Analysis of the Role of the Shc and Grb2 Proteins in Signal Transduction by the v-ErbB Protein

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The epidermal growth factor receptor, EGFR, has been implicated in cell transformation in both mammalian and avian species. The v-ErbB oncoprotein is an oncogenic form of the chicken EGFR. The tyrosine kinase activity of this oncoprotein is required for transformation, but no transformation-specific cellular substrates have been described to date. Recently activation of the *ras* signal transduction pathway by the EGFR has been shown to involve the Shc and Grb2 proteins. In this communication, we demonstrate that the Shc proteins are phosphorylated on tyrosine residues and are complexed with Grb2 and the chicken EGFR following ligand activation of this receptor. In fibroblasts and erythroid cells transformed by the avian erythroblastosis virus (AEV) strains H and ES4, the Shc proteins are found to be constitutively phosphorylated on tyrosine residues. The tyrosine-phosphorylated forms of the AEV strain H v-ErbB protein are found in a complex with Shc and Grb2, but the Shc proteins do not bind to the AEV strain ES4 v-ErbB protein. Mutant forms of the v-ErbB protein (in which several of the tyrosines that become autophosphorylated have been deleted by truncation) are unable to transform erythroid cells but can still transform fibroblasts. Analysis of cells transformed by one of these mutants revealed that the truncated v-ErbB protein could no longer bind to either Shc or Grb2, but this oncoprotein still gave rise to tyrosine-phosphorylated Shc proteins that complexed with Grb2 and led to activation of mitogen-activated protein (MAP) kinase. The results suggest that stable binding of Grb2 and Shc to the v-ErbB protein is not necessary to activate this signal transduction pathway and assuming that the mutants activate MAP kinase in erythroid cells in a manner similar to that of fibroblasts, that activation of this pathway is not sufficient to transform erythroid cells.

The epidermal growth factor receptor, EGFR, has been implicated in oncogenesis in both mammalian and avian species (for a review, see reference 14). In humans, amplification of the EGFR gene has been found in sarcomas, squamous cell carcinomas of the head and neck, and glioblastomas (13, 27, 28, 35), and the primary glioblastomas have frequently been found to contain rearranged as well as amplified EGFR genes (12, 27). In chickens, the oncogene *v-erbB* is responsible for the diseases induced by avian erythroblastosis viruses (AEV). The v-ErbB oncoprotein was shown to be homologous to the chicken equivalent of the EGFR (10). However, v-ErbB had sustained amino- and carboxy-terminal deletions as well as point mutations (for a review, see reference 14). Interestingly these truncations are very similar in structure to some of those seen in the human glioblastomas (12). The cell biology of the avian system is more amenable to genetic analysis than the human cell systems in which the EGFR has been implicated in tumorigenesis. Thus, most mechanistic studies addressing cell transformation have been performed with the *v-erbB* gene. Mutational analysis revealed that the tyrosine kinase activity of the v-ErbB oncoprotein was essential for cell transformation (33). Further studies localized important functions for erythroid cell transformation to the region located between the end of the kinase domain and the carboxyl terminus of the v-ErbB protein. For example, 3'-terminal truncations in the *v-erbB* gene from AEV strain ES4 (AEV-ES4) and AEV strain H (AEV-H) severely affected the capacity of the viruses to

transform erythroblasts, but these mutated forms of the v-ErbB oncoprotein still transform fibroblasts (8, 18, 41, 54).

Studies on the human EGFR identified a role for this carboxy-terminal domain in signal transduction. This domain contains the tyrosine residues that are autophosphorylated upon ligand binding (9). These phosphorylated tyrosine residues are responsible for the protein-protein interactions between the activated receptor and several cellular proteins, such as phospholipase C- γ , phosphatidylinositol 3-kinase, and the p21^{ras} GTPase-activating protein, GAP, that become phosphorylated upon tyrosine residues (1, 17, 29, 31, 47). All of these proteins contain Src homology 2 (SH2) domains, which bind to specific tyrosine-phosphorylated sites on other proteins such as the EGFR (20, 36). Thus, SH2 domains are responsible for the protein-protein interactions involved in the initiation of the signal transduction pathway. Since the v-ErbB proteins are constitutively autophosphorylated and retain at least three of these tyrosine residues (14), this region in the v-ErbB oncoprotein is a candidate for being involved in the interactions with protein substrates containing SH2 domains that could play important roles in cell transformation. However, to date, no SH2 domain-containing protein which is constitutively phosphorylated on tyrosine residues in AEV-transformed cells has been identified. Thus, while this carboxy-terminal region of v-ErbB is critical for erythroid transformation, the transformation-relevant substrates remain to be identified.

Recently the Shc proteins have been described as SH2 domain-containing proteins which interact with various growth factor receptors, including the EGFR, and nontransmembrane tyrosine kinases, such as v-Src and v-Fps (32, 38, 40, 43). Overexpression of the *shc* gene results in the malignant transformation of NIH 3T3 cells and induces *ras*-dependent

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neurite extensions in the PC12 pheochromocytoma neuronal cell line (38, 43). The *shc* gene encodes three proteins, p46^{shc}, p52^{shc}, and p66^{shc}, which are ubiquitously expressed, although the expression level of p66^{shc} varies from cell type to cell type (38). Activation of various growth factor receptor tyrosine kinases leads to the tyrosine phosphorylation of the Shc proteins, and upon tyrosine phosphorylation, the Shc protein binds to the Grb2 protein and the Ras GTP-GDP exchange protein Sos (11). Thus, the activation of the EGFR leads to the formation of a large complex, involving the receptor, the Shc and Grb2 proteins, Sos, and Ras, and this complex is postulated to bring all of these proteins together at the plasma membrane to initiate Ras activation and subsequent activation of mitogen-activated protein (MAP) kinase.

Cell transformation by various cytoplasmic tyrosine kinases has been shown to be dependent on Ras activity (48). Therefore, activation of the Shc proteins by tyrosine phosphorylation and subsequent activation of the *ras* signal pathway by the Grb2 protein is a potential mechanism that could be important for transformation by *v-erbB*. In this communication, we demonstrate that the Shc proteins are constitutively phosphorylated on tyrosine residues in AEV-H- and AEV-ES4-transformed fibroblasts and erythroblasts and are bound to Grb2. However, whereas Shc is bound to the tyrosine-phosphorylated forms of the wild-type (wt) v-ErbB protein of AEV-H, it does not bind to the AEV-ES4 v-ErbB protein. Carboxy-terminal truncations of the tyrosine residues in v-ErbB give rise to v-ErbB oncoproteins that can no longer efficiently transform erythroblasts. Although this form of v-ErbB can no longer bind to either Grb2 or Shc, it can still induce the tyrosine phosphorylation of the Shc proteins, and these proteins are in a complex with Grb2. These data indicate that activation of this signal transduction pathway does not require stable binding between v-ErbB and these proteins and that Ras activation may not be sufficient to induce the transformation of erythroblasts.

MATERIALS AND METHODS

Cell culture and viruses. Chicken embryo fibroblasts (CEF) were grown at 37°C with 7% CO₂ in Dulbecco modified Eagle medium supplemented with 8% fetal calf serum and 2% chicken serum. Primary CEF were obtained from 7- to 8-day-old chicken embryos (Spafas) as described previously (51). CEF overexpressing the chicken transforming growth factor α receptor (TGF- α R) (c-ErbB) protein were kindly provided by H. Beug, Institute of Molecular Pathology, Vienna, Austria. The AEV deletion mutants, *dl48* and *dl82*, were kindly provided by B. Vennstrom, Karolinska Institute, Stockholm, Sweden. The AEV-ES4-transformed erythroblast cell line HD2 has been described previously (4).

Antibodies. The monoclonal antibody 5E2 directed against phosphotyrosine was generously provided by Genentech, San Francisco, Calif. The rabbit anti-v-ErbB antibody kn14 was generated against a 588-bp *Bam*HI fragment encoding the v-ErbB kinase domain as described previously (15). The Shc antiserum directed against the SH2 domain of the Shc protein has been described previously (38). Monoclonal antibody against MAP kinase was kindly provided by M. Weber (University of Virginia, Charlottesville). Monoclonal antibody against the Grb2 protein was purchased from Transduction Laboratories, Lexington, Ky.

Growth factor stimulation. Cells were withdrawn from medium containing growth factor overnight and then stimulated with human TGF- α (10 ng/ml) (Promega) in complete growth medium for various times prior to lysis.

Immunoprecipitation and Western blotting (immunoblotting). All fibroblasts were lysed in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) (pH 8.0)–150 mM NaCl–10% glycerol–1% Triton X-100–0.1% sodium dodecyl sulfate (SDS)–1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)–1.5 mM magnesium chloride–1 mM sodium orthovanadate. Bone marrow cells were lysed in 1% Triton X-100–100 mM NaCl–50 mM Tris (pH 8)–1 mM sodium orthovanadate. Lysates were immunoprecipitated and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then immunoblotted as described previously (6).

In vitro binding studies. The bacterial proteins GST (glutathione *S*-transferase), GST-SHC(SH2) (GST fused to the SH2 domain of Shc), and GST-GRB2 (GST fused to Grb2) were induced and purified by binding to glutathione beads as described previously (38). Bacteria expressing the GST-GRB2 fusion protein were kindly provided by J. Bliska, Stony Brook, N.Y. The various cell lysates were incubated with these beads for 90 min at 4°C. The beads were washed three times in lysis buffer and then boiled for 5 min in SDS sample buffer. The eluted proteins were resolved by SDS-PAGE followed by Western blotting.

RESULTS

Ligand activation of c-ErbB leads to tyrosine phosphorylation of the Shc proteins in both chicken erythroblasts and fibroblasts. The Shc proteins are phosphorylated on tyrosine residues following ligand activation of the EGFR in mammalian cells (38). Therefore, we wanted to determine if activation of the chicken equivalent of the EGFR, that is, the c-ErbB protein, would also lead to tyrosine phosphorylation of the chicken Shc proteins. In addition, we wanted to assay this in the two cell types that are transformed by the v-ErbB oncoprotein, namely, CEF, and erythroblasts. CEF were infected with an avian retrovirus that expresses the c-ErbB protein, generating cells that overexpress this receptor. To determine if this pathway was functioning in response to activation of endogenous levels of receptor, we isolated normal erythroid precursor cells that express the endogenous receptor, TGF- α R (c-ErbB), and proliferate in response to the addition of TGF- α (the chicken receptor is preferentially activated by TGF- α [23]).

CEF that overexpress TGF- α R (c-ErbB), and the normal chicken erythroid cells were treated with TGF- α for various times, and whole-cell lysates were prepared and analyzed by Western blotting with an antiphosphotyrosine monoclonal antibody. As can be seen in Fig. 1A, following the addition of TGF- α , several proteins are phosphorylated on tyrosine residues in a rapid and sustained manner in both cell types. There appear to be proteins that are specific to the different cell types as well as proteins that are shared by the different cell types. In addition to the autophosphorylation of the 170,000-Da TGF- α R (c-ErbB) protein and unknown proteins in the 95- to 100,000-Da, molecular mass range, two prominent bands with approximate molecular masses of 48,000 and 56,000 Da were detected in both cell types. These apparent molecular masses are the same as those reported for the chicken Shc proteins (7).

To determine if these proteins were indeed the Shc proteins, the CEF lysates were immunoprecipitated with various antibodies followed by Western blotting with an antibody against phosphotyrosine. Figure 1B, lane 4, shows that antibodies against the Shc proteins precipitated these two phosphotyrosine-containing proteins from cells treated with TGF- α ,

FIG. 1. Tyrosine phosphorylation induced by TGF- α in both CEF and erythroblasts. (A) TGF- α -dependent erythroblasts or CEF overexpressing the TGF- α R (c-ErbB) protein were withdrawn from medium containing TGF- α for 16 h, and then TGF- α was added back at a concentration of 10 ng/ml for either 0, 1, 5, or 10 min as indicated under the lanes. Whole-cell lysates were prepared, and equal amounts of protein were subjected to SDS-PAGE followed by Western blotting with a monoclonal antibody against phosphotyrosine. The positions of the TGF- α R (c-ErbB) protein migrating at 170,000 Da (170 k) as well as the other shared substrates are indicated by the arrows. The positions (in kilodaltons) of the molecular mass markers are indicated on the left side of the gels in panels A and B. (B) Lysates were prepared from CEF overexpressing the TGF- α R (c-ErbB) protein that had been incubated either in the absence (–) of TGF- α or in the presence (+) of TGF- α (10 ng/ml) for 5 min and subjected to immunoprecipitation with normal rabbit serum (lanes 1), rabbit anti-ErbB serum (lanes 2), rabbit antiphosphotyrosine serum (lanes 3), or rabbit anti-SHC serum (lanes 4). The precipitated proteins were analyzed by SDS-PAGE followed by Western blotting with antiphosphotyrosine antibody. The positions of the TGF- α R (c-ErbB) protein and the two *shc*-encoded proteins are indicated on the right side of the gel.

indicated by the arrows. (Although these two Shc proteins are slightly larger than their mammalian counterparts, for simplicity's sake, we will retain the nomenclature established for the mammalian proteins and refer to them as p46^{shc} and p52^{shc}.) In addition to the p46^{shc} and p52^{shc} proteins, the anti-Shc antibodies coimmunoprecipitated a tyrosine-phosphorylated protein with the same molecular mass as that of the TGF- α R (c-ErbB) protein, (compare lanes 2 and 4 [with TGF- α]).

Previous studies have shown that the mammalian EGFR can be coprecipitated in a complex with the Shc proteins following ligand activation (38). Therefore, the filter used in Fig. 1B was reprobbed with antibodies against ErbB and this demonstrated that this band was indeed the TGF- α R (c-ErbB) protein (data not shown). It is interesting to note that the antibodies against the ErbB kinase domain (Fig. 1B, lane 2), do not coprecipitate the Shc proteins with the TGF- α R (c-erbB) protein. This result

FIG. 2. The Shc proteins are constitutively phosphorylated on tyrosine residues in AEV-H-transformed cells. (A) Whole-cell lysates were prepared from CEF overexpressing the TGF- α R (c-ErbB) protein that had been incubated either in the absence (–) of TGF- α or in the presence (+) of TGF- α (10 ng/ml) for 15 min or from AEV-H-transformed CEF. Equal amounts of protein were analyzed by SDS-PAGE followed by Western blotting with antiphosphotyrosine antibody. The positions of the v-erbB-encoded protein and the p52^{shc} protein are indicated. (B) Lysates prepared from AEV-H-transformed CEF were immunoprecipitated with normal rabbit serum or antiserum against v-ErbB or Shc, as indicated above the lanes. The proteins were analyzed by SDS-PAGE followed by Western blotting with antiphosphotyrosine antibody.

indicates that in this complex the kinase domain of the TGF- α R (c-ErbB) protein is no longer accessible to the antibody. Similar analysis of erythroblast lysates demonstrated that the p46^{shc} and p52^{shc} proteins were also phosphorylated on tyrosine residues in these cells following the addition of TGF- α (data not shown). These data demonstrate that ligand activation of the chicken TGF- α R (c-ErbB) leads to the tyrosine phosphorylation of the Shc proteins in both fibroblasts and erythroblasts.

The Shc proteins are constitutively phosphorylated on tyrosine in both chicken erythroblasts and fibroblasts transformed by v-erbB. Transformation of both CEF and erythroblasts by the v-ErbB oncoprotein is dependent on the constitutive tyrosine kinase activity of this protein. Therefore, we analyzed AEV-transformed CEF and erythroblasts to determine if the Shc proteins were constitutively phosphorylated in v-erbB-transformed cells. CEF transformed by the AEV-H virus were lysed, and the tyrosine-phosphorylated proteins were analyzed by Western blotting. As positive controls, TGF- α R (c-ErbB)-expressing CEF were stimulated with TGF- α and the lysates were analyzed in parallel. The results are shown in Fig. 2. In AEV-H-transformed fibroblasts, tyrosine-phosphorylated proteins with molecular masses identical to those of the p46^{shc} and p52^{shc} proteins could be detected (Fig. 2A, lanes 2 and 3).

To confirm that these proteins were indeed p46^{shc} and p52^{shc}, lysates from the AEV-H-transformed CEF were immunoprecipitated with antisera against v-ErbB and Shc and subjected to Western blotting with antiphosphotyrosine antibodies. Figure 2B shows that in AEV-H-transformed CEF, the Shc proteins are constitutively phosphorylated on tyrosine residues. Analysis of normal CEF failed to detect the presence of any tyrosine-phosphorylated Shc proteins (data not shown).

FIG. 3. Shc is phosphorylated on tyrosine residues in erythroid cells transformed by AEV-H and AEV-ES4 but binds only to the AEV-H ErbB protein. Lysates prepared from AEV-H- or AEV-ES4-transformed erythroid cells were immunoprecipitated with normal rabbit serum (NRS) or antiserum against v-ErbB or Shc as indicated above the lanes. The proteins were analyzed by SDS-PAGE followed by Western blotting with antiphosphotyrosine antibody.

It is interesting to note that the antisera against the Shc proteins also coprecipitated tyrosine-phosphorylated proteins with the molecular masses of the v-ErbB protein(s) (Fig. 2B). Reprobing with antisera against v-ErbB confirmed that these proteins were indeed the v-ErbB oncoprotein(s) (data not shown). Again, the antibodies against the ErbB kinase domain did not coprecipitate the Shc proteins, implying that the kinase domain is blocked from binding to the antibody by the Shc proteins (Fig. 2B). Thus, like the ligand-activated TGF- α R (c-ErbB) protein, the AEV-H v-ErbB protein can also complex with the p46^{shc} and p52^{shc} proteins. These data indicate that the AEV-H v-ErbB oncoprotein induces the constitutive tyrosine phosphorylation of the Shc proteins and forms a complex with these proteins.

We also wanted to determine if the Shc proteins were also phosphorylated on tyrosine residues in erythroid cells transformed by AEV. Thus, lysates were prepared from erythroblasts transformed by AEV-H and AEV-ES4 and immunoprecipitated with antibodies against ErbB or Shc and subjected to Western blotting with antiphosphotyrosine antibodies. Figure 3 demonstrates that the Shc proteins were phosphorylated to similar extents in erythroid cells transformed by both viruses. However, whereas the AEV-H v-ErbB protein coimmunoprecipitated with the Shc protein the AEV-ES4-encoded v-ErbB protein did not, even though the cells contained similar amounts of tyrosine-phosphorylated v-ErbB protein. These results are consistent with the absence of a specific binding site for Shc on the ES4 protein. These data indicate that while v-ErbB can induce the tyrosine phosphorylation of Shc, it does not have to be complexed with the Shc protein to do so.

Ligand-activated c-ErbB and AEV-H v-ErbB bind to the SH2 domain of the Shc proteins. Immunoprecipitation analysis of c-ErbB-overexpressing CEF (Fig. 1B) and AEV-H-transformed CEF and erythroid cells (Fig. 2B and 3) indicated that the Shc proteins were able to bind to the ligand-activated c-ErbB protein and the AEV-H v-ErbB protein. Previous analysis of the mammalian EGFR using bacterially expressed

FIG. 4. In vitro binding of the v-ErbB protein to the SH2 domain of Shc. Lysates from CEF expressing the TGF- α R (c-ErbB) protein that had been stimulated with TGF- α (10 ng/ml) for 5 min or CEF transformed by AEV-H were incubated with either the GST protein or GST-SHC protein attached to beads as described in Materials and Methods. The proteins were eluted from the beads, and bound proteins were identified by SDS-PAGE followed by Western blotting with antiphosphotyrosine antibody.

versions of the Shc protein has demonstrated that it is the SH2 domain of the Shc proteins that is responsible for the binding to the tyrosine-phosphorylated receptor (38) and that the major site for binding on the receptor is the phosphorylated tyrosine at residue 1173 (37). Since this residue is deleted in the v-ErbB oncoprotein encoded by AEV-H (14), we wanted to directly demonstrate the association between c-ErbB, AEV-H v-ErbB, and the Shc proteins. Cell lysates were prepared as described in Materials and Methods from AEV-H-transformed CEF and CEF expressing the c-ErbB protein, TGF- α R CEF. Lysates were incubated with bacterially ex-

pressed GST or GST-SHC(SH2). The proteins that bound to these bacterially expressed proteins were eluted and examined by Western blotting. Figure 4 shows that ligand-activated TGF- α R (c-ErbB) bound to the GST-SHC(SH2) fusion protein but not to GST. Similarly, the tyrosine-phosphorylated AEV-H v-ErbB protein also bound specifically to the GST-SHC(SH2) protein (Fig. 4). Reprobing of the same blot with antibodies against ErbB indicated that no specific binding of the non-tyrosine-phosphorylated v-ErbB proteins could be detected (data not shown).

AEV mutants that cannot transform erythroid cells still induce tyrosine phosphorylation of the Shc proteins. The above data demonstrate that the Shc proteins are constitutively phosphorylated on tyrosine residues in AEV-H- and -ES4-transformed erythroblasts and fibroblasts. Truncation of several of the tyrosines that are autophosphorylation sites within the carboxy terminus of the v-ErbB protein gives rise to AEV mutants that are capable of transforming CEF but are unable to transform erythroblasts. Two such AEV-ES4 mutants are *dl48* and *dl82*, which have deletions of 48 and 82 amino acids, respectively, compared with the AEV-ES4 v-ErbB protein (18), shown schematically in Fig. 5. Therefore, it was of interest to determine if these mutant forms of *v-erbB* led to phosphorylation of the Shc proteins. Erythroblasts must be transformed or stimulated by activation of the TGF- α R (c-ErbB) in order to grow (16). Since these mutants do not transform erythroblasts, it is impossible to test for Shc phosphorylation in erythroid cells. Therefore, we determined if the Shc proteins are phosphorylated on tyrosine in CEF transformed by *dl48* or *dl82*. First, we wanted to check the level of expression of the mutant proteins, since it has been reported that carboxy-terminal deletions give rise to v-ErbB proteins that do not accumulate to the same level that wt v-ErbB does (18). CEF infected with these mutants were lysed and analyzed by Western blotting for the expression of the *v-erbB*-encoded proteins. As reported

FIG. 5. Schematic representation of the different ErbB proteins analyzed. The presence of autophosphorylation sites in the different ErbB proteins is indicated by the letter P. The numbers to the left of the c-ErbB protein refer to the amino acid numbers of the tyrosine residues that serve as autophosphorylation sites.

FIG. 6. *v-erbB* mutants that cannot transform erythroblasts induce SHC phosphorylation. Lysates were prepared from CEF transformed by AEV-H or by the mutants *dl48* and *dl82*. (A) Equivalent amounts of lysate were subjected to SDS-PAGE followed by Western blotting with a monoclonal antibody against v-ErbB. (B) The same lysates were also subjected to immunoprecipitation with either normal rabbit serum (N.R.S.) or antisera against the Shc proteins. The tyrosine-phosphorylated proteins were identified by SDS-PAGE followed by Western blotting with antiphosphotyrosine antibody.

previously, Fig. 6A shows that although both mutant proteins transform CEF, they never accumulate to the levels of wt v-ErbB (both AEV-H and AEV-ES4 wt v-ErbB proteins accumulate to similar levels [data not shown]). To determine if Shc was phosphorylated on tyrosine, immunoprecipitates were formed with either normal rabbit serum or antibodies against Shc, followed by Western blotting with an antiphosphotyrosine antibody. As seen in Fig. 6B, the Shc proteins are phosphorylated in the cells expressing the mutant v-ErbB proteins. The level of phosphorylation was reduced from that seen in wt *v-erbB*-transformed cells, possibly reflecting the lower levels of v-ErbB protein in the mutant transformed CEF. It should be noted that this lower level of ErbB protein (and consequently, lower Shc phosphorylation) is still sufficient to transform CEF. Although it is possible that the lower efficiency of Shc phosphorylation may influence erythroid cell transformation, other mutations in the carboxy terminus that give rise to proteins that accumulate to levels equivalent to those encoded by *dl48* and *dl82* are not impaired in their ability to transform erythroid cells (24) (data not shown). This result indicates that the inability to transform erythroid cells is not simply explained by lower levels of ErbB protein and Shc phosphorylation. Thus, it

FIG. 7. In vitro binding of v-ErbB to GST-GRB2. Lysates from CEF infected with AEV-H, *dl48*, or *dl82* were incubated with either the GST protein (lanes 2) or the GST-GRB2 fusion protein (lanes 3) attached to beads as described in Materials and Methods. The proteins were eluted from the beads, and bound proteins were identified by SDS-PAGE followed by Western blotting with anti-ErbB antibody. Each lane 1 shows the amount of the whole-cell lysate used for binding to the beads.

appears that the truncated v-ErbB proteins encoded by the *dl82* and *dl48* mutants can induce phosphorylation of the Shc proteins, even though they cannot efficiently transform erythroid cells.

Interaction of Grb2 with v-ErbB. The activation of the Ras signal transduction pathway by the Shc proteins involves the Grb2 protein, and it is thought that complex formation due to Grb2 binding to both tyrosine-phosphorylated EGFR and Shc is important for the activation of the Ras pathway (30). The primary binding site for Grb2 on the human EGFR is tyrosine 1068 (45). This site is conserved in the c-ErbB protein, where it appears at tyrosine 1071. Interestingly, this tyrosine is deleted in the *dl82* protein but is still present in the *dl48* protein. The v-ErbB protein encoded by *dl48* is truncated at amino acid 1074, whereas the v-ErbB protein encoded by *dl82* is truncated at amino acid 1018 (18). Since the binding sites for the SH2 domain proteins are thought to include the three amino acids after the tyrosine, the *dl48* protein should retain this site (55). This is shown schematically in Fig. 5. However, since this site now exists at the extreme carboxy terminus of the protein, it was not clear how well the *dl48* protein would bind to Grb2. Thus, it was of interest to determine the ability of the Grb2 protein to bind to the *v-erbB*-encoded proteins of these mutants. To do this, we utilized GST fusion proteins as described above. Lysates from AEV-H; *dl82*- and *dl48*-infected CEF were incubated with either the GST protein (Fig. 7, lanes 2) or a GST-GRB2 fusion protein (Fig. 7, lanes 3). The bound proteins were eluted and analyzed by Western blotting with an anti-ErbB monoclonal antibody. An aliquot of the whole-cell lysate was run in lanes 1 to demonstrate that all the cells expressed *v-erbB*-encoded proteins. The AEV-H v-ErbB protein could be demonstrated to bind specifically to the GST-GRB2 fusion protein. Only the higher-molecular-weight forms of this wt v-ErbB protein bound, which have been shown to represent the tyrosine-phosphorylated forms (24). The *dl82*-encoded proteins did not bind to the GST-GRB2 fusion protein, whereas the *dl48*-encoded protein did, although the amount of protein binding was small (Fig. 7). Again, only the higher-molecular-weight forms of the *dl48*-encoded v-ErbB protein bound.

To determine if similar interactions took place in vivo, we immunoprecipitated the *v-erbB* and Shc proteins from CEF

FIG. 8. In vivo association of Grb2 with ErbB and the Shc proteins. Lysates were prepared from CEF infected with the mutants *dl48* and *dl82*. They were subjected to immunoprecipitation with normal rabbit serum (NRS), antiserum against v-ErbB, or antiserum to the Shc proteins. The immunoprecipitated proteins were subjected to SDS-PAGE followed by Western blotting with anti-Grb2 antibody. The position of the Grb2 protein is indicated.

infected with the *dl48* and *dl82* mutants. The proteins in the immune complexes were then analyzed by Western blotting with a monoclonal antibody against Grb2. Figure 8 demonstrates that in cells infected by *dl48*, the Grb2 protein is complexed to both the v-ErbB and the Shc proteins. Immunoprecipitates formed with normal rabbit serum did not contain any Grb2 protein. However, in cells infected by *dl82*, the Shc proteins were complexed to Grb2, whereas the v-ErbB protein was not, consistent with the results obtained with the GST-fusion proteins. Thus, the *dl82* v-ErbB protein cannot bind to Grb2, but it can still induce the tyrosine phosphorylation of the Shc proteins, and they can bind Grb2.

Activation of MAP kinase in *v-erbB*-transformed cells. Binding of the Grb2 protein to activated growth factor receptors and phosphorylation of the Shc proteins leads to activation of the *ras* signaling pathway, and one consequence of this is the activation of the enzyme, MAP kinase (43). Since the Grb2 protein can no longer bind to the *dl82*-encoded v-ErbB protein, it was of interest to see if the MAP kinase enzyme was constitutively activated in cells transformed by *dl48* and *dl82*. The MAP kinase enzyme is activated by phosphorylation on both tyrosine and threonine residues (2), which alters the mobility of the enzyme on SDS-polyacrylamide gels, such that the activated enzyme exhibits a slower migration (26). Therefore, the activation of the MAP kinase enzyme can be monitored by the formation of this slower-migrating species. As a control for MAP kinase activation, lysates were prepared from CEF and CEF stimulated by TGF- α . The lysates were analyzed by SDS-PAGE followed by Western blotting with antibodies against MAP kinase. As seen in Fig. 9, the addition of TGF- α to the TGF- α R (c-ErbB)-expressing CEF results in the appearance of the slower-migrating, activated form of MAP kinase, designated MAPK*, and a loss of most of the form of MAP kinase present in unstimulated CEF. In CEF transformed by both *dl48* and *dl82*, approximately 50% of the MAP kinase protein is constitutively present as the activated form of MAP kinase. Analysis of CEF transformed by wt *v-erbB* revealed that they also contained approximately 50% of the activated form of MAP kinase (data not shown). These data indicate that these two mutant forms of the v-ErbB oncoprotein are able to induce the activation of MAP kinase. Since the *dl82*-encoded protein cannot bind to Grb2, this indicates that stable binding between Grb2 and v-ErbB is not necessary to

FIG. 9. The deletion mutants *dl48* and *dl82* induce MAP kinase activation. Whole-cell lysates were prepared from CEF expressing the TGF- α R (c-ErbB) protein that had been incubated either in the absence (–) or presence (+) of TGF- α (10 ng/ml) for 5 min, from normal CEF, or from CEF transformed by the AEV mutants *dl48* and *dl82*. Equal amounts of protein were analyzed by SDS-PAGE followed by Western blotting with anti-MAP kinase antibody. MAPK*, activated form of MAP kinase.

activate the pathway leading to MAP kinase activation. Furthermore, *dl48* and *dl82* are unable to transform erythroblasts, which indicates that assuming that the mutants activate MAP kinase in erythroid cells in a manner similar to that in CEF, then the ability to activate this pathway may not be sufficient for erythroid cell transformation.

DISCUSSION

The v-ErbB oncoprotein is derived from the chicken homolog of the EGFR. Transduction of the *v-erbB* gene by AEV invariably leads to truncation of the gene, giving rise to a protein that has lost most of the extracellular domain. Passage of these viruses generates stocks with increased oncogenic potential, as judged by shorter latency periods and an increase in the spectrum of cell types that can be transformed (for a review, see reference 14). These changes can be attributed to additional mutations in the intracellular domain of the protein, including point mutations in the kinase domain and deletions in the region carboxy terminal to the kinase domain (18, 39, 46). From these analyses, a picture has emerged in which the loss of the extracellular domain leads to a constitutive tyrosine kinase activity that is essential for transformation and in which the carboxy-terminal domain plays regulatory roles in determining which cell types the virus can transform. In vitro mutagenesis studies have substantiated these conclusions (3, 25, 33, 39). However, in spite of extensive analysis, no substrates for the *v-erbB*-encoded tyrosine kinase have been identified that are relevant to cell transformation.

Overexpression of the Shc proteins is sufficient to transform NIH 3T3 cells and induce neurite outgrowth in PC12 cells (38, 43). Upon tyrosine phosphorylation, the Shc protein has been shown to bind to Grb2 and the Ras GTP-GDP exchange protein Sos (11). Grb2 has also been shown to bind to activated EGFR and bind to the Ras GTP-GDP exchange protein Sos (11, 42). These observations implicate tyrosine phosphorylation of the Shc proteins and Grb2 binding in the initiation of signal transduction from tyrosine kinases to the Ras signal transduction pathway. The Ras signal transduction pathway

can transmit signals for mitogenesis and differentiation from growth factor receptors and oncogenic cytoplasmic tyrosine kinases (48). Thus, in the studies reported here, activation of the Shc proteins by tyrosine phosphorylation and complex formation with Grb2 in *v-erbB*-transformed cells represents the first good candidate pathway for being involved in transformation by the *v-ErbB* protein.

The tyrosine-phosphorylated Shc proteins were found to be complexed to the wt *v-ErbB* protein of AEV-H. Recently the primary binding site for the Shc proteins to the human EGFR has been identified as tyrosine 1173 (37). This site is conserved in the chicken *c-ErbB* protein but is deleted in the AEV-H *v-ErbB* protein. Therefore, it appears that additional secondary binding sites on the *v-ErbB* protein exist for the Shc proteins. Although we have not yet mapped these secondary sites, the AEV-ES4 *v-ErbB* protein does not appear capable of stable binding to the Shc proteins. The AEV-ES4 protein induces the tyrosine phosphorylation of the Shc proteins to a degree similar to that of the AEV-H protein. This result indicates that stable binding is not necessary for phosphorylation and raises the interesting possibility that the *ErbB* kinase may not necessarily phosphorylate the Shc proteins directly. Recently it has been shown for a variety of receptor tyrosine kinases that they can induce mitogenic and differentiation signals via Src family tyrosine kinases (5, 21, 22, 50). Furthermore, interactions between the mammalian EGFR and *c-Src* have been documented (52, 53). Therefore, it is possible that the *v-ErbB* protein interacts with Src family tyrosine kinases to induce the tyrosine phosphorylation of the Shc proteins.

Examination of two deletion mutants of *v-erbB* that transform fibroblasts but are unable to transform erythroblasts demonstrated that these two mutant oncoproteins were still capable of inducing the tyrosine phosphorylation of the Shc proteins. One of these mutants, *dl82*, could no longer bind to Grb2 directly, but the tyrosine-phosphorylated Shc proteins did bind to Grb2. Furthermore, the MAP kinase enzyme, which is a downstream target in the Ras signal transduction pathway (34, 49), was found to be constitutively active in CEF infected by this *v-erbB* mutant. This indicates that stable binding of Grb2 to the *v-ErbB* oncoprotein is not necessary to activate this signal pathway and that activation of Grb2 can also be achieved by binding to Shc proteins. Although the Shc proteins are definitely involved in activation of the Ras signal transduction pathway, their precise role is unclear. To date, they have been shown to bind to both the EGFR and to the Grb2-Sos complex (11, 42, 43). However if binding of Grb2 to the EGFR and to Sos is all that is necessary to activate the Ras pathway, then it is not clear what role the Shc proteins play in this process. One possible role for the tyrosine-phosphorylated Shc proteins could be to receive the Grb2-Sos complex from the receptor while keeping it in an activated bound state and to present it to the Ras protein. At the present time, however, this would appear to be a redundant process, since the EGFR seems able to perform this task. Further experimentation will be necessary to investigate the mechanistic role of the Shc proteins in signaling via Ras.

The data presented here with the deletion mutants suggest that the activation of the Ras signal transduction pathway is not sufficient to induce transformation of erythroid cells by *v-erbB*. This interpretation suffers from the caveat that the activity of these two mutant forms of the *v-ErbB* oncoprotein cannot be readily be assayed in erythroid cells. However, activation of TGF- α R (*c-ErbB*) and expression of wt *v-ErbB* causes tyrosine phosphorylation of Shc proteins in both fibroblasts and erythroid cells, making it highly likely that the mutant forms would also function equivalently in both cell

types. If this is the case, then the data imply that activation of the Ras pathway may be sufficient to transform fibroblasts but, while possibly necessary, may not be sufficient to transform erythroid cells. This interpretation is consistent with the observation that overexpression of the oncogenic form of the *H-ras* oncogene causes only weak incomplete transformation of erythroid cells and requires the cooperation of the *v-ErbA* oncoprotein to give complete transformation (19).

It is becoming increasingly apparent that receptor-mediated signaling is cell type specific. For example, mutant forms of the platelet-derived growth factor receptor activate *ras* in BaF3 cells but not in CHO cells (44), presumably reflecting the availability of specific cellular substrates in the different cell types. It is clear that this is also the case for the TGF- α R (*c-ErbB*) protein. Activation of this receptor gives rise to mitogenesis in both fibroblasts and erythroblasts. However, examination of Fig. 1A clearly demonstrates cell type-specific substrates are being phosphorylated on tyrosine residues in response to activation of this protein. Presumably, cell type-specific transformation by the various *v-erbB* mutants is also a reflection of this phenomenon. These data strongly suggest that a complete understanding of physiologically relevant signal mechanisms will be achieved only when the studies are performed in the appropriate cellular context.

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REFERENCES

1. Anderson, D., C. A. Koch, L. Grey, C. Ellis, M. F. Moran, and T. Pawson. 1990. Binding of SH2 domains of phospholipase C gamma 1, GAP, and Src to activated growth factor receptors. *Science* **250**:979-982.
2. Anderson, N. G., J. L. Maller, N. K. Tonks, and T. W. Sturgill. 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature (London)* **343**:651-653.
3. Bassiri, M., and M. L. Privalsky. 1986. Mutagenesis of the avian erythroblastosis virus *erbB* coding region: an intact extracellular domain is not required for oncogenic transformation. *J. Virol.* **59**:525-530.
4. 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.* **1**(Suppl.):195-207.
5. Courtneidge, S. A., R. Dhand, D. Pilat, G. M. Twamley, M. D. Waterfield, and M. F. Roussel. 1993. Activation of Src family kinases by colony stimulating factor-1, and their association with its receptor. *EMBO J.* **12**:943-950.
6. Crowe, A. J., and M. J. Hayman. 1993. Post-translational modifications of the env-sea oncogene product: the role of proteolytic processing in transformation. *Oncogene* **8**:181-189.
7. Crowe, A. J., J. McGlade, T. Pawson, and M. J. Hayman. 1994. The SHC proteins are phosphorylated on tyrosine in both fibroblasts and erythroid cells transformed by the env-sea oncoprotein. *Oncogene* **9**:537-544.
8. Damm, K., H. Beug, T. Graf, and B. Vennström. 1987. A single point mutation in *erbA* restores the erythroid transforming potential of a mutant avian erythroblastosis virus (AEV) defective in

- both *erbA* and *erbB* oncogenes. *EMBO J.* **6**:375–382.
9. Downward, J., P. Parker, and M. D. Waterfield. 1984. Autophosphorylation sites on the epidermal growth factor receptor. *Nature (London)* **311**:483–485.
 10. Downward, J., Y. Yarden, E. Mayes, G. Scrace, 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 sequences. *Nature (London)* **307**:521–527.
 11. Egan, S. E., B. W. Giddings, M. W. Brooks, L. Buday, A. M. Sizeland, and R. A. Weinberg. 1993. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature (London)* **363**:45–51.
 12. Ekstrand, A. J., N. Sugawa, C. D. James, and V. P. Collins. 1992. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. *Proc. Natl. Acad. Sci. USA* **89**:4309–4313.
 13. Gusterson, B., G. Cowley, J. McIlhinney, B. Ozanne, C. Fisher, and B. Reeves. 1985. Evidence for increased epidermal growth factor receptors in human sarcomas. *Int. J. Cancer* **36**:689–693.
 14. Hayman, M. J., and P. J. Enrietto. 1991. Cell transformation by the epidermal growth factor receptor and *v-erbB*. *Cancer Cells* **3**:302–307.
 15. Hayman, M. J., G. Kitchener, J. Knight, J. McMahon, R. Watson, and H. Beug. 1986. Analysis of the autophosphorylation activity of transformation defective mutants of avian erythroblastosis virus. *Virology* **150**:270–275.
 16. Hayman, M. J., S. Meyer, F. Martin, P. Steinlein, and H. Beug. 1993. Self-renewal and differentiation of normal avian erythroid progenitor cells: regulatory roles of the *c-erbB*/TGF α receptor and the *c-kit*/SCF receptor. *Cell* **74**:157–169.
 17. Hu, P., B. Margolis, E. Y. Skolnik, R. Lammers, A. Ullrich, and J. Schlessinger. 1992. Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol. Cell. Biol.* **12**:981–990.
 18. Jansson, M., H. Beug, C. Gray, T. Graf, and B. Vennström. 1987. Defective *v-erbB* genes can be complemented by *v-erbA* in erythroblast and fibroblast transformation. *Oncogene* **1**:167–173.
 19. Kahn, P., L. Frykberg, C. Brady, I. Stanley, H. Beug, B. Vennström, and T. Graf. 1986. *v-erbA* cooperates with sarcoma oncogenes in leukemic cell transformation. *Cell* **45**:349–356.
 20. Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson. 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science* **252**:668–674.
 21. Kremer, N. E., G. D'Arcangelo, S. M. Thomas, M. DeMarco, J. S. Brugge, and S. Halegoua. 1991. Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of *src* and *ras* actions. *J. Cell Biol.* **115**:809–819.
 22. Kypta, R. M., Y. Goldberg, E. T. Ulug, and S. A. Courtneidge. 1990. Association between the PDGF receptor and members of the *src* family of tyrosine kinases. *Cell* **62**:481–492.
 23. Lax, I., A. Johnson, R. Howk, J. Sap, F. Bellot, M. Winkler, A. Ullrich, B. Vennstrom, J. Schlessinger, and D. Givol. 1988. Chicken epidermal growth factor (EGF) receptor: cDNA cloning, expression in mouse cells, and differential binding of EGF and transforming growth factor α . *Mol. Cell. Biol.* **8**:1970–1978.
 24. Lee, E. B., H. Beug, and M. J. Hayman. 1993. Mutational analysis of the role of the carboxy-terminal region of the *v-erbB* protein in erythroid cell transformation. *Oncogene* **8**:1317–1327.
 25. Lee, E. B., and M. J. Hayman. 1990. Analysis of the role of the extracellular domain of the *v-erbB* oncogene in cellular transformation. *Oncogene* **5**:1165–1172.
 26. Leever, S. J., and C. J. Marshall. 1993. Activation of extracellular signal-regulated kinase, ERK2, by p21^{ras} oncoprotein. *EMBO J.* **11**:569–574.
 27. Libermann, T. A., H. R. Nusbaum, N. Razon, R. Kris, I. Lax, H. Soreq, N. Whittle, N. M. D. Waterfield, A. Ullrich, and J. Schlessinger. 1985. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature (London)* **313**:144–147.
 28. Libermann, T. A., H. R. Nusbaum, N. Razon, R. Kris, I. Lax, H. Soreq, N. Whittle, N. M. D. Waterfield, A. Ullrich, and J. Schlessinger. 1985. Amplification and overexpression of the EGF receptor gene in primary human glioblastomas. *J. Cell Sci. Suppl.* **3**:161–172.
 29. Liu, X. Q., and T. Pawson. 1991. The epidermal growth factor receptor phosphorylates GTPase-activating protein (GAP) at Tyr-460, adjacent to the GAP SH2 domains. *Mol. Cell. Biol.* **11**:2511–2516.
 30. Lowenstein, E. J., R. J. Daly, A. G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, E. Y. 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.
 31. Margolis, B., N. Li, A. Koch, M. Mohammadi, D. R. Hurwitz, A. Zilberstein, A. Ullrich, T. Pawson, and J. Schlessinger. 1990. The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC- γ . *EMBO J.* **9**:4375–4380.
 32. McGlade, J., A. Cheng, G. Pelicci, P. G. Pelicci, and T. Pawson. 1992. Shc proteins are phosphorylated and regulated by the *v-Src* and *v-Fps* protein-tyrosine kinases. *Proc. Natl. Acad. Sci. USA* **89**:8869–8873.
 33. Ng, M., and M. L. Privalsky. 1986. Structural domains of the avian erythroblastosis virus *erbB* protein required for fibroblast transformation: dissection by in-frame insertional mutagenesis. *J. Virol.* **58**:542–553.
 34. Nishida, E., and Y. Gotoh. 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* **18**:128–131.
 35. Ozanne, B., C. S. Richards, F. Hendler, D. Burns, and B. Gusterson. 1986. Overexpression of the EGF receptor is a hallmark of squamous cell carcinomas. *J. Pathol.* **149**:9–14.
 36. Pawson, T., and G. D. Gish. 1992. SH2 and SH3 domains: from structure to function. *Cell* **71**:359–362.
 37. Pawson, T., and J. Schlessinger. 1993. SH2 and SH3 domains. *Curr. Biol.* **3**:434–442.
 38. Pelicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, I. Nicoletti, 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.
 39. Pelley, R. J., N. J. Maihle, C. Boerkoel, H. K. Shu, T. H. Carter, C. Moscovici, and H. J. Kung. 1989. Disease tropism of *c-erbB*: effects of carboxyl-terminal tyrosine and internal mutations on tissue-specific transformation. *Proc. Natl. Acad. Sci. USA* **86**:7164–7168.
 40. Pronk, G. J., J. McGlade, G. Pelicci, T. Pawson, and J. L. Bos. 1993. Insulin-induced phosphorylation of the 46- and 52-kDa Shc proteins. *J. Biol. Chem.* **268**:5748–5753.
 41. Royer-Pokora, B., S. Grieser, H. Beug, and T. Graf. 1978. Mutant avian erythroblastosis virus with restricted target cell specificity. *Nature (London)* **282**:750–752.
 42. Rozakis-Adcock, M., R. Fernley, J. Wade, T. Pawson, and D. Bowtell. 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature (London)* **363**:83–85.
 43. 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.
 44. 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.
 45. Schlessinger, J., and A. Ullrich. 1992. Growth factor signaling by receptor tyrosine kinases. *Neuron* **9**:383–391.
 46. Shu, H. K., R. J. Pelley, and H. J. Kung. 1990. Tissue-specific transformation by epidermal growth factor receptor: a single point mutation within the ATP-binding pocket of the *erbB* product increases its intrinsic kinase activity and activates its sarcomagenic potential. *Proc. Natl. Acad. Sci. USA* **87**:9103–9107.
 47. Skolnik, E. Y., B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich, and J. Schlessinger. 1991. Cloning of P13 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell* **65**:83–90.

48. **Smith, M. R., S. J. DeGudicibus, and D. W. Stacey.** 1986. Requirement for c-ras proteins during viral oncogene transformation. *Nature (London)* **320**:540–543.
49. **Thomas, G.** 1992. MAP kinase by any other name smells just as sweet. *Cell* **68**:3–6.
50. **Twamley-Stein, G. M., R. Pepperkok, W. Ansorge, and S. A. Courtneidge.** 1993. The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH-3T3 cells. *Proc. Natl. Acad. Sci. USA* **90**:7696–7700.
51. **Vogt, P. K.** 1969. Focus assay of Rous sarcoma virus, p. 198–211. *In* K. Habel and N. P. Salzman (ed.), *Fundamental techniques in virology*. Academic Press, New York.
52. **Wasilenko, W. J., D. M. Payne, D. L. Fitzgerald, and M. J. Weber.** 1991. Phosphorylation and activation of epidermal growth factor receptors in cells transformed by the *src* oncogene. *Mol. Cell. Biol.* **11**:309–321.
53. **Wilson, L. K., and S. J. Parsons.** 1990. Enhanced EGF mitogenic response is associated with enhanced tyrosine phosphorylation of specific cellular proteins in fibroblasts overexpressing c-src. *Oncogene* **5**:1471–1480.
54. **Yamamoto, T., H. Hihara, T. Nishida, S. Kawai, and T. Toyoshima.** 1983. A new avian erythroblastosis virus, AEV-H, carries *erbB* responsible for the induction of both erythroblastosis and sarcomas. *Cell* **34**:225–232.
55. **Zhou, S., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnoffsky, and R. J. Lechleider.** 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**:767–778.