Activation of Rho Is Required for Ligand-independent Oncogenic Signaling by a Mutant Epidermal Growth Factor Receptor*

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Mutations in the epidermal growth factor receptor have been identified in several human tumor types, including gliomas. These receptor mutants have deletions in their extracellular ligand-binding domains and are, therefore, no longer regulated by ligand, resulting in constitutive activation of the receptor kinase. These mutants have been proposed to transduce oncogenic signals via ligand-independent signaling pathways. Avian viral homologues of these oncogenic epidermal growth factor receptors exhibit structurally homologous deletions and form tumors in chickens. One such mutant, S3v-ErbB, transforms fibroblasts in vitro, and transformation has been correlated with the formation of a novel tyrosine phosphoprotein complex. V-ErbB-mediated complex formation and transformation have been shown to occur independently of Ras activation. The major aims of this study are to further characterize this ligand-independent v-ErbB oncogenic signaling pathway. Here we show that both v-ErbB-mediated phosphoprotein complex formation and transformation are inhibited by a dominant negative mutant of Rho. This inhibition is specific for dominant negative Rho; dominant negative mutants of Rac and Cdc42 have no effect on transformation or on tyrosine phosphorylation of the phosphoprotein complex. Based on these observations, we propose that S3v-ErbB stimulates a Rho-dependent tyrosine kinase, resulting in complex formation and ultimately oncogenic transformation.

An avian viral mutant of the EGFR, S3v-ErbB, transforms fibroblasts in vitro, and in vivo expression results in the development of fibrosarcomas and hemangiosarcomas (1). S3v-ErbB transformation of fibroblasts results in the loss of anchorage-dependent cell growth. This growth pattern has been correlated with specific cytoskeletal changes in v-ErbB-transformed fibroblasts (2, 38). Specifically, stress fibers are disassembled, and myosin light chain kinase activity is reduced (2, 38). In addition, two cytoskeletal associated proteins, i.e. caldesmon and p21-activated kinase, are uniquely tyrosine-phosphorylated in S3v-ErbB-transformed cells (2, 4, 38). Interestingly, Ras activation, a key aspect of ligand-dependent EGFR mitogenic signaling, is not required for S3v-ErbB-mediated transformation or for these cytoskeletal changes (5). Together, these studies suggest that ligand-independent oncogenic signaling occurs through a pathway that is distinct from the well characterized ligand-dependent mitogenic signaling pathway of EGFR.

Rho, Rac, and Cdc42 are small GTP-binding proteins important in the dynamic reorganization of the cytoskeleton of the cell. Rho is a crucial regulator of such cytoskeletal events and functions by controlling stress fiber assembly and focal adhesion formation (6). In contrast, Rac is important for membrane ruffling, lamellipodia formation, and focal complex formation (7–10), whereas Cdc42 is required for filopodia formation (11). The coordinated interplay of the activities of these proteins results in the control of complex biological events such as cell movement, cell-cell communication, cell growth, and cell death. When the regulatory activity of Rho, Rac, or Cdc42 is altered, dramatic changes in the actin-based cytoskeleton, as well as deregulation of these biological functions, occurs, resulting in pathologic effects such as malignant transformation.

Because we previously have shown that Ras activity is not required for S3v-ErbB transformation, in this study we have examined the role of Rho, Rac, and Cdc42 in S3v-ErbB-mediated primary fibroblast transformation. Our results demonstrate that Rho activity is essential for S3v-ErbB-mediated fibroblast transformation, whereas the loss of Rac or Cdc42 activity does not inhibit transformation. In support of this observation, expression of a dominant negative RhoA mutant inhibits the formation of a previously described transformation-associated tyrosine phosphoprotein complex. These data implicate Rho, as well as downstream mediators of Rho signaling, as important mediators of S3v-ErbB-mediated complex formation and transformation in fibroblasts. These results have important implications for ligand-independent signaling by receptor tyrosine kinases and may be particularly relevant to the development of novel therapeutics capable of uniquely targeting oncogenic signaling pathways.

MATERIALS AND METHODS

Cells and Viruses—Primary chicken embryo fibroblasts (CEF) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2% chick serum at 37 °C. RCAN-based retrovectors were used for coinfection studies (12). E1v-ErbB (a nontransforming, but constitutively active, form of v-ErbB) and S3v-ErbB were cloned into RCAN BH env subgroup A, and dominant neg-
ative Rho (N19RhoA, a gift from A. Hall), dominant negative Rac (N17Rac, a gift from A. Ridley), and dominant negative Cdc42 (N17Cdc42, a gift from M. Symons) were cloned into RCAS BH env subgroup B.

Retransfction Coinfection—Low passage CEF were infected with RCAS (B)-N19RhoA, N17Rac, N17Cdc42, or vector only for 3 days. Cells were passaged 1:3 and were subsequently infected with RCAN (A)-E1v-ErbB, S3v-ErbB, or vector in the presence of 2 mg/ml polybrene for 4 days as described previously (5).

Western Blot Analysis—Coinfected CEF were plated at 1 x 10⁶ cells per 100-mm² plate and were lysed in buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.5), 5 mM EDTA, 50 mM NaCl, 10 mM NaF, 50 mM NaF, 0.5% deoxycholate, 4 mM diisopropyl fluorophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate. Ten micrograms of total protein were separated by SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane. The membrane was blocked in Tris-buffered saline, 0.1% Tween dry milk for 1 h at room temperature and incubated with antibodies (diluted 1:500) against EGFR (13), Rho (Santa Cruz Biotechnology), or Myc tag (Upstate Biotechnology) for 1 h at room temperature. Membranes were washed three times for 5 min each in Tris-buffered saline, 0.1% Tween 20 at room temperature, followed by incubation in anti-mouse IgG horse-radish peroxidase and anti-rabbit IgG horseradish peroxidase (Am-ershaw Pharmacia Biotech) at a dilution of 1:2000 in blocking buffer for 1 h at room temperature. Membranes were washed again as above and incubated with chemiluminescence reagents (Pierce) for 5 min, followed by analysis using a lumia-imager (Gel Expert, Nucleotech).

pp75 Phosphoprotein Complex Formation—Equal amounts of lysates (500 μg) from CEF coexpressing v-ErbB and DNRho, DNRac, or DNCdc42 were immunoprecipitated with an anti-Shc antibody (1 μg/ml) for 1 h at 4 °C. Protein AG-agarose beads were added to the lysates for 30 min at 4 °C, and immunoprecipitates were then washed and analyzed as described previously.

Soft Agar Colony Formation—CEF coexpressing v-ErbB and DNRho, DNRac, and DNCdc42 were plated as previously described (14). Plates were supplemented with a few drops of media every 3 days, for a total culture time of 3 weeks. Colonies (>25 cells/colony) were counted by bright field light microscopy. Coinfected cultures of cells were maintained throughout the soft agar colony incubation. The average number of colonies for S3v-ErbB CEF was normalized to 100 for comparison between replicates. Each experiment consisted of four replicates and was repeated four times.

Rho Activity Assay—The ratio of GTP- to GDP-bound Rho was determined as described previously (15). Briefly, CEF and N19RhoA expressing CEF were serum-starved for 24 h, metabolically labeled with 1 μCi of [35S]pPI phospholinophoric acid/μl of phosphate-free media for 4 h at 37 °C, and stimulated with 50 mM transforming growth factor-α (TGαF). Cells were lysed in Rho extraction buffer (50 mM Tris (pH 7.5), 20 mM MgCl₂, 150 mM NaCl, 5% Nonidet P-40, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride) and immunoprecipitated with an antibody against Rho (Santa Cruz Biotechnology). GDP and GTP were eluted from the immunocomplexes in 1 M KH₂PO₄ (pH 3.4) at 100 °C for 3 min. Eluates were separated using thin-layer chromatography developed in 1 M KH₂PO₄ (pH 4.5), and chromatograms were exposed to x-ray film.

RESULTS

Coexpression of S3v-ErbB with N19Rho, N17Rac, or N17Cdc42—Dominant negative mutants of Rho (N19RhoA), Rac (N17Rac), or Cdc42 (N17Cdc42) were subcloned into the replication-competent avian retrovirus, RCAS, and these recombinant viruses were used to infect primary CEF. A coinfecion strategy for CEF has previously been developed such that sequential infection of CEF with two distinct envelope subtypes of RCAS allows for coexpression of two different genes of interest. Fig. 1 illustrates representative Western blot data for colonies of fibroblasts coexpressing a nontransforming but constitutively active form of v-ErbB, E1v-ErbB, the transforming S3v-ErbB and each of these dominant negative mutants. The expression of the v-ErbB mutants does not alter the endogenous expression levels of Rac, Cdc42, or Rho (Fig. 1).

N19RhoA Blocks the Formation of the Tyrosine Phosphoprotein Complex—CEF expressing S3v-ErbB form a tyrosinestyle phosphoprotein complex that coprecipitates with anti-Shc antibodies; this complex includes the proteins Grb2, Shc, and caldesmon (2). CEF lysates from cells coinfected with dominant negative mutants of Rho, Rac, or Cdc42 were immunoprecipitated with an anti-Shc antibody. Immunoprecipitates were resolved by SDS-PAGE followed by Western blot analysis with anti-phosphotyrosine antibodies (4G10, Upstate Biotechnology).

Fig. 2 shows that in the presence of vector alone, N17Rac, or N17Cdc42, the formation of this complex is not inhibited. In contrast, in CEF coexpressing N19RhoA and S3v-ErbB the formation of this complex is completely inhibited, and the tyrosine phosphorylation of Shc also is significantly reduced although the levels of immunoprecipitated Shc remained constant (data not shown and Fig. 2).

N19RhoA Inhibits S3v-ErbB-mediated Soft Agar Colony Formation—S3v-ErbB-expressing CEF form colonies in soft agar, a characteristic consistent with the ability of transformed cells to grow in the absence of cell adhesion. To determine whether N19RhoA, N17Rac, or N17Cdc42 expression would affect S3v-ErbB-induced anchorage-independent cell growth, coinfected CEF were plated in soft agar as previously described. After 3 weeks of growth, colonies were counted, and the number of colonies found in S3v-ErbB CEF was defined as 100% colony formation. As shown in Fig. 3, N19RhoA completely inhibits S3v-ErbB-mediated soft agar colony formation. Interestingly, N17Rac and N17Cdc42 also modestly reduce the number of soft colonies.
agar colonies formed, but never to a statistically significant extent. The reduction in soft agar colony formation is not the result of a reduction or inhibition of cell growth. Cultures of coinfected cells grown in parallel with the soft agar plates did not exhibit altered growth rates when compared with control cells (data not shown).

Rho, but Not Rac, Is Constitutively Active in S3v-ErbB-transformed Cells—To determine whether S3v-ErbB constitutive tyrosine kinase activity results in constitutive activation of Rho, CEF expressing E1v-ErbB or S3v-ErbB were plated, followed by serum starvation for 24 h. Cells were then stimulated with 50 nM TGFα and metabolically labeled with 32P. Cell lysates were immunoprecipitated with anti-Rho antibodies, and the associated nucleotides were separated by thin-layer chromatography. The resultant chromatogram was analyzed using computer-aided densitometry. Fig. 4 illustrates a graphical representation of these data. These results indicate that the level of Rho activity in CEF expressing S3v-ErbB is comparable with the level of Rho activation seen in a-stimulated cells. In contrast, cells expressing E1v-ErbB exhibit Rho activity levels comparable with the basal levels observed in serum-starved cells. These results suggest that constitutive tyrosine kinase activity (e.g. of the nontransforming E1v-ErbB mutant) is not sufficient to stimulate constitutive Rho activity. In addition, S3v-ErbB-expressing fibroblasts do not exhibit constitutive activation of Rac (data not shown). Taken together, these data suggest a unique role for Rho in S3v-ErbB-transformed fibroblasts.

**DISCUSSION**

S3v-ErbB has been shown to mediate fibroblast transformation through a Ras-independent signaling mechanism (5). In this study we have performed experiments to identify components of this ligand-independent oncogenic signaling pathway that might connect S3v-ErbB to its tyrosine-phosphorylated downstream mediators, such as caldesmon and p21-activated kinase (2–4). Our results suggest the existence of a ligand-independent oncogenic signaling pathway from S3v-ErbB to the small GTP-binding protein Rho. The activation of Rho apparently leads to the stimulation of at least one tyrosine kinase that phosphorylates components of a previously described transformation-associated phosphoprotein complex.

Here, we demonstrate that Rho activity is required for S3v-ErbB-mediated phosphoprotein complex formation. Of particular interest, Rho activation is required for the tyrosine phosphorylation of several components of this signaling complex including caldesmon and p21-activated kinase (data not shown). Careful observation also reveals a decrease in Shc tyrosine phosphorylation; Shc tyrosine phosphorylation is characteristically elevated in S3v-ErbB-transformed fibroblasts. These data, therefore, suggest the involvement of at least one tyrosine kinase downstream of Rho activation.

In this regard, several known tyrosine kinases have been implicated downstream of Rho activation, as well as downstream of cytoskeletal changes within the cell, particularly in transformed cells. One such tyrosine kinase, Src, has been shown to function downstream of Rho via several mechanisms; Src-phosphorylated proteins have been shown to be tyrosine-phosphorylated downstream of Rho, and Src and Rho share a common mediator linking them in this signaling pathway (16, 17). Specifically, Src has been shown to phosphorylate the cytoskeletal proteins focal adhesion kinase (Fak) and p130cas (16, 18–20). More significantly, recent investigations have linked Rho and Src activation through the Rho effector mDia (17). The Diaphanous-related formins, namely mDia1 and mDia2, have been shown to act as effectors for Rho activation and to link the tyrosine kinase activity of Src to Rho activation by acting as scaffolding or bridging proteins (17). Together, these results suggest that Src may be the critical kinase involved in tyrosine phosphorylation of components of our transformation-associated phosphotyrosine protein complex, and future studies will be directed toward testing this hypothesis.

Fak is another tyrosine kinase that has been shown to be required for the cytoskeletal changes observed as a consequence of Rho activation. Specifically, Fak has been shown to be a downstream Rho effector. Stimuli that activate Rho, e.g. sphingolipids, also result in the tyrosine phosphorylation of Fak (21–23). More directly, Fak has been shown to be auto-phosphorylated as well as phosphorylated by other kinases, e.g. by Src family kinases, in a Rho-dependent fashion (24, 25). Of additional interest relevant to this study, EGF can indirectly stimulate Rho activation, leading to increased activity of Src.
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as well as to the tyrosine phosphorylation of Fak. These observations suggest that Fak also might be an excellent candidate for the kinase responsible for phosphorylation of our Shc-based phosphoprotein complex (26).

Evidence for yet a third candidate tyrosine kinase, i.e., Abl, although less compelling, is still worth a mention. Abl has been shown to be critical for several of the actin-based cytoskeletal changes correlated with transformation. For example, in Ber-Abl-transformed cells, it is the kinase activity of Abl that is critical for the cytoskeletal changes resulting in morphological transformation (27). The specific cytoskeletal alterations correlated with v-Abl transformation of fibroblasts require Abl-binding partners and substrates, such as ArgB2 and c-Cbl (28, 29). Moreover, Abl itself contains an F-actin-binding domain that competes with gelsolin and is critical for F-actin bundling (30). These observations suggest that Abl also may be a tyrosine kinase downstream of Rho activation, providing yet another testable candidate for the kinase responsible for the tyrosine phosphorylation of the phosphoprotein complex observed in S3v-ErbB-transformed fibroblasts.

This study also demonstrates that Rho is constitutively activated in S3v-ErbB-transformed fibroblasts, suggesting that a constitutive signal from S3v-ErbB to Rho may be critical for expression of the phenotypes observed. In contrast, E1v-ErbB does not constitutively activate Rho, suggesting that more than just a constitutively active tyrosine kinase leads to Rho activation. These observations also demonstrate that although many pathways may be activated by constitutively active S3v-ErbB, only the pathway to Rho has escaped normal regulation by governing proteins, such as the GTPase-activating proteins. In this regard, studying aspects of regulating Rho activity in v-ErbB-transformed cells may be informative. Recently, the guanine nucleotide exchange factor Vav has been implicated in Rho activation via EGF stimulation of the EGFR (31). Specifically, tyrosine phosphorylation of Vav3 by the EGFR in response to EGF stimulation activates Rho (32). In contrast, other activators of Rho, i.e. Ost, Lbc, and Trio have not been shown to be regulated by EGFR. Additional studies will be needed to determine whether Vav family members are the missing link between S3v-ErbB onecogenic signaling and Rho activation.

Even though we demonstrate here that Rho activity is required for transformation of fibroblasts by S3v-ErbB, it remains a point of controversy whether expression of a constitutively active Rho is sufficient for fibroblast transformation. It has been shown, however, that constitutive activation of Rho can transform fibroblasts in the presence of constitutively active Raf (Raf-CAXX) (33). The authors of this particular study conclude that these two pathways are required for transformation of fibroblasts via Rho; one pathway is independent of Raf activity and regulates stress fiber dynamics, and a second pathway is dependent on Raf activity (33). In addition, Rho and Rac also have been shown to cooperate in the transformation of fibroblasts (34). Rho also has been implicated in the regulation of the EGFR endosomal trafficking, such that the EGFR is delayed from moving past the late endosome when Rho is constitutively active (35). The regulation of S3v-ErbB trafficking may perhaps provide an alternate mechanism for the contribution of Rho in S3v-ErbB-mediated transformation. Furthermore, constitutive activation of proteins shown to regulate the activity of Rho, such as the guanine nucleotide exchange factor Lbc, also have the ability to induce anchorage-independent cell growth (36).

In contrast, several investigators have demonstrated that the overexpression of a constitutively activated mutant Rho directly leads to the formation of tumors in nude mice (3, 37). Additional studies will be required to determine the basis for these discrepancies and to more precisely define the relationship between the role of Rho in regulating stress fiber dynamics versus transformation.

In conclusion, we believe that Rho plays a critical role in the transformation of fibroblasts by S3v-ErbB. We further hypothesize that Rho is constitutively activated by signals downstream of S3v-ErbB and that the normal regulatory proteins governing Rho activation are either outnumbered or disregulated themselves in S3v-ErbB-transformed fibroblasts. This constitutive activation of Rho leads to the constitutive activation of a nonreceptor tyrosine kinase, such as Src, Fak, or Abl, resulting in the tyrosine phosphorylation of a signaling complex of proteins including Shc, caldesmon, and p21-activated kinase. These tyrosine phosphorylation events not only result in cytoskeletal changes such as stress fiber disassembly, but also provide signals that lead to the transformation of fibroblasts. Together, these observations support the working hypothesis that ligand-independent oncogenic signaling by S3v-ErbB is distinct from the ligand-dependent mitogenic pathway that regulates normal cell division. Further delineation of this putative oncogenic signaling pathway may reveal new therapeutic targets unique to transformed cells.

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