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Individual Epidermal Growth Factor Receptor Autophosphorylation Sites Do Not Stringently Define Association Motifs for Several SH2-containing Proteins*

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To determine whether individual autophosphorylation sites in the epidermal growth factor (EGF) receptor define specific interaction sites for the *in vivo* association of signal transduction proteins that contain *src* homology 2 (SH2) domains, the capacity of wild-type and mutant EGF receptors to associate with several SH2 domain-containing proteins has been assayed. Mutants included receptors with single autophosphorylation site mutations at each of five autophosphorylation sites and receptors in which multiple autophosphorylation sites were removed by point mutation or deletion of carboxyl-terminal residues. Receptor association, as measured by coimmunoprecipitation, has been determined for phospholipase C-γ1, the *ras* GTPase-activating protein, the p85 subunit of phosphatidylinositol 3-kinase, and the *src* homology and collagen protein. In contrast to data obtained with single autophosphorylation site mutants of other receptor tyrosine kinases, none of the EGF receptor single site mutants was dramatically impaired in its capacity to associate with any of these SH2-containing proteins. However, association was completely abrogated when all five autophosphorylation sites were mutated or removed by deletion. These results indicate that individual autophosphorylation sites in the EGF receptor are not stringently required for the recognition and association of different SH2-containing substrates. Thus, EGF receptor autophosphorylation sites seem to be flexible and/or compensatory in their capacity to mediate association with these four SH2-containing substrates.

A key feature to the mechanism of signal transduction by receptor tyrosine kinase is the recognition of receptor motifs containing phosphotyrosyl residues by intracellular proteins that contain *src* homology 2 (SH2)[1] motifs (1, 2). This recognition event, the specificity of which is considered to depend on sequences surrounding the phosphotyrosyl residue as well as sequences within each SH2 domain, leads to high affinity association of activated receptors and SH2-containing molecules. While some SH2-containing molecules are used as phosphorylation substrates by receptor tyrosine kinases, others are not phosphorylated, but function as adaptors to mediate complex formation between receptors and other cellular proteins.

Current evidence indicates that many of these SH2-containing proteins mediate the signal transduction process initiated by growth factors. SH2-containing signal proteins that interact with the activated EGF receptor include enzymes such as phospholipase C-γ1, *ras* GTPase-activating protein (*ras*GAP) (3, 4), and the *syp* phosphotyrosine phosphatase (5, 6) as well as nonenzymatic adaptor molecules such as the p85 subunit of phosphatidylinositol 3-kinase (7, 8), the *src* homology and collagen protein (SHC) (9), GRB-2 (10), and Nck (11–13). Many of these SH2-containing proteins also associate with other activated receptor tyrosine kinases (1–12).

The basis of the interaction between SH2-containing proteins and receptor tyrosine kinases has been most extensively studied with autophosphorylation site mutants of the platelet-derived growth factor (PDGF) receptor, which contains eight known autophosphorylation sites. The results of these studies demonstrate that mutagenesis of one particular autophosphorylation site selectively and significantly abrogates the association of one (14–19) and, in one instance, two (20) SH2-containing molecules with the activated receptor. Thus, individual autophosphorylation sites in the PDGF receptor are stringently required for particular SH2-containing molecules to associate with and, in some cases, be phosphorylated by the PDGF receptors. Analyses of autophosphorylation site mutants in colony stimulating factor 1 (21), fibroblast growth factor (22, 23), and nerve growth factor (NGF) (24, 25) receptors have provided similar results.

When autophosphorylation site mutants of the EGF receptor were analyzed for their capacity to form complexes with *ras*GAP, no single autophosphorylation site mutant was dramatically deficient in this interaction, suggesting a lack of stringent selectivity (26). However, 10-fold less *ras*GAP was able to form an association complex with the activated wild-type EGF receptor compared with the activated PDGF receptor. This might indicate a low affinity interaction between *ras*GAP and the EGF receptor, perhaps due to the absence of a high affinity binding site.

In this study, we have analyzed the capacity of three additional SH2-containing substrates to form complexes with wild-type and autophosphorylation site mutants of the EGF receptor. The results indicate that the EGF receptor model for recognition of at least four SH2-containing substrates differs significantly from data acquired with other receptors and imply novel facets to the recognition mechanism between phosphotyrosyl-containing sequences and SH2-containing substrates.

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[1] The abbreviations used are: SH2, *src* homology 2; EGF, epidermal growth factor; *ras*GAP, *ras* GTPase-activating protein; SHC, *src* homology and collagen protein; PDGF, platelet-derived growth factor; NGF, nerve growth factor.

EXPERIMENTAL PROCEDURES

Materials—EGF was isolated from mouse submaxillary gland according to the method of Savage and Cohen (27). ^{125}I-Labeled goat anti-mouse IgG and ^{125}I-labeled protein A were obtained from ICN Radiochemicals. Protein A-Sepharose CL-4B was from Sigma. Protein G-Sepharose 4B, polyclonal antibodies to phosphotyrosine, and monoclonal antibodies to the intracellular domain of the human EGF receptor were from Zymed Laboratories, Inc. Monoclonal antibodies to the human EGF receptor extracellular domain and polyclonal antibodies to *ras*GAP and the p85 subunit of phosphatidylinositol 3-kinase were obtained from Upstate Biotechnology, Inc. Monoclonal antibodies to phospholipase C-γ1 (28) were kindly provided by Dr. Sue Goo Rhee (National Institutes of Health), and polyclonal antibodies were as described previously (29). Polyclonal antibodies to SHC were purchased from Transduction Laboratories.

EGF Receptor Mutants and Cell Culture—Human EGF receptor mutants were obtained using site-directed mutagenesis to substitute tyrosine residues with phenylalanine or to delete coding sequence for different carboxyl terminus fragments, previously described (26). Mouse NIH3T3 cells, which contain ~3000 endogenous EGF receptors/cell, were used for human receptor transfections. As determined by ^{125}I-EGF binding assay (29), the mutant receptors were expressed at high ($3–5 \times 10^5$) or low ($0.7–1.6 \times 10^5$) levels of receptors per cell. For comparative controls, either of two cell lines, wt-1 expressing high (4×10^5) or wt-2 expressing low (1×10^5) levels of wild-type receptor per cell, was used. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and gentamicin (50 μg/ml).

Growth Factor Treatment—Transfected NIH3T3 cells were grown to ~90% confluence in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and subsequently incubated overnight in Dulbecco's modified Eagle's medium containing 0.5% newborn calf serum. The cells were then incubated without or with EGF (100 ng/ml) for 1 h at 4 °C in Dulbecco's modified Eagle's medium supplemented with 20 mM Hepes, pH 7.4, and 0.1% bovine serum albumin. The capacity of EGF receptors to autophosphorylate, to phosphorylate cellular substrates, and to associate with SH2-containing substrates is not altered when cells are incubated at 4 °C. Control experiments demonstrated that maximal EGF-induced receptor-SH2-containing substrate association is achieved under these conditions.

Immunoprecipitation—After growth factor treatment, cultures were washed with ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline and solubilized in lysis buffer (1% Triton X-100, 10% glycerol, 50 mM Hepes, pH 7.2, 100 mM NaCl, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 544 μM iodoacetamide) for 15 min at 4 °C. The lysates were then clarified by centrifugation (12,000 × *g*, 10 min) at 4 °C. Receptor precipitation was performed using mouse monoclonal antibodies to the extracellular domain of the human EGF receptor (Clone LA1, Upstate Biotechnology, Inc.) and protein G-Sepharose 4B beads. Phospholipase C-γ1, *ras*GAP, the p85 subunit of phosphatidylinositol 3-kinase, and SHC were precipitated with specific rabbit antisera and protein A-Sepharose CL-4B beads. Controls showed that the antisera were not limiting. Immunocomplexes were washed four times with buffer containing 20 mM Hepes, pH 7.2, 100 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 1 mM Na_3VO_4. Samples were boiled in Laemmli buffer (30) prior to separation of proteins on 7.5% SDS-polyacrylamide gels and transfer to nitrocellulose paper.

Western Blot Analysis—The amount of receptor-associated phospholipase C-γ1, *ras*GAP, p85 subunit of phosphatidylinositol 3-kinase, or SHC was detected by Western blot analysis of receptor immunoprecipitates using monoclonal antibodies to phospholipase C-γ1 or polyclonal antibodies to *ras*GAP, p85, or SHC. The amount of EGF receptor immunoprecipitated was determined by immunoblotting using monoclonal antibodies to the intracellular domain of the human receptor (Clone Z025, Zymed Laboratories, Inc.). Since the receptor mutant Dc214 lacks the intracellular antigenic sequence of the Clone Z025 antibody, immunoblots of this receptor were performed with a monoclonal antibody to the extracellular domain of the human EGF receptor (Clone LA22, Upstate Biotechnology, Inc.). The amount of phosphorylated EGF receptor in the different SH2-containing substrate immunoprecipitations was detected by blotting with phosphotyrosine antibody.

Membranes were blocked for 1 h at room temperature in 5% nonfat dry milk/TBST (0.05% Tween 20, 150 mM NaCl, and 50 mM Tris, pH 7.4) and incubated with the indicated primary antibody for 2 h. After washing with TBST, blots were incubated for 1 h with ^{125}I-protein A or with ^{125}I-labeled goat anti-mouse IgG to detect primary rabbit polyclonal or mouse monoclonal antibodies, respectively. Blots were then washed with TBST. Reactive bands were quantitated using a PhosphorImager (Molecular Dynamics, Inc.).

TABLE I
Quantitation of SH2-containing substrate-activated EGF receptor complexes

NIH3T3 cells expressing transfected human wild-type EGF receptor were serum-starved overnight, incubated without or with EGF (100 ng/ml) for 1 h at 4 °C, and then solubilized in lysis buffer. The EGF receptor, phospholipase C-γ1, *ras*GAP, p85, or SHC was immunoprecipitated, electrophoresed, and transferred to nitrocellulose membranes as indicated under "Experimental Procedures." SH2-containing substrates that coprecipitated with the EGF receptor or phosphorylated receptor that coprecipitated with each SH2-containing substrate was analyzed by immunoblotting. To determine the amount of each coprecipitating protein relative to the total cellular content of that protein, increasing amounts of whole cell lysate were electrophoresed, transferred to nitrocellulose membranes, and analyzed by Western blotting to construct a standard curve for each protein.

SH2 substrate	Total cell substrate associated with receptor	Total phosphorylated receptor associated with SH2 substrate
	%	%
PLC-γ1[a]	0.8	0.5
*ras*GAP	0.1	ND
p85	1.0	1.0
SHC	10.0	2.0

[a] PLC-γ1, phospholipase C-γ1; ND, not detectable.

RESULTS AND DISCUSSION

Before analyzing the influence of autophosphorylation site mutations on the formation of association complexes between the EGF receptor and the SH2-containing substrates phospholipase C-γ1, *ras*GAP, p85, and SHC, experiments were performed to quantitate these associations in cells expressing wild-type receptors. The coimmunoprecipitation data in Table I indicate the amount of each SH2-containing protein present in immunoprecipitation complexes with activated EGF receptors relative to the total cellular level of that protein. A similar quantitation is made for the amount of phosphorylated EGF receptor. Under these conditions, at least 50% of the total cellular pool of EGF receptors is phosphorylated. Clearly, these complexes represent very small fractions of the total cellular pool of each molecule. The data suggest that among the four SH2-containing proteins tested, SHC may form the largest and *ras*GAP the smallest amount of association complex with the EGF receptor.

Certain coimmunoprecipitation experiments with the PDGF and NGF receptors have concluded that multiple substrates may be associated simultaneously with one receptor molecule, creating a multimeric complex (25, 31). We attempted to detect such complexes with the EGF receptor. However, when cells treated with EGF are immunoprecipitated with antisera to one SH2-containing protein, we detect only that protein associated with the EGF receptor. Attempts to detect the presence of a second SH2-containing protein in these immunoprecipitates have been unsuccessful. These experiments have included immunoprecipitation of phospholipase C-γ1, *ras*GAP, or SHC from EGF-treated cells and subsequent Western blotting with antibodies to the two heterologous substrates. Since the data in Table I indicate that only very small amounts of receptor (<3%) are ever associated with one SH2-containing protein, it seems unlikely that EGF receptor complexes with multiple SH2-containing substrates are formed.

Analysis of Single Autophosphorylation Sites—To determine whether an individual autophosphorylation site in the EGF receptor is essential for the association of each of four SH2-containing proteins, coprecipitation experiments were performed with cells expressing wild-type or mutant human EGF receptors having a single Tyr → Phe substitution at each of the

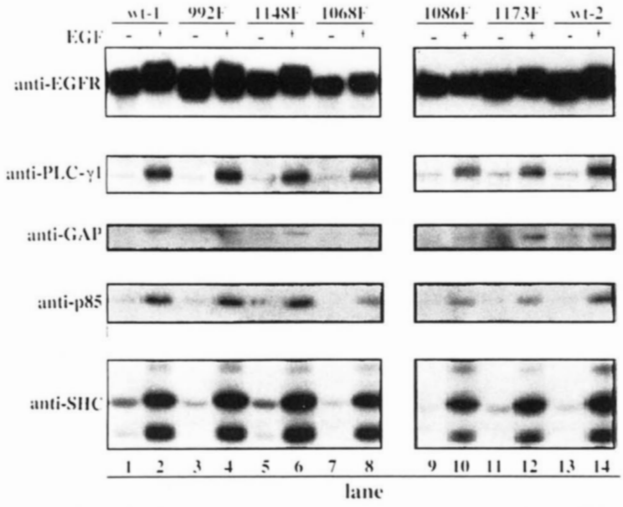

FIG. 1. **Association of SH2-containing substrates with EGF receptor single autophosphorylation site mutants.** Cells expressing EGF receptor single autophosphorylation site mutants were treated without or with EGF (100 ng/ml) for 1 h at 4 °C and then solubilized in lysis buffer. The EGF receptor was immunoprecipitated as indicated under "Experimental Procedures," electrophoresed, and transferred to nitrocellulose membranes. Immunoprecipitated EGF receptor (*EGFR*) and receptor-associated phospholipase C-γ1 (*PLC-γ1*), *ras*GAP, p85, and SHC were analyzed by immunoblotting. *1173F*, *1148F*, *1086F*, *1068F*, and *992F* are EGF receptor mutants with phenylalanine substituted for tyrosine. A transfected cell line expressing high numbers of wild-type EGF receptors (wt-1) was matched with cell lines expressing similar high numbers of mutant receptors (Y992F, Y1148F, and Y1068F). Cell lines expressing low numbers of the receptor mutants Y1086F and Y1173F were matched with a cell line expressing a similar low number of wild-type EGF receptors (wt-2). Autoradiograms of the latter group of cell lines were exposed for a longer period of time, so the data appear equivalent to the results from cells expressing a high number of receptors.

five known autophosphorylation sites (tyrosines 1173, 1148, 1086, 1068, and 992). After growth factor treatment, EGF receptors were immunoprecipitated and analyzed for the presence of phospholipase C-γ1, *ras*GAP, p85, and SHC by Western blotting. Since receptor immunoprecipitations were performed with an antibody specific for human (but not mouse) EGF receptors, the low number of endogenous EGF receptors present in these cells does not contribute to the coprecipitations.

The data in Fig. 1 show Western blots for phospholipase C-γ1, *ras*GAP, p85, and SHC in receptor immunoprecipitates. Also, the blots show the amount of EGF receptor in each immunoprecipitate. The primary data from these Western blots are quantitated and normalized for the amount of immunoprecipitated receptors in Table II. These results demonstrate that no single autophosphorylation site mutant was dramatically impaired in its capacity to associate with any of the four SH2-containing proteins analyzed. The only statistically significant decreases were 35 and 37% for phospholipase C-γ1 and p85 association with mutant Y1173F, respectively. No other mutant-substrate pair exhibited a statistically significant decrease in association capacity. Tyrosine 1173 has been reported to be the major EGF receptor autophosphorylation site in intact cells (32).

These results contrast with the very substantial reductions (>80% or nondetectable levels) observed with single autophosphorylation site mutants of the PDGF receptor and phospholipase C-γ1 (14, 15), *ras*GAP, p85 (16–18), *syp* (19), Nck (20), and *src* (33). SHC association with the PDGF receptor has not been reported. Also, a single mutation of the fibroblast growth factor receptor significantly disrupts phospholipase C-γ1 association (22, 23), and single mutations of the NGF receptor produce substantial decreases in association capacity with phospholipase C-γ1 (24) and p85 and SHC (25). For the EGF receptor, different methods of analysis have suggested the association of the SH2-containing proteins phospholipase C-γ1 (34) and GRB-2 (35) with Tyr-992 and Tyr-1068, respectively. The phospholipase C-γ1 data are derived from an *in vitro* measurement of the protection by EGF receptor-associated phospholipase C-γ1 SH2 domains of different receptor autophosphorylation sites against dephosphorylation by exogenous phosphatase. Our data do not necessarily contradict this result. While receptor-associated phospholipase C-γ1 may be preferentially associated with one or two autophosphorylation sites in the wild-type receptor, our data show that in the intact cell, the association-recognition basis in the EGF receptor is sufficiently flexible that other sites are able to compensate when Tyr-992 is not available.

TABLE II

Quantitation of association of SH2-containing substrates with EGF receptor single autophosphorylation site mutants

Cells expressing the indicated EGF receptors (described in the legend to Fig. 1) were assayed for phospholipase C-γ1, *ras*GAP, p85, and SHC association as indicated for Fig. 1. The amount of each SH2-containing substrate associated with immunoprecipitated receptors was corrected for the amount of immunoprecipitated receptor. Data are expressed as percent of SH2-containing substrate associated with the wild-type receptor and correspond to the mean ± S.E. of at least three independent experiments. Since the amounts of receptor and/or receptor-associated SH2-containing substrate are very low, the error inherent in these measurements is expectedly high and is reflected in the values obtained from different experiments.

EGF receptor	Receptor-associated substrate (% of wild-type)			
	PLC-γ1[a]	*ras*GAP[b]	p85	SHC
Wild-type	100	100	100	100
Y1173F	65 ± 10[c]	83 ± 32	63 ± 2[d]	82 ± 23
Y1148F	110 ± 14	62 ± 19	125 ± 27	181 ± 31
Y1086F	110 ± 26	89 ± 37	189 ± 10	173 ± 46
Y1068F	81 ± 10	72 ± 15	82 ± 26	127 ± 32
Y992F	114 ± 26	76 ± 27	118 ± 23	148 ± 16

[a] Phospholipase C-γ1.
[b] From Ref. 26.
[c] $p < 0.5$ (Student's t test).
[d] $p < 0.001$ (Student's t test).

These results indicate that individual autophosphorylation sites in the EGF receptor are not stringently required for the association of at least these four SH2-containing signaling substrates. These results are in agreement with prior studies of these individual autophosphorylation site mutants, which have shown no decrease in EGF-dependent growth (36) and transformation (37)[2] capacities relative to the wild-type EGF receptor.

Influence of Multiple Autophosphorylation Site Mutations—To determine how the loss of multiple autophosphorylation sites might affect the capacity of the activated EGF receptor to interact with phospholipase C-γ1, *ras*GAP, p85, or SHC, the coimmunoprecipitation experiments shown in Fig. 2 and quantitated in Table III were performed. In the F5 and Dc214 mutants, all five autophosphorylation sites have been removed by site-directed mutagenesis to phenylalanine or by truncation at residue 972, respectively. The data show that neither of these receptor mutants forms association complexes with any of the four SH2-containing proteins examined. Therefore, autophosphorylation sites are required for the observed associations.

The other two mutants examined in Table III are a double mutant (F2) affecting Tyr-1173 and Tyr-1148 and a triple mutant (F3) altering Tyr-1173, Tyr-1148, and Tyr-1068. For phospholipase C-γ1, these data together with the results on mutant Y1173F (Table II) show a progressive decrease in association

[2] L. Beguinot, unpublished results.

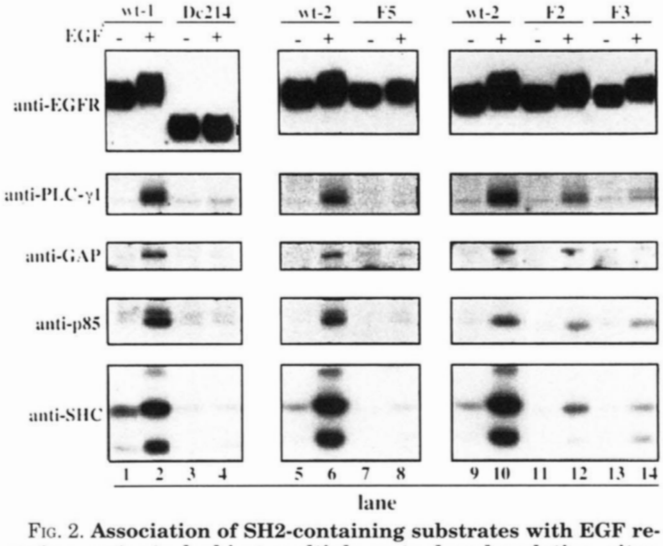

FIG. 2. **Association of SH2-containing substrates with EGF receptor mutants lacking multiple autophosphorylation sites.** Cells expressing the indicated wild-type and mutant EGF (*EGFR*) receptors were tested for phospholipase C-γ1 (*PLC-γ1*), *ras*GAP, p85, and SHC association as described for Fig. 1. The F2 receptor mutant has phenylalanine substitutions at Tyr-1173 and Tyr-1148; the F3 receptor mutant has phenylalanine substitutions at Tyr-1173, Tyr-1148, and Tyr-1068; and the F5 receptor mutant has phenylalanine substitutions at Tyr-1173, Tyr-1148, Tyr-1086, Tyr-1068, and Tyr-992. The Dc214 receptor mutant lacks the last 214 carboxyl-terminal amino acid residues, which include all five known autophosphorylation sites. As in Fig. 1, cell lines expressing high or low numbers of mutant EGF receptors were matched with cell lines expressing high (wt-1) or low (wt-2) numbers of EGF receptors. Autoradiogram exposure time for blots derived from cells expressing low numbers of EGF receptors was longer to produce autoradiogram intensities equivalent to those for the blots from cells expressing high numbers of receptors.

TABLE III

Quantitation of association of SH2-containing substrates with EGF receptors having mutations at multiple autophosphorylation sites

Cells expressing the indicated EGF receptors (described in the legend to Fig. 2) were tested for phospholipase C-γ1, *ras*GAP, p85, and SHC association as indicated for Fig. 1. The amount of each SH2-containing substrate associated with immunoprecipitated receptors was corrected for the amount of immunoprecipitated receptor. Data are expressed as percent of SH2-containing substrate associated with the wild-type receptor and correspond to the mean ± S.E. of at least three independent experiments.

EGF receptor	Receptor-associated substrate (% of wild-type)			
	PLC-γ1[a]	*ras*GAP[b]	p85	SHC
Wild-type	100	100	100	100
F2	51 ± 12[c]	35 ± 9[d]	86 ± 12[e]	25 ± 4[d]
F3	20 ± 11[d]	18 ± 12[d]	49 ± 17[e]	17 ± 9[d]
F5	ND	6 ± 9[d]	ND	ND
Dc214	ND	ND	ND	ND

[a] PLC-γ1, phospholipase C-γ1; ND, not detectable.
[b] From Ref. 26.
[c] $p < 0.001$ (Student's t test).
[d] $p < 0.001$ (Student's t test).
[e] $p < 0.05$ (Student's t test).

capacity with the loss of multiple autophosphorylation sites: Tyr-1173 (35%), Tyr-1173 and Tyr-1148 (49%), and Tyr-1173, Tyr-1148, and Tyr-1068 (80%). These data agree with the previously described effect of these mutations on EGF-induced phosphoinositide hydrolysis and intracellular Ca^{2+} increase (38).

A significant autophosphorylation site for phospholipase C-γ1 appears to be Tyr-1173, which has a sequence motif similar to that of the unique phospholipase C-γ1 association sites in the NGF (24) and fibroblast growth factor (22, 23) receptors, but different from that of the stringent phospholipase C-γ1 association site in the PDGF receptor (14, 15). The EGF receptor Tyr-992 sequence motif is similar to that of the PDGF receptor phospholipase C-γ1 association site. However, phospholipase C-γ1 association with the EGF receptor is unaffected by mutation at Tyr-992. Our single autophosphorylation site mutant data (Table II) and the *in vitro* results of others (39) using phosphotyrosine-containing peptides to each autophosphorylation site do not suggest a unique phospholipase C-γ1-binding site in the EGF receptor. Rather, the phospholipase C-γ1 association data in Table III suggest a cumulative requirement of autophosphorylation sites for maximal phospholipase C-γ1 association with the EGF receptor.

The data with *ras*GAP from Tables II and III seem similar to the phospholipase C-γ1 data in that a progressive reduction in EGF receptor association capacity is recorded with the loss of Tyr-1173 (27%), Tyr-1173 and Tyr-1148 (65%) and Tyr-1173, Tyr-1148, and Tyr-1068 (82%).

In contrast to SHC, *ras*GAP, and phospholipase C-γ1, the association of p85 with the EGF receptor is significantly less affected in the F2 and F3 mutants (Table III). Interestingly, no EGF receptor autophosphorylation site has the consensus motif Y(M/V)XM, which is stringently required for p85 association with PDGF (16, 18), colony stimulating factor 1 (21), and NGF (25) receptors. Recently, a novel motif (YVXV) for p85 association with the hepatocyte growth factor receptor has been identified (40). The sequence at Tyr-1173 of the EGF receptor is YLRV, and it may sufficiently resemble the p85 motif in the hepatocyte growth factor receptor to explain the small decrease observed with the single mutation at this site. Nevertheless, the association data with the F2 and F3 mutants indicate that other autophosphorylation sites are able to mediate p85 association effectively in the absence of Tyr-1173.

Of the four SH2-containing proteins assayed, only the association of SHC is dramatically reduced (75%) in the F2 mutant (Table III), suggesting that the coordinate loss of Tyr-1173 and Tyr-1148 cannot be compensated by the other three autophosphorylation sites. Since single mutations at Tyr-1173 or Tyr-1148 did not significantly reduce SHC association, it seems that either Tyr-1173 or Tyr-1148 can mediate significant levels of SHC association with the EGF receptor. A SHC-binding site has been identified only in the NGF receptor (25) and has a sequence motif of NPQYFSD. This sequence has some similarity to the Tyr-1148 autophosphorylation site (NPDYQQD) of the EGF receptor, but not to the Tyr-1173 site. Alternatively, SHC may be the most sensitive of the four SH2-containing proteins tested to the loss of two autophosphorylation sites because it has a single SH2 domain. The other three proteins tested have two different SH2 domains, which may provide additional flexibility in phosphotyrosine-SH2 interactions.

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