INTRODUCTION

Ligand stimulation of the EGFR leads to the activation of many signaling proteins, including Ras (Kamata and Feramisco, 1994). Ras is a small GTP-binding protein, which is activated by guanine nucleotide exchange factors (GNEFs), such as Sos, and is down regulated by GTPase activating proteins, such as Ras-GAP (reviewed by Joneson and Bar-Sagi, 1997). The mitogenic signaling pathway by which EGFR activates Ras is well established and involves the adapter proteins Shc and Grb2, and the GNEF Sos (Cai et al., 1990; Li et al., 1993; Lowenstein et al., 1992). The EGFR also can signal cell growth independent of ligand-stimulation, which has been correlated with malignant transformation (Maihle and Kung, 1988; Moscatello et al., 1996).

It is less clear, however, that ligand-independent oncogenic signaling by the EGFR involves the Ras signal transduction pathway. We previously have demonstrated in fibroblasts expressing a ligand-independent EGFR mutant, that there is no increase in urokinase plasminogen activator (uPA) transcription, even though EGF rapidly stimulates uPA transcription via a Ras-dependent pathway (Bell et al., 1993). Moreover, Ras is not activated in cells expressing a ligand-independent human EGFR mutant (Moscatello et al., 1996).

SUMMARY

Mutations in the ligand-binding domain of the epidermal growth factor receptor have been identified in several types of human cancers, including malignant gliomas. These mutations render signaling by this receptor to be constitutively ligand-independent. In fibroblasts transformed with ligand-independent epidermal growth factor receptor mutants, there is a correlation between the formation of a unique phosphotyrosine protein complex and oncogenic transformation. This phosphoprotein complex includes Grb2, Shc, Sos, tyrosine-phosphorylated form of caldesmon, and two, as yet, unidentified proteins. The presence of Grb2, Shc, and Sos in this complex implicates Ras in ligand-independent signaling by these oncogenic epidermal growth factor receptor mutants. We, therefore, have used retroviral co-infections of cultured primary fibroblasts to determine if Ras activation is required for phosphoprotein complex formation, stress fiber loss, or transformation. As predicted, expression of a dominant-negative Ras mutant (N17Ras) completely abrogates ligand-stimulated soft agar colony growth of primary fibroblasts. In contrast, N17Ras expression has no effect on v-ErbB mediated stress fiber disassembly, soft agar colony growth, or phosphoprotein complex assembly. In addition, our data suggest that ligand-dependent Ras activation may be suppressed by oncogenic v-ErbB expression. Together these observations suggest that oncogenic signaling by v-ErbB does not require Ras activation, and implicate an alternative signal transduction pathway in ligand-independent epidermal growth factor receptor oncogenic signaling.

Key words: v-ErbB, Dominant-negative Ras, Chicken embryo fibroblast, Stress fiber

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In addition, Shc and Grb2 are not stably associated with ligand-independent EGFR mutants, although Shc is constitutively tyrosine phosphorylated in cells expressing these mutants (McManus et al., 1997; Meyer et al., 1994). Together, these studies suggest that ligand-independent oncogenic signaling by mutant forms of the EGFR may not require Ras activation.

In this regard, our laboratory has established a model system in which to study ligand-independent EGFR oncogenic signaling. We previously have identified and characterized two constitutively active, ligand-independent EGFR mutants, i.e. E1v-ErbB and S3v-ErbB (Raines et al., 1988b). Interestingly, S3v-ErbB transforms primary fibroblasts in culture, whereas E1v-ErbB does not (Raines et al., 1988a). This system has been very useful in allowing us to compare differences between ligand-dependent vs ligand-independent EGFR biological endpoints in cultures of primary fibroblasts and tumor cells. Using this experimental system we have identified a novel tyrosine phosphorylated protein complex that is correlated with oncogenic transformation, and that is not seen under ligand-stimulated conditions (McManus et al., 1997). This complex contains Grb2, Shc, Sos, and a tyrosine phosphorylated form of caldesmon (McManus et al., 1997). The presence of Grb2,
Shc, and Sos in this complex suggests that Ras activation may be involved in ligand-independent EGFR oncogenic signaling. The studies in this report, therefore, were designed to specifically determine if Ras activation was required for ligand-independent EGFR signaling and fibroblast transformation.

In this study, we demonstrate that ligand-independent v-ErbB mediated primary fibroblast transformation occurs in a Ras-independent fashion. Specifically, we demonstrate by co-expression of either E1v-ErbB or S3v-ErbB with a dominant-negative mutant of Ras, i.e. N17Ras, that Ras activity is not required for v-ErbB mediated soft agar colony formation or for the formation of a novel transformation-associated phosphoprotein complex that we recently have identified (McManus et al., 1997). Moreover, the loss of stress fibers characteristic of S3v-ErbB transformed fibroblasts is not reversible by expression of N17Ras. These observations suggest that ligand-independent oncogenic signaling by EGFR may occur through an alternative, Ras-independent signaling pathway.

MATERIALS AND METHODS

Cells and viruses
Primary chicken embryo fibroblasts (CEF) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2% chick serum at 37°C. RCAN based retroviral vectors were used for co-infection studies (Hughes and Kosik, 1984). E1v-erbB and S3v-erbB were cloned into RCAN BH env subgroup A and dominant negative Ras (N17Ras) was cloned into RCAS BH env subgroup B (Aftab et al., 1997; Pelley et al., 1988).

Retroviral co-infection
Low passage CEF were infected with RCAS (B) - N17Ras, or with vector only, for three days. The cells were passaged 1:3 and were subsequently infected with RCAN (A) - E1v-erbB, S3v-erbB, or vector only in the presence of 2 mg/ml polybrene for four days as described previously (Aftab et al., 1997).

Analysis of v-ErbB and N17Ras expression
Co-infected CEF were plated at 1×10⁶ cells per 100 mm² plate and lysed in buffer containing 1% Triton X-100, 50 mM HEPES, pH 7.5, 5 mM EDTA, 50 mM NaCl, 10 mM NaPPi, 50 mM NaF, 0.5% deoxycholate, 4 mM DFP, 1 mM PMSF, 10 μg/ml aprotinin, and 1 mM NaOVoA. 30 μM of total protein were separated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in TBS, 0.1% Tween-20, 5% non-fat dry milk for 1 hour at room temperature and incubated with antibodies against the EGFR and Ras, diluted 1:500 in blocking buffer (Maiblle et al., 1988, and Transduction Labs, respectively) for 1 hour at room temperature. The membrane was washed three times for 5 minutes each in TBS, 0.1% Tween-20 at room temperature, incubated in anti-mouse IgG HRP and anti-rabbit IgG HRP (Amersham) at a dilution of 1:2000 in blocking buffer for 1 hour at room temperature, washed again as above, and incubated with chemiluminescence reagents for 1 minute, and exposed to x-ray film.

Stress fiber integrity assay
Co-expressing CEF were plated at 50,000 cells/coverslip in 24-well plates. After 24 hours, cells were fixed to the coverslips by incubation with 4% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.1% Triton X-100, and incubated with rhodamine-phalloidin for 30 minutes at room temperature. Nuclei were labeled with DAPI and stress fibers were visualized using an epifluorescence microscope.

pp75 phosphoprotein complex formation
Equal amounts of lysates (300 μg) from co-expressing CEF were immunoprecipitated with an anti-Shc antibody (1 μg/ml) for 1 hour at 4°C. Protein A/G agarose beads were added to the lysates for 30 minutes at 4°C and the immunoprecipitates were washed and prepared as described previously (McManus et al., 1997). Proteins were transferred to PVDF membrane, and western blotted.

Ras activity assay
The ratio of GTP to GDP bound Ras was determined as described previously (Johnson et al., 1996). Briefly, CEF and N17Ras expressing CEF were metabolically labeled with 1 mCi [32P]orthophosphoric acid/4 ml phosphate free medium for 4 hours at 37°C. Cells were lysed in Ras extraction buffer (50 mM Tris, pH 7.5, 20 mM MgCl2, 150 mM NaCl, 5% NP-40, 1 mM aprotinin, 1 mM PMSF) and immunoprecipitated with an antibody against Ras (Transduction Labs). GDP and GTP were eluted from the immunocomplexes in 1 M KH2PO4, pH 3.4, at 100°C for three minutes. Eluants were separated using thin layer chromatography, developed in 1 M KH2PO4, pH 4.5, and exposed to x-ray film.

Raf binding assay
E1v-erbB, S3v-erbB, and N17Ras expressing CEF were lysed in RIPA buffer (as previously described), immunoprecipitated with GST-Raf Ras binding domain (RBD) linked to glutathione beads, washed 3× with RIPA buffer, and then boiled in sample buffer (de Roolj and Bos, 1997). Proteins were then separated by SDS-PAGE, transferred to PVDF, and western blotted for Ras as described above.

RESULTS

Co-expression of v-ErbB and N17Ras in CEF
In these studies, we have utilized two ligand-independent mutants of the EGFR, i.e. E1v-ErbB and S3v-ErbB, both of which are deleted in the extracellular ligand-binding domain (Fig. 1). S3v-ErbB has an additional in-frame deletion within the intracellular domain, eliminating three potential autophosphorylation sites (Fig. 1, and Raines et al., 1988b).

Both mutant receptors have constitutive tyrosine kinase activity and are constitutively dimerized (Adelsman et al., 1996; Nair et al., 1992). However, unlike E1v-ErbB, S3v-ErbB transforms fibroblasts and endothelial cells, resulting in the development of fibrosarcomas and hemangiosarcomas in chickens (Raines et al., 1988a,b).

It is well established that the small GTP-binding protein Ras is important in mediating EGF’s diverse cellular effects (Mulcahy et al., 1985). In order to determine if Ras also plays a role in ligand-independent v-ErbB mediated fibroblast...
transformation, we have used a dominant negative mutant of Ras (N17Ras; Aftab et al., 1997; Feig and Cooper, 1988). Ras is active when bound to GTP and inactive when associated with GDP (Bourne et al., 1991). The N17Ras mutant has a single point mutation in the GTP-binding domain, which prevents GTP from associating with Ras, thereby locking this Ras mutant in an inactive conformation (Feig and Cooper, 1988).

In order to co-express v-ErbB and N17Ras, we have used the RCAN/RCAS helper-independent vectors, which were derived from avian leukosis virus (ALV; Hughes and Kosik, 1984). The availability of variant forms of these retroviral vectors, which express unique viral envelope subtypes, has allowed us to perform co-expression studies in primary chicken embryo fibroblasts (CEF). Once a virus of a distinct envelope subtype has infected a cell, the cell undergoes a process referred to as receptor interference. This phenomenon prevents that cell from being further infected by another virus of the same envelope subtype. Therefore, we have used two distinct envelope subtypes, A and B, to co-infect fibroblasts with retroviral vectors expressing two different genes i.e. v-ErbB and N17Ras.

Chicken embryo fibroblasts were infected with medium containing RCAS (B) expressing N17Ras, or vector only, for three days followed by infection with medium containing RCAN (A) expressing E1v-ErbB, S3v-ErbB or vector alone in the presence of polybrene for four days. Lysates were western blotted with (A) anti-ErbB and (B) anti-Ras antibodies. The N17Ras is not epitope tagged; therefore, Ras detected by the anti-Ras antibody in this western blot analysis represents both endogenous and introduced Ras and N17Ras expression levels. (C) Anti-Ras antibody western blot analysis using an antibody that does not recognize chicken Ras. Therefore, the bands detected in the ‘N17Ras +’ lanes represent the level of N17Ras expression.

**N17Ras inhibits Ras function in CEF**

To confirm that N17Ras is acting as a competitive inhibitor of endogenous Ras function in CEF, we infected CEF with N17Ras viral medium or RCAS (B) alone for three days. Cells were split and infected cells were co-infected with E1v-ErbB, S3v-ErbB or RCAN (A) alone in the presence of polybrene for four days. Lysates were western blotted with (A) anti-ErbB and (B) anti-Ras antibodies. The N17Ras is not epitope tagged; therefore, Ras detected by the anti-Ras antibody in this western blot analysis represents both endogenous and introduced Ras and N17Ras expression levels. (C) Anti-Ras antibody western blot analysis using an antibody that does not recognize chicken Ras. Therefore, the bands detected in the ‘N17Ras +’ lanes represent the level of N17Ras expression.

**Fig. 2. Co-expression of N17Ras and E1v-ErbB or S3v-ErbB in CEF.** CEF were infected with N17Ras viral medium or RCAS (B) alone for three days. Cells were split and infected cells were co-infected with E1v-ErbB, S3v-ErbB or RCAN (A) alone in the presence of polybrene for four days. Lysates were western blotted with (A) anti-ErbB and (B) anti-Ras antibodies. The N17Ras is not epitope tagged; therefore, Ras detected by the anti-Ras antibody in this western blot analysis represents both endogenous and introduced Ras and N17Ras expression levels. (C) Anti-Ras antibody western blot analysis using an antibody that does not recognize chicken Ras. Therefore, the bands detected in the ‘N17Ras +’ lanes represent the level of N17Ras expression.

**S3v-ErbB mediated stress fiber loss occurs in a Ras-independent fashion**

The loss of stress fiber integrity has been correlated with...
fibroblast transformation (Boschek et al., 1981). In previous studies, we have demonstrated that expression of the oncogenic S3v-ErbB mutant reduces the number and length of stress fibers observed in primary fibroblasts (McManus et al., 1997). Since many EGF-dependent signaling events associated with reorganization of the actin cytoskeleton are Ras-dependent, we tested for the involvement of Ras in ligand-independent oncogenic signaling by v-ErbB. CEF co-expressing N17Ras and S3v-ErbB or E1v-ErbB were plated on glass coverslips. Cells were fixed with 4% paraformaldehyde, permeabilized with Triton X-100, and labeled with rhodamine-phalloidin to visualize stress fibers. Fig. 4 demonstrates that expression of N17Ras does not interfere with the loss of actin stress fibers associated with S3v-ErbB fibroblast transformation. However, the expression of N17Ras does result in the loss of stress fiber integrity in primary fibroblasts grown in serum, an indication that N17Ras expression levels are sufficient to block endogenous Ras signaling. Interestingly, N17Ras co-expression in E1v-ErbB expressing fibroblasts also results in stress fiber disassembly (Fig. 4).

**S3v-ErbB mediated anchorage independent growth occurs in a Ras-independent fashion**

Since alterations in the actin cytoskeleton often are associated with changes in transformation potential, we examined the effect of N17Ras on S3v-ErbB fibroblast transformation. N17Ras was co-expressed with either S3v-ErbB, or with E1v-ErbB, in CEF, and cells were plated in soft agar. Three weeks later colonies were counted. The colony numbers observed in these experiments are displayed graphically in Fig. 5A as a ratio of counted colonies to cells plated. These results indicate that N17Ras does not interfere with the ability of S3v-ErbB to mediate anchorage-independent growth in primary fibroblasts. Moreover, N17Ras had no effect on the number of colonies formed in CEF or CEF expressing E1v-ErbB in the presence of serum (Fig. 5A). However, when fibroblasts were plated in agar under low serum conditions and stimulated with excess ligand (50 nM TGFα), they formed colonies in soft agar (Ginsburg and Vonderhaar, 1985). As predicted based on our understanding of ligand-dependent signaling mechanisms, N17Ras does inhibit ligand-stimulated anchorage-independent growth in these cells (Fig. 5B).

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**Fig. 4.** Stress fiber loss in CEF co-expressing N17Ras and E1v-ErbB or S3v-ErbB. Co-infected CEF were plated on glass coverslips, fixed with 4% PFA, permeabilized with 0.5% Triton X-100, and stained with rhodamine-phalloidin. Stress fibers were visualized with an epi-fluorescence microscope. This experiment was repeated three times, and three separate coverslips were analyzed for each experiment. The shown data shown are representative of the results in all three experiments.

**Fig. 5.** Soft agar colony formation in CEF co-expressing N17Ras and E1v-ErbB or S3v-ErbB. Co-infected cells were plated in 0.55% agar with a 0.65% and 0.45% agar sandwich and cultured for three weeks either in the presence of (A) 10% serum or (B) under low serum conditions (2% serum). Colonies were counted with the aid of a light microscope. This experiment was repeated three times, and similar ratios were obtained in each experiment. The results illustrated in A represent four independent plates per experimental point, and the error bars represent the standard error within each sample/trial, as calculated using Student’s t-test.
pp75 phosphoprotein complex formation is not inhibited by N17Ras

We previously have shown that fibroblasts expressing oncogenic S3v-ErbB exhibit a unique pp75 phosphoprotein complex in Triton X-100 cell lysates (McManus et al., 1997). In order to determine if Ras is required for the assembly of this complex we co-expressed N17Ras and E1v-ErbB or S3v-ErbB in primary fibroblasts, immunoprecipitated cell lysates with anti-Shc antibodies, and then western blotted with anti-phosphotyrosine. Fibroblasts co-expressing N17Ras and S3v-ErbB maintain the ability to form this complex (Fig. 6A), while CEF and CEF expressing E1v-ErbB still show no evidence of complex formation. These data suggest that pp75 phosphoprotein complex formation is either independent of Ras, or perhaps that complex assembly occurs upstream of Ras activation.

TGFα-stimulated Ras activation is down-regulated by oncogenic S3v-ErbB expression

To test directly for Ras activation in S3v-ErbB transformed fibroblasts, serum starved cells were stimulated with TGFα, lysed and precipitated with a GST-Raf Ras binding domain (RBD) fusion protein, followed by western blot analysis with anti-Ras. Both of these experiments were repeated three times with the result shown here illustrated the best representation of the observed results. Relative levels of GTP-bound Ras were determined by computer aided densitometry.

DISCUSSION

Extensive analysis of mitogenic signaling by the EGFR clearly has established the Ras-MAPK pathway as the major route of ligand-dependent EGFR signal transduction (reviewed by Hackel et al., 1999). Moreover, it has been inferred from these studies that ligand-independent signaling by oncogenic mutant forms of the receptor also utilize this signaling pathway (reviewed by Schlessinger, 1989). Recent studies in our laboratory, and also by others, however, suggest that signaling molecules and pathways unique to ligand-independent oncogenic signaling may exist (Bell et al., 1993; McManus et al., 1995, 1997; Meyer et al., 1994; Moscatello et al., 1996). We, therefore, have studied tyrosine phosphorylation events in v-ErbB transformed cells that may be specifically correlated with ligand-independent EGFR oncogenic signaling (Adelman et al., 1996; McManus et al., 1995, 1997). Such phosphorylation events do exist, and the results of these studies have implicated Ras as well as other proteins in ligand-independent oncogenic signaling.

To test directly for the involvement of Ras in mutant EGFR signaling we have examined three biological endpoints associated with ligand-independent EGFR mediated oncogenic transformation. We have found that Ras activation is not required for S3v-ErbB mediated stress fiber disassembly, anchorage-independent cell growth, or pp75 phosphoprotein complex formation. These observations suggest that Ras activation is not required for ligand-independent EGFR oncogenic signaling. Moreover, our studies demonstrate that the constitutively activated receptor mutant encoded by E1v-ErbB apparently mimics ligand-dependent EGFR signaling, exhibiting stress fiber integrity, anchorage-dependent growth, and constitutive Ras activation. When N17Ras is co-expressed in E1v-ErbB infected fibroblasts there is a reduction in the number of stress fibers and no change in anchorage-
independent cell growth, similar to our observations in ligand-stimulated CEF. In addition, Ras is constitutively activated in CEF expressing E1v-ErbB, suggesting that the constitutive tyrosine kinase activity of E1v-ErbB results in sustained activation of a signal transduction pathway that is very similar to the ligand-stimulated pathway. In spite of this prolonged and elevated level of tyrosine kinase activity and Ras activation, however, fibroblasts are not transformed by E1v-ErbB, and this mutant receptor is not tumorigenic (Raines et al., 1988b). Therefore, these results provide an example of constitutive activation of a mitogenic signaling pathway that does not lead to transformation.

Our data also suggest that ligand-dependent EGFR-mediated Ras activation is moderated by S3v-ErbB expression. This observation suggests that S3v-ErbB may have the ability to suppress ligand-dependent EGFR signaling through a mechanism that is not yet well characterized. In this regard, Carter et al. (1995) previously have demonstrated that E1v-ErbB has the ability to suppress v-ErbB mediated transformation of chicken embryo fibroblasts. Together, these studies suggest that these mutant receptors may be able to interact with each other or with distinct downstream signaling molecules. While we previously have demonstrated the ability of E1v-ErbB to interact with S3v-ErbB and as well as with the wild-type receptor in a ligand-independent fashion, the functional effect of these interactions, if any, on receptor signaling has not yet been determined (Adelsman et al., 1996).

A model summarizing the results of these studies and graphically illustrating our hypothesis is illustrated in Fig. 8.

The results presented in this study demonstrate that E1v-ErbB signaling in CEF is a constitutive version of ligand-dependent EGFR mitogenic signaling; E1v-ErbB is Ras-dependent (Fig. 8A,B). In contrast, we propose that S3v-ErbB signaling in CEF occurs through a unique, Ras-independent, signaling mechanism (Fig. 8C). We further hypothesize that the biological endpoints that we have focussed on in this study, i.e. pp75 phosphoprotein complex formation, stress fiber loss, and anchorage-independent growth are not just correlative, but, rather, are functionally related. Specifically, we hypothesize that pp75 phosphoprotein complex formation leads to the loss of stress fiber number and length, as well as to anchorage-independent cell growth.

The results of this study are consistent with two recent reports demonstrating that Ras activation is not required by the nonreceptor tyrosine kinase v-Src. Specifically, Aftab et al. (1997) have shown that v-Src mediated anchorage-independent growth can occur in the absence of Ras activation. Similarly, Oldham et al. (1998) have reported that Ras, but not Src transformation of RIE-1 cells, is inhibited by MEK and farnesyltransferase inhibitors. Therefore, while ligand-dependent, anchorage-independent cell proliferation clearly requires Ras activation, oncogenic signaling by both receptor and non-receptor tyrosine kinases does not.

These studies support the hypothesis that oncogenic transformation is not simply the result of constitutive mitogenic signaling. Conceptually, this is a relatively novel paradigm; it is, however, supported by the results of several other recent studies focused on both receptor and nonreceptor tyrosine kinases. For example, Hongo et al. (1996) have shown that regions of the insulin-like growth factor I receptor (IGF-IR) that are required for transformation are not required for mitogenic signaling. Inque et al. (1998) have used the expression of an exogenous gene, i.e., drs ('downregulated by v-Src'), to suppress v-Src mediated transformation of primary rat embryo fibroblasts. While drs expression has no effect on cell proliferation, it can significantly reduce v-Src mediated soft agar colony formation (Inque et al., 1998). And finally, McIntyre et al. (1998) have shown that the phosphatase inhibitors orthovanadate and pervanadate have the ability to enhance the tyrosine kinase activity of the EGFR and to increase cell proliferation under submitogenic conditions (EGF<500 pg/ml); treatment with these inhibitors, however, does not result in transformation as measured by soft agar colony growth. Together, the results presented in these studies provide strong evidence that oncogenic transformation is not simply mediated via constitutive activation of mitogenic signaling pathways, but rather occurs through a process that involves unique signaling molecules and pathways.

Fig. 8. Model of oncogenic transformation pathway vs mitogenic pathway. Several differences and similarities can be noted between ligand-independent oncogenic and ligand-dependent mitogenic EGFR signaling, leading to distinct biological endpoints. E1v-ErbB and ligand-stimulated EGFR associate with a Shc/Grb2/Sos complex to activate Ras, resulting in mitogenesis and stress fiber assembly. S3v-ErbB fibroblasts form a Shc/Grb2/Sos/Caldesmon/pp72/pp75 complex that does not associate with S3v-ErbB and does not activate Ras, resulting in transformation and a reduction in stress fiber number and length.
In this regard, we recently have shown that the novel, calcium-dependent, actin and myosin binding protein caldesmon becomes tyrosine phosphorylated in transformed fibroblasts, but not in ligand-stimulated fibroblasts (McManus et al., 1997). In addition, caldesmon can associate with Shc and Grb2, but based on the results presented here, the association of caldesmon with these proteins does not require Ras activation. Therefore, caldesmon, as well as other tyrosine phosphorylated proteins that associate with Shc and Grb2 in a transformation-dependent manner are likely candidates for proteins that may be involved in oncogenic signaling by mutant EGFRs. Two other proteins that also may be involved in Ras-independent transformation are phosphatidylinositol-3-kinase and Stat3; these proteins recently have been implicated in Ras-independent transformation by v-Src (Bromberg et al., 1998; Penuel and Martin, 1999; Turkson et al., 1998).

In conclusion, in this study we have shown that ligand-independent signaling by the EGFR may involve pathways not requiring Ras activation. Our evidence demonstrates that in primary fibroblasts a dominant negative mutant of Ras does not interfere with ligand-independent EGFR mediatied stress fiber loss, anchorage-independent cell growth, nor with formation of the transformation-associated phosphoprotein complex, pp75. However, N17Ras does interfere with the ligand-mediated EGFR signaling of stress fiber loss and anchorage-independent cell growth. Together, these results suggest that distinct signaling pathways may exist that are specifically involved in ligand-independent oncogenic EGFR signaling. Support for the hypothesis that mitogenic and oncogenic signaling pathways are qualitatively distinct has profound clinical implications for future drug design and potential targeting of novel chemotherapeutics. Therefore, studies further delineating these oncogenic signaling pathways, and the extent of their overlap with normal mitogenic signaling events are clearly warranted.

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