert the transformed state of NM1 expressing cells. The inhibition of ER2-induced colonies can not be fully accounted for by general inhibition of mitogenicity by the dnStat3. These results imply that Stat3 signaling function(s)

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plays a differential role in Ros- and NM1-induced anchorage independent growth versus cell growth in monolayer. The observed growth inhibition of the ER2 and dnStat3 co-expressing cells in monolayer can not fully account for their dramatic reduction in both number and size of colonies in soft agar whereas the growth inhibition of the NM1 and dnStat3 co-expressing cells is more in accordance with their reduced colony size in soft agar.

ER2- and NM1-Induced Stat3 Activation Is Blocked by Dominant Negative Stat3 To further characterize the inhibition of colony formation by dnStat3, the DNA binding and transcriptional activities of Stat3 were examined. First, I compared the DNA binding activity of Stat3 proteins obtained from the ER2 or ER2 and dnStat3 mutant co-expressing cells. Cell extracts from either stable lines or transiently transfected cells were analyzed for Stat3 DNA binding activity. The result revealed that both stable lines and transiently transfected cells expressing dnStat3 displayed reduced Stat3 DNA binding activity upon activation of Ros comparing to that of the ER2 expressing cells (Fig.4-6A,B). DNA binding activity of both Stat3 homodimer and Stat3/Stat1 heterodimer was greatly increased after Ros activation in ER2 expressing cells (Fig.4-6 A, B). Co-expression of ER2 and either of the dnStat3 mutants resulted in a dramatic reduction of SIFA and SIF B (Fig.4-6B). When exogenous wild type Stat3 was co-transfected, inhibition by the dnStat3 was also observed (Fig.4-6C)

Next, we assessed the transcriptional activity of Stat3. Using two distinct Stat3 binding site-containing luciferase reporter constructs (Turkson etal., 1998; Wen et al., 1995). These constructs were introduced separately by transfection

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along with various other expression vectors. Ros activation was capable of driving increased transcription of the Stat3 reporter gene in NIH 3T3 cells (Fig.4-7A,B). This Ros-mediated stimulation of Stat3 transcriptional activity was inhibited by dnStat3 in a dose dependent manner (Fig.4-7C). Similar results were obtained with NM1 expressing cells, the dnStat3 mutants also greatly decreased the Stat3-mediated transcriptional activity in NM1 cells (Fig.4-7D). It is concluded that both Ros and NM1 are able to induce activation of Stat3 function which could be blocked by the presence of dnStat3. Overall these results strongly suggest that activation of Stat3 by these oncogenic RPTKs plays an important role in their transforming function.

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Discussion

Our studies indicate that activation of Stat3 by Ros and NM1 plays an important role in establishment and maintenance of cell transformation by these oncogenic receptor PTKs. For Ros, Stat3 mediated signaling appears to be more critical for anchorage independent growth than for cell growth in monolayer. It is likely that certain adhesion triggered signaling can be fulfilled by Stat3 mediated function when cells are grown in soft agar. Together with the studies by Zong et al. (1995), Turkson et al. (1998) and Bromberg et al. (1998), we have provided the initial direct evidence for the involvement of Stat3 in the process of cell transformation by oncogenic PTKs.

While the mechanism by which various cytokines induce activation of STAT proteins has been well characterized through the use of JAK knock out cell lines (Muller et al., 1993; Velazquez et al., 1992; Watling et al., 1993), the mechanism for STATs activation via growth factors that siganl through RPTKs is less clear. Recent evidences suggest that JAKs may not be essential in growth factor receptor-mediated activation of STATs (Barahamand-Pour et al., 1995; Chen et al., 1997; Patel et al., 1996; Vignais et al., 1996). V-Src protein has been shown to be associated in a complex with Stat3 implying that Stat3 can be directly phosphorylated by v-Src (Cao et al., 1996). We have recently reported that the cytoplasmic domain of insulin receptor (IR) could interact with Stat5 in a yeast two hybrid assay and that this interaction appears to require PTK activity of IR (Chen et al., 1997). Moreover, Stat5 can be tyrosine phosphorylated by purified IR PTK in

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vitro (Chen et al., 1997). Although those observations strongly suggest that RPTKs may be able to directly interact and phosphorylate STATs, the possibility of indirect activation via JAKs is not excluded. JAK family kinases were shown to be constitutively activated in Src-transformed fibroblasts (Campbell et al., 1997). The two mechanisms of STATs activation are not mutually exclusive, and may actually coexist and operate in parallel in oncogenic PTKs transformed cells.

The precise role of Stat3 in RPTK-induced cell transformation remains an intriguing and challenging question. The evidence for its involvement in cell transformation is consistent with various published observations for its functioning in growth and protection from apoptosis (Fukada et al., 1996; Tekeda et al., 1997). Since the only known function of STATs is their role in transcriptional regulation, it is logical to assume that modulation of certain gene(s) by Stat3 is essential for those PTK oncogenes-induced cell transformation. It has been shown that Stat3 is not activated in v-Ras transformed cells and that dnSta3 has no effect on Ras induced transformation of NIH 3T3 cells (Bromberg et al., 1998; Turkson et al., 1998). Therefore, Ras must be able to signal through a Stat3-independent pathway to modulate those target genes. Alternatively, Ras may be capable of activating other genes with equivalent functions as the Stat3 target genes induced by those PTK oncogenes. In either case, modulation of those genes must be via a Ras-specific signaling pathway which is Stat3 independent and is not shared by the Src, Ros and IGFR PTK oncogenes. Much is still to be learned about the activation and down regulation of specific genes required for initiation and maintenance of cell transformation. A requirement of Stat3 for RPTK-induced transformation provides

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a clue to search for those genes.

The observation that Ros-induced growth of cells in monolayer is less sensitive to the inhibition of Stat3 suggests that blockage of Stat3 signaling can be partially compensated by adhesion of cells to substratum and extracellular matrix. This raises a possibility that Stat3 may signal to regulate the expression of those components involved in integrin and cytoskeleton mediated signaling. A protein kinase C and Ras transformed NIH 3T3 cell revertant line was shown to display transformed properties including enhanced growth and focus formation in monolayer, but they were unable to form colonies in soft agar (Krauss et al., 1992). Subsequently, it was found that the revertant cells had a diminished expression level of cyclin A when they were grown in soft agar medium and that the defectiveness can be rescured by transfection with a cyclin A expression vector (Kang and Krauss, 1996). In this instance, the revertant cells are impaired in expression of cyclin A when they are deprived of adhesion. It is possible that activation of Stat3 by those oncogenic PTKs may allow this transcriptional factor to induce the expression of genes such as cyclins required for growth in the absence of adhesion.

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Fig. 4-1. **Activation of STATs by Ros and Src in CEF and NIH 3T3 cells.** A, control CEF and cells infected with NY68 or UR2 ts251 were maintained at 41°C. Half of the culture were shifted down to 35°C 2 h before protein extraction with RIPA. 200mM Na_3VO_4 was added to all cultures two hours before extraction. 800 μg of the total protein extract of each sample was immunoprecipitated (IP) with anti-Stat1 or anti-Stat3 serum as indicated. Each IP was divided into duplicate aliquots and run in duplicate gels, one was subjected to Western blotting with anti-P-Tyr antibody, the other was reacted with the respective original anti-Stat antibodies as indicated at the bottom of each panel. B, Control NIH 3T3, ER1 and ER2 expressing 3T3 cells were starved for serum overnight. Half of the cultures were then stimulated with EGF(100ng/ml) for 15 min. The total protein extracts were prepared. 800 μg of the total protein from each sample was IP with anti-Stat1, anti-Stat3 or anti-Stat5. The immunoprecipitates were divided into duplicates and analyzed in Western blotting with anti-P-Tyr or anti-Stat antibodies as in A. C, Control and ER2 expressing 3T3 cells were treated as in B and subjected to extraction and fractionation into cytosolic and nuclear fractions. 20 μg protein of each fraction was used for DNA binding and EMSA analysis using the M67 SIE probe containing binding sites for Stat1 and Stat3 as described in Chapter 2. SIFA, SIFB and SIFC indicate the electrophoretic positions of Stat3/Stat3, Stat3/Stat1 and Stat1/Stat1 dimers respectively.

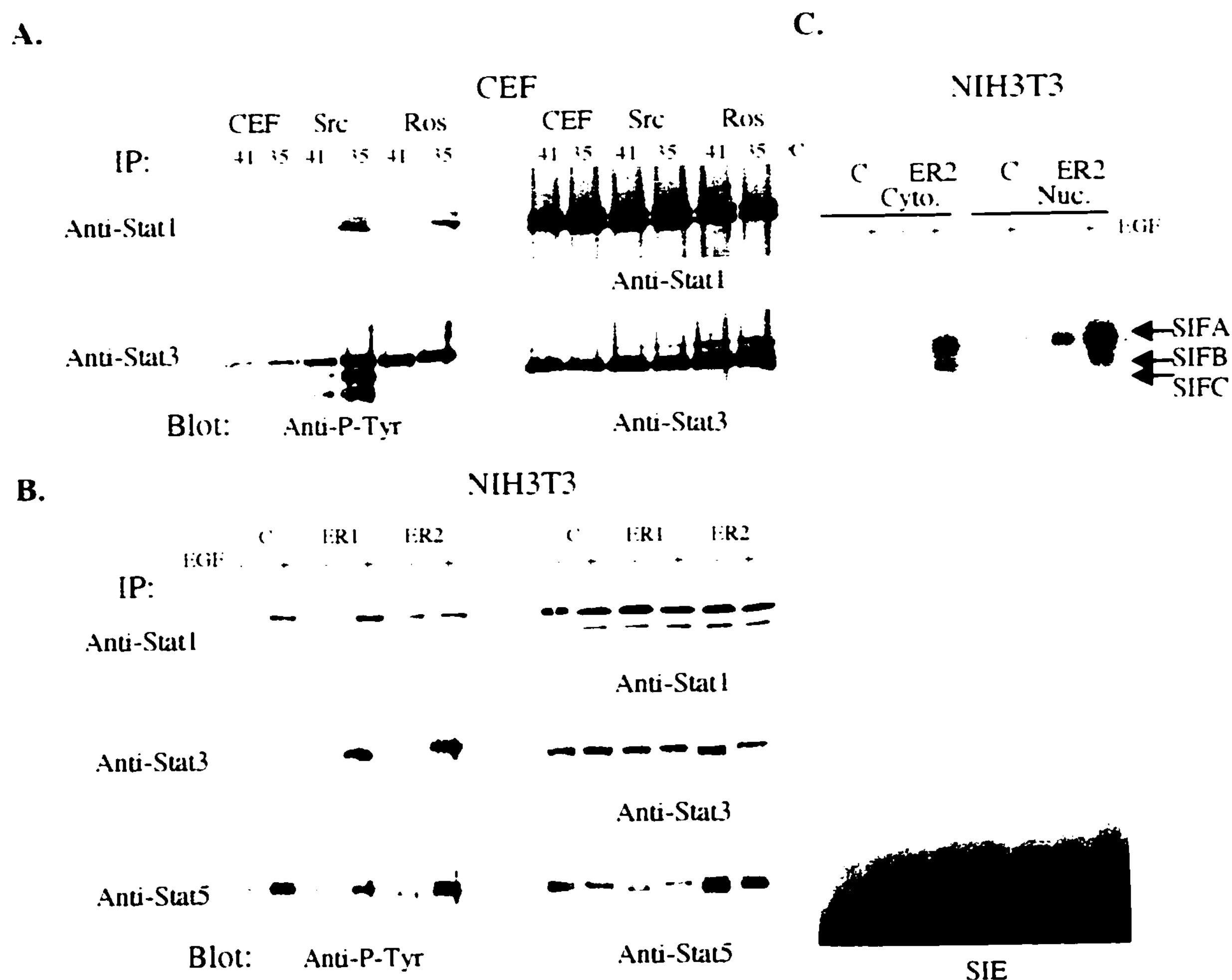

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Fig. 4-2. **Inhibition of ER2 induced-colony formation by dnStat3 mutants in stable expressing lines.** A and B, Three independent stable NIH 3T3 lines expressing ER2 alone and three lines co-expressing ER2 and dnStat3-1 were assessed for their colony forming ability. 1X10^5 cells from each clone were seeded in 60mm plate with DEM soft agar medium containing 5% calf serum and with or without 50ng/ml EGF . Pictures were taken 3 weeks after plating. C, Parallel ER2 and ER2/dnStat3-1 cultures were starved for serum overnight, stimulated with EGF (100ng/ml) for 15 min and 500 μg of the total lysate of each was analyzed for tyrosine phosphorylation of the ER2 protein. D, 15 μg each of the total cell lysates from each clone was analyzed for the protein level of ER2 and Stat3 by direct Western blotting.

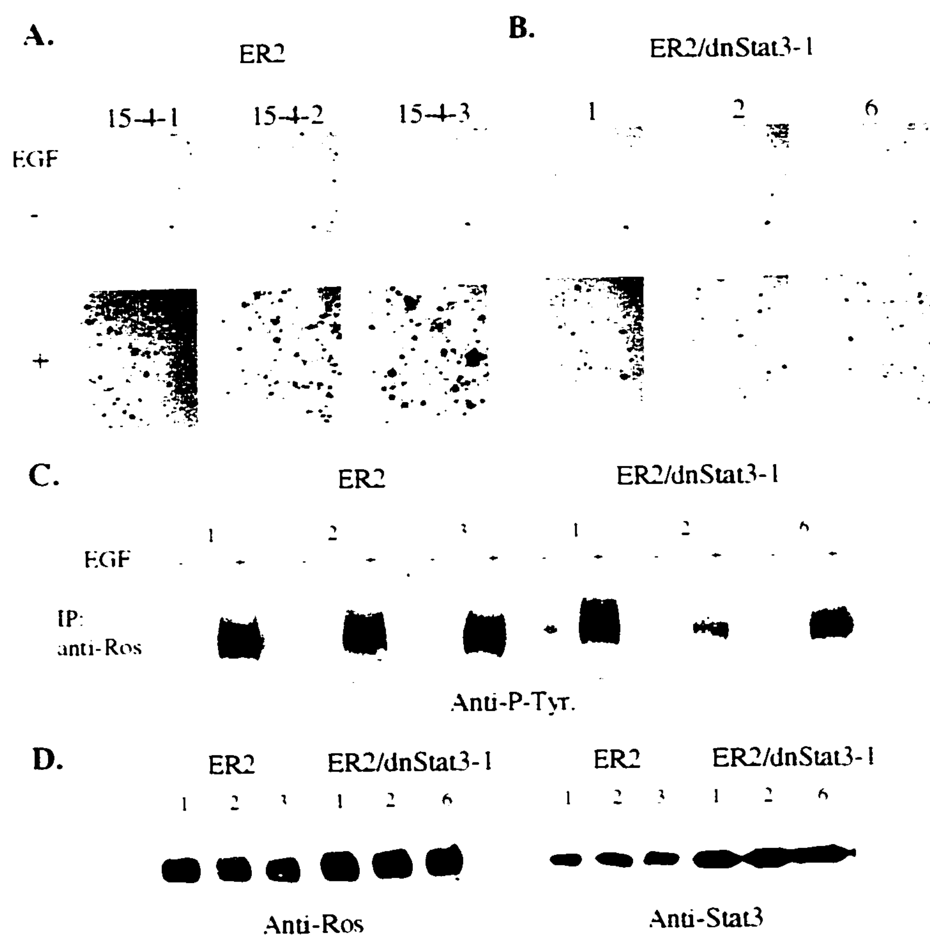

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Fig. 4-3. **Inhibition of colony forming ability of the ER2 expressing lines transiently transfected with dnStat3-2.** Three independent ER2 expressing 3T3 lines were transfected with either 10 μg of the control (RcCMV) or dnStat3-2 plasmid. Forty eight hours later, cells were used for colony formation assay. Histograms in A represent the average values of three experiments. B, parallel culture in each transfection experiment was subjected to analysis for the ER2 and dnStat3-2 protein expression. 10 μg each of the protein extracts was subjected to direct Western blotting with the indicated antisera, Stat3-2 is Flag-tagged. The result from one of the experiments is shown here.

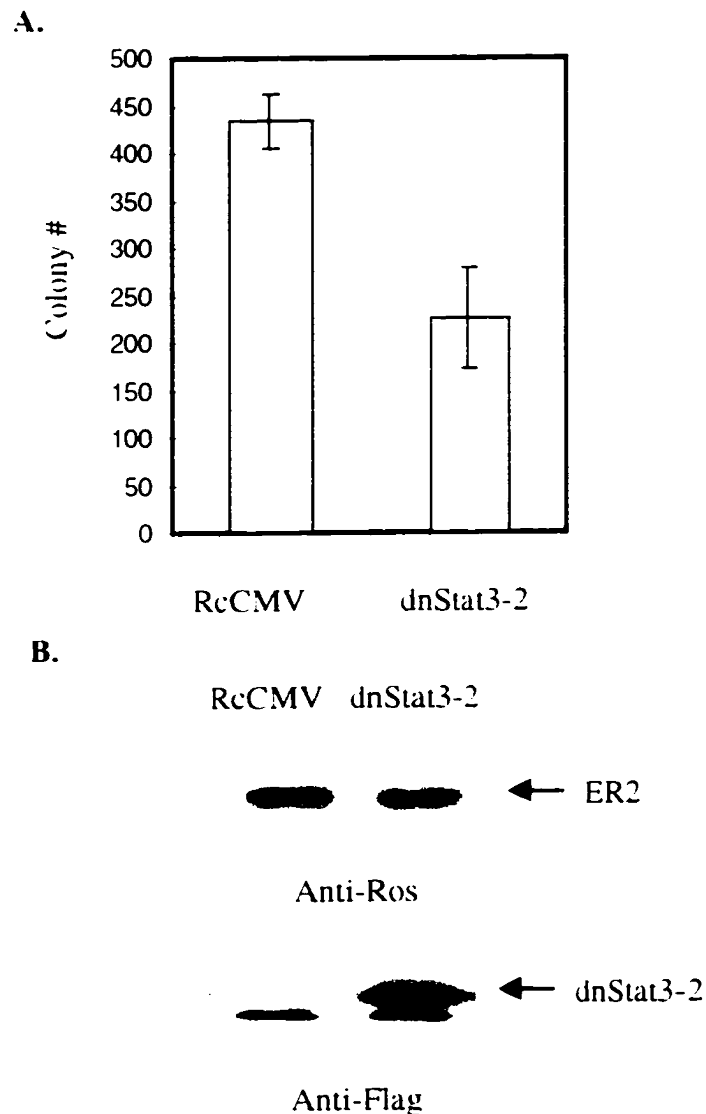

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Fig. 4-4. **Inhibition of NM1-induced colony formation by dnStat mutants.** A, B, and C, a stable NM1 expressing 3T3 line was transfected with control or individual dnStat3 mutant plasmid together with a puromycin encoding plasmid pBABE. Drug resistant clones were selected, amplified and characterized for expression of NM1 and Stat3 mutant proteins. Those with equivalent NM1 and dnStat protein expression levels were selected and compared for their colony forming ability. Colony assay was similar to that described in Fig.4-2 except no EGF was added. The numbers indicate individual clones. Pictures were taken 3 weeks after plating of the cells. D, parallel cultures were used for analyzing the protein expression. 15 μg of total cell lysate of each was analyzed for expression level of NM1, Stat3-1 and Stat3-2 proteins by direct Western blotting. The lower bands of the doublet in the upper two panels are the background signals that we sometime observed in blots with this anti-serum. The arrows point to the NM1 and Stat protein bands.

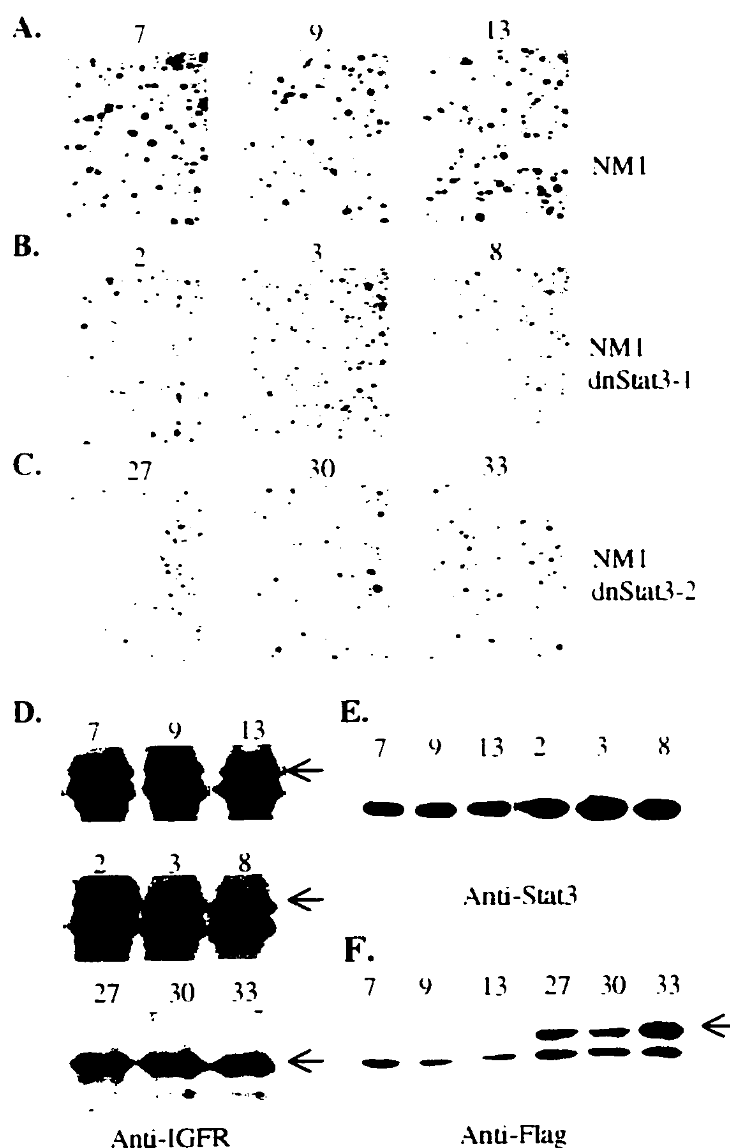

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Fig. 4-5. **Effect of the dnStat mutant on growth rate of the ER2 and NM1 expressing cells.** A, two control plasmid transfected ER2 expressing lines(PB1, PB4) and 3 ER2 lines co-expressing ER2 and dnStat3-1 (dnst2, 6, 9) were compared for their growth rate in DMEM containing 5% serum. $1X10^5$ cells were seeded per 60mm dish at the beginning of the assay. Duplicate dishes were counted for cell numbers at each time point. B, A stable NM1 expressing 3T3 line was transfected with control or dnStat3-1 plasmid and drug-resistant clones were selected as in Fig. 5 and pooled to form the control NM1 expressing mass culture (NM1 PB) and the dnStat3-1 co-expressing mass culture (NM1 dnst). The growth rate assay was similar to that in A.

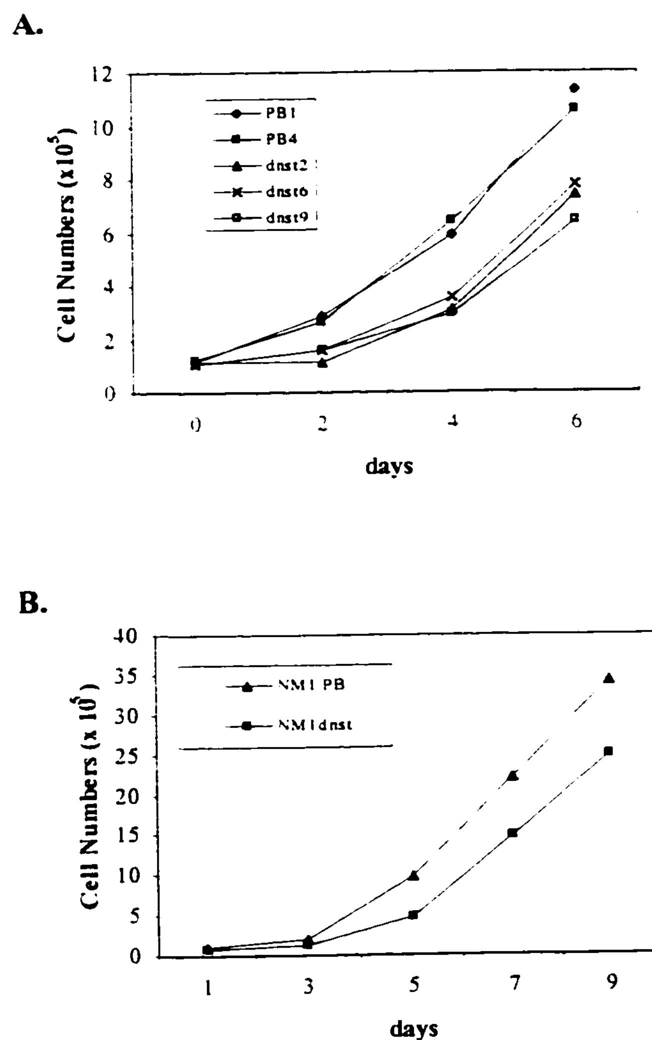

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Fig. 4-6. **Inhibition of ER2-induced activation of the Stat3 DNA binding activity by dnStat3 mutants.** A, a control ER2 expressing lines (C-1) and two dnStat3-1 co-expressing ER2 lines (dnStat3-1-1,2) were analyzed for activation of the DNA binding activity of the endogenous Stat3 upon activation of Ros by EGF. The cells were starved in serum free medium overnight and then with or without EGF treatment for 15 min. 20 μg of each total cell lysate was subjected to DNA binding by EMSA using the M67SIE probe as described in Fig. 1. B, NIH 3T3 cells were transfected with control (phEF), phEFER2 (5 μg each) or phEFER2 plus dnStat3-2 (20 ug) plasmid. Twenty four hours later, cells were changed to serum free medium overnight followed by EGF stimulation. 20 ug each of the total cell lysates was subjected to DNA binding analysis as in A. C, 3T3 cells were transfected with ER2 plus wild type Stat3 or with ER2 plus wtStat3 and dnStat3-2, and analyzed as in B.

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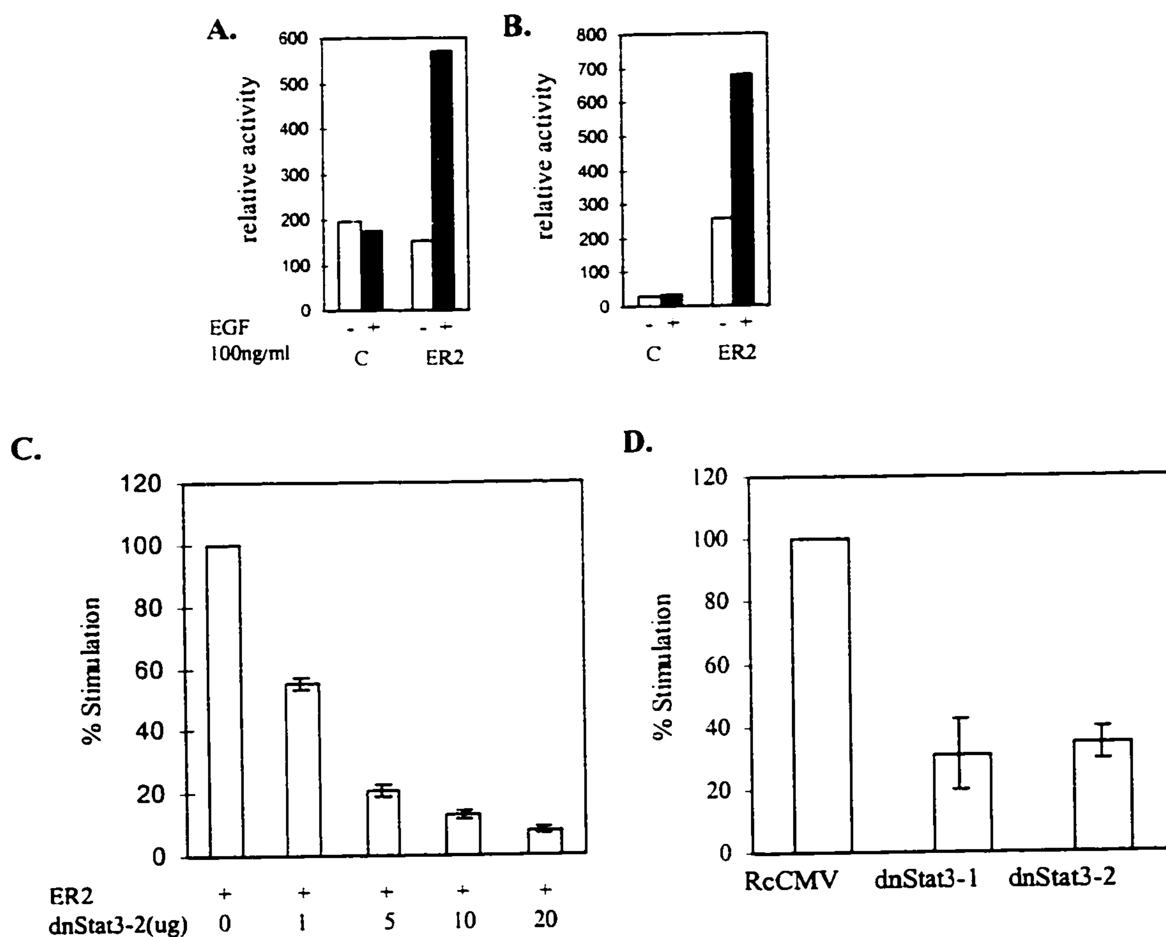
Fig. 4-7. **Inhibition of the ER2 and NM1 activated Stat3 transcriptional activity by dnStat3 mutants.** A, NIH 3T3 cells were transfected with 5 μg of the control or ER2 plasmid together with 5 μg of the reporter plasmid TKS3 (A) or Ly6E (B) and 0.4 μg of a β-gal expressing plasmid. They were subjected to serum starvation as described in Fig. 7 and subsequent EGF stimulation for 6 h. Cell lysates were prepared and aliquots subjected to assays for luciferase and β-galactosidase activities. The luciferase activity was normalized to the β-galactosidase activity. C, 3T3 cells were transfected with ER2 (5 μg) plus control plasmid (10 μg) or increasing amount of dnStat3-2 plasmid and analyzed similarly as in B. Average values of 3 experiments are shown. D, 3T3 cells were transfected with 5 μg of NM1 plus 25 μg of control or either of the dnStat3 mutant plasmids, 5 ug of the reporter TKS3 and 0.4 ug of the β-gal plasmid and subjected to luciferase and β-galactosidase assays as in B except without starvation and EGF stimulation. Average values of 3 experiments are shown.



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Chapter 5

General Discussion and Future Perspectives

In this study I have used site-specific mutants of the oncogenic Ros RPTK and dominant negative mutants of Stat3 to elucidate the signaling pathways required for Ros-mediated cell growth and transformation.

Cell transformation represents multiple changes of a variety of properties within a cell. These changes can be divided into morphogenic and mitogenic changes. Morphogenic transformation is characterized by the rearrangement of the cytoskeleton, altered interaction with the extracellular matrix (ECM) as well as changes in the ECM leading to altered cell shape. Mitogenic transformation includes increased proliferation rate, immortalization, growth in low serum, loss of contact growth inhibition, and acquisition of anchorage independent growth. Morphological transformation and enhanced proliferation are frequently linked events in tumorigenesis. In some cases these two aspects of cell transformation can be dissociated.

It is well established that an oncogenic RPTK is capable of eliciting multiple and parallel pathways to cause cell transformation. The Ras/MAP kinase pathway has been shown to be involved in mitogenic activation. The role of other pathways involving PLC-γ, PI3 kinase, and JAK/STATs is less clear. The functional role of cytoskeleton-mediated signaling is not very clearly defined either. Studies so far suggest that a combination of all these pathways would lead to full fledged cell

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transformation, but each pathway may be specifically required for a given parameter in cell transformation.

The mutants that display differential impairment of transformation are particularly useful for dissecting and identifying the specific pathways essential for certain properties of cell transformation. F419 and DI described in Chapter 3 fit this category in that they have lost the ability to grow in anchorage-independent conditions, but retain the ability to grow in anchorage-dependent conditions are allowed adhesion to surface substratum. These mutants allowed us to identify that IRS-1 and PI3 kinase as potential mediators of Ros-induce anchorage independent growth, but activation of those pathways alone is insufficient to support this transforming function. Cell-matrix and cell-cell interaction triggered signaling appears to be indispensable for the growth of normal cells. Therefore, both growth factor- and cytoskeleton-mediated signaling events are required for cell growth and furthermore, oncogenic transformation is able to relax or abrogate such requirements.

F419 and DI provided us with the tools to assess whether specific molecules in the IRS-1/PI3 kinase pathway are needed for anchorage-independent growth. For example, one could use the constitutively activated signaling molecule(s) downstream of PI3 kinase to determine if they are able to rescue the defects of the mutants.

The ability of Ros to activate certain components in the adhesion plaque- and /or cell-cell interaction-mediated signaling is impaired in F419 and DI mutants. To substantiate that the components which can no longer be activated by these Ros

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mutants are indeed important for Ros-mediated transformation, one could use the activated mutants of those cytoskeleton signaling pathway components to assess their ability to rescue the defects of the F419 and DI mutants. One obvious area to investigate in this regard is the role of Rho family GTP-binding proteins in Ros-induced anchorage-independent growth. For this purpose, constitutively activated or dominant negative mutants of Rho, Rac, Cdc42 and of various upstream and downstream components of their signaling pathways will be particularly instrumental. They can be used to rescue the mutants or to block wild type Ros promoted cell transformation.

To date, no mutants with a phenotype opposite to that of F419 and DI, namely retaining colony forming ability in soft agar but not mitogenicity in monolayer, have been identified. It would be interesting to see if a constitutively activated PI3 kinase can abrogate the requirement of the MAP kinase pathway in the growth of cells in monolayer. Similarly it would also be interesting to see if activated Rho, Rac, or Cdc42 mutants could abrogate either the IRS-1/PI3 kinase or MAP kinase pathways for cell growth in soft agar and monolayer.

Activation of STATs by normal and oncogenic RPTKs raises a question of the role of STATs in cell growth and transformation. Using a ts Ros mutant, an EGF-inducible EGFR-Ros chimera and a constitutively activated gag-IGFR fusion receptor, our study demonstrated that Ros and IGFR are able to activate STAT proteins in a cell type dependent manner and that activation of Stat3 is required for the transformation of NIH 3T3 cells by these RPTKs. The requirement for the activation of Stat3 signaling in transformation so far has been observed only for

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oncogenic PTKs including Src, Eyk, Ros, and IGFR. Since Stat3 activation is not required Ras transformation (Bromberg et al., 1998; Turkson et al., 1998), it would be interesting to identify which of the Ras-initiated signaling pathways is capable of substituting for the Stat3 function. It was shown that Ras is required for Src-induced cell transformation (Smiths et al., 1986). Therefore, both Stat3- and Ras-mediated signaling downstream of Src are essential for cell transformation by Src. Our results show that Stat3 function is required for the initiation and maintenance of transformation.

An obvious question is: what are the target gene(s) of Stat3 that are required for cell transformation by those RPTKs? The inducible EGFR-Ros chimera will be useful in attempting to identify the target gene(s) of Stat3. Differential display or subtractive cDNA cloning can be used to identify the potential target genes involved. Since F419 and DI are impaired in their ability to promote cell growth in soft agar, it would be interesting to see if these Ros mutant are able to activate Stat3. It would be intriguing to see if expression of certain Rho/Rac/Cdc42- or cytoskeleton-mediated signaling molecules are regulated by Stat3.

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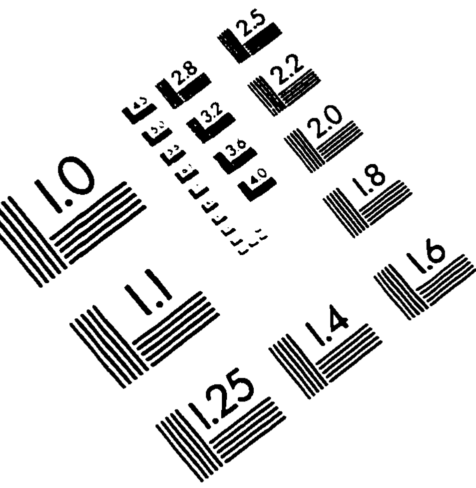
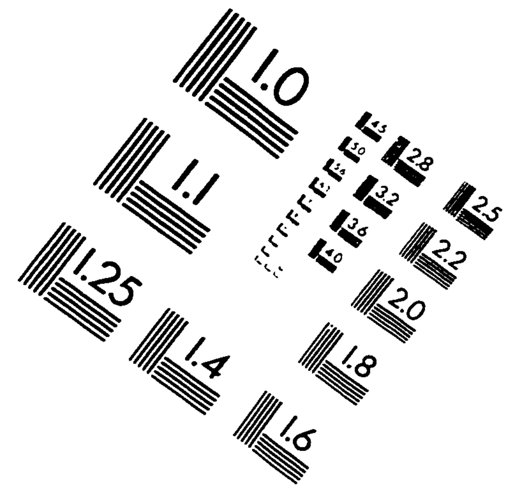
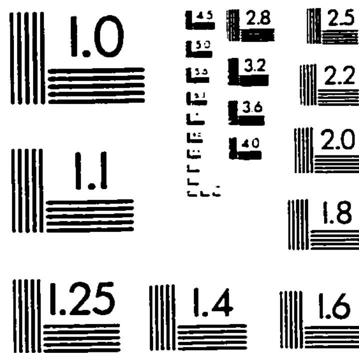
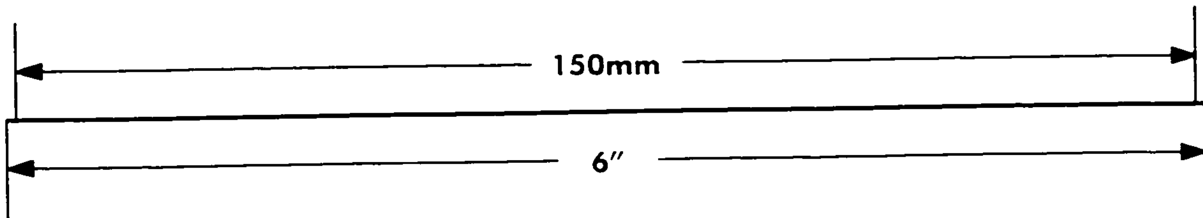
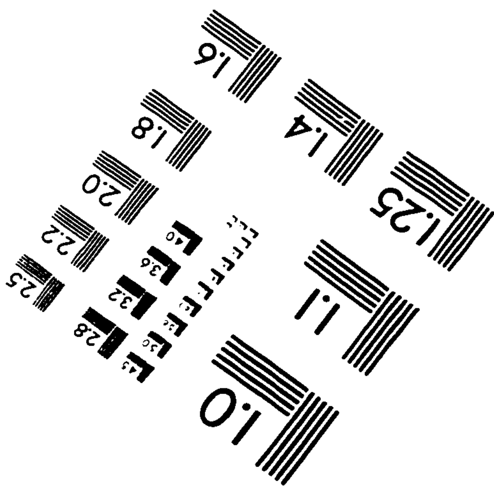
IMAGE EVALUATION TEST TARGET (QA-3)

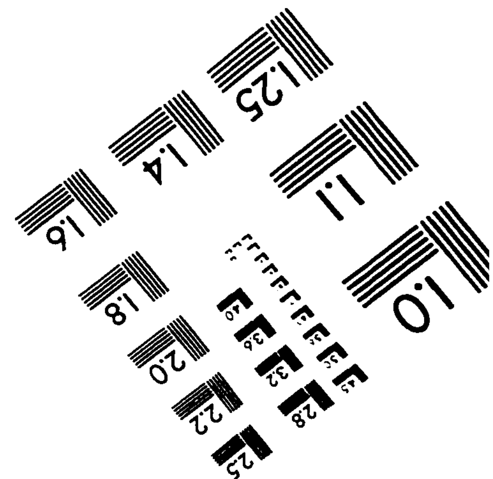












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