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The Src Homology 2 and Phosphotyrosine Binding Domains of the ShcC Adaptor Protein Function as Inhibitors of Mitogenic Signaling by the Epidermal Growth Factor Receptor*

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Upon ligand activation, the epidermal growth factor receptor (EGFR) becomes tyrosine-phosphorylated, thereby recruiting intracellular signaling proteins such as Shc. EGFR binding of Shc proteins results in their tyrosine phosphorylation and subsequent activation of the Ras and Erk pathways. Shc interaction with activated receptor tyrosine kinases is mediated by two distinct phosphotyrosine interaction domains, an NH_2-terminal phosphotyrosine binding (PTB) domain and a COOH-terminal Src homology 2 (SH2) domain. The relative importance of these two domains for EGFR binding was examined by determining if expression of the isolated SH2 or PTB domain of ShcC would inhibit EGFR signaling. The SH2 domain potently inhibited numerous aspects of EGFR signaling including activation of Erk2 and the Elk-1 transcription factor as well as EGFR-dependent transformation. Furthermore, the SH2 domain inhibited focus formation by the Neu oncoprotein, another EGFR family member. Surprisingly, inhibition of the EGFR by the SH2 domain did not involve stable association with the receptor. In contrast, the PTB domain associated quite well with the receptor yet had little effect on EGFR signaling. Although the EGFR cytoplasmic tail contains consensus binding sites for the PTB and SH2 domains of ShcC, and both domains of ShcC interact with the receptor *in vitro*, the SH2 domain is more potent for inhibiting receptor function *in vivo*. However, inhibition is not due to stable association with the receptor, suggesting that the SH2 domain is binding to a heretofore unknown protein(s) necessary for proper EGFR function.

The regulation of intracellular signaling cascades through the binding of peptide growth factors by membrane-bound RTKs[1] is important for regulation of cellular growth, differentiation, apoptosis, and oncogenesis. RTKs regulate a multitude of intracellular signaling pathways. One such pathway involves the Shc adaptor proteins. Shc proteins are part of a diverse family of proteins encoded by at least three separate genes, *shcA*, *shcB*, and *shcC* (1–4). All Shc proteins share a similar structural arrangement that includes an NH_2-terminal phosphotyrosine binding domain (PTB), a central Gly- and Pro-rich region (CH1), and a COOH-terminal Src homology 2 domain (SH2). In addition, each gene encodes multiple splice forms that may possess distinct activities (5, 6).

In particular, Shc proteins are thought to link RTKs with regulation of the Ras pathway (7). Shc proteins bind the adaptor protein Grb2, which, like Shc proteins, also possesses an SH2 domain in addition to two flanking SH3 domains. Grb2 physically associates with the Ras guanine nucleotide exchange factor, Sos, through its SH3 domains. Upon activation of the EGFR, Shc binds the phosphorylated receptor and itself becomes tyrosine-phosphorylated, leading to the recruitment of the Grb2-Sos complex to the plasma membrane in close proximity to Ras, thus leading to Ras activation.

Although the above model provides a molecular basis for our understanding of how RTKs lead to Ras activation, a number of important questions remain unanswered. First, the importance of Shc in EGFR activation of Ras is not clear, since the EGFR can bind Grb2 directly in lieu of a Shc intermediary. Second, the presence of two distinct phosphotyrosine binding domains in Shc (PTB *versus* SH2) suggests that Shc may link to tyrosine-phosphorylated proteins in different manners. Furthermore, the EGFR contains two binding sites for Shc, an SH2 binding site (Tyr^{1173}) and a PTB binding site (Tyr^{1148}), whereas other receptors, such as the nerve growth factor receptor, contain only a PTB binding site, suggesting that Shc proteins may function differently with different RTKs. However, the importance of direct binding of Shc to the EGFR is unclear given that mutant versions of the EGFR that lack the tyrosine autophosphorylation sites, and hence the Shc binding sites, are still mitogenic and capable of activating Shc, suggesting that abrogation of Shc interaction with the EGFR may not affect Ras activation by the receptor (8–10). Finally, recent data have indicated that Shc may also function in Ras-independent pathways, suggesting that Shc may perform other functions besides regulation of the Ras/Erk pathway (11–20).

To define the importance of Shc in EGFR function, we assessed the ability of the isolated PTB and SH2 domains of ShcC to act as dominant negative inhibitors of EGFR signaling and transformation. We found that the SH2 domain inhibited EGFR activation of Erk-2 and Elk-1 and the growth of EGFR-transformed cells in soft agar in the absence of stable association with the receptor. In contrast, we found that the PTB domain formed a stable complex with the activated receptor yet did not significantly inhibit the activation of Erk-2 or Elk-1 by the receptor. These observations suggest that the ShcC SH2, rather than the PTB, mediates EGFR activation of Erk/Elk-1 and growth transformation. Furthermore, we suggest that the ShcC SH2 domain may promote this function via interaction

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[1] The abbreviations used are: RTK, receptor tyrosine kinase; SH2 and SH3, Src homology 2 and 3, respectively; PTB, phosphotyrosine binding; EGF, epidermal growth factor; EGFR, EGF receptor; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; GAP, GTPase activating protein.

with an as yet to be defined cellular component other than the EGFR.

EXPERIMENTAL PROCEDURES

Cell Lines and Constructs—293-T cells, hereafter referred to as 293 cells (kindly provided by Dr. Brian Howell, Fred Hutchinson Cancer Research Center), are a human embryonic kidney cell line expressing simian virus 40 T antigen. 293 cells and NIH/3T3 cells (kindly provided by Dr. Edison Liu, University of North Carolina) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin. The murine luekemia virus-EGFR expression construct encodes the wild type human EGFR and was kindly provided by Dr. Alan Wells (University of Alabama at Birmingham). The pSV2-NeuT plasmid encodes a point-mutated, activated Neu oncoprotein (21) and was kindly provided by Dr. Ming-Chi Huang (MD Anderson Cancer Center, University of Texas).

The ShcC PTB and SH2 domains were subcloned into the pCGN-hyg expression vector, which encodes a hemagglutinin (HA) epitope sequence at the 5′-end of the subcloned cDNA fragments (22). Each construct was made as follows. The PTB domain expressed in these experiments extends from the second residue of ShcC (2). The cDNA sequence encoding the ShcC PTB domain (amino acids 2–212) was PCR-amplified to create a *Bam*HI site in frame with codon 2. This fragment was subcloned into the pCRII vector (Stratagene), sequenced to check for mutations, and then subcloned into the *Bam*HI site of pCGN-hyg as a *Bam*HI–*Bgl*II fragment. The cDNA sequence encoding the SH2 domain of ShcC was PCR-amplified as described previously (2). The resulting product was subcloned into the pCRII cloning vector and sequenced to check for mutations. The 5′-PCR oligonucleotide for the SH2 domain contained a *Bam*HI site, which, in conjuction with the *Bam*HI site in pCRII, was used to subclone the SH2 domain into the *Bam*HI site of pCGN-hyg.

The pCGN Grb2 SH2 (kindly provided by Dr. Lawrence Quilliam, University of Indiana) encodes amino acids 56–155 of human Grb2, a sequence that corresponds to the isolated SH2 domain. The pCGN $p120^{GAP}$ SH2-SH3-SH2 construct (kindly provided by Dr. Geoff Clark, University of North Carolina) encodes amino acids 181–443 of human $p120^{GAP}$. The construct contains a stop codon between the sequence encoding the HA epitope tag and the initiating Met of the GAP sequence; therefore, the resulting protein product is not recognized by the HA monoclonal antibody. The SrcY527F construct encodes a constitutively activated avian Src mutated at amino acid 527 and was kindly provided by Dr. Brian Howell. The NPM-ALK construct encodes the NPM-ALK translocation (23) and was kindly provided by Dr. Steve Morris (St. Jude Children's Research Hospital).

Gal-Luciferase and MAPK Activation Assays—Elk-1 activation was measured using a transient transcriptional activation assay essentially as described previously (24). Briefly, 293 cells were transfected with constructs encoding a Gal-Elk fusion protein along with a Gal-luciferase reporter construct. In addition to these constructs, cells were cotransfected with the various dominant negative constructs. 293 cells (1.25×10^5 per well) were plated in six-well tissue culture plates. On the following day, a mixture of the following DNAs was transfected into each well of a six-well tissue culture plate by the calcium phosphate precipitation method: 2.5 μg of 5× Gal-luciferase, 0.5 μg of Gal-Elk, 1 μg of dominant negative and 1 μg of calf thymus DNA (Boehringer Mannheim). DNAs were mixed in H_20 (112.5 μl/well). To the mixture was added dropwise with gentle mixing 12.5 μl of 2.5 M $CaCl_2$. The DNA/$CaCl_2$ mixture was then added dropwise with gentle agitation to an equal volume of 2× HEPES-buffered saline (280 mM NaCl, 50 mM HEPES, 1.5 mM $Na_2HPO_4 \cdot 12H_2O$, pH 7.05). This mixture was allowed to incubate for 20–30 min and then added dropwise to the cells. After incubation of the cells with DNA for 4–5 h, the media were removed and replaced with fresh media. Cells were not glycerol-shocked. On the following day, the cells were serum-starved overnight in 0.1% fetal bovine serum. On the second day following transfection, cells were stimulated with EGF (100 ng/ml) for 5 h at 37 °C or left untreated. Cells were then gently washed with phosphate-buffered saline, which had been warmed to 37 °C to prevent dislodging of the cells from the dish. Cells were then processed as described previously (24). Lysates (5 μl) were read on a MONOLIGHT 2010 luminometer using enhanced chemiluminescent reagents (Analytical Luminescence, San Diego, CA). All assays were performed in duplicate, and the results presented are the average of at least three independent transfections.

For analysis of Erk activity, 10^6 293 cells in a 100-mm tissue culture plate were transfected with an expression construct encoding an HA epitope-tagged Erk-2 along with the various dominant negative constructs. On the following day, cells were starved overnight in 0.1% fetal bovine serum. On the second day following transfection, cells were either stimulated with EGF (100 ng/ml for 5 min) or left untreated. Cells were washed in phosphate-buffered saline warmed to 37 °C and lysed in 1 ml of cold PLC-LB (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM magnesium chloride, 100 mM sodium flouride) supplemented with 1 mM vanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Insoluble debris was pelleted in a microcentrifuge for 3–5 min at 4 °C. Equal amounts of protein from each sample were then immunoprecipitated with a hemagglutinin antibody (Babco; 1:200 dilution). These immunoprecipitates were fractionated on SDS-PAGE and transferred to Immobilon-P filters. The resulting filters were stained with Ponceau S to visualize the proteins and then cut just below the immunoglobulin heavy chain. This step decreases the background obtained from reactivity of the heavy chain protein with the secondary antibody. The filters were then probed with an antibody that recognizes the dually phosphorylated (phosphothreonine and phosphotyrosine), activated form of MAPK (Anti-ACTIVE MAPK polyclonal antibody; Promega). The blots were stripped according to the manufacturer's protocol for the ECL kits and reprobed with an anti-HA monoclonal antibody to determine the amount of expressed HA-tagged Erk-2. The ratio of activated Erk-2 to total Erk-2 was determined by densitometric analysis. Results were standardized against the vector-transfected cells without EGF treatment.

Generation of EGFR-transformed Cell Lines—EGFR-transformed NIH/3T3 cells were generated by transfection of a murine leukemic virus-EGFR construct into cells and selection in growth medium supplemented with 400 μg/ml G418. The resulting G418-resistant colonies were then passaged once as a population and allowed to grow to confluence. Transformed foci emerged within 10–14 days after passaging. The resulting transformed population of cells were further transfected with expression constructs either corresponding to vector alone or encoding ShcC SH2 or PTB, Grb2 SH2, or p120 Ras GAP SH2-SH3-SH2. Cells were selected in growth medium supplemented with 400 μg/ml hygromycin and 400 μg/ml G418, and the resulting drug-resistant colonies were pooled together and analyzed as described.

Transformation Assays—To test for anchorage-independent growth in soft agar, 10^3 cells from each of the EGFR-transformed lines transfected with pCGN-hyg, pCGN-ShcC PTB, or pCGN ShcC SH2 were plated in triplicate in media containing 0.3% soft agar essentially as described (25). Colonies were examined after 3–4 weeks. For the focus formation assays, NIH/3T3 cells (2.5×10^5) cells were plated in 60-mm dishes and on the following day were transfected with 0.5 μg of pSV2 NeuT plasmid along with 2 μg of the various dominant negatives. Each condition was tested in triplicate, and the results were averaged.

Western Blot Analysis of the EGFR-transformed Cell Lines—EGFR-transformed cell lines expressing the various dominant negative proteins were lysed in PLC-LB as described above. Equal amounts of protein were fractionated on a 12.5% SDS-PAGE and transferred to Immobilon-P membranes. The membranes were then cut into different portions and probed with antibodies directed against the EGFR (Upstate Biotechnology, Inc.), ShcA (Upstate Biotechnology), or the HA epitope (Babco). Reactive bands were visualized using the Enhanced Chemiluminescent kit (Amersham Pharmacia Biotech).

For analysis of 293 cels, 1.25×10^6 cells/100-cm dish were transfected with constructs encoding the various dominant negative proteins (5 μg/dish). Cells were then starved overnight in 0.1% fetal bovine serum. On the following day, cells were stimulated with EGF (100 ng/ml) for 5 min and then lysed in PLC-LB. Equal amounts of protein (1 mg) from each sample were subjected to immunoprecipitation with an antibody specific to the human EGFR (Ab3, NeoMarkers, Inc.). Immunoprecipitates were then fractionated on a 12.5% SDS-PAGE, transfected to Immobilon-P membranes, and probed with an antibody to ShcA (Upstate Biotechnology). Bands were visualized using the Enhanced Chemiluminescent kit (Amersham Pharmacia Biotech).

RESULTS

Expression of ShcC SH2 but Not PTB Domain Inhibits EGF Activation of MAP Kinase—Activation of the EGFR by EGF results in the recruitment of the Shc-Grb2-Sos complex to the activated receptor, thereby leading to Ras activation (7). Activated Ras, in turn, activates the Raf-Erk cascade. Given the ability of ShcC to bind to the activated EGFR receptor through both its SH2 and PTB domains *in vitro* (2), we tested whether expression of either of these domains individually could inhibit the function of the EGFR (Fig. 1). As shown in Fig. 1*A*, each of

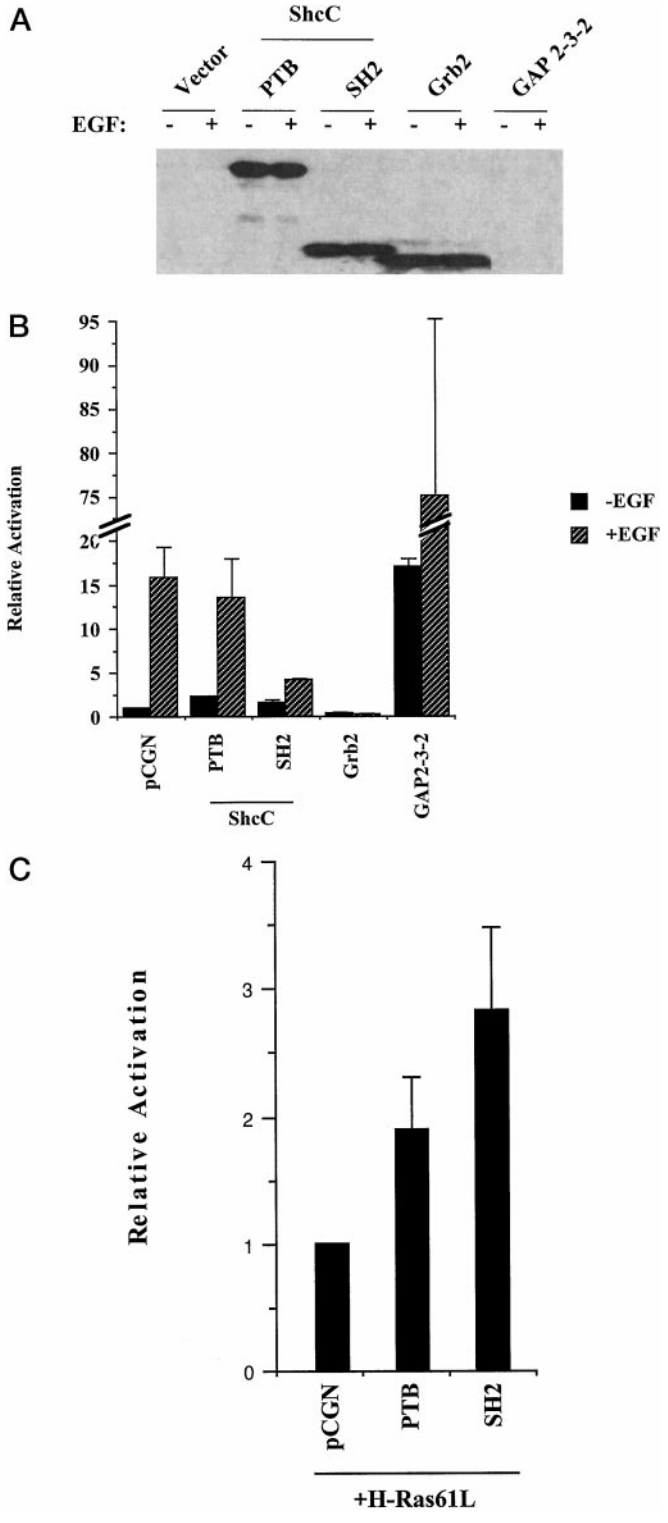

FIG. 1. **ShcC dominant negative proteins inhibit Elk-1 activation by the EGFR.** *A*, Western blot analysis of 293 cells transfected with dominant negative adaptors. Equivalent amounts of proteins were fractionated on SDS-PAGE, transferred to filters, and then Western blotted with an antibody directed against the HA epitope or an antibody directed against the GAP SH3 domain (data not shown). *B*, constructs encoding the various dominant negative proteins were transfected into 293 cells to measure their effect on the EGF activation of Elk-1 as described under "Experimental Procedures." The results represent the average of at least three independent transfections each performed in duplicate. *Error bars* represent S.E. *C*, ShcC dominant negatives were tested for their effect on Ras activation of Elk-1. Cells were transfected with constructs encoding the ShcC dominant negatives along with an expression vector encoding an activated Ras protein (Ha-Ras(61L)). Cells were serum-starved overnight and lysed in luciferase buffer, and then lysates were measured for luciferase activity. The results represent the average of four independent transfections each performed in duplicate. *Error bars* represent S.E.

the dominant negatives is expressed at similar levels in 293 cells. Since the GAP SH2-SH3-SH2 construct lacked an HA epitope, we performed Western blot analysis with a GAP-specific antiserum, which indicated that this protein was also expressed in these cells (data not shown).

Next we tested if expression of either the ShcC PTB or SH2 domain could inhibit EGF induction of Erk activity. Since the p42/p44 Erks activate the transcription factor Elk-1 through phosphorylation of the transcriptional activation domain, we utilized a transient reporter assay in which we measure activation of a chimeric Gal4-Elk-1 transcription factor consisting of the Gal4 DNA binding domain linked to the Elk-1 transcriptional activation domain (24). Expression of the ShcC SH2 domain blocked greater than 70% of the EGF-dependent activation of Elk-1, whereas expression of the PTB domain did not appear to significantly impact on Elk-1 activation by EGF (Fig. 1*B*). The inhibition by the SH2 domain was dependent on phosphotyrosine binding as mutation of an Arg to Lys in the conserved FLVRES sequence abolished inhibition (data not shown). Expression of the SH2 domain of Grb2 also inhibited EGF activation of Elk-1 as previously shown (26), since Grb2 can interact with the activated EGFR, either directly or through Shc, via its SH2 domain (27, 28). In contrast, no inhibition of Elk-1 was seen when the isolated SH2-SH3-SH2 region of p120 Ras GAP was expressed (Fig. 1*B*) (29), indicating that the inhibition seen with the ShcC SH2 was not a nonspecific property of all SH2 domains. Furthermore, neither the SH2 nor the PTB domains inhibited Elk-1 activation by activated Ha-Ras(61L), suggesting that inhibition occurs at a point upstream of Ras (Fig. 1*C*). Also, this result demonstrates that the inhibition of EGFR signaling by the ShcC SH2 domain was not due to nonspecific toxicity. In addition, expression of neither the SH2 nor the PTB domain inhibited activation of Elk-1 by the NPM-ALK oncoprotein, a member of the insulin family of RTKs (23, 30, 31) (data not shown). Together these results suggest that ShcC mediates EGFR activation of MAPKs primarily through its SH2 domain.

Given that Elk-1 can be activated by a number of MAPK family members, including Erk, p38, and Jnk (32, 33), we tested whether expression of the ShcC mutants could inhibit EGF-induced activation of Erk in 293 cells. Cells were transfected with an expression construct encoding an HA epitope-tagged MAPK/Erk2 protein (34) along with expression constructs encoding the various dominant negative proteins. Following stimulation with EGF, HA epitope-tagged Erk-2 was immunoprecipitated and analyzed for activation using a phosphospecific antibody that recognizes the dually phosphorylated, activated form of Erk-2. As shown in Fig. 2, stimulation of Erk-2 phosphorylation was significantly inhibited by expression of the SH2, but not PTB, domain of ShcC. As with the transient transcriptional assays, expression of the Grb2 SH2 domain also inhibited Erk-2 activation. In contrast to the transient assays, expression of the SH2-SH3-SH2 region of p120 Ras GAP inhibited EGF activation of Erk-2, suggesting that the activation of Elk-1 shown in Fig. 1 occurs through an Erk-independent pathway possibly through the activation of Jnk or p38.[2] These results support the notion that the ShcC SH2 domain is a potent inhibitor of the EGF activation of Erks.

Expression of the ShcC SH2 Domain Inhibits Soft Agar Growth of EGFR-transformed NIH/3T3 Cells—We next deter-

[2] J. P. O'Bryan, G. Clark, and C. J. Der, manuscript in preparation.

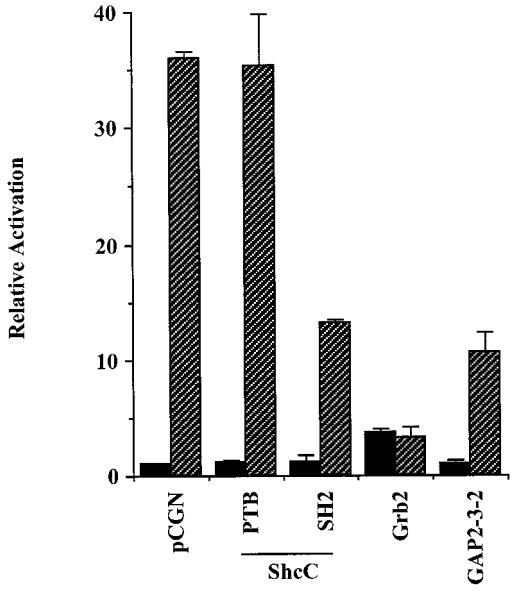

FIG. 2. **Expression of ShcC dominant negative proteins inhibits Erk-2 activation by EGF.** 293 cells were transfected with an expression vector encoding an HA epitope-tagged Erk-2 along with the various dominant negative constructs. Erk-2 activity was assayed as described under "Experimental Procedures." The results shown represent the average of two independent transfections. *Error bars* represent S.E. ■, −EGF; ▨, +EGF.

mined if expression of the SH2 or PTB domains of ShcC inhibited aspects of EGFR-mediated transformation. To address this possibility, we isolated mass transfected populations of EGFR-transformed NIH/3T3 cells stably expressing the isolated ShcC SH2 or PTB domains (see Fig. 5). The growth rates on plastic of the resulting populations of cells were comparable with the control, vector-transfected population of EGFR-transformed cells. Thus, neither domain caused a significant inhibition of cell proliferation, suggesting that inhibition of Erk activation is separable from inhibition of growth. Similar results were seen in 293 cells in that expression of the ShcC dominant negative proteins did not appear to affect the proliferation of these cells (data not shown). Although expression of the SH2 or PTB domain did not cause a significant reversion in the transformed morphology of cells, we did observe an impairment in their ability to form colonies in soft agar (Fig. 3). Whereas control vector-transfected cells formed large colonies in soft agar, the SH2 domain-expressing cells showed a striking impairment in their ability to form colonies. Although PTB domain-expressing cells did form colonies, the efficiency was reduced (approximately 50%), and the colonies were smaller than those formed by the vector-transfected EGFR-transformed cells (Fig. 3*B*). Taken together, these results suggest that the ShcC SH2 domain, and to a lesser degree the PTB domain, mediates EGFR signaling pathways that promote anchorage-independent growth but not morphologic transformation by the EGFR. Indeed, overexpression of Shc proteins was found to promote growth in soft agar with little effect on the morphology of NIH/3T3 cells (3).

The relative importance of the SH2 and PTB domains in mediating growth transformation signaling pathways was also observed for another EGFR family member, the ErbB2/Neu RTK. Co-transfection of an expression construct encoding a constitutively activated and transforming mutant of Neu (21) along with the SH2 encoding plasmid resulted in a 75% reduction in Neu focus-forming activity (Fig. 4). In contrast, expression of the PTB domain resulted in only a 25% reduction in Neu focus-forming activity. This inhibition was specific, since co-expression of the ShcC SH2 or PTB domains did not significantly impair the focus-forming activity of an activated Src nonreceptor tyrosine kinase (SrcY527F) or the NPM-ALK transforming protein (data not shown), both of which have been shown to activate Shc proteins (23, 35).

The SH2 Domain Inhibits EGFR Signaling without Stable Association with the Receptor—The EGFR contains two binding sites for Shc proteins, Tyr^{1148} for the PTB domain and Tyr^{1173} for the SH2 domain (36, 37). We hypothesized that the dominant negative proteins were exerting their inhibitory effect through competition with endogenous Shc proteins for binding to the activated EGFR. Therefore, we analyzed the EGFR-transformed cell lysates described above for the association of the activated receptor with the various mutant ShcC proteins. As shown in Fig. 5*A*, all of the cell lines expressed approximately equal amounts of the EGFR. However, there was a marked difference in the association of the dominant negative proteins with the receptor. As predicted, the ShcC PTB and Grb2 SH2 proteins were both found in complexes with the receptor. However, the ShcC SH2 domain was only weakly associated with the receptor. Analysis of whole cell lysates indicated that the various dominant negatives were expressed at similar levels, suggesting that the difference in association of the EGFR with the different proteins was not due to differences in levels of expression (Fig. 5*B*). These results suggest that although the SH2 domain is the more potent inhibitor of EGFR function, this inhibition occurs in the absence of stable association with the receptor.

In addition, we examined the association of endogenous ShcA with the activated EGFR in the presence of the various dominant negative proteins. Surprisingly, the dominant negatives did not cause significant impairment of endogenous ShcA association with the activated EGFR (Fig. 5). In the case of the SH2 domain, this result is not surprising, given the lack of stable association with the receptor *in vivo*. However, for the PTB domain, the amount of ShcA may be limiting, given the overexpression of the transfected EGFR. In this case, we may not be able to detect a loss of endogenous ShcA binding, since the PTB domain may be binding to a different pool of receptors than endogenous ShcA. To test this possibility, we examined the ability of the various dominant negative proteins to block ShcA association with the EGFR in 293 cells, since these cells express higher levels of the dominant negative proteins as compared with NIH/3T3 cells. As shown in Fig. 5*C*, expression of the ShcC SH2, Grb2 SH2, and GAP SH2-SH3-SH2 proteins did not affect association of endogenous ShcA with the activated EGFR. However, expression of the PTB domain did partially block ShcA binding to the EGFR. These results suggest that the PTB domain is the region of Shc proteins that directs association with the EGFR and that the SH2 domain may interact with additional cellular components important for EGFR function.

DISCUSSION

In this report, we describe a set of dominant negative versions of the ShcC adaptor protein and their effects on EGFR function. Previous work from our laboratory (2, 38) and others (1, 4) has shown that ShcC is a neural cell-specific adaptor protein that, like the initially identified Shc protein, ShcA, also binds receptor tyrosine kinases such as EGFR and TrkA. *In vitro* binding studies suggest that specific tyrosine residues within the cytoplasmic tail of the EGFR are important for Shc binding via its PTB and/or SH2 domain. However, the relative importance of these interactions for EGFR function *in vivo* remains unclear. To address this question, we expressed the isolated SH2 or PTB domain of ShcC to determine if they served as dominant negative inhibitors of EGFR function. Of

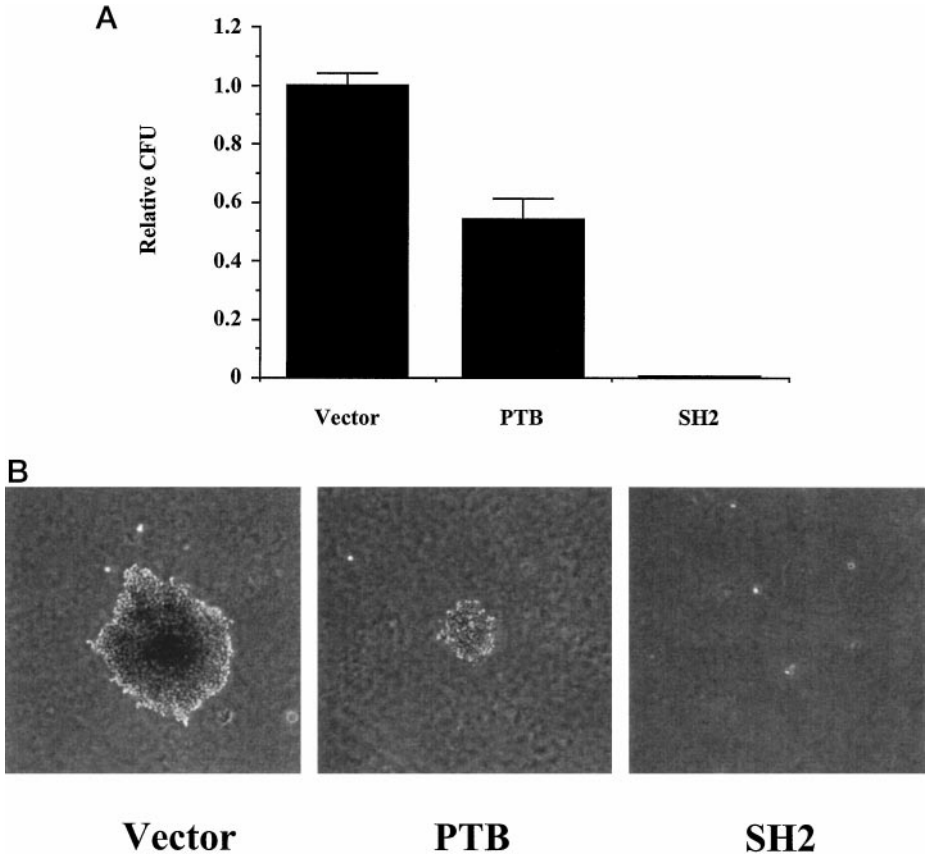

FIG. 3. **ShcC SH2 and PTB domains inhibit soft agar growth of EGFR-transformed NIH/3T3 cell lines.** EGFR-transformed NIH/3T3 cells were stably transfected with empty vector or with PTB or SH2 expression constructs. Stable cell lines were then tested for their ability to form colonies in soft agar as described under "Experimental Procedures." *A*, colonies were stained and then counted. The result shown represents the average of three wells for each cell line. S.E. are indicated with *bars*. *B*, colonies formed by EGFR-transformed cells expressing the ShcC PTB domain are smaller than those formed by vector control cells.

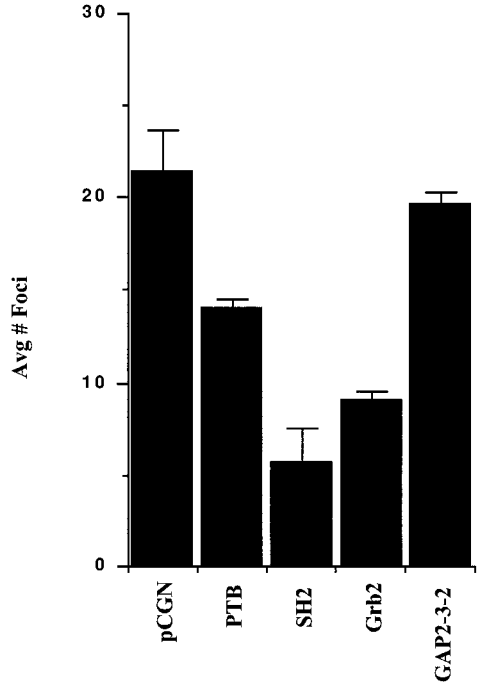

FIG. 4. **ShcC SH2 and PTB domains inhibit Neu-induced focus formation.** NIH/3T3 cells were co-transfected with 0.5 μg of an activated Neu expression vector per dish along with 2 μg of the indicated dominant negative expression construct. Foci were stained after 28 days and counted. The results shown are the average of triplicate transfections. S.E. values are indicated with *bars*.

the two ShcC dominant negatives, the SH2 domain appears to be the more efficacious inhibitor of EGFR function. This domain potently blocks EGF-induced activation of Elk-1 and Erk-2. The inhibition by the SH2 domain was dependent on the ability of this domain to bind tyrosine-phosphorylated proteins. Additionally, the SH2 and PTB domains of ShcC inhibited both focus formation and soft agar growth induced by overexpression of the EGFR family of RTKs. Expression of either the SH2 or PTB domain inhibited the growth of EGFR-transformed cells in soft agar. Furthermore, both domains inhibited the formation of transformed foci induced by an activated form of HER2/Neu.

During the preparation of this manuscript, we became aware of the results of Kaiyi, *et al.*[3] This group utilized a deletion mutant in the CH1 domain of ShcA to block Neu-induced transformation. As with our results, this Shc mutant did not inhibit the association of endogenous Shc with the receptor. However, in contrast to our findings, they did not see any effect of this Shc mutant on Neu activation of MAPK, suggesting a mechanism of inhibition distinct from that suggested by our results with the EGFR.

Consistent with our finding that the SH2 domain inhibits EGFR function, others have shown that DNA synthesis induced by both EGF and platelet-derived growth factor, but not insulin, is inhibited by microinjection of the isolated ShcA SH2 domain (39–42). In contrast, microinjection of the PTB domain appeared to inhibit insulin-induced DNA synthesis (42); however, its effect on EGFR signaling was less clear. There are conflicting reports as to the ability of the ShcA PTB domain to block EGF-induced DNA synthesis (8, 42).

Although both the PTB and SH2 domains inhibited EGFR-mediated transformation, only the PTB domain formed stable complexes with the receptor. Our results are consistent with the notion that the PTB domain promotes Shc protein interaction with the EGFR, leaving the SH2 domain to interact with additional cellular components necessary for EGFR signaling. This idea is supported by several lines of evidence. First, the

[3] K. Li, R. Shao, and M.-C. Hung, personal communication.

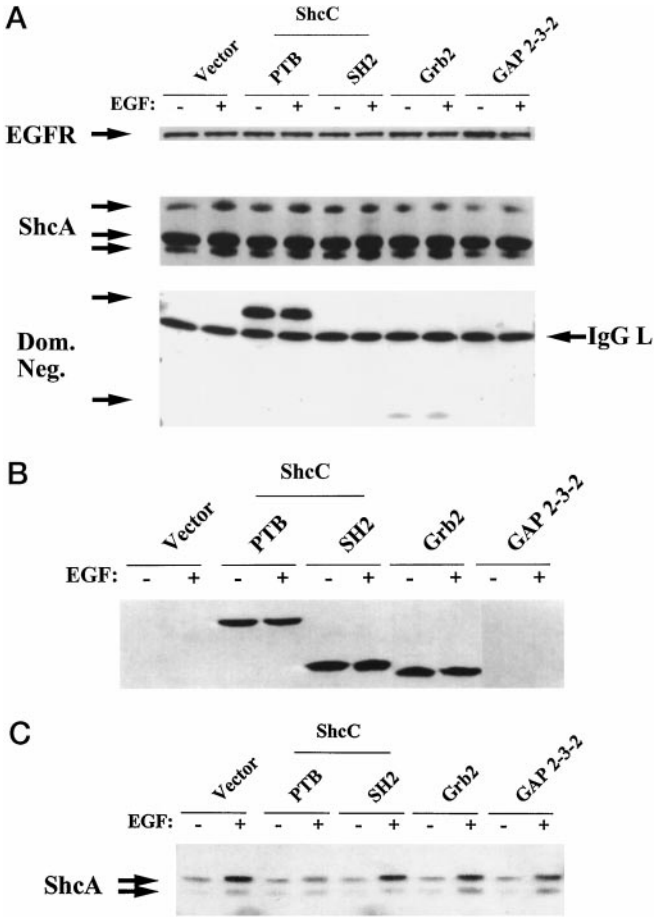

FIG. 5. **The ShcC SH2 domain inhibits EGFR signaling independent of receptor binding.** *A*, the EGFR was immunoprecipitated from cells using an anti-EGFR monoclonal antibody. The immunoprecipitates were fractionated on SDS-PAGE and then analyzed by Western blot for EGFR, ShcA, or HA. The GAP samples appear negative for expression, because this protein is not HA-tagged; however, Western blot analysis with a GAP-specific antibody did recognize this protein (data not shown). *B*, Western blot analysis of whole cell lysates used in *A*. Equal amounts of proteins were fractionated on SDS-PAGE and then probed with HA epitope-specific antibodies. *C*, Western blot analysis of transiently transfected 293 cells. Cells were transfected with the indicated expression constructs and processed as described under "Experimental Procedures." EGFR immunoprecipitates were fractionated on SDS-PAGE then analyzed by Western blot for ShcA association using a ShcA polyclonal antibody.

PTB binding site of the EGFR, Tyr^{1148}, appears to be the predominant Shc binding site as assessed through mutational analysis of the EGFR (37) as well as comparison of the binding affinities of both the SH2 and PTB domains of ShcA for tyrosine-phosphorylated phosphopeptides (43). Second, deletions or mutations of the carboxyl terminus of the EGFR that remove the autophosphorylation sites abolish interaction of the mutant receptors with Shc yet inhibit neither the phosphorylation of Shc by the mutant receptors nor the signaling functions of the mutant receptors (8–10). Third, mutations in the ShcA PTB domain that impair binding to phosphotyrosine inhibit interaction of ShcA with the EGFR although a functional SH2 domain is present (44). Fourth, as stated above, microinjection of a GST-SH2 fusion protein of ShcA into cells inhibits EGF-induced mitogenesis (39, 40, 42). However, the SH2 domain continues to inhibit even when injected 10 min following stimulation with EGF, suggesting that the SH2 domain interactions are important for EGF-induced signals distal to the receptor (40). And finally, *in vitro* binding experiments with the isolated SH2 domains from all three Shc family members indicate that this domain interacts with a number of tyrosine-phosphorylated cellular proteins (2).

Our data support the premise that the SH2 domain is important for signaling events downstream of the receptor. These events may involve interaction with either membrane-bound proteins, cytosolic proteins, or both. We believe there may be a cytosolic component(s) to which the isolated SH2 domain binds *in vivo*, thereby blocking interaction with the endogenous Shc proteins. This idea is supported by our finding that the isolated ShcC SH2 domain is found predominantly in the cytosol of cells both in the presence and absence of EGF stimulation.[4] Furthermore, the interaction of Shc with this additional protein may be important for activation of Ras and hence MAPK.

Although the ShcC dominant negative proteins inhibited two members of the EGFR family of RTKs (EGFR and Neu), expression of these domains does not inhibit all RTKs, suggesting that the ShcC dominant negatives possess specificity in their inhibitory action. Coexpression of the ShcC dominant negative proteins with an activated form of the ALK RTK, a member of the insulin receptor family (23, 30, 31), did not block signaling as measured either by activation of Elk-1 in transient reporter assays or focus formation assays in NIH/3T3 cells.[4] Although the ALK protein contains a consensus Shc PTB binding site in the cytoplasmic tail, deletion of this region does not impair transforming activity, suggesting that interaction with Shc proteins is not required for transformation by the receptor (23). Furthermore, since the SH2 domain was not inhibitory, this result suggests that the proposed target of the SH2 domain that is important for EGFR signaling is not important for ALK signaling. Together, these findings suggest that the ShcC dominant negative proteins exhibit specificity with regard to inhibition of RTKs.

The data presented here suggest that Shc proteins function in a complex manner to regulate signaling events. In the case of the EGFR, our results are consistent with the notion that the PTB domain mediates the interaction of Shc with the receptor while the SH2 domain contacts additional cellular components important for signaling by the receptor. This paradigm may not apply for all RTKs as discussed above for ALK. Future experiments will help define the downstream targets of Shc proteins and their importance in signal transduction cascades, particularly those mediated by the EGFR.

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REFERENCES

1. Nakamura, T., Sanokawa, R., Sasaki, Y., Ayusawa, D., Oishi, M., and Mori, N. (1996) *Oncogene* **13,** 1111–1121
2. O'Bryan, J. P., Songyang, Z., Cantley, L., Der, C., and Pawson, T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 2729–2734
3. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pelicci, P. G. (1992) *Cell* **70,** 93–104
4. Pelicci, G., Dente, L., De Giuseppe, A., Verducci-Galletti, B., Giuli, S., Mele, S., Vetriani, C., Giorgio, M., Pandolfi, P. P., Cesareni, G., and Pelicci, P. G. (1996) *Oncogene* **13,** 633–641
5. Migliaccio, E., Mele, S., Salcini, A., Pelicci, G., Lai, K.-M. V., Superti-Furga, G., Pawson, T., Di Fiore, P. P., Lanfrancone, L., and Pelicci, P. G. (1997) *EMBO J.* **16,** 706–716
6. Okada, S., Kao, A. W., Ceresa, B. P., Blaikie, P., Margolis, B., and Pessin, J. E. (1997) *J. Biol. Chem.* **272,** 28042–28049
7. Bonfini, L., Migliaccio, E., Pelicci, G., Lanfrancone, L., and Pelicci, P. G. (1996) *Trends Biochem. Sci.* **21,** 259–261
8. Gotoh, N., Tojo, A., Muroya, K., Hashimoto, Y., Hattori, S., Nakamura, S., Takenawa, T., Yazaki, Y., and Shibuya, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 167–171
9. Li, N., Schlessinger, J., and Margolis, B. (1994) *Oncogene* **9,** 3457–3465
10. Soler, C., Alvarez, C. V., Beguinot, L., and Carpenter, G. (1994) *Oncogene* **9,** 2207–2215

[4] J. P. O'Bryan, unpublished observations.

11. Damen, J. E., Liu, L., Rosten, P., Humphries, R. K., Jefferson, A. B., Majerus, P. W., and Krystal, G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 1689–1693
12. Gotoh, N., Tojo, A., and Shibuya, M. (1996) *EMBO J.* **15,** 6197–6204
13. Habib, T., Herrera, R., and Decker, S. J. (1994) *J. Biol. Chem.* **269,** 25243–2546
14. Harrison-Findik, D., Susa, M., and Varticovski, L. (1995) *Oncogene* **10,** 1385–1391
15. Hill, R. J., Zozulya, S., Lu, Y.-L., Hollenbach, P. W., Joyce-Shaikh, B., Bogenberger, J., and Gishizky, M. L. (1996) *Cell Growth Differ.* **7,** 1125–1134
16. Matoskova, B., Wong, W. T., Salcini, A. E., Pelicci, P. G., and Di Fiore, P. P. (1995) *Mol. Cell. Biol.* **15,** 3805–3812
17. Lioubin, M. N., Algate, P. A., Tsai, S., Carlberg, K., Aebersold, A., and Rohrschneider, L. R. (1996) *Genes Dev.* **10,** 1084–1095
18. Okabayashi, Y., Sugimoto, Y., Totty, N. F., Hsuan, J., Kido, Y., Sakaguchi, K., Gout, I., Waterfield, M. D., and Kasuga, M. (1996) *J. Biol. Chem.* **271,** 5265–5269
19. Thomas, D., Patterson, S. D., and Bradshaw, R. A. (1996) *J. Biol. Chem.* **270,** 28924–28931
20. Xu, Y., Guo, D.-F., Davidson, M., Inagami, T., and Carpenter, G. (1997) *J. Biol. Chem.* **272,** 13463–13466
21. Youming, X., Li, K., and Hung, M.-C. (1995) *Oncogene* **10,** 2409–2413
22. Tanaka, M., and Herr, W. (1990) *Cell* **60,** 375–386
23. Bischoff, D., Pulford, K., Mason, D. Y., and Morris, S. W. (1997) *Mol. Cell. Biol.* **17,** 2312–2325
24. Hauser, C. A., Westwick, J. K., and Quilliam, L. A. (1995) *Methods Enzymol.* **255,** 412–426
25. Clark, G., Cox, A. D., Graham, S. M., and Der, C. J. (1995) *Methods Enzymol.* **255,** 395–412
26. Tanaka, M., Gupta, R., and Mayer, B. (1995) *Mol. Cell. Biol.* **15,** 6829–6837
27. Hashimoto, Y., Matuoka, K., Takenawa, T., Muroya, K., Hattori, S., and Nakamura, S. (1994) *Oncogene* **9,** 869–875
28. Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G., Schlessinger, J., and Pawson, T. (1992) *Nature* **360,** 689–692
29. Medema, R. H., DeLaat, W. L., Martin, G. A., McCormick, F., and Bos, J. L. (1992) *Mol. Cell. Biol.* **12,** 3425–3430
30. Morris, S. W., Kirstein, M. N., Valentine, M. B., Dittmer, K. G., Shapiro, D. N., Saltman, D. L., and Look, A. T. (1994) *Science* **263,** 1281–1284
31. Fujimoto, J., Shiota, M., Iwahara, T., Seki, N., Satoh, H., Mori, S., and Yamamoto, T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 4181–4186
32. Whitmarsh, A. J., Shore, P. S., Sharrocks, A. D., and Davis, R. J. (1995) *Science* **269,** 403–407
33. Whitmarsh, A. J., Yang, S.-H., Su, M. S.-S., Sharrocks, A. D., and Davis, R. J. (1997) *Mol. Cell. Biol.* **17,** 2360–2371
34. Reuter, C. W. M., Catling, A. D., and Weber, M. J. (1995) *Methods Enzymol.* **255,** 245–256
35. McGlade, J., Cheng, A., Pelicci, G., Pelicci, P. G., and Pawson, T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 8869–8873
36. Batzer, A. G., Rotin, D., Urena, J. M., Skolnick, E. Y., and Schlessinger, J. (1994) *Mol. Cell. Biol.* **14,** 5192–5201
37. Batzer, A. G., Blaikie, P., Nelson, K., Schlessinger, J., and Margolis, B. (1995) *Mol. Cell. Biol.* **15,** 4403–4409
38. O'Bryan, J. P., Martin, C. B., Zhou, S., Cantley, L. C., and Der, C. J. (1996) *J. Biol. Chem.* **271,** 11787–11791
39. Gotoh, N., Muroya, K., Hattori, S., Nakamura, S., Chida, K., and Shibuya, M. (1995) *Oncogene* **11,** 2525–2533
40. Ricketts, W. A., Rose, D. A., Shoelson, S., and Olefsky, J. M. (1996) *J. Biol. Chem.* **271,** 26165–26169
41. Roche, S., McGlade, J., Jones, M., Gish, G., Pawson, T., and Courtneidge, S. A. (1996) *EMBO J.* **15,** 4940–4948
42. Sasaoka, T., Ishihara, H., Sawa, T., Ishiki, M., Morioka, H., Imamura, T., Usui, I., Takata, Y., and Kobayashi, M. (1996) *J. Biol. Chem.* **271,** 20082–20087
43. Zhou, M.-M., Harlan, J. E., Wade, W. S., Crosby, S., Ravichandran, K. S., Burakoff, S. J., and Fesik, S. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **270,** 31119–31123
44. Yajnik, V., Blaikie, P., Bork, P., and Margolis, B. (1996) *J. Biol. Chem.* **271,** 1813–1816