An Oncogenic Epidermal Growth Factor Receptor Signals via a p21-activated Kinase-Caldesmon-Myosin Phosphotyrosine Complex*

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Many ligand-independent receptor tyrosine kinases are tumorigenic. The biochemical signals that mediate ligand-independent transformation of cells by these transmembrane receptors are poorly defined. In this report, we demonstrate that a constitutively activated mutant epidermal growth factor receptor (v-ErbB) induces the formation of a transformation-specific signaling module that complexes with myosin II. The components of this signaling complex include the signal adapter proteins Shc, Grb2, and Nck, and tyrosine-phosphorylated forms of p21-activated kinase (Pak), caldesmon, and myosin light chain kinase. Transformation-specific, tyrosine phosphorylation of Pak enhances the catalytic activity of this serine/threonine kinase. Furthermore, the tyrosine phosphorylation of Pak is Rho-, but not Ras-, Rac-, or Cdc42-dependent. These results demonstrate that a ligand-independent epidermal growth factor receptor mutant can transduce oncogenic signals that are distinct from ligand-dependent, mitogenic signals. In addition, these data provide evidence for the coupling of oncogenic receptor tyrosine kinases with the actomyosin molecular motor. This myosin-associated signaling module may mediate some of the biochemical changes of myosin found in v-ErbB-transformed fibroblasts, thereby contributing to the regulation of the mechanical forces governing cellular adhesion, cytoskeletal tension, and, hence, anchorage-independent cell growth.

Mutations in receptor tyrosine kinases may result in ligand-independent, constitutive kinase activity and tumorigenesis (1–3). The biochemical signals that mediate transformation by these ligand-independent receptors are poorly defined, including the signals that influence the reorganization of the actomyosin-based cytoskeleton and anchorage-independent cell growth. Two fundamentally distinct mechanisms of oncogenic signaling may be at work: (i) the persistent stimulation of mitogenic signaling pathways by the kinase, or (ii) the stimulation of novel, transformation-specific, signaling networks arising from altered substrate specificity of the kinase. To distinguish between these alternative models, we have studied fibroblast transformation by v-ErbB, a ligand-independent, oncogenic homolog of the human epidermal growth factor receptor (EGFR).1

Our previous studies provided compelling evidence that there are qualitatively distinct signaling pathways associated with v-ErbB-mediated transformation compared with ligand-dependent, mitogenic signaling. Specifically, we identified a novel, transformation-associated phosphotyrosine protein complex (4). This Src homologous collagen protein (Shc)-phosphotyrosine multiprotein complex forms in fibroblasts expressing a transforming v-ErbB protein, but not in cells stimulated with ligand, or in cells expressing a non-transforming v-ErbB mutant with a constitutively active kinase domain (4). In addition to the signal adapter protein Shc, this protein complex includes growth factor receptor binding protein-2 (Grb2), a 78-kilodalton (kDa) tyrosine-phosphorylated form of the actin-myosin regulatory protein caldesmon, and unidentified tyrosine-phosphorylated proteins migrating at 210, 68–75, and 47 kDa (refer to Fig. 1A, lane S3; see also Ref. 5).

Although Shc commonly transduces signals through Ras-regulated pathways (6), we recently showed that the formation of the transformation-specific Shc-phosphoprotein complex is Ras-independent (7). In addition, anchorage-independent cell growth of v-ErbB-transformed fibroblasts is not inhibited by the expression of dominant-negative Ras (7). More recently, we have shown that the formation of this transformation-specific Shc-phosphoprotein signaling module is dependent on the activation of the guanosine triphosphatase Rho A and is associated with actin stress fiber disassembly and anchorage-independent cell growth.2 Our current studies demonstrate that a tyrosine-phosphorylated form of the serine/threonine kinase p21-activated kinase (Pak) is a component of this signaling complex. Specifically, tyrosine-phosphorylated Pak associates with Shc, Nck, caldesmon, myosin, and a 210-kDa isoform of myosin light chain kinase. Furthermore, we show that the transformation-specific tyrosine phosphorylation of Pak activates its catalytic domain. Taken together, the transformation-specific tyrosine phosphorylation events and the formation of

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1 The abbreviations used are: EGFR, epidermal growth factor receptor; CEF, chicken embryo fibroblast; Grb2, growth factor receptor binding protein-2; MLC, myosin light chain; MLCK, myosin light chain kinase; Pak, p21-activated kinase; Shc, Src homologous, collagen homologous protein; v-ErbB, viral erythroblastosis oncprotein; IP, immunoprecipitation; GST, glutathione S-transferase; DN, dominant negative; PAGE, polyacrylamide gel electrophoresis; TGFRα, transforming growth factor-α; MOPS, 4-norbornepropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; SHP, SH2-domain tyrosine phosphatase.

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this phosphotyrosine protein complex demonstrates that oncogenic signaling is distinct from mitogenic signaling, and favors an altered substrate model for ligand-independent signaling by the epidermal growth factor receptor.

**EXPERIMENTAL PROCEDURES**

**EGFR Oncogenes and Retrosilviral Infection**—These studies use two v-erbB-encoded epidermal growth factor receptors, designated E1-v-ErbB and S3-v-ErbB. E1-v-ErbB has a truncated extracellular ligand-binding domain resulting in a constitutively active tyrosine kinase domain, and transforms erythroblasts but not fibroblasts. S3-v-ErbB has the same ligand-binding domain truncation and constitutively active kinase domain as E1-erbB and, in addition, has a 139-amino acid in-frame deletion in its carboxyl-terminal end. Due to this COOH-terminal deletion, S3-erbB has lost its leukemogenic potential, but has gained the ability to transform fibroblasts in vitro and to cause avian fibrosarcomas and hemangiosarcomas in vivo (8). v-erbB cDNAs encoding these mutant oncoproteins were cloned into avian leukemia virus-derived retroviral vectors as described previously (9). Primary cultures of chick embryo fibroblasts were infected with these v-erbB helper-independent retroviruses, cultured, and assayed for protein expression of E1-v-ErbB and S3-v-ErbB by immunoblotting as described previously (4). Protein expression levels of these two v-erbB oncoproteins were consistently equivalent (Fig. 4).

**Dominant Negative Rho Constructs and Retrosilviral Co-infection**—Dominant negative (DN) RhoA (T19N) was a gift from Anne Ridley (Ludwig Institute for Cancer Research, London, United Kingdom) and was subcloned into RCAS envelope subtype B retroviral vectors. S3-erbB and E1-erbB were subcloned into RCAN envelope subtype A retroviral vectors (9). Low passage chick embryo fibroblasts were infected with DN RhoA-containing retroviral vectors, then infected with v-erbB-containing retroviral vectors in the presence of Polybrene (10).

**GST-Grb2 Affinity Chromatography—Glutathione S-transferase fusion proteins of Grb2 were produced and purified as described previously (5). The lysate from S3-v-ErbB-transformed CEF (500 mg) was passed over a column of GST-Grb2-glutathione-agarose beads equilibrated with 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, then eluted with 100 mM phosphotyrosine in Tris-buffered saline, pH 7.4. Fractions were collected and monitored for phosphotyrosine content by anti-phosphotyrosine immunoblotting. Phosphotyrosine-containing fractions were pooled, concentrated, and desalted in Centricron-10 centrifuge tubes (Amicon, Beverly, MA); they were then lyophilized and solubilized in 2× Laemmli sample buffer. Proteins were separated by SDS-PAGE, and immunoprecipitated and analyzed by immunoblot analysis as described previously (4).

**Immunoprecipitation and Immunoblotting Assays—**These methods were performed as described previously (5). Antibodies for immunoprecipitation and immunoblotting included monoclonal anti-phosphotyrosine IgG (4G10) and polyclonal a-Shc (Upstate Biotechnology, Inc.), polyclonal a-MLCK (Covance/Babco), monoclonal a-MLCK and monoclonal a-MLC IgM (Sigma), polyclonal a-Pak (C-19) and monoclonal a-Rho, monoclonal a-N-Ras and monoclonal a-calmodulin (Transduction Laboratories), polyclonal antibody specific for MLCK-210 (gift from D. M. Watterson, Northwestern University, Chicago, IL), polyclonal anti-phosphoserine-19 MLC phosphospecific antibody (11), and polyclonal anti-erbB (4).

**Myosin Light Chain Kinase Assay—**For the MLCK assay, cells were lysed in a Triton X-100-based lysis buffer (4). Lysates (500 μg of protein) were immunoprecipitated in 50 mM MOPS, pH 7.0, 10 mM MgCl2 using a-MLCK antibodies (Sigma), followed by the addition of protein A/G-agarose beads (Pierce). Beads were washed in 50 mM MOPS, pH 7.0, 10 mM MgCl2, and resuspended in 50 μl of 50 mM MOPS, pH 7.0, 10 mM MgCl2, 0.3 mM CaCl2-2H2O, 1 mM calmodulin, and 2 mM dithiothreitol (12, 13). Purified rat aorta smooth muscle regulatory MLC (gift of P. de Lanerolle, University of Illinois, Chicago, IL) was added to a final concentration of 10 μM, and the reaction mixture was rocked at room temperature for 5 min. The reaction was initiated by the addition of 75 μM (final concentration) adenosine 5′-triphosphate (ATP) and 10 μCi of [γ−32P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences). The reaction tubes were rocked at room temperature, and aliquots of the supernatant were removed at various time points over 40 min. The assay was terminated by the addition of 2× SDS-PAGE sample buffer and heating at 100 °C for 3 min. Proteins were separated by 12.5% acrylamide SDS-PAGE. Incorporation of 32P into regulatory MLC was detected by autoradiography. The regulatory MLC bands were excised and counted in a Beckman LS 5000TD liquid scintillation system. For the analysis of the phosphorylation of MLCK-210, the immunoprecipitated MLCK bound to protein A/G-agarose beads (remaining at the conclusion of the MLCK assay described above) was solubilized in Laemmli sample buffer, heated, and separated by 7.5% acrylamide SDS-PAGE. Incorporation of 32P into MLCK-210 was detected by autoradiography. We interpreted the phosphorylation of MLCK-210 under these conditions to result from the catalytic activity of co-precipitating Pak (Fig. 1D, and Ref. 12), as it is a ligand-independent signaling activity under saturating concentrations of Ca2+/calmodulin is known to be severely depressed (14). Phosphorylation of MLCK-210 by a co-precipitating serine/threonine kinase other than Pak or a tyrosine kinase cannot be ruled out.

**Pak Kinase Assay—**Pak catalytic activity was measured in an immunoprecipitation kinase assay using myelin basic protein as the substrate, as described previously (15). N,N-Dimethylsphingosine (Sphingosine) in ethanol was dried under nitrogen, resuspended in 20 mM HEPES, pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, and 2 mM dithiothreitol, and solubilized by water bath sonication prior to its use in the Pak assays (16). The tyrosine phosphatase SHP was purified from GST-SHP (a gift from Tao Lin Yi, Lerner Research Institute, Cleveland, OH) by activated factor X. Pak was dephosphorylated by 1 μg of SHP in 25 mM PIPES (pH 7.0), 5 mM EDTA, 10 mM dithiothreitol for 1 h at 37 °C (17). Protein immunoblot analysis confirmed that equal amounts of Pak were immunoprecipitated from each sample (Fig. 1E, middle panel; data not shown).

**RESULTS**

**Formation of a Transformation-specific Signaling Complex in v-ErbB-transformed Fibroblasts—**The novel, phosphotyrosine protein complex in v-ErbB-transformed fibroblasts is composed of the signal adapter proteins Shc and Grb2, a tyrosine-phosphorylated form of the actomyosin regulatory protein, caldesmon, and several unknown phosphotyrosine proteins (5). The formation of this phosphoprotein complex is correlated with transformation by v-ErbB; it does not form in cells stimulated with transforming growth factor-α, or in cells expressing a kinase-active, non-transforming v-ErbB mutant (Fig. 1A and Refs. 4 and 5). Of note, this tyrosine-phosphorylated protein complex does not form in normal fibroblasts as a result of actin microfilament disruption by cytochalasin D or by inhibitors of Ras or Rho family GTTPases (7). The unidentified tyrosine-phosphorylated proteins in this complex migrate at 210, 68–75, and 47 kDa (Fig. 1A, lane S3).

**Tyrosine-phosphorylated p21-activated Kinase Is a Component of the Complex—**To identify the 68–75-kDa phosphotyrosyl protein components, we used glutathione S-transferase-Grb2 affinity chromatography followed by phosphotyrosine elution (5). One of the Coomassie-stained protein bands isolated by this method migrated at 75 kDa. Subsequent microsequence analysis of the peptides generated from this 75-kDa protein matched conserved sequences in the kinase domain of p21-activated kinases 1, 2, and 3 (Pak) (data not shown). Next, we used immunoprecipitation followed by immunoblot analysis to verify the identity of Pak as a component of the Shc-phosphoprotein complex. Shc co-immunoprecipitation assays detected Pak in association with Shc in both normal and transformed fibroblasts (Fig. 1B). Similarly, anti-Pak antibodies co-immunoprecipitated the 55- and 52-kDa isoforms of avian Shc in both normal and transformed fibroblasts (Fig. 1C). To verify that the 68–75-kDa phosphotyrosyl protein bands in Fig. 1A (lane S3) contained tyrosine-phosphorylated isoforms of Pak, we precleared Pak from lysate of v-ErbB-transformed fibroblasts prior to anti-Shc immunoprecipitation and anti-phosphotyrosine immunoblotting. Fig. 1D (lane S3 preclear a-Pak) shows that preclearing Pak by immunoprecipitation removes greater than 90% of the 68–75-kDa tyrosine-phosphorylated bands, as determined by densitometry. In addition, this pre-clearing with anti-Pak antibodies removes the 210- and 47-kDa phosphoproteins and significantly diminishes the phosphotyrosine signal at 78, 55, and 52 kDa. Comparison of the densi-
A transformation-specific, tyrosine-phosphorylated, multiprotein complex in v-ErbB-transformed fibroblasts contains Shc, Nck, Pak, caldesmon, and MLCK. A, lysates from normal CEF, CEF expressing a kinase-active, non-transforming v-ErbB mutant (E1), and CEF transformed by v-ErbB (S3) were immunoprecipitated with α-Shc antibodies followed by α-phosphotyrosine (α-p tyr) immunoblotting. B, lysates from CEF and S3 were immunoprecipitated with α-Shc antibodies followed by α-Pak immunoblotting. C, lysates from CEF and S3 were immunoprecipitated with α-Pak antibodies followed by α-Shc immunoblotting (note: α-Shc migrates slower in acrylamide gels than the corresponding mammalian Shc isoforms p46Shc and p52Shc). D, lysates from S3 were pre-cleared by immunoprecipitation with protein A/G-agarose beads alone (S3 lane) or with anti-Pak antibodies (S3 preclear α-Pak lane), followed by α-phosphotyrosine immunoblotting. Lysates from CEF and S3 were immunoprecipitated with α-Pak antibodies followed by E, α-phosphotyrosine immunoblotting; F, immunoblotting with antibodies to MLCK-210 (top panel), Pak (middle panel), or Nck (bottom panel); and G, α-caldesmon immunoblotting (CaD, caldesmon). All immunoprecipitations started with equivalent amounts of protein from each cell type. All experiments were repeated at least three times and gave reproducible results. Solid arrowhead, Pak isoforms; open arrowhead, Shc isoforms.

p21-activated Kinase Is Tyrosine-phosphorylated in a Transformation-associated Manner—The tyrosine-phosphorylated 68–75-kDa proteins found in v-ErbB-transformed fibroblasts are not present in fibroblasts overexpressing wild type epidermal growth factor receptor and grown in the presence of transforming growth factor-α, nor are they present in fibroblasts overexpressing a constitutively active, non-transforming v-ErbB mutant (Fig. 1A and Ref. 4). To verify that it is Pak that is tyrosine-phosphorylated in a transformation-associated manner, we immunoprecipitated Pak, followed by anti-phosphotyrosine immunoblotting. Fig. 1E (middle panel, lane S3) demonstrates that Pak is a tyrosine-phosphorylated only in transformed fibroblasts. In addition, the 47-kDa tyrosine-phosphorylated protein in this complex (Fig. 1E, bottom panel) was identified as the signal adapter protein Nck (Fig. 1F, bottom panel). In contrast to Pak, Nck is tyrosine-phosphorylated in both normal and transformed fibroblasts (Fig. 1E, bottom panel), consistent with previous reports (18, 19).

The She-Pak Complex Associates with Actomyosin Regulatory Proteins—Guided by our previous results suggesting that this phosphoprotein complex regulates the actomyosin-based cytoskeleton, we used immunoprecipitation and immunoblot analyses to identify the 210-kDa tyrosine-phosphorylated protein that associates with the protein complex in v-ErbB-transformed fibroblasts. Fig. 1F (top panel) shows that Pak coprecipitates with the 210-kDa isoform of myosin light chain kinase (MLCK-210). MLCK-210 is a recently described major, nonmuscle isoform of MLCK that contains an additional 934 amino-terminal residues compared with smooth muscle MLCK (20). Phosphotyrosine immunoblot analysis revealed that MLCK-210, like Pak and caldesmon, is only tyrosine-phosphorylated in v-ErbB-transformed fibroblasts (Fig. 1E, top panel). The observation that MLCK-210 is associated with the She-Grb2-caldesmon-Nck-Pak complex suggests that one specific function of this transformation-associated signaling complex is to regulate the phosphorylation state of myosin.

It is clear that the phosphotyrosine protein complex in v-ErbB-transformed fibroblasts contains several components that potentially can regulate the actomyosin-based cytoskeleton. One such regulatory protein is the tyrosine-phosphorylated form of caldesmon, which is a stable component of a Shc-Grb2 signal adapter complex in v-ErbB-transformed fibroblasts (21). To determine if this Shc-Grb2-caldesmon phosphoprotein complex is a stable subunit of an even larger multiprotein module involving Nck and Pak, we looked for a caldesmon-Pak

FIG. 1. A transformation-specific, tyrosine-phosphorylated, multiprotein complex in v-ErbB-transformed fibroblasts contains Shc, Nck, Pak, caldesmon, and MLCK. A, lysates from normal CEF, CEF expressing a kinase-active, non-transforming v-ErbB mutant (E1), and CEF transformed by v-ErbB (S3) were immunoprecipitated with α-Shc antibodies followed by α-phosphotyrosine (α-p tyr) immunoblotting. B, lysates from CEF and S3 were immunoprecipitated with α-Shc antibodies followed by α-Pak immunoblotting. C, lysates from CEF and S3 were immunoprecipitated with α-Pak antibodies followed by α-Shc immunoblotting (note: α-Shc migrates slower in acrylamide gels than the corresponding mammalian Shc isoforms p46Shc and p52Shc). D, lysates from S3 were pre-cleared by immunoprecipitation with protein A/G-agarose beads alone (S3 lane) or with anti-Pak antibodies (S3 preclear α-Pak lane), followed by α-phosphotyrosine immunoblotting. Lysates from CEF and S3 were immunoprecipitated with α-Pak antibodies followed by E, α-phosphotyrosine immunoblotting; F, immunoblotting with antibodies to MLCK-210 (top panel), Pak (middle panel), or Nck (bottom panel); and G, α-caldesmon immunoblotting (CaD, caldesmon). All immunoprecipitations started with equivalent amounts of protein from each cell type. All experiments were repeated at least three times and gave reproducible results. Solid arrowhead, Pak isoforms; open arrowhead, Shc isoforms.
interaction by co-precipitation assay. Fig. 1G demonstrates that there is a marked increase in Pak association with caldesmon in v-ErbB-transformed fibroblasts compared with normal fibroblasts. In fact, Pak antibodies co-immunoprecipitate the majority of caldesmon found in lysates of v-ErbB-transformed fibroblasts (data not shown and Ref. 5). In summary, we have identified a multiprotein signaling module that forms in v-ErbB-transformed fibroblasts that consists of the signal adapter proteins Shc, Grb2, and Nck, and tyrosine-phosphorylated forms of caldesmon, myosin light chain kinase, and Pak.

Pak Kinase Is Activated in a Ligand-independent Manner in v-ErbB-transformed Fibroblasts—To analyze the effect of tyrosine phosphorylation on Pak kinase activity, fibroblasts transformed with v-ErbB and control fibroblasts were incubated in the presence or absence of transforming growth factor-α (Fig. 2A). Using a Pak immunocomplex kinase assay, Pak activation by growth factor stimulation of the control fibroblasts (Fig. 2A, CEF), or fibroblasts expressing a kinase-active, non-transforming v-ErbB mutant (data not shown), could be detected as early as 1 min after treatment. In contrast, Pak is constitutively active in serum-starved v-ErbB-transformed fibroblasts (Fig. 2A, S3-CEF). To investigate the mechanism of Pak activation in v-ErbB-transformed cells, we used the recently described Pak inhibitor N,N-dimethylsphingosine (DMS).

Pak Kinase Is Activated by Tyrosine Phosphorylation—To determine if the catalytic activity of Pak is regulated by tyrosine phosphorylation, we immunoprecipitated Pak from v-ErbB-transformed fibroblasts and dephosphorylated it’s tyrosine residues with a constitutively activated, amino-terminal truncation mutant of the Src homology protein-tyrosine phosphatase SHP-1 (17). Fig. 3A shows that recombinant SHP can efficiently dephosphorylate Pak in vitro. Subsequently, an in vitro kinase assay was used to measure the catalytic activity of tyrosine-phosphorylated and dephosphorylated forms of Pak derived from v-ErbB-transformed fibroblasts. Fig. 3B shows that there is a significant (consistently 2–3-fold) decrease in Pak kinase activity with SHP dephosphorylation of tyrosine residues. In contrast, growth factor-induced Pak kinase activity in normal chick embryo fibroblasts is not affected by the SHP tyrosine phosphatase (data not shown).

Tyrosine Phosphorylation of Pak Is Rho-dependent—Recently, we have shown that the formation of the Shc-Grb2-Nck-Pak-caldesmon-MLCK complex and anchorage-independent cell growth in v-ErbB-transformed fibroblasts is disrupted by the expression of a dominant negative Rho mutant. To test whether the tyrosine phosphorylation of Pak also is disrupted by DNRho expression, we co-infected fibroblasts with transforming or non-transforming v-ErbB mutants and a DNRho mutant using avian retroviral vectors. Fig. 4A demonstrates the expression of DNRho (top panel) and both v-ErbB products (middle panel) in the co-infected fibroblasts. Immunoprecipitation of Pak and anti-phosphotyrosine immunoblot analysis of lysates from v-ErbB-infected fibroblasts demonstrate that Pak tyrosine phosphorylation is inhibited by the expression of DNRho (Fig. 4A, bottom panel, + DNRhoA, + S3-v-ErbB lane). In addition, co-precipitation of Pak with the tyrosine-phosphorylated isoforms of Shc is inhibited by DNRhoA.

The Shc-Caldesmon-Pak-MLCK Complex Is a Transformation-associated Myosin-binding Module—Fibroblast contractility and tension are regulated by the action of kinases and phosphatases on myosin regulatory light chains and by the direct interaction of myosin with caldesmon (22). We hypothesize that the tyrosine-phosphorylated forms of Pak, MLCK, and caldesmon regulate the actomyosin molecular motor in v-ErbB-transformed fibroblasts, thereby influencing contractility, tension, and anchorage-independent growth in transformed cells. To determine if there is a direct interaction between the phospho-myosin protein complex and the myosin molecular motor assembly, we used anti-myosin immunoprecipitation and anti-phosphotyrosine immunoblot analysis. These experiments revealed an association between myosin heavy chain and several tyrosine-phosphorylated proteins migrating at 210, 68–78, 55, and 47 kDa (Fig. 5A). This association only occurs in the v-ErbB-transformed fibroblasts (Fig. 5A, lane S3) and is not seen in control fibroblasts or in fibroblasts expressing the kinase-active, non-transforming v-ErbB mutant (Fig. 5A, lanes CEF
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on-transforming v-ErbB mutant (S3-v-ErbB), and dominant negative Rho (DNRho) as indicated. Equal amounts of protein from cell lysates were immunoblotted with α-Rho antibodies (top panel) and α-ErbB antibodies (middle panel). Next, equal amounts of protein from cell lysates were assayed by α-Pak immunoprecipitation (IP α-Pak) followed by α-phosphotyrosine (α-ptyr) immunoblotting.

In addition to the regulatory role of myosin-binding proteins, tyrosine-phosphorylated isoforms of the serine/threonine kinase Pak is tyrosine-phosphorylated in a Rho-dependent manner in v-ErbB-transformed fibroblasts. Chick embryo fibroblasts were co-infected with retroviral vectors encoding a kinase-active, non-transforming v-ErbB mutant (E1-v-ErbB) or a kinase-active, transforming v-ErbB mutant (S3-v-ErbB), and dominant negative Rho (DNRho) as indicated. Equal amounts of protein from cell lysates were immunoblotted with α-Rho antibodies (top panel) and α-ErbB antibodies (middle panel). Next, equal amounts of protein from cell lysates were assayed by α-Pak immunoprecipitation (IP α-Pak) followed by α-phosphotyrosine (α-ptyr) immunoblotting.

and E1, respectively). These transformation-specific, myosin-associated, tyrosine-phosphorylated proteins were identified as MLCK (210-kDa isoform), caldesmon (78-kDa isoform), Pak (68–75-kDa isoforms), Shc (55-kDa isoform), and Nck (47-kDa form) by immunoblot analysis (Fig. 5B, middle and bottom panels; data not shown). Also noted in these experiments was the dramatic increase in myosin content in the Triton-soluble fraction of v-ErbB-transformed fibroblasts (Fig. 5B, top panel). In addition, we have shown that the myosin-associated phosphoprotein complex does not co-precipitate with actin under these lysis conditions (data not shown). Because myosin-binding proteins regulate the solubility and localization of myosin II in non-muscle cells (23), we suggest that this myosin-binding complex influences the conformation, solubility, and actin-binding ability of myosin.

MLCK Catalytic Activity, Myosin Light Chain Phosphorylation, and Myosin Localization Are Altered in v-ErbB-transformed Cells—To further evaluate the affect of v-ErbB-mediated transformation on myosin localization, we immunostained v-ErbB-transformed and control cells using anti-myosin heavy chain antibodies. Immunofluorescence microscopy revealed a dramatic reorganization of myosin in v-ErbB-transformed fibroblasts compared with normal fibroblasts. As expected, myosin is localized periodically along actin stress fibers and within the membrane ruffles at the leading edge of normal fibroblasts (Fig. 5C, CEF). In contrast, in v-ErbB-transformed fibroblasts (Fig. 5C, S3) myosin is diffusely localized throughout the cytoplasm with some areas of focal cytoplasmic accumulation. This observation is consistent with the increased Triton X-100-solubility of myosin in v-ErbB-transformed cells (Fig. 5B, top panel; data not shown) and the association of myosin with the cytoplasmic signal adapter protein Shc (Fig. 5B, bottom panel). Together, these data suggest that the myosin-associated phosphotyrosine protein complex alters the solubility and localization of myosin in these transformed fibroblasts.

In addition to the regulatory role of myosin-binding proteins, the phosphorylation state of myosin has a profound effect on its function. In v-ErbB-transformed fibroblasts, the phosphorylation state of myosin II may be regulated by the Pak and MLCK components of the transformation-associated phosphoprotein complex (12). In this model, constitutively activated Pak could down-regulate MLCK activity by direct phosphorylation (12). Consequently, the decreased phosphorylation of regulatory MLCK on serine 19 by MLCK would promote the disassembly of myosin filaments and dampen myosin ATPase activity (23). To test this hypothesis, we determined the phosphorylation status and catalytic activity of MLCK and the phosphorylation status of regulatory MLCK in v-ErbB-transformed cells. First, we performed a MLCK immunocomplex phosphorylation assay on MLCK-210. This study revealed that, although MLCK-210 is expressed at similar levels in normal versus transformed cells (Fig. 6A, top panel), MLCK-210 is extensively phosphorylated only in v-ErbB-transformed cells (Fig. 6A, bottom panel). Next, we assessed the catalytic activity of MLCK in normal versus transformed fibroblasts by the ability of MLCK to phosphorylate purified MLCK, and by assaying for the in vivo phosphorylation status of regulatory MLCK. In vitro analysis of MLCK catalytic activity shows that MLCK activity in v-ErbB-transformed cells is approximately 60% less than in normal cells (Fig. 6B). The phosphorylation status of MLCK in normal versus transformed fibroblasts corroborates this observation (Fig. 6C). Specifically, immunoprecipitation and immunoblot analysis of MLCK show that, although there are equivalent levels of regulatory MLCK in transformed versus normal cells (Fig. 6C, left panel), MLCK is significantly phosphorylated on serine 19 only in normal cells (Fig. 6C, middle panel). Furthermore, the overall phosphorylation level of MLCK in vivo is decreased in transformed cells (Fig. 6C, right panel). In summary, Pak is constitutively activated by tyrosine phosphorylation, MLCK is hyperphosphorylated with decreased catalytic activity, and regulatory MLCK phosphorylation is significantly decreased in v-ErbB-transformed fibroblasts.

DISCUSSION

Evidence is mounting that dominant transforming genes contribute to the development of human cancers. For genes encoding receptor tyrosine kinases, these dominant mutations commonly disrupt the ligand-binding ability of the receptor and, consequently, cause constitutive activation of the catalytic domain (2). Examples of such ligand-independent oncoproteins in human malignancies include the Ret tyrosine kinase in multiple endocrine neoplasia syndromes (24–26) and the epidermal growth factor receptor in malignant astrocytomas (27–29). The ligand-independent signaling pathways downstream of these dominant transforming tyrosine kinases continue to be defined; however, the transformation-specific nature of these transduced signals has been difficult to establish.

By investigating the signaling pathways of well characterized oncogenic and non-oncogenic mutants of the epidermal growth factor receptor, we have demonstrated ligand-independent, transformation-associated signal transduction mediated by a receptor tyrosine kinase. Specifically, we demonstrate the formation of a myosin-binding, phosphoryrosine protein complex in v-ErbB-transformed fibroblasts. Components of this complex include: (i) the signal adapter proteins Shc, Nck, and Grb2; (ii) novel, tyrosine-phosphorylated isoforms of the serine/threonine kinase Pak; (iii) tyrosine-phosphorylated MLCK-210; and (iv) tyrosine-phosphorylated caldesmon. The formation and tyrosine phosphorylation of this protein complex represent signaling events that are distinct from the Ras-dependent, mitogenic signal transduction pathways stimulated by the ligand-dependent epidermal growth factor receptor (7). In fact, we recently have shown that actin stress fiber disassembly, anchorage-independent cell growth, and formation of...
this phosphoprotein complex in v-ErbB-transformed fibroblasts are Rho-dependent events that are independent of the activation of Ras, Rac, and Cdc 42 (7). It is important to note that the constitutively activated (but non-transforming in fibroblasts) E1-v-ErbB mutant does not induce the formation of this phosphoprotein complex. Our evidence for novel signaling by an oncogenic receptor tyrosine kinase not only supports a model in which oncogenesis involves altered substrate specificity of the kinase and the formation of transformation-specific signaling modules, but also suggests that these modules may be ideal targets for the development of cancer-specific molecular therapeutic agents.

The Pak component of this signaling module is of particular interest. In v-ErbB-transformed fibroblasts Pak is resistant to the inhibitor N,N-dimethylsphingosine. This observation suggests that the mechanism of Pak activation in v-ErbB-transformed fibroblasts, in contrast to transforming growth factor-α (TGFα)-dependent Pak activation, does not require either GTPase binding or phospholipid targeting. Given the tyrosine phosphorylation of Pak in v-ErbB-transformed cells, we speculate that the constitutive activation of Pak may be related to its tyrosine phosphorylation. In fact, our findings demonstrate that tyrosine dephosphorylation of Pak results in a subsequent decrease in Pak kinase activity in direct support of this hypothesis. Based on these observations, we propose that the transformation-associated tyrosine phosphorylation of Pak may mimic the autoregulatory effect of serine/threonine phosphorylation. This idea is supported by the observation that the replacement of serine/threonine residues with acidic amino acids at known phosphorylation sites in the catalytic domain can directly activate Pak (30). Alternatively, the conformation of the amino-terminal negative regulatory domain of Pak may be altered by tyrosine phosphorylation, in a manner similar to the allosteric modulation of Pak associated with binding to GTPases (31).

Irrespective of the mechanism, the constitutive activation of Pak may play a vital role in establishing and maintaining the transformed phenotype of cells. Specifically, it has been shown that constitutively active Pak can disrupt actin stress fibers and focal adhesions (30). Pak also has been shown to regulate upstream components of the actomyosin molecular motor assembly by phosphorylating myosin light chain kinase (MLCK) (12), LIM kinase (32), and caldesmon (33). In addition, Pak has recently been shown to phosphorylate and inactivate the death-promoting factor Bad (34). Therefore, the tyrosine phosphorylation and activation of Pak may allow the EGFR oncoprotein to influence not only cytoskeletal mechanics, but block pro-apoptotic pathways as well. Additional studies will be needed to address these potential contributions of Pak to the transformed phenotype.

Increasingly, signaling modules are being recognized as a new level of biological organization and regulation (35). Signaling modules that associate with the actomyosin-based cytoskeleton, such as the module we describe in v-erbB-transformed fibroblasts, could have wide ranging influences on microfilament mechanics, cytoskeletal tension generation, focal adhesion complex formation, anchorage-independent cell growth, and cell cycle progression (36–38). In v-ErbB-transformed fibroblasts, there appears to be a cascade of events involving the ligand-independent activation of Rho and subsequent Pak tyrosine phosphorylation, the formation of the myosin-binding signaling module, the down-regulation of MLCK activity, the dephosphorylation of myosin regulatory subunits, and the reorganization of myosin II. In these transformed fibroblasts, we hypothesize that such modulation of mechanochemical signal transduction events induces anchorage-independent cell growth and other elements of the transformed phenotype (39). Our findings directly support this notion. When dominant negative Rho inhibits Pak tyrosine phosphorylation and inhibits assembly of the multiprotein complex, fibroblasts lose their...
and immunoprecipitated with chain kinase and dephosphorylation of myosin in transformed S3 transformed fibroblasts (sayed by autoradiography for S3 and S3 were metabolically labeled blotted with Pak-MLCK protein complex that associates with myosin. This receptor mutants can transduce transformation-specific scaffolding, and/or assembly of this complex.

cytoskeleton by DNRho inhibits kinase localization, proper migration is necessary for proper protein-protein interactions tyrosine kinase phosphorylates Pak, and that this phosphorylation does not occur in the presence of complex will require further study; however, the fact that tyrosine phosphorylation of Pak does not occur in the presence of ability to grow in an anchorage-independent manner. Details of the link between Rho and the formation of this phosphoprotein complex will require further study; however, the fact that tyrosine phosphorylation of Pak does not occur in the presence of dominant negative Rho suggests either that a Rho-dependent tyrosine kinase phosphorylates Pak, and that this phosphorylation is necessary for proper protein-protein interactions within the complex, or that localized disruption of the actin cytoskeleton by DN Rho inhibits kinase localization, proper scaffolding, and/or assembly of this complex.

In summary, ligand-independent epidermal growth factor receptor mutants can transduce transformation-specific signals that are distinct from ligand-dependent, mitogenic signals. In this study, we show that these transformation-specific events include the tyrosine phosphorylation-dependent activation of Pak and the formation of a Src-Grb2-caldesmon-Nck-Pak-MLCK protein complex that associates with myosin. This novel signaling pathway couples an oncogenic receptor tyrosine kinase with regulatory components of the actomyosin-based cytoskeleton, thus potentially allowing the oncoprotein to alter the balance of mechanical forces governing cellular adhesion, cytoskeletal tension, cell cycle progression, and anchorage-independent cell growth (36, 39).

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