Tissue- and Transformation-Specific Phosphotyrosyl Proteins in *v-erbB*-Transformed Cells

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To understand the mechanism of tissue-specific and transformation-specific signaling by the v-ErbB oncoprotein, we have investigated signaling pathways downstream of this transmembrane tyrosine kinase. In this report, we describe tissue-specific patterns of phosphotyrosyl proteins in three distinct cell types transformed by the *v-erbB* oncogene: fibroblasts, erythroblasts, and endothelial cells. In addition, we describe transformation-specific tyrosine phosphorylation events and signal complex formation in *v-erbB*-transformed fibroblasts. Two patterns of phosphotyrosyl proteins have been detected in *v-erbB*-transformed cells. The first is a fibroblast-specific pattern which includes unique phosphotyrosyl proteins of 170 kDa (c-ErbB1), 158 kDa, and 120 kDa (the catenin-like protein p120^{cas}). The second is an erythroblast/endothelial cell-specific pattern which includes a prominent unidentified phosphotyrosyl protein of 120 kDa. Evaluation of the phosphotyrosyl proteins p120^{cas} and SHC in chicken embryo fibroblasts infected with transforming and nontransforming *v-erbB* mutants reveals transformation-specific patterns of tyrosine phosphorylation. One corollary of these phosphorylation events in *v-erbB*-transformed fibroblasts is the formation of a complex involving SHC, growth factor receptor-bound protein 2, and a novel 75-kDa phosphotyrosyl protein. The results of these studies suggest that the v-ErbB oncoprotein can couple to multiple signal transduction pathways, that these pathways are tissue specific, and that *v-erbB*-mediated transformation involves specific tyrosine phosphorylation events.

Studying the tissue specificity of tumor formation by oncogenes is one approach to understanding the signal transduction pathways controlling oncogenesis. Why is the tumorigenicity of a particular oncogene limited to one tissue type, whereas a mutant of the same oncogene may cause tumors in distinctly different tissues? The study of avian retroviruses offers an opportunity to address this question. Independent isolates of the acutely transforming avian erythroblastosis virus (AEV) can induce tumor formation in more than one tissue type. The primary site of oncogenesis associated with AEV infection is the bone marrow, resulting in the development of erythroleukemia; however, fibrosarcomas, hemangiosarcomas, angiosarcomas, and renal adenocarcinomas can also occur in AEV-infected birds (17, 31, 38).

AEV isolates contain various forms of the transforming oncogene *v-erbB*. The cellular homolog of *v-erbB* is the avian *c-erbB1* gene. The *c-erbB1* gene encodes a 170-kDa tyrosine kinase growth factor receptor that is structurally homologous to the human epidermal growth factor receptor (7). Mutant protein products encoded by *v-erbB* genes are all transmembrane receptors that have sustained truncation of their extracellular ligand-binding domains. In addition, some of these *v-erbB* genes have sustained further mutations in their intracytoplasmic domains (17). We have previously correlated these structural changes with the ability of *v-erbB* products to transform three distinct cell types: fibroblasts, erythroblasts, and endothelial cells (26, 27). In this study, we have used *v-erbB* mutants isolated and characterized in our laboratory to test the hypothesis that tissue-specific *v-erbB*-mediated transformation is correlated with the tyrosine phosphorylation of tissue-specific substrates. Furthermore, we have compared transforming

and nontransforming *v-erbB* mutants to look for transformation-specific tyrosine phosphorylation events.

This study shows that tyrosine phosphorylation patterns in *v-erbB*-transformed cells are tissue specific and can be categorized into a fibroblast-specific pattern and an erythroblast/endothelial cell-specific pattern. We identify transformation-specific differences in the patterns of tyrosine phosphorylation of the Src tyrosine kinase substrate p120^{cas} (cadherin-associated Src substrate) (28, 29) and the signal adapter protein SHC (Src homologous and collagen homologous) (24). Furthermore, we identify a complex involving SHC, GRB-2 (growth factor receptor-bound protein 2) (16), and a novel 75-kDa phosphotyrosyl protein which specifically forms in *v-erbB*-transformed fibroblasts. Taken together, our results offer evidence for tissue-specific and transformation-specific tyrosine phosphorylation events, thus providing insight into the signal transduction pathways utilized by *v-erbB*-encoded oncoproteins.

MATERIALS AND METHODS

Cell culture and transfection. Primary cultures of chicken embryo fibroblasts (CEF) were prepared from day 10 line 0 chicken embryos obtained from the USDA Avian Disease and Oncology Laboratory, East Lansing, Mich. CEF were maintained in Dulbecco modified Eagle medium (DMEM) containing 4.5 g of glucose per liter supplemented with 10% fetal calf serum (FCS), 1% chicken serum, 100 U (each) of penicillin-streptomycin per ml, and 0.1% amphotericin B (Fungizone). The *v-erbB* mutants were used to infect CEF as previously described (4, 25) and were all in the RCAN (replication-competent avian leukosis virus with no splice acceptor) vector, which is a helper-independent retroviral vector derived from Rous sarcoma virus (11), except for AEV strain R (AEV-R). The *v-erbB*-transformed chick erythroblasts are derived from AEV-R-infected chick erythroid precursors (6C2 cells) (1) and were maintained in DMEM with 10% FCS and 1% chicken serum. The REB-S3-transformed endothelial cells were derived from hemangiosarcomas which developed in vivo in chicks infected with the REB-S3 retroviral vector (4) and were cultured in DMEM with 15% FCS. For transient overexpression of *c-erbB1*, CEF were transfected by calcium phosphate precipitation (41) with eucaryotic expression vector pCMV (Invitrogen, San Diego, Calif.) alone or with the pCMV vector containing a *NotI*-to-*KpnI* fragment of *c-erbB1* cloned into the *XbaI* site of the vector.

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Growth factor stimulation of CEF. Subconfluent CEF were serum starved in DMEM without FCS for 24 h; human transforming growth factor alpha (TGF- α ; Gibco) was then added to a concentration of 50 ng/ml; the cells were incubated for 1 h at 37°C in the presence of 50 μ M sodium orthovanadate (Johnson Matthey) and then lysed for Western blot (immunoblot) analysis.

Antiphosphotyrosine antibodies. *O*-Phospho-L-tyrosine (Sigma) was coupled to keyhole limpet hemocyanin (Sigma) by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma) (12). Phosphotyrosine-keyhole limpet hemocyanin was used to immunize rabbits as previously described (19). Immunoglobulin fractions were precipitated from rabbit serum with ammonium sulfate and purified on phosphotyrosine-linked Affi-Gel 10/15 agarose beads (Bio-Rad) (12, 20). Cross-reacting antibodies were eluted from the column with 5 mM each phosphoserine and phosphothreonine (Sigma); 40 mM phenyl phosphate was used to elute off antiphosphotyrosine antibodies. The commercially available antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc. [UBI], Lake Placid, N.Y.) was also used in selected Western immunoblot analyses.

Cell lysis. Cells were pretreated with 50 μ M sodium orthovanadate for 1 h at 37°C, then kept at 4°C, and scraped from the plate in the presence of cold phosphate-buffered saline containing 4 mM diisopropyl fluorophosphate (DFP; Sigma) and 100 μ M vanadate. Cells were lysed for 30 to 60 min at 4°C in lysing buffer (1% Triton X-100, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 5 mM EDTA, 50 mM NaCl, 10 mM NaPP_i) containing 100 mM vanadate, 4 mM DFP, 1 mM phenylmethylsulfonyl fluoride, 10 μ g each of aprotinin, pepstatin A, and leupeptin per ml and then centrifuged at 14,000 rpm for 15 min at 4°C. The orthovanadate and DFP were used to inhibit phosphatases and thereby enhance the detection of tyrosine-phosphorylated proteins. Protein concentrations of lysates were determined by using bicinchoninic acid protein reagents (Pierce).

Western immunoblotting. Cell lysates were prepared in standard Laemmli sodium dodecyl sulfate (SDS) sample buffer (15) plus 200 mM dithiothreitol; samples were then heated at 100°C for 3 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), using 8 to 12.5% acrylamide as indicated. Proteins were then transferred to nitrocellulose in a Hoefer Transphor apparatus (Hoefer Scientific Instruments) at 0.5 A for 4 to 6 h at 4°C (39). The nitrocellulose was blocked with blocking buffer (TNA buffer [1 M Tris HCl [pH 7.4], 150 mM NaCl, 0.01% sodium azide] plus 5% bovine serum albumin) overnight. Western blotting was done with an affinity-purified polyclonal antiphosphotyrosine antibody (1 μ g/ml) or monoclonal antiphosphotyrosine antibody 4G10 (1 μ g/ml), in blocking buffer, for 1 to 2 h at 4°C. A monoclonal antibody to p120^{cas} (2B12) (14) was used at 1:1,000 dilution. A polyclonal antibody to SHC (24) (UBI) was used at 1:1,000 dilution. A polyclonal antibody to GRB-2 (UBI) was used at 1:500 dilution. A monoclonal antibody to GRB-2 (UBI) was used at 1 μ g/ml. ¹²⁵I-protein A (0.5 μ Ci/ml; Amersham) was used to detect rabbit-derived antibodies; ¹²⁵I-anti-mouse antibody (1.0 μ Ci/ml; Amersham) was used to detect mouse-derived antibodies. Blots were extensively washed after each antibody incubation with TNA buffer plus 0.3% Nonidet P-40, with three to four buffer changes over 3 h. Blots were exposed to X-ray film (Kodak X-Omat AR) at -70°C for the specified period of time. For blots detected by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, Ill.), azide was removed from all Western blotting reagents; the horseradish peroxidase (HRP)-linked anti-mouse and anti-rabbit secondary antibodies and HRP-linked protein A were used at 1:20,000 dilution for 1 h at 4°C, and the blots were extensively washed in Tris-buffered saline (20 mM Tris HCl [pH 7.4], 150 mM NaCl) with 1% Tween 20.

Immunoprecipitation. Immunoprecipitation was performed in immunoprecipitation buffer A (190 mM NaCl, 50 mM Tris [pH 7.4], 6 mM EDTA, 2.5% Triton X-100) in a total volume of 1 ml; anti-p120^{cas} (2B12) and anti-SHC antibodies were used at 1 to 2 μ l/250 μ g of protein; samples were rocked at 4°C for 1 h; 50 μ l of ImmunoPure Protein A/G beads (Pierce) was added to samples, which were rocked for 1 h and then centrifuged at 14,000 rpm for 30 s to recover the beads. Protein A/G beads were preincubated with rabbit anti-mouse antibody (Jackson ImmunoResearch) as a bridging antibody for immunoprecipitations involving mouse monoclonal antibodies. Protein A/G beads were washed three times with immunoprecipitation buffer B (150 mM NaCl, 10 mM Tris [pH 9.0], 5 mM EDTA, 0.1% Triton X-100) and once with Tris-buffered saline (10 mM Tris HCl [pH 7.4], 150 mM NaCl). Two-fold SDS-PAGE sample buffer plus 200 mM dithiothreitol was then added to each sample, and samples were heated at 100°C for 3 min.

RESULTS

Characterization of v-erbB-transformed cells. The v-ErbB proteins expressed by tissue-specific transforming mutants of AEV used in this study are depicted in Fig. 1. Although c-ErbB1 overexpression in fibroblasts has been correlated with ligand-dependent soft agar colony formation in vitro (5, 30), c-ErbB1 overexpression has not been demonstrated to be tumorigenic in vivo. In contrast, truncation of the ligand-binding domain of avian c-ErbB1 by either insertional activation (8, 22)

FIG. 1. Schematic comparison of the structures and tumorigenic potentials of the *c-erbB1* product and proteins encoded by *v-erbB* within AEV-R and the recombinant retroviral constructs REB-C and REB-S3. The ability of each virus to induce erythroblastosis (Ery), fibrosarcomas (Sarc), or hemangiosarcomas (Hem) in vivo is indicated. Deleted amino acid (aa) sequences are indicated by dotted lines. [hatched box], ligand-binding domain; TM, transmembrane domain; KINASE, tyrosine kinase domain. Adapted from reference 27.

or transduction (26, 27) results in the expression of a v-ErbB protein that is leukemogenic in vivo. This leukemogenic v-ErbB protein has been expressed in the context of a helper-independent retroviral vector (RCAN) (11, 25) and is referred to as REB-C (Fig. 1). REB-C infection does not result in transformation of CEF in vitro and is not sarcomagenic in vivo (Fig. 1) (25). The S3-v-*erbB* gene was identified in avian leukosis virus-infected birds as the transforming element of a novel AEV (27). Expression of the S3-v-ErbB protein results in transformation of fibroblasts and endothelial cells; S3-v-ErbB has, however, lost its ability to transform erythroblasts (26). We have recently demonstrated that S3-v-*erbB*, when expressed in the context of the RCAN vector (referred to as REB-S3; Fig. 1), retains this pattern of associated tumorigenicity (4). The structure of S3-v-ErbB is illustrated in Fig. 1; in addition to having a truncated extracellular ligand-binding domain, S3-v-ErbB has sustained a 139-amino-acid in-frame deletion in the C-terminal portion of the protein (27). The v-*erbB* gene within AEV-R expresses a c-ErbB1-related protein which has a truncated ligand-binding domain, two separate C-terminal deletions, and multiple point mutations (Fig. 1) (3, 35). This v-*erbB* product can induce erythroleukemia and fibrosarcomas in vivo and can transform erythroblasts and fibroblasts in vitro. Cells infected with these viruses, or primary cell cultures and cell lines derived from v-*erbB*-induced tumors, were routinely monitored during these studies for v-ErbB expression by Western immunoblot analysis and consistently expressed v-ErbB proteins of the predicted mobilities (Fig. 2A, lane 3 [REB-C v-ErbB, 70 to 95 kDa], lanes 4 and 6 [REB-S3 v-ErbB, 63 to 75 kDa], and lanes 2 and 5 [AEV-R v-ErbB, 65 to 75 kDa]). As evident in Fig. 2A, the v-ErbB proteins migrate as broad bands on acrylamide gels. This is due to extensive N-linked glycosylation. Previous studies in our laboratory have demonstrated differential glycosylation of v-ErbB both within a given cell type and between different cell types (18). Furthermore, it should be noted that AEV-R v-ErbB does not appear to be as intensely tyrosine phosphorylated as REB-S3 v-ErbB because the C-terminal deletion in AEV-R v-ErbB includes two (of the five) major autophosphorylation sites (17).

Ligand-stimulated CEF and v-erbB-transformed fibroblasts exhibit distinct patterns of tyrosine phosphorylation. As illustrated in Fig. 2B, lane 1, uninfected CEF contain very low levels of tyrosine-phosphorylated proteins when grown in 10% FCS and 1% chick serum; faint bands at 120 and 95 kDa are detectable. In REB-S3-transformed CEF, several new phos-

FIG. 2. Western immunoblot analysis of *v-ErbB* expression and tyrosine phosphorylation in normal and retrovirus-infected cells. One hundred micrograms of lysate from each cell type was separated by SDS-PAGE (8% acrylamide gel) and then transferred to nitrocellulose. Panel A was blotted with polyclonal anti-ErbB antibodies; panel B was blotted with polyclonal antiphosphotyrosine antibodies. Both blots were incubated with ^{125}I -protein A and exposed to X-ray film for 1.5 days except panel B, lane 6, which was exposed for 5.5 days. (A) Lanes: 1, CEF; 2, AEV-R-transformed CEF; 3, REB-C-infected CEF; 4, REB-S3-transformed CEF; 5, AEV-R-transformed erythroblasts; 6, REB-S3-transformed endothelial cells (primary cultures of REB-S3-induced hemangiosarcomas). (B) Lanes: 1, CEF; 2, serum-starved CEF plus TGF- α ; 3, REB-C-infected CEF; 4, REB-S3-transformed CEF; 5, AEV-R-transformed erythroblasts; 6, REB-S3-transformed endothelial cells. Sizes are indicated in kilodaltons.

phosphotyrosyl proteins and more prominent phosphorylation of existing phosphotyrosyl proteins are detectable compared with uninfected CEF (Fig. 2B, lanes 1 and 4). The most prominent new tyrosine-phosphorylated protein in REB-S3-transformed CEF migrates at 63 to 75 kDa. This 63 to 75-kDa phosphotyrosyl protein is identified as S3-*v-ErbB*, for it comigrates with the higher-molecular-weight forms of S3-*v-ErbB* on the anti-ErbB Western blot (Fig. 2A, lane 4) and can be detected by stripping and reprobing the blot in Fig. 2B with an anti-ErbB antibody (data not shown). There are also new phosphotyrosyl proteins migrating at 170, 158, 52, and 46 kDa in REB-S3-transformed CEF compared with uninfected CEF (Fig. 2B, lanes 1 and 4). Furthermore, there are proteins more prominently tyrosine phosphorylated in REB-S3-transformed CEF compared with uninfected CEF migrating at 110 to 120 and 95 kDa (Fig. 2B, lanes 1 and 4).

Stimulation of CEF with human TGF- α results in tyrosine phosphorylation of the native c-ErbB1 receptor migrating at 170 kDa and the appearance of a phosphotyrosyl protein migrating at approximately 158 kDa (Fig. 2B, lane 2). CEF overexpressing c-ErbB1 show increased tyrosine phosphorylation of these same proteins both in the presence and in the absence of TGF- α compared with uninfected CEF (data not shown). Comparison of these phosphotyrosyl protein patterns in CEF (with or without TGF- α and with or without *c-erbB1* overexpression) with those of REB-S3-transformed CEF reveals that the *v-erbB*-transformed fibroblasts express several new or more prominent phosphotyrosyl proteins which migrate at 120, 63 to 75 (S3-*v-ErbB*), 52, and 46 kDa (Fig. 2B, lanes 2 and 4, and data not shown).

CEF expressing transforming and CEF expressing non-transforming *v-ErbB* proteins exhibit subtle differences in the patterns of phosphotyrosyl proteins. The pattern of tyrosine-phosphorylated proteins in REB-C-infected CEF, which express an active *v-ErbB* protein unable to transform fibroblasts, is subtly different from the pattern of tyrosine-phosphorylated proteins detectable in REB-S3-transformed CEF (Fig. 2B,

lanes 3 and 4). Specifically, the 52- and 46-kDa tyrosine-phosphorylated proteins in REB-S3-transformed CEF are not detectable by antiphosphotyrosine Western blot analysis in REB-C-infected CEF. The prominent phosphotyrosyl protein detected at 85 to 95 kDa in REB-C-infected CEF is *v-ErbB*. Because of the mobility of *v-ErbB* in REB-C-infected CEF, a comparison cannot be made in Fig. 2B between the 95-kDa band in REB-S3-transformed CEF (lane 4) and potentially corresponding bands in REB-C-infected CEF (lane 3).

Tissue-specific patterns of phosphotyrosyl proteins in *v-erbB*-transformed fibroblasts, erythroblasts, and endothelial cells. As illustrated by the antiphosphotyrosine Western blot analysis in Fig. 2B, the patterns of tyrosine-phosphorylated proteins in the different cell types transformed by *v-erbB* show qualitative and quantitative differences (Fig. 2B, lanes 4 to 6). Two patterns emerge: a fibroblast-specific pattern (Fig. 2B, lane 4) and an erythroblast/endothelial cell-specific pattern (Fig. 2B, lanes 5 and 6). Apart from tyrosine-phosphorylated *v-ErbB* migrating at 63 to 75 kDa, the major phosphotyrosyl protein band in *v-erbB*-transformed fibroblasts is a broad band migrating at 110 to 120 kDa (Fig. 2B, lane 4). In contrast, in *v-erbB*-transformed erythroblasts and endothelial cells, the most prominent tyrosine-phosphorylated proteins migrate at 52 and 46 kDa (Fig. 2B, lanes 5 and 6). Further comparison reveals that there are no tyrosine-phosphorylated proteins at 170 and 158 kDa in the erythroblasts or endothelial cells (Fig. 2B; compare lanes 5 and 6 with lane 4).

To further illustrate the tissue-specific nature of these phosphotyrosyl protein patterns, different *v-erbB* mutants transforming the same cell type and the same *v-erbB* mutant transforming different cell types were analyzed by antiphosphotyrosine Western blotting. Figure 3A shows identical phosphotyrosyl protein patterns (apart from the differences in migration of the two tyrosine-phosphorylated *v-erbB* products) in CEF transformed by two different *v-erbB* mutants (AEV-R and REB-S3). In contrast, Fig. 3B shows tissue-specific phospho-

FIG. 3. Tissue-specific patterns of phosphotyrosyl proteins in *v-erbB*-transformed cells. Panel A shows the fibroblast-specific pattern; panel B contrasts the fibroblast and erythroblast (erythro)-specific patterns. In each lane, 100 μ g of lysate was separated by SDS-PAGE (8% acrylamide gel), transferred to nitrocellulose, blotted with antiphosphotyrosine monoclonal antibody 4G10, and detected with an HRP-conjugated anti-mouse secondary antibody and ECL. Sizes are indicated in kilodaltons.

tyrosyl protein patterns in fibroblasts and erythroblasts transformed by the same *v-erbB* mutant (AEV-R).

Identification of phosphotyrosyl proteins in *v-erbB*-transformed cells. (i) p120^{cas} is a major tyrosine-phosphorylated protein in *v-erbB*-transformed fibroblasts. Since the phosphotyrosyl protein pattern in REB-S3-transformed CEF was only subtly different from the pattern in nontransformed REB-C-infected CEF (Fig. 2B, lanes 3 and 4), we sought to identify individual phosphotyrosyl proteins and analyze them for transformation-specific tyrosine phosphorylation. In an effort to identify specific phosphotyrosyl proteins in the broad protein band migrating at 110 to 120 kDa in REB-S3-transformed CEF, we used antibodies to known tyrosine-phosphorylated substrates migrating in this molecular mass range. One such phosphotyrosyl protein is the tyrosine kinase substrate referred to as p120^{cas} (see Discussion) (13, 28, 29). Figure 4A shows that p120^{cas} is expressed at approximately equivalent levels in CEF, REB-C-infected CEF, and REB-S3-transformed CEF (lanes 1 to 3) but is not expressed in *v-erbB*-transformed eryth-

FIG. 4. Expression and tyrosine phosphorylation status of p120^{cas} in *v-erbB*-transformed fibroblasts, erythroblasts, and endothelial cells. Equal amounts of cell lysates were immunoprecipitated with anti-p120^{cas} and Western blotted as follows. (A) Blotted with anti-p120^{cas} and detected with ¹²⁵I-labeled anti-mouse immunoglobulin G. Lanes: 1, CEF; 2, REB-C-infected CEF; 3, REB-S3-transformed CEF; 4, AEV-R-transformed erythroblasts; 5, REB-S3-transformed endothelial cells. (B) Blotted with antiphosphotyrosine antibody and detected with ¹²⁵I-protein A. Lanes: 1, CEF; 2, REB-C-infected CEF; 3, REB-S3-transformed CEF; 4, AEV-R-transformed erythroblasts. Arrowheads indicate p120^{cas}.

FIG. 5. Expression and tyrosine phosphorylation of SHC in *v-erbB*-transformed erythroblasts (AEV-R erythroblasts). Lanes: 1, 100 μ g of whole cell lysate, blotted with antiphosphotyrosine antibody; 2, immunoprecipitation with polyclonal anti-SHC antibody followed by Western blotting with anti-SHC antibody; 3, immunoprecipitation with anti-SHC antibody followed by Western blotting with antiphosphotyrosine antibody; 4, whole cell lysate precleared with anti-SHC antibody followed by Western blotting with antiphosphotyrosine antibody. ¹²⁵I-protein A was used as the detecting antibody; immunoglobulin G heavy chains migrate at $\sim$ 50 kDa, bind ¹²⁵I-protein A, and obscure p46^{shc} in the immunoprecipitation lanes.

roblasts or endothelial cells (lanes 4 and 5). On the basis of this expression pattern, it should be noted that the phosphotyrosyl protein migrating at approximately 120 kDa on the antiphosphotyrosine Western blots of erythroblasts and endothelial cells (Fig. 2B, lanes 5 and 6) is not p120^{cas}. The antiphosphotyrosine Western blot illustrated in Fig. 4B demonstrates that p120^{cas} is not tyrosine phosphorylated in uninfected CEF (lane 1) but is tyrosine phosphorylated in REB-S3-transformed CEF (lane 3) and in AEV-R-transformed CEF (data not shown). p120^{cas} is also tyrosine phosphorylated in nontransformed REB-C-infected CEF (lane 2). It is interesting, however, that the migration of p120^{cas} (Fig. 4A) and the intensity of tyrosine phosphorylation of p120^{cas} (Fig. 4B) are distinct when REB-C-infected CEF are compared with REB-S3-transformed CEF. Specifically, p120^{cas} in REB-S3-transformed CEF consistently migrates more slowly (Fig. 4A, lane 3) and is more intensely tyrosine phosphorylated (Fig. 4B, lane 3) compared with p120^{cas} in REB-C-infected CEF. This observation could be explained by hyperphosphorylation of p120^{cas} and/or by phosphorylation of additional tyrosine residues on p120^{cas} in the transformed fibroblasts. Preliminary experiments using V-8 protease digestion of p120^{cas} and phosphotyrosine peptide immunoprecipitation suggest that at least one additional tyrosine phosphorylation site may become phosphorylated on p120^{cas} in REB-S3-transformed fibroblasts (data not shown).

(ii) SHC is a tyrosine-phosphorylated protein in *v-erbB*-transformed fibroblasts, erythroblasts, and endothelial cells. There are tyrosine-phosphorylated proteins found in *v-erbB*-transformed fibroblasts, erythroblasts, and endothelial cells which migrate at 46 and 52 kDa (Fig. 2B, lanes 4 to 6). We have determined that the 46- and 52-kDa proteins in these *v-erbB*-transformed cells correspond to two of the proteins encoded by the *shc* gene (24). Figure 5 shows that p46^{shc} and p52^{shc} are expressed in *v-erbB*-transformed erythroblasts (AEV-R erythroblasts; Fig. 5, lane 2), are tyrosine phosphorylated (Fig. 5, lane 3), and comigrate with the 52- and 46-kDa phosphotyrosyl proteins in the whole cell lysates (Fig. 5, lane 1). When SHC is precleared from the AEV-R-transformed erythroblast lysate by immunoprecipitation, the tyrosine-phosphorylated bands at 52 and 46 kDa are no longer detectable (Fig. 5, lane 4), indicating that the SHC proteins are the only tyrosine-phosphorylated proteins migrating at 52 and 46 kDa in these transformed erythroblasts. It is also worth noting that we have found no

FIG. 6. Expression of the signal adapter proteins SHC and GRB-2 in 100 μ g of cell lysate of three *v-erbB*-transformed cell types. (A) Cell lysates were blotted with polyclonal anti-SHC antibody and detected with HRP-conjugated protein A and ECL except that lane 5 was detected with ¹²⁵I-protein A. (B) Cell lysates were blotted with monoclonal anti-GRB-2 antibody and detected with HRP-conjugated anti-mouse antibody and ECL. Lanes: 1, CEF; 2, REB-C-infected CEF; 3, REB-S3-transformed CEF; 4, AEV-R-transformed erythroblasts; 5, REB-S3-transformed endothelial cells.

evidence (from immunoprecipitation and Western blot analyses) that *v-ErbB* coimmunoprecipitates with SHC in AEV-R-transformed erythroblasts (data not shown).

Expression of the adapter proteins SHC and GRB-2 in three *v-erbB*-transformed cell types. To investigate whether the specificity of *v-erbB*-mediated transformation is dependent on tissue-specific differences at the level of expression of known signal transduction molecules, we compared the levels of SHC and GRB-2 in cell lysates of *v-erbB*-transformed fibroblasts, erythroblasts, and endothelial cells. Figure 6A shows that the levels of p52^{shc} expression, as detected by immunoblotting, are similar in fibroblasts (uninfected CEF, REB-C-infected CEF, and REB-S3-transformed CEF) and endothelial cells and are severalfold higher in erythroblasts when normalized to total protein levels for all cell lysates. p46^{shc} is expressed in a pattern similar to that for p52^{shc} but at markedly lower levels. p66^{shc} is not expressed at all in any of these avian cells. Similarly, the levels of GRB-2 expression are approximately equivalent in fibroblasts and endothelial cells and are consistently severalfold higher in erythroblasts (Fig. 6B).

Selective tyrosine phosphorylation of SHC proteins in *v-erbB*-transformed and nontransformed cells. Since SHC proteins are expressed in all three tissues of interest, we proceeded to compare the tyrosine phosphorylation patterns of SHC in *v-erbB*-transformed and nontransformed cells. Figure 7A shows that neither p52^{shc} nor p46^{shc} is tyrosine phosphorylated in uninfected CEF (lane 1). The p52^{shc} protein is, however, tyrosine phosphorylated in REB-S3-transformed CEF, AEV-R-transformed erythroblasts (Fig. 7A, lanes 3 and 4, respectively), and AEV-R-transformed CEF (data not shown). Similarly, p46^{shc} is tyrosine phosphorylated in these *v-erbB*-

FIG. 7. Tyrosine phosphorylation of SHC and signal protein complex formation in *v-erbB*-transformed cells. (A) Immunoprecipitation of equal amounts of lysate with anti-SHC antibody followed by Western blotting with monoclonal antiphosphotyrosine antibody; detected with HRP-conjugated anti-mouse and ECL. Lanes: 1, CEF; 2, REB-C-infected CEF; 3, REB-S3-transformed CEF; 4, AEV-R-transformed erythroblasts. The arrowhead indicates a 75-kDa phosphotyrosyl coimmunoprecipitating protein. (B) Immunoprecipitation of equal amounts of lysate with anti-SHC antibody followed by Western blotting with monoclonal anti-GRB-2 antibody; detected with HRP-conjugated anti-mouse and ECL. Lanes are as in panel A. (C) Coimmunoprecipitation of a 75-kDa tyrosine-phosphorylated protein (arrowhead) with anti-SHC antibody in REB-S3-transformed CEF. Lanes: 1, anti-ErbB Western blot of REB-S3-transformed CEF; 2 and 3, immunoprecipitation of REB-S3-transformed CEF with anti-SHC antibody followed by blotting with either anti-ErbB (lane 2; immunoglobulin G heavy chains migrate in a broad band at ~50 kDa) or with an antiphosphotyrosine monoclonal antibody (lane 3).

transformed cell types (Fig. 7A, lanes 3 and 4, and data not shown). In fact, considering the significantly lower level of p46^{shc} than of p52^{shc} expressed in these cells (Fig. 6A) and the marked intensity of the tyrosine-phosphorylated p46^{shc} signal (Fig. 7A, lanes 3 and 4), p46^{shc} appears to be hyperphosphorylated in *v-erbB*-transformed fibroblasts and erythroblasts compared with p52^{shc}. In contrast, the tyrosine phosphorylation pattern of SHC proteins in REB-C-infected CEF is strikingly different (Fig. 7A, lane 2). In these nontransformed, *v-ErbB*-expressing fibroblasts, p52^{shc} is tyrosine phosphorylated to a variable but always lesser degree than in *v-erbB*-transformed fibroblasts and erythroblasts. Furthermore, p46^{shc} is not tyrosine phosphorylated at all in REB-C-infected CEF

(Fig. 7A, lane 2). It should be noted that the level of tyrosine phosphorylation of p52^{shc} in REB-C-infected CEF is at the lower limit of detectability by direct Western blotting techniques and at times could not be detected at all (as in Fig. 2B, lane 3). Repeated experiments using antibodies to SHC to immunoprecipitate the protein followed by antiphosphotyrosine Western blotting (as in Fig. 7A) consistently revealed the quantitative and qualitative differences in SHC phosphorylation patterns described here.

Signal protein complex formation in *v-erbB*-transformed cells. To investigate the possibility of complex formation between proteins in *v-erbB*-transformed cells, we looked for interactions between SHC and the adapter protein GRB-2. Figure 7B shows that GRB-2 coimmunoprecipitates with SHC in *v-erbB*-transformed fibroblasts and erythroblasts and to a lesser extent in REB-C-infected CEF. In addition, we have consistently observed a protein of approximately 75 kDa which coimmunoprecipitates with anti-SHC antibodies exclusively in REB-S3-transformed CEF (Fig. 7A, lane 3, arrowhead). This 75-kDa phosphotyrosyl protein is not detectable by using an antibody to either the kinase or C-terminal domain of c-ErbB1 (Fig. 7C, lane 2), and its mobility (Fig. 7C, lane 3, arrowhead) is consistently slower than the mobility of the S3-v-ErbB protein (Fig. 7C, lane 1). This 75-kDa protein is also not detectable with antibodies to potential candidate phosphotyrosyl proteins in this molecular mass range (Raf, 74 kDa; ezrin, 75 to 80 kDa; paxillin, 68 to 75 kDa [data not shown]). Thus, this phosphotyrosyl protein which coimmunoprecipitates with anti-SHC antibodies only in the presence of tyrosine-phosphorylated p46^{shc} and only in S3-v-ErbB-transformed fibroblasts remains unidentified.

DISCUSSION

We have recently demonstrated that tyrosine kinase activity is essential for *v-erbB*-mediated tumorigenicity (4). We have now used various *v-ErbB* tyrosine kinase oncoproteins (Fig. 1) to study both tissue-specific and transformation-specific tyrosine phosphorylation events. What has emerged from these studies is the identification of two patterns of tyrosine-phosphorylated proteins in *v-erbB*-transformed cells: a fibroblast-specific pattern containing unique phosphotyrosyl proteins of 170 kDa (the c-ErbB1 receptor), 158 kDa (unknown), and 120 kDa (the catenin-like protein p120^{cas}) (28) and an erythroblast/endothelial cell-specific pattern containing an unidentified phosphotyrosyl protein of 120 kDa. The use of transforming *v-erbB* mutants expressed in more than one tissue type has allowed us to show that these phosphotyrosyl protein patterns are indeed tissue specific and not *v-erbB* mutant specific. This is illustrated by comparison of phosphotyrosyl protein patterns in AEV-R-transformed fibroblasts and erythroblasts (Fig. 3B) and REB-S3-transformed fibroblasts and endothelial cells (Fig. 2B, lanes 4 and 6). The presence of these tissue-specific phosphotyrosyl protein patterns implies that the *v-ErbB* protein can couple to multiple tissue-specific signaling pathways. There is evidence that other tyrosine kinase growth factor receptors may also couple to multiple signaling pathways, depending on the specific cellular environment; for example, the platelet-derived growth factor receptor can activate Ras via different cell-specific signaling pathways (34), and activation of fetal liver kinase 2 causes cell-specific differences in tyrosine phosphorylation of the p85 subunit of phosphatidylinositol 3'-kinase and SHC (6).

To gain an understanding of the signaling specificity required for tissue-specific *v-ErbB*-mediated transformation, we sought to identify individual phosphotyrosyl proteins. It is gen-

erally believed that tyrosine phosphorylation of proteins by tyrosine kinase growth factor receptors plays an essential role in the transformation process (2, 40). In the avian *v-erbB* system, however, there has been a dearth of information on the identity of specific phosphotyrosyl proteins, and there have been no reports of transformation-specific tyrosine phosphorylation events. The major phosphotyrosyl proteins identified in *v-erbB*-transformed CEF, thus far, have been subunits of the fibronectin receptor (140 and 120 kDa) (10) and p42 mitogen-activated protein kinase (9, 32, 36). The signal adapter protein SHC has recently been shown to be tyrosine phosphorylated in *v-erbB*-transformed fibroblasts and erythroblasts (21, 36). Here, we report that the catenin-like protein p120^{cas} is tyrosine phosphorylated in *v-erbB*-transformed CEF, and we expand the spectrum of *v-erbB*-transformed cells in which SHC is tyrosine phosphorylated to include endothelial cells. Furthermore, the availability of CEF expressing equivalent levels of transforming and nontransforming *v-ErbB* proteins (i.e., REB-S3-transformed and REB-C-infected CEF) has enabled us to look for transformation-specific tyrosine phosphorylation events. It is clear from our results that there are transformation-specific differences in the tyrosine phosphorylation of p120^{cas} and SHC. Specifically, in *v-erbB*-transformed CEF, there is a distinct phosphorylation difference and mobility shift in p120^{cas} and a differential phosphorylation of the 46- and 52-kDa species of SHC.

The protein p120^{cas} is a tyrosine kinase substrate localized to cell-cell junctions (13, 23, 29) and was recently identified as a catenin-type protein, hence its designation as Cas (cadherin-associated Src substrate) (28). Our studies show that p120^{cas} is a tissue-specific tyrosine-phosphorylated substrate in *v-erbB*-transformed fibroblasts. Furthermore, studies comparing REB-C-infected CEF with REB-S3-transformed CEF indicate that the phosphorylation of p120^{cas} occurs in a transformation-specific manner. In REB-C-infected fibroblasts, in which *v-ErbB* possesses an active kinase but no ability to transform fibroblasts, there is phosphorylation of p120^{cas}. It is important to note, however, the consistently greater intensity of the phosphotyrosyl signal and the slower mobility of p120^{cas} in REB-S3-transformed CEF than in nontransformed REB-C-infected CEF (Fig. 4). This p120^{cas} mobility shift has also been seen in *v-src*-transformed CEF (13). The data presented here, as well as our preliminary phosphopeptide mapping data, suggest that p120^{cas} becomes phosphorylated on at least one additional tyrosine site in REB-S3-transformed CEF. Therefore, the site and/or the degree of tyrosine phosphorylation of the catenin protein p120^{cas} may be correlated with *v-erbB*-mediated transformation.

The second phosphotyrosyl protein identified in our studies is SHC. The gene for SHC encodes protein products migrating at 66, 52, and 46 kDa (24). Because of its SH2 domain and its ability to associate with growth factor receptors and GRB-2, SHC is thought to act as an adapter molecule that couples tyrosine kinases to downstream signaling molecules, specifically in the *ras* signaling pathway (33). In this study, we show that two of these SHC proteins, p46^{shc} and p52^{shc}, are differentially tyrosine phosphorylated in transformed and nontransformed *v-erbB*-infected fibroblasts. The tyrosine phosphorylation of p52^{shc} occurs in nontransformed REB-C-infected CEF as well as in REB-S3-transformed CEF and thus is not transformation specific (Fig. 7A, lanes 2 and 3, respectively). In contrast, p46^{shc} is tyrosine phosphorylated in a transformation-specific manner (Fig. 7A, lanes 2 and 3) and appears to be hyperphosphorylated compared with p52^{shc} (as judged from expression levels and phosphotyrosyl signal intensities of the two SHC species; Fig. 6A and 7A, respectively). Although it

has been reported that p46^{shc} and p52^{shc} are produced by alternative usage of translation initiation sites (24), there is currently no information regarding the possible functional significance of the expression of alternate forms of SHC, let alone an explanation for the differential phosphorylation of these alternate SHC species.

From our observations in fibroblasts of the contrasting patterns of tyrosine phosphorylation of p52^{shc} versus p46^{shc} and the coimmunoprecipitation of a novel 75-kDa phosphotyrosyl protein only in the presence of tyrosine-phosphorylated p46^{shc}, we would hypothesize that these alternate SHC species interact differently with tyrosine kinases and other elements of the signal transduction machinery active in *v-erbB*-transformed fibroblasts. It has recently been postulated that receptor tyrosine kinase signaling specificity may be related to the interaction between SH2 domains and specific sequence motifs surrounding tyrosine-phosphorylated residues (37). Usage of the putative translation initiation site ATG218 to produce p46^{shc} reportedly truncates the N terminus of SHC by 45 amino acids (compared with p52^{shc}) (24). Since this 45-amino-acid sequence contains neither tyrosine residues nor SH2 or SH3 motifs, our data suggest that this region of the SHC protein may be an additional protein motif important in signal transduction pathways used during *v-erbB*-mediated transformation. The expression of alternate SHC translation products in *v-erbB*-transformed cells may represent a novel mechanism whereby structural variations in an adapter protein impart specificity to a signaling complex. Specifically, we would propose that a signaling complex including tyrosine-phosphorylated p46^{shc}, GRB-2, and the phosphotyrosyl protein p75 plays an important role in S3-*v-erbB*-mediated transformation in fibroblasts.

In conclusion, the ability of mutant avian *c-erbB1* products to transform three separate cell types, fibroblasts, erythroblasts, and endothelial cells, has allowed us to begin to explore fundamental aspects of signal transduction by this receptor tyrosine kinase. This study shows that there are tissue-specific patterns of tyrosine phosphorylated proteins in *v-erbB*-transformed cells, implying that *v-ErbB* can couple to multiple, tissue-specific signaling pathways. Furthermore, we have identified p120^{cas} as a tissue-specific phosphotyrosyl protein and have described transformation-specific tyrosine phosphorylation events involving p120^{cas}, SHC, and a novel 75-kDa phosphotyrosyl protein. Together, these results show that the *v-ErbB* oncoprotein's ability to couple to multiple signaling pathways, the tissue-specific availability of tyrosine kinase substrates, and subtle differences in tyrosine phosphorylation events may all play an important role in signal transduction during *v-erbB*-mediated transformation.

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REFERENCES

1. Beug, H., G. Doederlein, C. Freudenstein, and T. Graf. 1982. Erythroblast cell lines transformed by a temperature-sensitive mutant of avian erythroblastosis virus: a model system to study erythroid differentiation in vitro. *J. Cell. Physiol. Suppl.* 1:195-207.
2. Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell* 64:281-302.
3. Choi, O. R., C. Trainor, T. Graf, H. Beug, and J. D. Engel. 1986. A single amino acid substitution in *v-erbB* confers a thermolabile phenotype to ts167 avian erythroblastosis virus-transformed erythroid cells. *Mol. Cell. Biol.* 6:1751-1759.
4. Connolly, D. C., S. L. Toutenhoofd, and N. J. Maible. 1994. Tyrosine kinase activity may be necessary but is not sufficient for *c-erbB1*-mediated tumorigenicity. *J. Virol.* 68:6804-6810.
5. DiFiore, P. P., J. H. Pierce, T. P. Fleming, R. Hazan, A. Ullrich, C. R. King, J. Schlessinger, and S. A. Aaronson. 1987. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. *Cell* 51:1063-1070.
6. Dosil, M., S. Wang, and I. R. Lemischka. 1993. Mitogenic signalling and substrate specificity of the FLK2/FLT3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells. *Mol. Cell. Biol.* 13:6572-6585.
7. Downward, J., Y. Yarden, E. Mayers, G. Scrase, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield. 1984. Close similarity of epidermal growth factor receptor and *v-erbB* oncogene protein sequence. *Nature (London)* 307:521-527.
8. Fung, Y. K. T., W. G. Lewis, L. B. Crittenden, and H. J. Kung. 1983. Activation of the cellular oncogene *c-erbB* by LTR insertion: molecular basis for induction of erythroblastosis by avian leukosis virus. *Cell* 33:357-368.
9. Gilmore, T., J. E. DeClue, and G. M. Martin. 1985. Phosphorylation at tyrosine is induced by the *v-erbB* gene product in vivo and in vitro. *Cell* 40:609-618.
10. Hirst, R., A. Horwitz, C. Buck, and L. Rohrschneider. 1986. Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 83:6470-6474.
11. Hughes, S. H., J. J. Greenhouse, C. J. Petropoulos, and P. Sutcliffe. 1987. Adapter plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* 61:3004-3012.
12. Kamps, M. P., and B. M. Sefton. 1988. Identification of multiple novel polypeptide substrates of the *v-src*, *v-yes*, *v-fps*, *v-ros* and *v-erbB* oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. *Oncogene* 2:305-315.
13. Kanner, S. B., A. B. Reynolds, and J. T. Parsons. 1991. Tyrosine phosphorylation of a 120-kilodalton pp60^{src} substrate upon epidermal growth factor and platelet-derived growth factor stimulation and in polyomavirus middle-T-antigen-transformed cells. *Mol. Cell. Biol.* 11:713-720.
14. Kanner, S. B., A. B. Reynolds, R. R. Vines, and J. T. Parsons. 1990. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 87:3328-3332.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
16. Lowenstein, E., R. Daly, A. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, E. Skolnik, D. Bar-Sagi, and J. Schlessinger. 1992. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* 70:431-442.
17. Maible, N. J., and H. J. Kung. 1988. *c-erbB* and the epidermal growth-factor receptor: a molecule with dual identity. *Biochim. Biophys. Acta* 948:287-304.
18. Maible, N. J., M. A. Raines, T. W. Flickinger, and H. J. Kung. 1988. Proviral insertional activation of *c-erbB*: differential processing of the protein products arising from two alternate transcripts. *Mol. Cell. Biol.* 8:4868-4876.
19. Maible, N. J., and B. H. Satir. 1986. Identification of a biochemical marker for the secretory pathway in *Tetrahymena thermophila*. *J. Cell. Biochem.* 31:195-202.
20. McCune, B. K., and H. S. Earp. 1989. The epidermal growth factor receptor tyrosine kinase in liver epithelial cells. *J. Biol. Chem.* 264:15501-15507.
21. Meyer, S., K. LaBudda, J. McGlade, and M. J. Hayman. 1994. Analysis of the role of the SHC and GRB2 proteins in signal transduction by the *v-ErbB* protein. *Mol. Cell. Biol.* 14:3253-3262.
22. Nilsen, T. W., P. A. Maroney, R. G. Goodwin, F. M. Rottman, L. B. Crittenden, M. B. Raines, and H. J. Kung. 1985. *c-erbB* activation in ALV induced erythroblastosis: novel RNA processing and promoter-insertion result in the expression of an amino-truncated EGF receptor. *Cell* 41:719-726.
23. Peifer, M., S. Berg, and A. B. Reynolds. 1994. A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell* 76:789-791.
24. Pelicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Fornì, I. Nicoletti, F. Grignani, T. Pawson, and P. G. Pelicci. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* 70:93-104.
25. Pelley, R. J., C. Moscovici, S. Hughes, and H. J. Kung. 1988. Proviral-activated *c-erbB* is leukemogenic but not sarcomagenic: characterization of a replication-competent retrovirus containing the activated *c-erbB*. *J. Virol.* 62:1840-1844.
26. Raines, M. A., N. J. Maible, C. Moscovici, L. Crittenden, and H. J. Kung. 1988. Mechanism of *c-erbB* transduction: newly released transducing viruses retain poly(A) tracts of *erbB* transcripts and encode C-terminally intact *erbB* proteins. *J. Virol.* 62:2437-2443.

27. Raines, M. A., N. J. Maihle, C. Moscovici, M. G. Moscovici, and H. J. Kung. 1988. Molecular characterization of three *erbB* transducing viruses generated during avian leukosis virus-induced erythroleukemia: extensive internal deletion near the kinase domain activates the fibrosarcoma- and hemangioma-inducing potentials of *erbB*. *J. Virol.* **62**:2444–2452.
28. Reynolds, A. B., J. Daniel, P. D. McCrea, M. J. Wheelock, J. Wu, and Z. Zhang. 1994. Identification of a new catenin: the tyrosine kinase substrate p120^{cas} associates with E-cadherin complexes. *Mol. Cell. Biol.* **14**:8333–8342.
29. Reynolds, A. B., D. J. Roesel, S. B. Kanner, and J. T. Parsons. 1989. Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular *src* gene. *Mol. Cell. Biol.* **9**:629–638.
30. Riedel, H., S. Massaglia, J. Schlessinger, and A. Ullrich. 1988. Ligand activation of overexpressed epidermal growth factor receptors transforms NIH 3T3 mouse fibroblasts. *Proc. Natl. Acad. Sci. USA* **85**:1477–1481.
31. Robinson, H. L., S. E. Tracy, N. Nair, C. Taglienti-Sian, and D. C. Gamett. 1992. Characterization of an angiosarcoma-inducing mutation in the *erbB* oncogene. *Oncogene* **7**:2025–2030.
32. Rossomando, A. J., D. M. Payne, M. J. Weber, and T. W. Sturgill. 1989. Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. *Proc. Natl. Acad. Sci. USA* **86**:6940–6943.
33. Rozakis-Adcock, M., J. McGlade, G. Mbamalu, G. Pelicci, R. Daly, W. Li, A. Batzer, S. Thomas, J. Brugge, P. G. Pelicci, J. Schlessinger, and T. Pawson. 1992. Association of the SHC and GRB2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature (London)* **360**:689–692.
34. Satoh, T., W. J. Fantl, J. A. Escobedo, L. T. Williams, and Y. Kaziro. 1993. Platelet-derived growth factor receptor mediates activation of RAS through different signaling pathways in different cell types. *Mol. Cell. Biol.* **13**:3706–3713.
35. Sealy, L., M. L. Privalsky, G. Moscovici, C. Moscovici, and J. M. Bishop. 1983. Site-specific mutagenesis of avian erythroblastosis virus: *erb-B* is required for oncogenicity. *Virology* **130**:155–178.
36. Shu, H. K., C. M. Chang, L. Ravi, L. Ling, C. M. Castellano, E. Walter, R. J. Pelley, and H. J. Kung. 1994. Modulation of *erbB* kinase activity and oncogenic potential by single point mutations in the glycine loop of the catalytic domain. *Mol. Cell. Biol.* **14**:6868–6878.
37. Songyang, Z., S. E. Shoelson, J. McGlade, P. Olivier, T. Pawson, X. R. Bustelo, M. Barbacid, H. Sabe, H. Hanafusa, T. Yi, R. Ren, D. Baltimore, S. Ratnoffsky, R. A. Feldman, and L. C. Cantley. 1994. Specific motifs recognized by the SH2 domains of Csk, 3BP2, Fps/Fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol. Cell. Biol.* **14**:2777–2785.
38. Taglienti-Sian, C. A., B. Banner, R. J. Davis, and H. L. Robinson. 1993. Induction of renal adenocarcinoma by a nonmutated *erbB* oncogene. *J. Virol.* **67**:1132–1136.
39. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
40. Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**:203–212.
41. Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of mammalian cells with genes from prokaryotes and leukocytes. *Cell* **16**:777–785.