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Hierarchy of Binding Sites for Grb2 and Shc on the Epidermal Growth Factor Receptor

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We analyzed the binding site(s) for Grb2 on the epidermal growth factor (EGF) receptor (EGFR), using cell lines overexpressing EGFRs containing various point and deletion mutations in the carboxy-terminal tail. Results of coimmunoprecipitation experiments suggest that phosphotyrosines Y-1068 and Y-1173 mediate the binding of Grb2 to the EGFR. Competition experiments with synthetic phosphopeptides corresponding to known autophosphorylation sites on the EGFR demonstrated that phosphopeptides containing Y-1068, and to a lesser extent Y-1086, were able to inhibit the binding of Grb2 to the EGFR, while a Y-1173 peptide did not. These findings were confirmed by using a dephosphorylation protection assay and by measuring the dissociation constants of Grb2's SH2 domain to tyrosine-phosphorylated peptides, using real-time biospecific interaction analysis (BIAcore). From these studies, we concluded that Grb2 binds directly to the EGFR at Y-1068, to a lesser extent at Y-1086, and indirectly at Y-1173. Since Grb2 also binds Shc after EGF stimulation, we investigated whether Y-1173 is a binding site for the SH2 domain of Shc on the EGFR. Both competition experiments with synthetic phosphopeptides and dephosphorylation protection analysis demonstrated that Y-1173 and Y-992 are major and minor binding sites, respectively, for Shc on the EGFR. However, other phosphorylation sites in the carboxy-terminal tail of the EGFR are able to compensate for the loss of the main binding sites for Shc. These analyses reveal a hierarchy of interactions between Grb2 and Shc with the EGFR and indicate that Grb2 can bind the tyrosine-phosphorylated EGFR directly, as well as indirectly via Shc.

Tyrosine autophosphorylation plays a crucial role in determining the selectivity of signaling pathways activated by growth factor receptors, such as the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors (EGFR and PDGFR) (reviewed in reference 59). Receptor activation leads to tyrosine autophosphorylation resulting in the association of the receptor with cytoplasmic target proteins containing a conserved noncatalytic region of approximately 100 amino acids, referred to as the Src homology 2 (SH2) domain (reviewed in reference 48). Both binding experiments and structural studies indicate that high-affinity binding of SH2 domains requires interactions with both the phosphotyrosine moiety and the immediate flanking amino acid residues (13, 16, 17, 42, 43, 45, 47, 55, 61, 65, 69, 70).

We have previously identified a small ubiquitously expressed protein, Grb2, that is composed of one SH2 domain flanked by two SH3 domains (36). Both genetic studies in *Caenorhabditis elegans* and *Drosophila melanogaster* and biochemical studies in mammalian cells indicate that Grb2 and its homologs from lower eukaryotes play an important role in a highly conserved process for the control of Ras signaling by receptor and cytoplasmic tyrosine kinases (reviewed in reference 58). It has been shown that Grb2 binds activated growth factor receptors and other tyrosine-phosphorylated proteins through its SH2 domain and the guanine nucleotide-releasing factor Sos through its SH3 domains (8, 14, 19, 34, 56, 60, 61). This leads to translocation of Sos to the plasma membrane where Ras is located, thus increasing the exchange of GDP for GTP on Ras. The GTP-bound active form of Ras then triggers the activation of a kinase cascade leading to phosphorylation of nuclear proteins involved in transcriptional control (reviewed in reference 4).

Shc is an SH2 domain-containing protein that is tyrosine phosphorylated in response to a variety of growth factors and by oncogenic protein tyrosine kinases (7, 41, 49, 52, 57, 61). Overexpression of Shc leads to transformation of fibroblasts and neuronal differentiation of PC12 cells (49, 57). The finding that Shc-induced differentiation of PC12 cells is blocked by expression of a dominant inhibitory Ras mutant, together with the demonstration that the SH2 domain of Grb2 forms a stable complex with tyrosine-phosphorylated Shc, suggests that a Grb2-Shc complex is involved in Ras activation by both receptor and nonreceptor tyrosine kinases (14, 56, 57, 60, 61).

In this study, we determined the binding sites for Grb2 and Shc on the EGFR. Both Grb2 and Shc bind with high affinity to a single site on the EGFR and to a lesser extent to a secondary site. Moreover, Grb2 can couple to the activated EGFR both directly and indirectly, via the adaptor molecule Shc. In contrast with other receptor tyrosine kinases that bind SH2 domain-containing proteins at a single site, the EGFR can interact with a single SH2 domain at multiple sites.

MATERIALS AND METHODS

Cell lines, immunoprecipitation, and immunoblotting. NIH 3T3 lines lacking endogenous EGFR (2.2 cells) were engineered to overexpress either wild-type or mutant EGFRs. Cell lines overexpressing the human EGFR or mutant EGFRs used in this study were HER14, Y1068F, Y1148F, Y1173F (21), CD63, and CD126 (38). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum. Prior to stimulation, the cells were starved for 18 h in Dulbecco's modified Eagle's medium containing 0.5% calf serum. Cells were then stimulated with EGF (275 ng/ml) for 3 min at room temperature, washed with ice-cold phosphate-buffered saline,

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and lysed in lysis buffer {50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 7.5], 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 50 μM $ZnCl_2$, 250 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 50 μg of trypsin inhibitor from soybean per ml, 50 μg of pepstatin per ml}. Lysate protein content was normalized as described previously (5). Cell lysis, immunoprecipitation, and immunoblotting using ^{125}I-protein A (ICN) were performed as previously described (40).

Antibodies. Anti-EGFR antibodies used in this study were monoclonal antibody (MAb) 108, a mouse MAb directed against domain III of the extracellular domain (2), and RK2, a rabbit antibody directed against a C-terminal peptide (residues 984 to 996) (33). RK2 and MAb 108 were used for immunoblotting and immunoprecipitation, respectively. Rabbit anti-Grb2 antibody (antibody 86) was used for immunoblotting (36). Polyclonal antiphosphotyrosine (antibody 72) and anti-glutathione *S*-transferase (GST) (antibody 23) antibodies were raised in rabbits against alanine-glycine-phosphotyrosine polymers coupled to keyhole limpet hemocyanin (25) and purified GST, respectively.

Peptide synthesis. A 9-fluorenylmethoxycarbonyl (Fmoc)-based strategy for peptide synthesis was used in conjunction with standard side chain-protecting groups as described previously (1). Fmoc-Tyr(PO_3Me_2)-OH (Bachem Bioscience) was used for incorporation of phosphotyrosine. Peptides were purified by ether precipitation and preparative reverse-phase high-pressure liquid chromatography (HPLC). Analytical HPLC demonstrated that the products were purified to homogeneity; amino acid analysis (ABI model 420 sequencer) confirmed the identities of the peptides. The sequences of the EGFR-derived phosphopeptides were as follows: phosphopeptide Y-992 (pY992), DADEpYLIPQQGFFSK; pY992-8Y4, DDVVDADEpYLIPQ; pY1068, PVPEpYINQSVP KRK; pY1086, QNPVpYHNQPLNPAPSK; pY1086-8Y3, AGSVQNPVpYHNQ; pY1148, DNPDpYQQDFFPKEAK; pY1148-7Y4, ISLDNPDpYQQDF; pY1173, ENAEpYLRV APQSSEK; and pY1173-8Y4, GSTAENAEpYLRVA.

Protein preparation. GST fusion proteins were purified on glutathione-agarose as described by Smith and Johnson (63) except that 20 mM triethanolamine (pH 7.5)–150 mM NaCl–0.1% β-mercaptoethanol (buffer A) was used as the buffer. When necessary, the proteins were eluted with 15 mM glutathione in buffer A (pH 8.0), concentrated, and stored at −70°C in the presence of 20% glycerol. Grb2 was cloned into pET3a (Novagen), using the *Nde*I and *Bam*HI sites. *Escherichia coli* pLysS expressing Grb2 was induced with 0.5 mM isopropythiogalactopyranoside (IPTG) and grown for 3 h. The bacterial pellet was resuspended in buffer A, and the bacteria were broken by sonication. After centrifugation at 10,000 × *g* for 10 min, glycerol was added to 20%, and aliquots were stored at −70°C. The amount of Grb2 was estimated by Coomassie blue staining of lysates and comparison with known standards.

Peptide competition assays. For the competition experiments, the EGFR was immunoprecipitated from lysates of A431 cells, washed four times with lysis buffer, and subjected to in vitro autophosphorylation in the presence of 0.2 mM ATP and 10 mM $MnCl_2$ for 5 min at room temperature. After washing, the autophosphorylated EGFR was divided into equal aliquots (approximately 0.5 pmol) and incubated with 20 pmol of either Grb2 or Shc SH2-GST in the absence or presence of 1 nmol of the various phosphopeptides in a volume of 100 μl for 1 h at 4°C. After three washes with lysis buffer, the samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and then immunoblotted as described above. The amount of bound protein was then quantified with a PhosphorImager. One hundred percent binding was defined as the amount of protein bound in the absence of competing phosphopeptide. To determine phosphopeptide concentrations necessary for half-maximal inhibition of SH2 domain binding (ID_{50}), competition experiments were performed in the presence of increasing amounts of phosphopeptides from 0 to 20 nmol.

EGFR protection assays. Identification of the tyrosine phosphorylation site to which Grb2 or the SH2 domain of Shc binds was carried out essentially as described previously (55). Briefly, the EGFR was immunoprecipitated from A431 cell lysate and phosphorylated in vitro with 10 μCi of [γ-^{32}P]ATP (per assay), 2 μM unlabeled ATP, and 10 mM $MnCl_2$. The phosphorylated receptor was washed four times with ice-cold 20 mM HEPES (pH 7.5) solution and then dephosphorylated for 20 min at 37°C with A431 cell lysates (without phosphatase inhibitors) in the presence of 0, 0.5, or 1 μM Grb2-GST (36) or 0, 5, or 30 μM Shc SH2-GST fusion protein (residues 366 to 473 [49]). Proteins were then separated by SDS-PAGE (8% gel) and transferred to nitrocellulose, and the band corresponding to the EGFR was excised. The receptor was then digested with trypsin as detailed previously (55), and the tryptic fragments were purified by HPLC using a C18 reverse-phase column with 0.1% trifluoroacetic acid in H_2O (buffer A) or in acetonitrile (buffer B), at a flow rate of 1 ml/min. The following gradients were used: for Grb2-GST, from 0 to 10 min in 0% buffer B, from 10 to 50 min in a linear gradient to 40% buffer B, and from 50 to 51 min an increase to 60% buffer B; and for Shc SH2-GST, from 0 to 10 min in 0% buffer B, from 10 to 15 min in a linear gradient to 30% buffer B, from 15 to 55 min in a linear gradient to 50% buffer B, and from 55 to 56 min an increase to 80% buffer B. The assignment of tryptic fragments separated by HPLC to the autophosphorylation sites of the EGFR was described earlier (12, 38, 55, 71).

BIAcore measurements. Real-time biospecific interaction analysis measurements were made by using BIAcore (Pharmacia Biosensor AB, Uppsala, Sweden) with CM5 sensor chips. For analysis, HBS buffer (10 mM HEPES [pH 7.5], 150 mM NaCl, 3.4 mM EDTA) containing 0.05% BIAcore surfactant P20 was used. The measurements are based on changes in surface plasmon resonance signal from chips coated with carboxymethylated dextran to which the peptides are covalently cross-linked (24). The changes in the signal are measured in real time, reflecting the changes in the refractive index due to increased protein concentration on the surface of the chip (15). Peptides were immobilized as previously described (17). Briefly, carboxyl groups of the matrix were activated with 50 mM *N*-hydroxysuccinimide and 200 mM *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide for 1 min, and then the peptides at 1 mg/ml in 50 mM HEPES (pH 7.5)–1 M NaCl–3.4 mM EDTA were injected onto the matrix for 3 min at a flow rate of 5 μl/min. Remaining activated groups were blocked with 1 M ethanolamine (pH 8.5) for 6 min. The equilibrium dissociation constants (K_Ds) were calculated either from Scatchard analysis of steady-state binding or from kinetic parameters.

RESULTS

Interaction of Grb2 with EGFR mutants. We have previously demonstrated that Grb2 binds to the autophosphorylated EGFR via its SH2 domain (36). The C-terminal tail of the EGFR contains at least five autophosphorylation sites (12, 38, 71) and has been shown to be the primary binding site for SH2

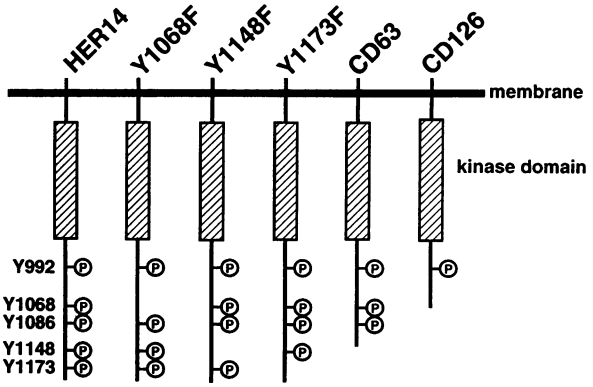

FIG. 1. Schematic representation of the intracellular domains of the wild-type and mutant EGFR. HER14, wild-type receptor; Y1068F, Y1148F, and Y1173F, mutant EGFRs in which the indicated tyrosines have been mutated to phenylalanine; CD63, EGFR with a deletion of 63 amino acids from the C terminus; CD126, EGFR with a deletion of 126 amino acids from the C terminus; P, autophosphorylation site. The cell lines express approximately 300,000 (HER14), 200,000 (Y1068F), 80,000 (Y1148F), 250,000 (Y1173F), 300,000 (CD63), and 150,000 (CD126) receptors per cell, respectively.

domain-containing proteins (23, 36, 37, 40, 55, 62). We analyzed the binding site(s) of Grb2 on the EGFR by using cell lines expressing either the wild-type EGFR or various EGFR mutants. The mutant EGFRs used in this study include receptors containing point mutations at autophosphorylation sites Y-1068, Y-1148, and Y-1173 (Y to F) and two deletion mutants lacking either 63 (CD63) or 126 (CD126) carboxy-terminal amino acids. The various EGFR mutants used in this study are depicted in Fig. 1.

Lysates from various cells lines that were either untreated or treated with EGF were subjected to immunoprecipitation with MAb 108 against the EGFR. Each sample was divided in half and after separation by SDS-PAGE was immunoblotted with the various antibodies as indicated. Appropriate strips of the nitrocellulose filters were treated with anti-EGFR, antiphosphotyrosine, or anti-Grb2 antibodies (Fig. 2A). The EGFR was immunoblotted with anti-EGFR RK2 antibodies which recognize both the wild-type and mutant EGFRs. In agreement with previous studies (36), Grb2 coimmunoprecipitates with the wild-type EGFR in stimulated cells (Fig. 2A). The autophosphorylation of unstimulated CD63 mutant receptor was due to starvation conditions (18 h with 0.5% serum); starvation for 24 h with 0.1% serum abolished autophosphorylation of this EGFR mutant. The cell lines used in this study express different amounts of EGFRs ranging from approximately 300,000 to 80,000 receptors per cell (Fig. 1). To quantitate the relative amount of Grb2 which coimmunoprecipitated with the different EGFRs, the bands corresponding to EGFR or Grb2 were excised, and the amount of radioactivity in each band was quantitated by counting in a gamma counter. A ratio between the number of counts obtained for the EGFR and Grb2 was determined; the ratio obtained for the association between Grb2 and the wild-type EGFR was arbitrarily set at 100%. The results presented in Fig. 2B summarize the means ± standard deviations of four independent experiments. Elimination of tyrosine 1068 or 1173 consistently led to a decrease in the binding of Grb2 to the EGFR (Fig. 2B). The Y1173F EGFR and the CD63 mutant EGFR (also lacking Y-1173) bound only 40% as much Grb2 as that bound by the wild-type EGFR. Removal of Y-1068 also reduced the binding by approximately

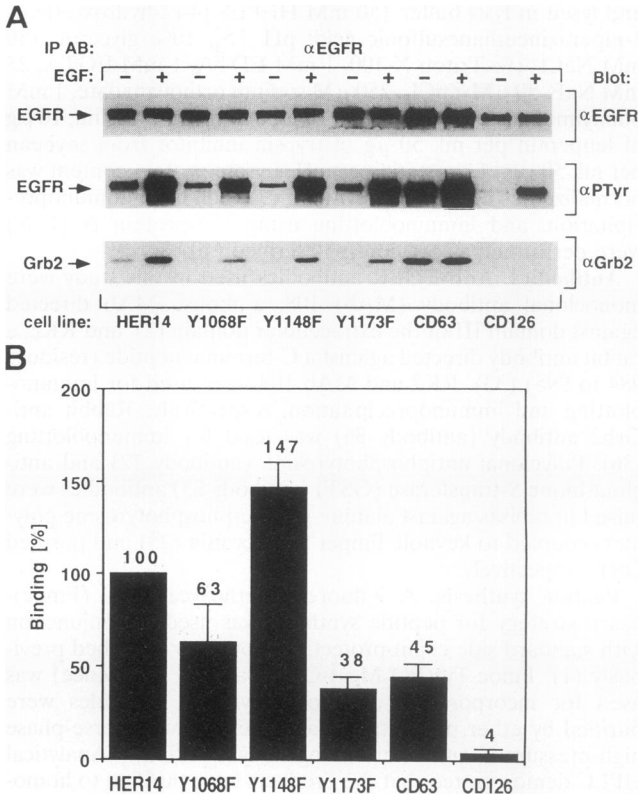

FIG. 2. Coimmunoprecipitation of Grb2 with the wild-type and mutant EGFR. Cells were incubated in the absence (−) or presence (+) of EGF for 3 min at 23°C; 3 mg of lysate protein were subjected to immunoprecipitation (IP) with 10 μg of anti-EGFR (αEGFR) MAb 108 (AB) as described in Materials and Methods. The samples were split in half, loaded on separate SDS-gels, and transferred to nitrocellulose. (A) The nitrocellulose filters were cut and immunoblotted with anti-EGFR, antiphosphotyrosine (αPTyr), and anti-Grb2 (αGrb2) antibodies. (B) The bands corresponding to Grb2 and to the EGFR were excised, and the pieces were counted in a gamma counter. A ratio between the number of counts obtained for the receptor and Grb2 was determined. The ratio obtained for the association between Grb2 and the wild-type EGFR was arbitrarily set as 100%. The results shown represent the means ± standard deviations of four independent experiments.

40% (Fig. 2B). When both Y-1068 and Y-1173 were eliminated (CD126), essentially no binding of Grb2 to the autophosphorylated receptor was detected (Fig. 2B). These results suggest that phosphorylation of Y-1068 and Y-1173 is required for the binding of Grb2 to the EGFR.

Analysis of Grb2 binding sites by using a dephosphorylation protection assay. To further examine the binding sites for Grb2 on the EGFR, we used a dephosphorylation protection assay (55). We have previously shown that the binding of SH2 domains to phosphorylated tyrosine residues protects them from dephosphorylation by cellular tyrosine phosphatases (55). The ability of the SH2 domain of Grb2 to block dephosphorylation of specific tyrosine residue on the EGFR can therefore be used to determine the binding site of GRB2 on the EGFR.

The EGFR was immunoprecipitated from A431 cells, phosphorylated in vitro with [γ-^{32}P]ATP, and then subjected to a dephosphorylation reaction by cellular phosphatases in the presence of increasing amounts of Grb2-GST fusion protein.

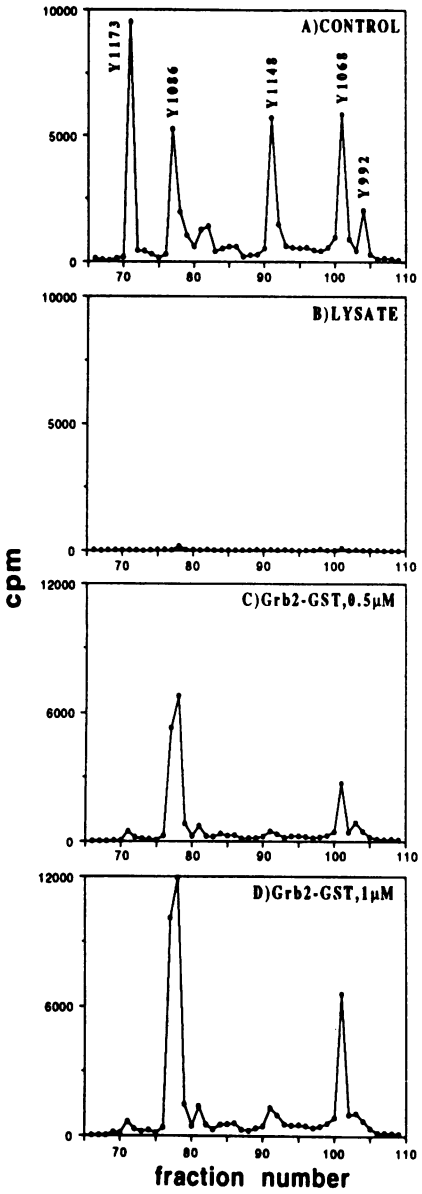

FIG. 3. Dephosphorylation protection assay using Grb2-GST fusion protein. The dephosphorylation protection assay was performed as described in Materials and Methods. (A) Control; (B) lysate; (C) 0.5 μM Grb2-GST plus lysate; (D) 1 μM Grb2-GST plus lysate. The following gradient was used: from 0 to 10 min in 0% acetonitrile, from 10 to 50 min in a linear gradient to 40% acetonitrile, and from 50 to 51 min an increase to 60% acetonitrile.

After separation by SDS-PAGE and transfer to nitrocellulose, the band corresponding to the EGFR was excised, digested with trypsin, and separated by HPLC. The fractions were collected, and the radioactivity in each fraction was determined by scintillation counting. Figure 3A shows the typical five tryptic fragments corresponding to the five autophosphorylation sites of the EGFR at Y-1173, Y-1086, Y-1148, Y-1068, and Y-992 (12, 38, 55, 71). These phosphopeptide fragments were absent from the dephosphorylated EGFR preparation subjected to dephosphorylation by the cellular phosphatases (Fig. 3B). Addition of 0.5 or 1 μM Grb2-GST fusion protein led to strong protection of Y-1068 and Y-1086 from dephosphorylation, while Y-1173, Y-1148, and Y-992 were poorly protected (Fig. 3C and D).

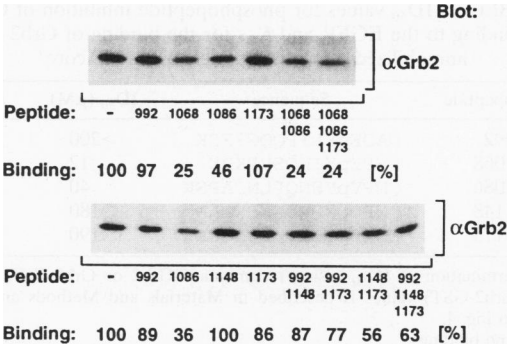

FIG. 4. Inhibition of Grb2 binding to the EGFR with synthetic phosphopeptides. The EGFR was immunoprecipitated from A431 cells, autophosphorylated in vitro, and incubated with bacterially expressed Grb2 (0.2 μM) in the absence or presence of 10 μM phosphopeptides indicated below the lanes. When two or three peptides were used together, each was at 10 μM. Blots were quantitated with a PhosphorImager. A sample without phosphopeptide (−) served as a 100% standard for each panel. αGrb2, anti-Grb2 antibody.

Inhibition of Grb2 binding to the EGFR by phosphotyrosine-containing peptides and BIAcore analysis of Grb2 binding to phosphopeptides. To determine which phosphotyrosine in the C terminus of the EGFR interacts directly with the SH2 domain of Grb2, we compared the capacity of synthetic phosphopeptides, corresponding to EGFR autophosphorylation sites, to block binding of Grb2 to the autophosphorylated EGFR. The EGFR was immunoprecipitated from A431 cells, subjected to autophosphorylation, and then incubated with Grb2 protein (0.2 μM) in the absence or presence of various phosphopeptides (10 μM) (Fig. 4). The amount of Grb2 associated with the EGFR was determined by immunoblotting with anti-Grb2 antibodies. The upper part of the nitrocellulose filters was immunoblotted with antiphosphotyrosine antibodies to control for the amount of phosphorylated EGFR that is present in each lane (data not shown). The results presented in Fig. 4 show that pY1068 inhibits Grb2 binding to the EGFR best (25% binding). pY1086 also inhibited Grb2 binding to the EGFR (41% ± 5%), while essentially no inhibition was detected with pY992 (93% ± 6%), pY1148 (100%), and pY1173 (97% ± 15%). Competition with various combinations of phosphopeptides gave a consistent picture; only mixtures containing pY1068 or pY1086 blocked binding of Grb2 to the EGFR.

To quantitate the potency of the various phosphopeptides, we determined the ID_{50} values for Grb2 binding to the EGFR. The ID_{50} values for pY1068 and pY1086 were 12 and 40 μM, respectively, while the ID_{50} values for pY992, pY1148, and pY1173 were >200, 180, and 190 μM, respectively (Table 1). In agreement with these findings, BIAcore analysis (15, 24) demonstrated that a Grb2-GST fusion protein bound pY1068 with a K_D of approximately 30 nM and that the K_D for pY1086 was approximately 60 nM. No binding to pY992, pY1148, or pY1173 was detected (Table 1). These results indicate that Y-1068 and to a lesser extent Y-1086 are responsible for direct binding of Grb2 to the EGFR. We have recently demonstrated that the discrepancy in the binding affinities determined by competition experiments and the BIAcore analysis is mainly due to the dimerization of the GST-SH2 fusion proteins mediated by GST. Recombinant SH2 domains without GST bind with lower affinity to the same immobilized phosphopep-

TABLE 1. ID_{50} values for phosphopeptide inhibition of Grb2 binding to the EGFR and K_Ds for the binding of Grb2 to immobilized phosphopeptides, using BIAcore[a]

Phosphopeptide	Sequence	ID_{50} (μM)	K_D (nM)
pY992	DADEpYLIPQQGFFSK	>200	NB[b]
pY1068	PVPEpYINQSVPKRK	12	30
pY1086	QNPVpYHNQPLNPAPSK	40	60
pY1148	DNPDpYQQDFFPKEAK	180	NB
pY1173	ENAEpYLRVAPQSSEK	190	NB

[a] Determination of ID_{50} (using recombinant Grb2 or Grb2-GST) and K_D (using Grb2-GST) values is described in Materials and Methods and in the legend to Fig. 4.
[b] NB, no binding.

tide compared with the binding of GST-SH2 fusion proteins (unpublished results). The dissociation constants for phosphopeptide-recombinant SH2 interactions are closer to the values obtained from the competition experiments.

The association of Grb2 with the Y1173F receptor mutant was strongly decreased in lysates from living cells (Fig. 2). On the other hand, there was no protection of Y-1173 from dephosphorylation by Grb2 (Fig. 3); pY1173 was unable to inhibit the binding of Grb2 to the autophosphorylated EGFR in vitro (Fig. 4), and no binding of Grb2 to pY1173 was detected with the BIAcore system (Table 1). We therefore suspected that Grb2 may bind to Y-1173 indirectly via Shc. Shc has been shown to associate with the EGFR and to become tyrosine phosphorylated upon EGF stimulation (57). Furthermore, the SH2 domain of Grb2 binds with high affinity to a phosphopeptide encompassing the phosphorylation site of Shc, Y-317 (61).

Analysis of Shc binding sites by using a dephosphorylation protection assay. We first analyzed the binding sites for Shc on the EGFR by using a dephosphorylation protection assay (55). Figure 5A shows the five tryptic fragments corresponding to the five autophosphorylation sites of the EGFR at Y-1173, Y-1148, Y-1086, Y-1068, and Y-992 (12, 38, 55, 71). These phosphopeptide fragments were absent from the dephosphorylated EGFR preparation subjected to dephosphorylation by the cellular phosphatases (Fig. 5B). Addition of 5 μM Shc SH2-GST fusion protein markedly inhibited the dephosphorylation of Y-1173 (Fig. 5C). Higher concentrations of Shc SH2 domain (30 μM) led to a greater protection of Y-1173 and to weak protection of Y-992 and Y-1086 from dephosphorylation (Fig. 5D).

Inhibition of Shc binding to the EGFR by using phosphotyrosine-containing peptides and BIAcore analysis of Shc binding to phosphopeptides. Phosphopeptide inhibition experiments of Shc binding to the EGFR were performed similarly to those described for Grb2. The EGFR was immunoprecipitated from A431 cells, autophosphorylated in vitro, and then incubated with Shc SH2 fusion protein in the absence or presence of synthetic phosphopeptides corresponding to various EGFR autophosphorylation sites (Fig. 6). The amount of Shc SH2 fusion protein associated with the EGFR was determined by immunoblotting with anti-GST antibodies. The upper part of the nitrocellulose filters was immunoblotted with antiphosphotyrosine antibodies to control for the amount of phosphorylated EGFR that is present in each lane (data not shown). The results presented in Fig. 6 show that pY1173 had the strongest inhibitory effect on Shc SH2 binding (16% ± 9% binding), followed by pY992 (33% ± 5%). However, pY1068, pY1086, and pY1148 only minimally inhibited the binding of Shc to the EGFR (Fig. 6). Addition of pY1068 and pY1086

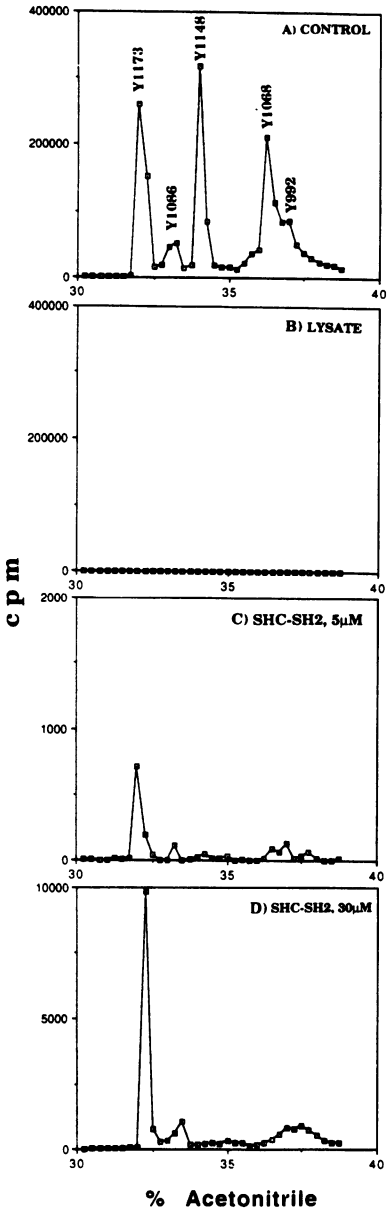

FIG. 5. Dephosphorylation protection assay using Shc SH2-GST fusion protein. The dephosphorylation protection assay was performed as described in Materials and Methods. (A) Control; (B) lysate; (C) 5 μM Shc SH2-GST plus lysate; (D) 30 μM Shc SH2-GST plus lysate. The following gradient was used: from 0 to 10 min in 0% acetonitrile, from 10 to 15 min in a linear gradient to 30% acetonitrile, from 15 to 55 min in a linear gradient to 50% acetonitrile, and from 55 to 56 min an increase to 80% acetonitrile.

together had a strong inhibitory effect on Grb2 binding to the EGFR but had only a minor effect on Shc binding to the EGFR. Complete inhibition of Shc binding to the EGFR was observed in the presence of a mixture of pY992, pY1148, and pY1173. It is possible, therefore, that Shc interacts to some extent with pY1148.

We next determined the ID_{50} values for phosphopeptide inhibition of Shc binding to the EGFR (Table 2). The ID_{50} values for pY1173 and pY992 were 2 and 6 μM, respectively, while the ID_{50} values for pY1068, pY1086, and pY1148 were

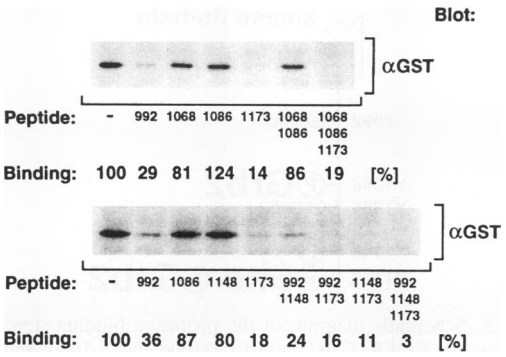

FIG. 6. Inhibition of Shc binding to the EGFR with synthetic phosphopeptides. The EGFR was immunoprecipitated from A431 cells, autophosphorylated in vitro, and then incubated with Shc SH2-GST (0.2 μM) in the absence or presence of 10 μM phosphopeptides indicated below the lanes. When two or three peptides were used together, each was at 10 μM. Blots were quantitated with a Phosphor Imager. A sample without phosphopeptide (−) served as a 100% standard for each panel. αGST, anti-GST antibody.

from 45 to 85 μM. However, using the BIAcore system, we did not detect binding of the Shc SH2 domain to pY1173 or pY992, although the same phosphopeptides were active in the competition analysis (Table 2). Recently the binding site for Shc on the nerve growth factor receptor (NGFR) has been identified (46). Comparison of the amino acid sequence flanking the binding site of Shc on the NGFR with the amino acid sequence around Y-1173 on the EGFR revealed that both tyrosines have a glutamic acid and an asparagine residue at positions −3 and −4, respectively, from the phosphotyrosine moiety. It is possible, therefore, that attachment of the phosphopeptides to the biosensor matrix through their amino-terminal amino acids may have rendered the key residues of the phosphopeptides inaccessible for interaction with the SH2 domain. To overcome this problem, we synthesized new phosphopeptides that contained four additional residues N terminal to the tyrosine moiety but lacked five to eight of the C-terminal residues (Table 2). These phosphopeptides were similarly active in the competition experiments (Table 2). However, analysis with the BIAcore system demonstrated that unlike the short peptide, the long pY1173 peptide bound the Shc SH2 fusion protein with a K_D of approximately 65 nM (Table 2). This result is consistent with the assignment of Y-1173 as the primary binding site for Shc on the EGFR and suggests that residues N terminal to Y-1173 are important for defining the specificity of the SH2 domain of Shc toward its binding site.

TABLE 2. ID_{50} values for phosphopeptide inhibition of Shc SH2 binding to the EGFR and K_Ds for the binding of Shc SH2 to immobilized phosphopeptides, using BIAcore[a]

Phosphopeptide	Sequence	ID_{50} (μM)	K_D (nM)
pY992	DADEpYLIPQQGFFSK	6	NB[b]
pY1068	PVPEpYINQSVPKRK	85	NB
pY1086	QNPVpYHNQPLNPAPSK	45	NB
pY1148	DNPDpYQQDFFPKEAK	70	NB
pY1173	ENAEpYLRVAPQSSEK	2	NB
pY 992-8Y4	DDVVDADEpYLIPQ	6	NB
pY1068-8Y3	AGSVQNPVpYHNQ	45	NB
pY1148-7Y4	ISLDNPDpYQQDF	45	NB
pY1173-8Y4	GSTAENAEpYLRVA	2.5	65

[a] Determination of ID_{50} (using Shc SH2-GST) and K_D (using Shc SH2-GST) values is described in Materials and Methods and in the legend to Fig. 6.
[b] NB, no binding.

We have analyzed the interaction between Shc and the various EGFR mutants by coimmunoprecipitation-immunoblotting experiments. Elimination of the carboxy-terminal tail of the EGFR or mutation of all tyrosine autophosphorylation sites in the carboxy terminus abolished binding of Shc to the EGFR (35, 64). Elimination of individual autophosphorylation sites including Y-1173 had only a minor effect on Shc binding to the EGFR (reference 64 and data not shown). Elimination of both Y-1173 and Y-1148, however, reduced the binding of Shc to EGFR by 75%, while elimination of Y-1173, Y-1148, and Y-1068 reduced the binding of Shc to the EGFR by 83% (64). Although Y-1173 is clearly able to bind Shc, and most likely represents the primary binding site of Shc on the EGFR, other phosphorylation sites within the carboxy-terminal tail of the EGFR are able to compensate for the loss of Y-1173. We have previously shown that phospholipase Cγ (PLCγ) is able to interact with at least three autophosphorylation sites in the EGFR carboxy-terminal tail (55). Similarly, the binding of Ras GTPase-activating protein (GAP) and p85 is strictly dependent upon tyrosine phosphorylation of the carboxy terminus of the EGFR (39, 62). However, we could not assign major binding sites for these proteins on the EGFR (reference 64 and unpublished results).

DISCUSSION

Autophosphorylation may provide different regulatory roles in the control of signaling by receptor tyrosine kinases. Earlier studies have shown that tyrosine autophosphorylation sites on the EGFR can compete with exogenous substrates for the substrate binding site in the catalytic domain, thus providing an autoinhibitory function (3, 22, 71). On the other hand, autophosphorylation of receptor tyrosine kinases, such as the insulin receptor, can provide an autostimulatory function; autophosphorylation potentiates kinase activity by enhancing the V_{max} of the phosphorylation reaction (54). The autophosphorylation sites responsible for activating the kinase activity of the insulin receptor are located within the catalytic domain (18, 66, 72). However, most known tyrosine autophosphorylation sites are located within noncatalytic regions of receptors and function as specific binding sites for the SH2 domains of signaling molecules (48). The β-PDGFR, for example, contains two sites in the juxtamembrane region that serve as binding sites for members of the *src* family (44). It also possesses binding sites for phosphatidylinositol-3 kinase (PI-3 kinase), GAP, and Nck in the kinase insert region and binding sites for PLCγ and the tyrosine phosphatase PTP1D in the carboxy-terminal tail (9, 16, 26, 27, 29–32, 45, 53, 67).

In contrast to the PDGFR, all five tyrosine autophosphorylation sites of the EGFR are clustered in the carboxy-terminal tail region (12, 38, 71). The tyrosine-autophosphorylated EGFR has thus far been shown to associate with PLCγ, GAP, Grb2, Shc, and to a lesser extent PI-3 kinase (23, 36, 37, 39, 40, 57). Whereas binding sites for PLCγ on the EGFR could be determined (55), we have been unable to assign specific binding sites for GAP or PI-3 kinase (unpublished results).

Various independent approaches have been used to identify the binding site(s) of Grb2 on the EGFR. Analysis of the interaction between Grb2 and the EGFR mutants in vivo indicated that phosphorylation of Y-1068 and Y-1173 is essential for binding of the SH2 domain of Grb2 to the EGFR. Mutation of either of these two tyrosines to phenylalanine led to a decreased association of Grb2 with the mutant receptors,

EGFR-1173	TAE**NA**E**pY**LRVA
NGFR-490	IIE**NP**Q**pY**FSDA
middle T-250	LLS**NP**T**pY**SVMR

FIG. 7. Alignment of Shc binding sites on the EGFR, NGFR, and polyomavirus middle T antigen.

while binding of Grb2 to a deletion mutant of the EGFR lacking Y-1068 and Y-1173 was essentially abolished. To address whether the SH2 domain of Grb2 binds directly to the EGFR at Y-1068 and Y-1173, we used a dephosphorylation protection assay. Only Y-1068 and Y-1086 were protected from dephosphorylation. Furthermore, we determined whether phosphopeptides corresponding to the EGFR autophosphorylation sites compete the binding of Grb2 to the EGFR and used BIAcore analysis to study the interaction of Grb2-GST with immobilized phosphopeptides. On the basis of these analyses, we concluded that the main binding site for Grb2 on the EGFR is Y-1068 and a secondary binding site is Y-1086.

The assignment of Y-1068 as the major binding site for Grb2 to the EGFR is consistent with the recent identification of the binding sites of Grb2 on tyrosine-phosphorylated IRS1, Shc, and BCR/ABL (50, 61). Comparison of the amino acid sequences corresponding to Grb2 binding sites to the EGFR (Y-1068, YINQ), to IRS1 (Y-895, YVNI), to Shc (Y-317, YVNV), and to BCR/ABL (Y-177, YVNV) defines a YV/IN sequence as the core motif recognized by the SH2 domain of Grb2. It has been recently reported that the colony-stimulating factor 1 (CSF-1) receptor contains an autophosphorylation site at Y-697, YKNI, which serves as a binding site for Grb2 (68). Elimination of this site by site-directed mutagenesis abolished Grb2 binding to the CSF-1 receptor and CSF-1-induced mitogenesis. A similar sequence, YL/V/I/MN, was obtained by screening degenerate phosphopeptide libraries with the SH2 domain of the Sem5 protein (65). The experiments presented in this report indicate that Y-1068 and Y-1086 can serve as binding sites of GRB2, with some preference for binding to the Y-1068 site. However, the stoichiometry of the autophosphorylation sites of EGFR in living cells is not known.

Since pY1173 did not inhibit the binding of Grb2 to the EGFR, we reasoned that Grb2 binds indirectly to pY1173 and this binding is mediated by another adaptor protein, probably Shc. A number of approaches have been used to determine the binding site of Shc on the EGFR. Both the dephosphorylation protection assay and phosphopeptide inhibition experiments gave a consistent picture regarding the binding sites of Shc on the EGFR. The major binding site for Shc on the EGFR is Y-1173, with a secondary binding site at Y-992. However, additional weaker interaction with Y-1086 or Y-1148 cannot be ruled out. Comparison of the N-terminal sequence of Y-1173 (AENAEY) with amino acid sequence N terminal to the binding site of Shc on the NGFR (Y-490, IENPQY) (46) and middle T antigen (Y-250, LSNPTY) (11) reveals sequence similarity (Fig. 7). All three sequences contain a conserved asparagine residue at positions −3 from the phosphotyrosine and a hydrophobic amino acid, proline or alanine, at position −2. We have shown that pY992 and pY1173 containing four amino acids residues N terminal to the phosphotyrosine were able to compete for binding of Shc SH2 to the EGFR (Fig. 6; Table 2). However, the Shc SH2 did not bind to these peptides when immobilized via their N termini to the biosensor matrix. Therefore, a new set of longer phosphopeptides containing four additional residues N terminal to the phosphotyrosine was

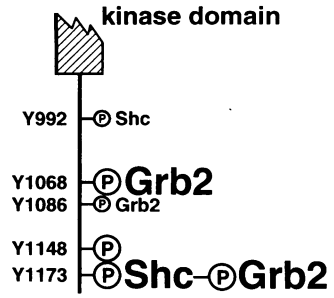

FIG. 8. Schematic diagram of the proposed binding sites of Grb2 and Shc on the EGFR. Grb2 binds to Y-1068 and Y-1086; Shc binds to Y-1173 and Y-992. Large and small site designations indicate major and minor binding sites; large and small P indicate major and minor autophosphorylation sites.

synthesized. Like the shorter phosphopeptides, the new phosphopeptides were able to block the binding of Shc SH2 domain to EGFR in the competition experiments (Table 2). However, extension of the N-terminal amino acids enabled the Shc SH2 to bind to an immobilized pY1173 peptide with high affinity. This analysis provides further support for the assignment of Y-1173 as a major binding site for Shc on the EGFR and suggests that residues N terminal to Y-1173 are important for the generation of a specific binding site for the SH2 domain of Shc. The finding that a recognition sequence for the SH2 domain of Shc may include residues amino terminal to the phosphorylated tyrosine could explain the failure to identify a binding motif for the SH2 domain of Shc by screening random phosphopeptide libraries; the random phosphopeptide library contained only residues C terminal to the phosphotyrosine moiety (65).

Figure 7 contains a comparison of binding sites of Shc in the NGFR, EGFR, and middle T antigen. It appears from this comparison that an NP/AXpY motif is responsible, at least in part, for binding of the SH2 domain of Shc. It is possible that additional residues are also required to generate a high-affinity binding site for Shc's SH2 domain; Y-1086 and Y-1148, which also contain an NPXpY motif, exhibit weak and no interaction, respectively, with the SH2 domain of Shc both in the protection assay and in the phosphopeptide inhibition analysis. Immunoprecipitation experiments using additional EGFR autophosphorylation site mutants indicate that Shc exhibits less stringent selectivity in the context of other phosphorylation sites at the carboxy-terminal tail of the EGFR (64).

On the basis of these analyses, we propose that both Grb2 and Shc have multiple binding sites on the EGFR (Fig. 8). Shc binding has some preference towards autophosphorylated Y-1173 and Y-992 (Y-1173 > Y-992). Grb2 binds to the EGFR directly at Y-1068 and Y-1086 (Y-1068 > Y-1086) and indirectly at Y-1173 through Shc. However, because Y-1173 is the most highly phosphorylated tyrosine in vivo, followed by Y-1148 and Y-1068 (12), the secondary binding sites at Y-992 and Y-1086 for Shc and Grb2, respectively, may play only minor roles as binding sites for these adaptors in the context of living cells.

Given the complicated nature of interactions of these two proteins with the EGFR, it is not surprising that a simple picture did not emerge in the in vivo coimmunoprecipitation experiments using the EGFR mutants. Elimination of the major direct binding site Y-1068 did not decrease the binding of Grb2 by more then 40% (Fig. 2B), nor could elimination of Y-1173 abolish binding of Shc to the receptor (reference 64

and data not shown). It is feasible that following the mutation of a single site, a secondary site may become more phosphorylated and be used to a higher extent. A change in the stoichiometry of phosphorylation of the remaining sites, therefore, could obscure the effect of a single mutation (e.g., the increased binding of Grb2 to the Y1148F mutant; Fig. 2A).

The interaction of SH2 domain-containing proteins with autophosphorylation sites on the PDGFR appears to be much more restricted than the interaction of the same proteins with the EGFR. As mentioned above, the tyrosine-autophosphorylated EGFR binds PLCγ, GAP, Grb2, Shc, and to a lesser extent PI-3 kinase (23, 36, 37, 39, 40, 57). However, in contrast to the PDGFR, we could not assign exclusive binding sites for these proteins on the EGFR. For example, among the five autophosphorylation sites of the EGFR, we were unable to a assign specific binding site(s) for GAP or PI-3 kinase (unpublished results). Moreover, PLCγ (55) and, as shown in this report, Grb2 and Shc exhibit a hierarchy of binding toward autophosphorylation sites on the EGFR. The main binding site of PLCγ to the EGFR is at Y-992, although PLCγ also interacts less strongly with Y-1173 and Y-1086 (55). The reason for the selectivity of PDGFR binding versus the relative promiscuity of EGFR binding warrants further discussion. The difference in the interaction between the same signaling molecules and these two receptors may lie in the different distributions of autophosphorylation sites in these two receptors. All five autophosphorylation sites are clustered within a stretch of less than 190 amino acids in the carboxy-terminal tail of the EGFR (12, 38, 71). However, the seven autophosphorylation sites of PDGFR are spread over the entire cytoplasmic domain: two sites in the juxtamembrane region, three sites in the kinase insert, and two sites in the carboxy-terminal tail (27, 28, 44, 53). Clearly, the local concentration of autophosphorylation sites in the EGFR is much higher than the local concentration of autophosphorylation sites in the PDGFR. The high local concentration of autophosphorylation sites in the EGFR may have a cooperative effect on the binding of SH2 domain-containing proteins to the EGFR. Since the dissociation rates of SH2 domains toward tyrosine-phosphorylated peptides are very high (17), a local high concentration of tyrosine phosphorylation sites may enable multiple phosphotyrosines to compete for the binding of a dissociated SH2 domain; after dissociation, a SH2 domain may rebind to adjacent phosphotyrosines. In this regard, the binding affinity of an SH2 domain toward a specific site such as p85 binding to a pYMXM peptide is only approximately 30- to 100-fold higher than the binding affinity of the same SH2 domain to phosphotyrosine flanked by a random peptide sequence (51). Moreover, recent studies suggest that individual SH2 domains are able to bind two different phosphotyrosine-containing peptides (44, 65). Alternatively, for proteins containing two SH2 domains, such as PLCγ, GAP, and p85, each SH2 domain may bind to different autophosphorylation sites on the same molecule or neighboring EGFR molecules.

Grb2 binds to the guanine nucleotide-releasing factor Sos through its SH3 domains, thus linking receptor and nonreceptor tyrosine kinases to the Ras signaling pathway (6, 8, 14, 19, 34, 56). Experiments presented in this report indicate that the Grb2-Sos complex probably binds to the EGFR directly through phosphorylated Y-1068, with an additional site at Y-1086. However, Grb2 also interacts with tyrosine-phosphorylated Shc, which binds mainly to Y-1173 of the EGFR and to a lesser extent to Y-992. It is not yet clear whether a single EGFR simultaneously binds Grb2-Sos complexes directly as well as indirectly through Shc. It is also possible that one population of receptors interacts directly with Grb2-Sos and another population interacts with a Shc-Grb2-Sos complex. The biological significance of the ability of the EGFR to interact directly and indirectly with Grb2-Sos is not yet understood. Recent studies indicate that EGFRs lacking all five autophosphorylation sites, and therefore unable to interact directly with Grb2 or Shc, are still able to promote tyrosine phosphorylation of Shc, induce formation of Grb2-Shc complexes, and mediate signaling events necessary for mitogenesis (10, 20, 35). Analysis of the biological activities of these mutant receptors indicates that these receptors are able to recruit a similar repertoire of SH2-containing adaptor proteins by auxiliary mechanisms which do not involve direct binding to the EGFR (35).

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