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Direct analysis of the binding of Src-homology 2 domains of phospholipase C to the activated epidermal growth factor receptor

(protein-tyrosine kinase/phosphorylation/Scatchard analysis)

GUOCHANG ZHU[†‡], STUART J. DECKER[‡§], AND ALAN R. SALTIEL[†‡¶]

Departments of [†]Physiology and [§]Microbiology, University of Michigan, School of Medicine, Ann Arbor, MI 48109; and [‡]Department of Signal Transduction, Parke–Davis Pharmaceutical Research Division, Warner–Lambert Company, Ann Arbor, MI 48105

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ABSTRACT **A number of proteins involved in intracellular signaling contain regions of homology to the product of the *src* oncogene that are termed Src-homology (SH) 2 domains. SH2 domains are believed to mediate the association of these proteins with various tyrosine-phosphorylated receptors in a growth factor-dependent manner. We have examined the kinetic characteristics of one of these interactions, the binding of the SH2 domains of phospholipase Cγ1 with the receptor for epidermal growth factor (EGF). Bacterial fusion proteins were prepared containing the two SH2 domains of PLCγ1 and labeled metabolically with [^{35}S]methionine/cysteine. A fusion protein containing both SH2 domains bound to the purified EGF receptor from EGF-treated cells, whereas no binding to receptors from control cells was detected. Binding was rapid, reaching apparent equilibrium by 10 min. Dissociation of the complex occurred only in the presence of excess unlabeled SH2 protein and exhibited two kinetic components. Similarly, analysis of apparent equilibrium binding revealed a nonlinear Scatchard plot, further indicating complex binding kinetics that may reflect cooperative behavior. The binding of the fusion protein containing both SH2 domains was inhibited by a fusion protein containing only the amino-terminal SH2 domain, although at concentrations an order of magnitude higher than that observed with the complete fusion protein. Fusion proteins containing SH2 domains from the GTPase-activating protein, the p85 regulatory subunit of phosphatidylinositol 3′-kinase, or the Abl oncoprotein competed less effectively. Binding of the PLCγ1 SH2 fusion protein to a mutant EGF receptor lacking the two carboxyl-terminal tyrosine phosphorylation sites exhibited a significantly lower affinity than that observed with the wild type, suggesting that this region of the receptor may play an important role. This binding assay represents a means with which to evaluate the pleiotropic nature of growth factor action.**

Src-homology (SH) 2 domains are noncatalytic regions of ≈100 amino acids found in Src-family tyrosine kinases that are conserved among a variety of functionally distinct proteins, including phospholipase C (PLC) γ1 and γ2 (1, 2), the GTPase-activation protein (GAP) of Ras (3, 4), the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase (5, 6), the v-*crk* oncogene product (7–9), protein-tyrosine phosphatase 1C (10), the oncoprotein Vav (11), and others. The critical feature common to proteins containing SH2 domains is their ability to bind to tyrosine-phosphorylated proteins (12–16), including a number of receptor tyrosine kinases that undergo autophosphorylation, such as receptors for epidermal growth factor (EGF) (17, 18), platelet-derived growth factor (PDGF) (19–22), colony-stimulating factor 1 (23), and nerve growth factor (NGF) (24). These interactions may play an important role in mediating the mitogenic or differentiative responses to certain growth factors, and some of these interactions may result in the deregulation of cellular growth associated with transformation (4, 5, 8, 11, 12, 25).

Immunoprecipitation experiments have indicated that SH2-containing proteins may discriminate in their binding to tyrosine-phosphorylated proteins (26, 27). For instance, the colony-stimulating factor 1 receptor can be coprecipitated with PI 3-kinase, but not with GAP or PLCγ1 (28, 29); the NGF pp140$^{c\text{-}trk}$ receptor coprecipitates with PLCγ1 (24), but not with GAP (24) or PI 3-kinase (48); and c-Kit binds PI 3-kinase and PLCγ1 but not GAP (30). Moreover, both the PDGF (31–34) and EGF (18, 33, 35) receptors associate with all three effector proteins, whereas the insulin receptor does not directly coprecipitate with any, although it does associate with PI 3-kinase through its phosphorylation substrate, pp185 IRS-1 (36). In addition to coimmunoprecipitation experiments, numerous receptors directly bind *in vitro* in a growth factor-dependent manner to isolated fusion proteins containing SH2 domains, although some of these associations do not strictly correlate with the known biological actions mediated by these receptors (12, 16). Such observations suggest that interactions between receptors and SH2 domains may exhibit different affinities and, further, that these differences may account for the involvement of different signaling pathways in growth factor action. Thus, to explore the precise kinetic characteristics of the tyrosine kinase–SH2 interaction in more detail, we have established a simple and sensitive binding assay.

MATERIALS AND METHODS

Materials. Mouse EGF was from Collaborative Research. Mouse anti-EGF receptor antiserum (Ab-1) was from Oncogene Sciences (Mineola, NY). Tran^{35}S-label was purchased from ICN. L-Glutathione and L-glutathione-agarose CL-4B were from Fluka. Cells expressing the p85 PI 3-kinase–glutathione *S*-transferase (GST) fusion protein were a gift of J. Schlessinger (New York University). Cells expressing the Abl–GST fusion protein were a gift of Bruce Mayer (Rockefeller University). All other reagents were purchased from Sigma and were the highest quality available.

Molecular Cloning of PLCγ1 SH2 Domains. A cDNA clone encoding the PLCγ1 SH2 domains was amplified by polymerase chain reaction (PCR), using oligonucleotides with *Sma* I (5′)–3′ *Eco*RI linkers. The purified *Sma* I–*Eco*RI DNA fragments from PCR products were ligated into *Sma* I/*Eco*RI-digested pGEX-KT (37) vector in the case of PLCγ1 or pGEX-2T vector in the case of p85. Plasmids were generated that encoded peptides fused to the carboxyl ter-

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Abbreviations: EGF, epidermal growth factor; GAP, GTPase-activating protein; GST, glutathione *S*-transferase; PI, phosphatidylinositol; PLC, phospholipase C; SH, Src homology; PDGF, platelet-derived growth factor.

¶To whom reprint requests should be addressed.

minus of GST from *Schistosoma japonicum*. The PLCγ1 SH2 fusion protein bearing both SH2 domains (NC*SH) contained residues 550–756 from PLCγ1, the amino-terminal SH2 (N*SH) contained residues 550–657, and the carboxyl-terminal SH2 (C*SH) contained residues 668–756. The amino-terminal SH2 domain of human GAP containing residues 171–278 was cloned by the same strategy. Briefly, PCR primers had 5′ *Bam*HI and 3′ *Eco*RI sites, and PCR products were digested with the two enzymes and cloned into *Eco*RI/*Bam*HI-digested pGEX-KT vector.

Expression and Purification of Fusion Proteins. Expression and purification of fusion proteins were performed basically as described (37). A 100-ml overnight culture was inoculated into 2 liters of LB broth or 2× YT medium containing ampicillin (100 μg/ml). The culture was incubated at 37°C with shaking until reaching an OD_{600} of 1. Isopropyl β-D-thiogalactopyranoside (IPTG, 0.2 mM) was then added and the culture was grown for an additional 3 hr. The culture was centrifuged, and the pellet was suspended in phosphate-buffered saline (150 mM NaCl/16 mM NaH_2PO_4/4 mM Na_2HPO_4, pH 7.3) with 1% (wt/vol) Triton X-100 and 0.1% 2-mercaptoethanol. The cells were lysed by sonication and the lysate was centrifuged at 10,000 × *g* for 10 min. The supernatant was mixed with 8 ml of 50% (vol/vol) glutathione-agarose at 4°C for 30 min. The beads were washed three times with phosphate-buffered saline containing 1% Triton X-100 and twice with 50 mM Tris·HCl (pH 8.0) containing 5 mM glutathione. To prepare the ^{35}S-labeled PLCγ1 fusion proteins, a 20-ml overnight culture was added to 180 ml of LB broth with ampicillin (100 μg/ml). After the culture reached an OD_{600} of 1, IPTG (0.2 mM) was added for 15 min, followed by the addition of 2 mCi (74 MBq) of Tran^{35}S-label for 90 min at 37°C.

Purification of the Phosphorylated EGF Receptor. NIH 3T3 mouse cells expressing the wild-type human EGF receptor (3T3/hEGFr) or expressing the human EGF receptor with the 63-amino acid carboxyl-terminal deletion (3T3/hEGFrHT) (38) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells grown to confluence in 150-mm dishes were treated with EGF (150 ng/ml) for 10 min at 37°C. After two washes with 10 ml of ice-cold phosphate-buffered saline, cells were lysed in HY buffer (50 mM Hepes, pH 7.4/150 mM NaCl/10% (vol/vol) glycerol/1% (wt/vol) Triton X-100/2.5 mM $MgCl_2$/1 mM EGTA/10 mM sodium pyrophosphate/100 mM NaF/200 μM Na_3VO_4 with leupeptin and aprotinin at 10 μg/ml (38). Lysates were centrifuged at 12,000 × *g* for 6 min, and the supernatant was incubated for 45 min with anti-EGF receptor antiserum. Then protein A-Sepharose beads were added and the solutions were mixed for 30 min. Immune complexes bound to the beads were washed three times with HY buffer.

SH2–Receptor Binding Assay. Binding of ^{35}S-labeled SH2 fusion proteins to the immunoprecipitated EGF receptor was assayed in HY buffer. The binding reaction mixture contained 0.1 ml of phosphorylated EGF receptor–bead complex (≈1 μg of receptor per assay) and the indicated concentration of labeled and unlabeled fusion protein. Unless otherwise indicated, the reaction mixture (0.4 ml) was incubated at 4°C for 20 min. Bound ^{35}S-labeled fusion protein was separated from free by microcentrifugation. The beads were washed three times by centrifugation and resuspension, and radioactivity was determined by scintillation counting. For dissociation experiments, binding was allowed to proceed to equilibrium in 2 ml using 85 nM [^{35}S]PLCγ1 NC*SH. Bound ligand was separated from free ligand by centrifugation, and the beads were resuspended in an equal volume of HY buffer in the presence or absence of 8 mM unlabeled ligand and allowed to dissociate at room temperature for the designated times.

RESULTS

To evaluate the binding of a SH2 domain to a tyrosine-phosphorylated protein, we have characterized the interaction of the EGF receptor with the SH2 domains of PLCγ1. Three fragments of PLCγ1 containing either both amino- and carboxyl-terminal SH2 domains (NC*SH), or the separate amino (N*SH)- or carboxy (C*SH)-terminal domains alone were cloned into GST fusion proteins. These three proteins were expressed in *Escherichia coli* and purified by affinity chromatography on glutathione-agarose. For some experiments, the proteins were labeled by incubating bacteria with Tran^{35}S-label prior to purification of the fusion proteins. To assess their purity and specific activity, the labeled fusion proteins were electrophoresed in 10% polyacrylamide gels and detected by Coomassie blue staining (Fig. 1*A*) and autoradiography (Fig. 1*B*). The resulting proteins were >99% pure, based upon staining of the gel, and the purification yielded 2–5 mg of fusion protein per liter of culture broth. The ^{35}S-labeled proteins exhibited a specific activity of 15,000–20,000 dpm/μg.

The binding of the three fusion proteins to the EGF receptor was evaluated. NIH 3T3 cells overexpressing the human EGF receptor (3T3/hEGFr) were treated with or without EGF for 10 min, and receptors were purified by immunoprecipitation with an anti-EGF receptor antiserum directed toward the extracellular domain (Table 1). The immunoprecipitated receptors were then incubated with the ^{35}S-labeled SH fusion proteins in the presence or absence of 100-fold excess unlabeled protein. After the incubation, bound ligand was separated from free by centrifugation, and binding was determined by scintillation counting of the pellet. Approximately 10% of the fusion protein containing both SH2 domains (NC*SH) bound to the receptor from EGF-treated cells. This binding was almost completely blocked by the addition of excess unlabeled ligand. In contrast, only background binding was detected in receptor from untreated cells. The labeled amino (N*SH)- and carboxyl (C*SH)-terminal SH2 fusion proteins bound to the receptor in an EGF-dependent manner, although this binding was only 10% of that seen with the NC*SH protein. However, these two ligands were not displaced by excess unlabeled ligand, indicating that this binding, although dependent upon tyrosine phosphorylation, was of such low affinity as to be considered nonspecific.

Some of the characteristics of the binding of NC*SH to the EGF receptor were studied. Addition of divalent cations such as Ca^{2+}, Mg^{2+}, or Mn^{2+} at concentrations up to 10 mM had no effect. Moreover, the pH dependence of binding was not striking; varying the pH from 6 to 9 had no influence on

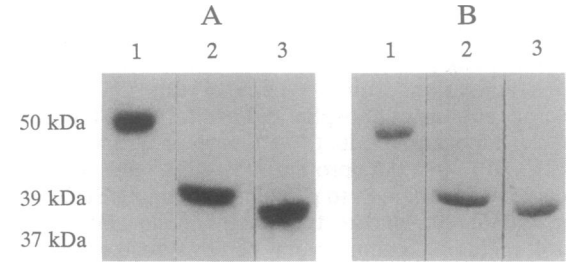

FIG. 1. Purification of the PLCγ1 SH2 fusion proteins. ^{35}S-labeled PLCγ1 SH2–GST fusion proteins were purified by affinity chromatography on glutathione-agarose. The resulting proteins were electrophoresed in SDS/10% polyacrylamide gels, and proteins were stained with Coomassie brilliant blue (*A*) and subsequently exposed to Kodak XAR film for 2 hr (*B*). NC*SH, N*SH, and C*SH fusion proteins were 50, 39, and 37 kDa, respectively. Lanes: 1, 0.8 μg of the NC*SH fusion protein; 2, 1 μg of the N*SH fusion protein; 3, 1 μg of the C*SH fusion protein.

Table 1. Binding of ^{35}S-labeled PLCγ1 SH2 fusion proteins to the EGF receptor

Ligand	Total dpm	Bound, dpm: EGF-treated, Label alone	Bound, dpm: EGF-treated, Excess unlabeled	Bound, dpm: Control, Label alone	Bound, dpm: Control, Excess unlabeled
NC*SH	15,000	4120 ± 17	85 ± 8	82 ± 7	79 ± 3
C*SH	40,000	850 ± 2	521 ± 16	80 ± 0	82 ± 7
N*SH	60,000	519 ± 26	550 ± 2	133 ± 1	57 ± 5

3T3/hEGFr cells were treated with or without EGF (150 ng/ml) for 10 min at 37°C. The EGF receptor was immunoprecipitated from cell lysates, and the resulting immune complexes were incubated for 30 min with the indicated ^{35}S-labeled fusion proteins in the presence or absence of 100-fold excess unlabeled protein. Bound ligand was separated from free by centrifugation. Results are expressed as the means ± SD of triplicate determinations, and identical results were obtained in three separate experiments. Specific activities of the fusion proteins were 22,000 dpm/μg for NC*SH, 13,000 dpm/μg for N*SH, and 14,400 dpm/μg for C*SH.

binding, although it was greatly reduced at pH 5 and not detectable at pH 4.

The time course of binding of the NC*SH protein to the receptor was evaluated (Fig. 2). The binding of labeled intact SH2 fusion protein to receptors from cells treated with EGF was assayed as a function of time of incubation. This binding was rapid, reaching 50% of maximum by 1–2 min and attaining apparent equilibrium by 10 min. Virtually no binding was detected to receptors from untreated cells (see Table 1).

The kinetics of dissociation of the NC*SH–EGF receptor complex were also evaluated (Fig. 3). The labeled fusion protein was incubated with the purified receptor derived from EGF-treated cells, and binding was allowed to reach equilibrium. The SH2–receptor complex was separated from unbound ligand by centrifugation, and dissociation was followed as a function of time after dilution in the presence or absence of excess unlabeled NC*SH or N*SH. The dilution of the receptor–SH2 complex did not cause an appreciable dissociation. Moreover, addition of excess amino-terminal SH fusion protein or the carboxyl-terminal protein (data not shown) was also without effect. However, addition of excess unlabeled NC*SH fusion protein produced a marked enhancement of the dissociation rate. Dissociation was biphasic, with a rapidly dissociating component ($t_{1/2}$ of 1–2 min) and an apparently nondissociable component. No differences in the dissociation profile were observed when experiments were performed at 4°C, 22°C, or 37°C (data not shown).

To further evaluate the affinity of SH2–EGF receptor binding, a competition experiment was performed under apparent equilibrium binding conditions (Fig. 4). ^{35}S-labeled NC*SH was incubated with the activated receptor in the presence of various concentrations of unlabeled NC*SH, C*SH, or N*SH or of GST fusion proteins containing the amino-terminal SH2 domains of GAP, the p85 subunit of PI 3-kinase, or the SH2 domain of Abl. Displacement of bound NC*SH with unlabeled ligand revealed a shallow competition curve, with an IC_{50} of ≈100 nM. Bound NC*SH was also displaced by the amino-terminal SH2 protein, although the IC_{50} for this binding was ≈1 μM. The amino-terminal SH2 domain of GAP was ≈100 times less potent in inhibiting binding. Interestingly, the carboxyl-terminal SH2 domain of PLCγ1, the amino-terminal SH2 domain of p85, and the SH2 domain of Abl produced only weak inhibition of binding, with no appreciable displacement achieved up to 10 μM concentrations of these fusion proteins. These results suggest a considerable degree of specificity in the SH2–EGF receptor interaction.

To further explore the specificity of binding of the NC*SH fusion protein, we used the competition binding assay to compare the affinity of NC*SH for wild-type and a well-characterized mutant EGF receptor (Fig. 5). Scatchard analysis of SH2 binding to the wild-type human EGF receptor revealed a curvilinear plot, with two apparent binding sites: a high-affinity site with a K_d of ≈70 nM and a B_{max} of ≈8 pmol/μg of receptor protein, and a low-affinity site with a K_d of ≈2.5 μM and a B_{max} of ≈50 pmol/μg of receptor. Inter-

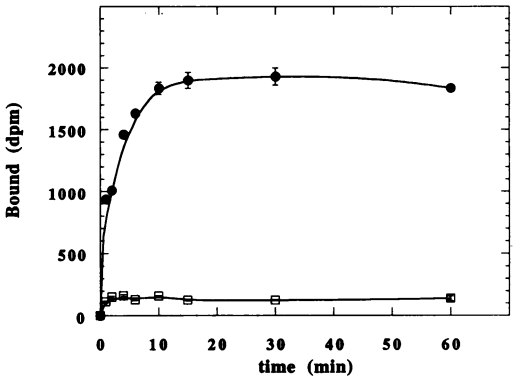

FIG. 2. Rate of association of the NC*SH2 fusion protein with the activated EGF receptor. 3T3/hEGFr cells were treated with (●) or without (□) EGF (150 ng/ml) for 10 min. Receptors were immunoprecipitated and the immune complexes were incubated with 25 nM [^{35}S]NC*SH in 0.4 ml of HY buffer for the indicated times. Bound ligand was separated from free by centrifugation. Results are expressed as the means ± SD of triplicate determinations, and identical results were obtained in four separate experiments.

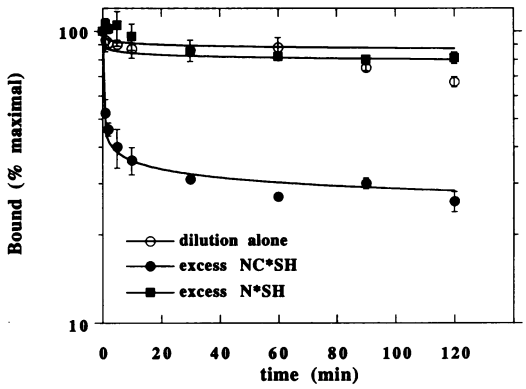

FIG. 3. Dissociation of NC*SH2 fusion protein–EGF receptor complex. Immunoprecipitated EGF receptor from EGF-treated 3T3/hEGFr cells was incubated with 85 nM [^{35}S]NC*SH in 2 ml of HY buffer for 20 min. Following centrifugation, the bound precipitated complexes were diluted in 5.5 ml of HY buffer alone (○) or with 8 μM unlabeled NC*SH (●) or N*SH (■). Aliquots (0.25 ml) were removed at the indicated times and centrifuged to separate bound ligand from free. Results are expressed as percent of binding at time zero and are the means ± SD of triplicate experiments, which have been reproduced in three separate experiments.

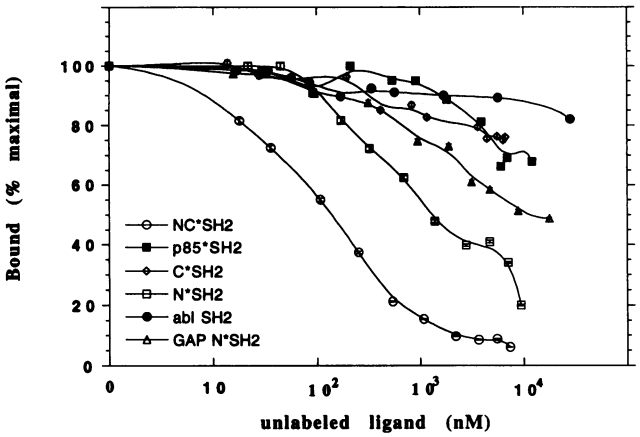

FIG. 4. Equilibrium binding of NC*SH2 fusion protein to the activated EGF receptor. Immunoprecipitated EGF receptor from EGF-treated 3T3/hEGFr cells was incubated with 25 nM [^{35}S]NC*SH in the presence of the indicated concentrations of unlabeled NC*SH (○), N*SH (□), C*SH (◇), or a GST fusion protein containing the amino-terminal SH2 domains of PI 3-kinase p85 (■) or GAP (△) or the SH2 domain of Abl (●). The incubation was carried at 4°C for 20 min in HY buffer, and bound ligand was separated from free. Results are expressed as percent of maximal binding and are the means ± SD of triplicate determination. Identical results were reproduced in four separate experiments.

estingly, the characteristics of the high-affinity site suggested an approximate 1:1 molar stochiometry for this binding. To determine the importance of different phosphorylation sites on the receptor, we examined binding to a mutant human EGF receptor in which 63 amino acids of the carboxyl terminus were deleted, thus removing Tyr^{1148} and Tyr^{1173} (38). These receptors are similar to wild-type receptors in EGF binding, metabolism, internalization, and tyrosine kinase activity (38). Analysis of NC*SH binding to this truncated receptor revealed a single class of binding sites with significantly lower affinity (K_d of ≈6 μM) than that observed with wild-type receptors.

DISCUSSION

The recent observations that growth factor receptor tyrosine kinases may bind to a number of different intracellular proteins containing SH2 domains have provided a new perspective with which to explore the mechanisms of pleiotropism in signal transduction. In early experiments (20, 21), observed differences in the identity and extent to which SH2 proteins associate with specific receptors suggested that differences in the affinities of these interactions might be a critical determinant not only in defining a specific pathway of signaling but also in the modulation of growth control. Thus, variations in cell type, presence of other hormones or their receptors, or relative concentrations of different effector molecules, especially kinases and phosphatases, could markedly influence the nature of receptor–SH2 interactions. For example, the stimulation of PI hydrolysis by EGF is generally restricted to cells overexpressing receptors (33, 39–41). This observation correlates with the degree of PLCγ1–receptor association, as well as with tyrosine phosphorylation of PLCγ1 (16–18). In contrast, even when overexpressed, the insulin receptor does not mediate stimulation of PI turnover and does not associate with PLCγ1 (33, 41), whereas the PDGF receptor virtually always exhibits this behavior, even in cells expressing lower numbers of receptors (33, 41–43). Thus, the regulation of receptor or tyrosine kinase concentration may be critical to determining which signaling pathways are activated by a growth factor or oncogene.

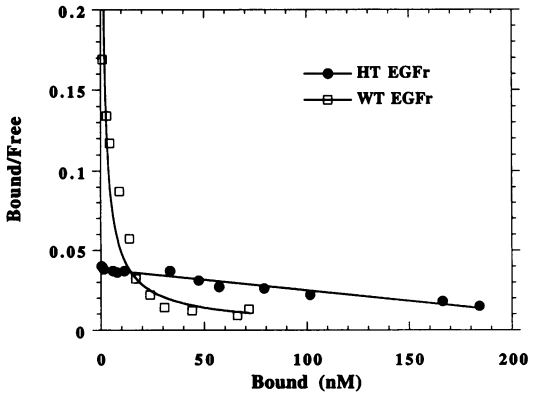

FIG. 5. Scatchard analysis of NC*SH2 fusion protein binding to wild-type and mutant EGF receptors. 3T3/hEGFr (□) or 3T3/hEGFrHT (●) cells were treated with EGF (100 ng/ml) for 10 min. EGF receptors were immunoprecipitated, and equilibrium binding of NC*SH to the immune complexes was assayed for Fig. 4. The results were transformed to Scatchard plots, and data were subjected to nonlinear regression analysis. Results are means of triplicate determinations in which variation was <2%.

To study the dynamics of tyrosine kinase–SH2 interactions, we have established a simple quantitative assay. Analysis of the binding of a pure metabolically labeled fusion protein containing the two SH2 domains of PLCγ1 to a purified EGF receptor revealed high-affinity, low-capacity binding that was absolutely dependent upon the phosphorylation of the receptor produced by EGF. Labeled fusion proteins containing the separate amino- and carboxyl-terminal SH2 domains also bound to the phosphorylated receptors, although the bound protein was not displaceable with unlabeled protein, indicating a very low-affinity interaction that most likely reflects nonspecific binding of SH2 domains to phosphotyrosine. Such observations highlight the importance of quantitative analysis of tyrosine kinase–SH2 binding, to avoid the potential pitfalls associated with interpreting results of coprecipitation experiments.

Although it is difficult to interpret the significance of binding affinities predicted by the analyses described here, this technology has allowed a straightforward comparison of the binding of SH2 domains to different tyrosine phosphorylated proteins. Moreover, we have also used this binding assay to evaluate the dynamics of this interaction. Although this binding is rapid, reaching apparent equilibrium within 10 min, the kinetics are complex. There was little dissociation of the labeled NC*SH2 fusion protein induced by dilution, although considerable dissociation occurred in the presence of excess unlabeled ligand, suggesting cooperative site–site interactions. Similarly, Scatchard analysis of equilibrium binding also indicated nonlinear kinetics, suggesting negative cooperativity among a single class of sites, or two (or more) discrete classes of binding sites. Although the molecular basis for these observations is not completely understood, it is clear that both ligand and receptor have more than one region involved in binding. The PLCγ1 fusion protein contains two SH2 domains. Analysis of competition binding data with the labeled NC*SH fusion protein, as well as direct binding of the isolated SH2 domains, suggests that these regions differentially contribute to binding in a cooperative fashion. Moreover, different phosphorylation sites in the receptor are also differentially involved in this binding. The marked decrease in affinity exhibited by the truncated EGF receptor indicates that the carboxyl-terminal domain containing Tyr^{1148} and Tyr^{1173} is critical for NC*SH binding, although the fusion protein can bind with lower affinity to a receptor lacking this region. However, experiments with this mutant receptor should not be interpreted to indicate that these are the precise binding sites on the EGF receptor for PLCγ1. Recent studies

examining protection of receptor dephosphorylation by PLCγ1 SH2 fusion proteins indicated that Tyr^{992} is a crucial residue for this binding (44). These findings are consistent with the specific inhibition of NC*SH–EGF receptor binding with phosphopeptides modeled around this site (G.Z., D. McNamara, E. Dobrussin, S.J.D., and A.R.S., unpublished work). However, results presented here suggest that the carboxyl-terminal tyrosines play some role in receptor–SH2 binding, and are consistent with findings that the truncated receptor is significantly impaired in tyrosine phosphorylation of PLCγ1 (45), stimulation of PI hydrolysis (46), and calcium mobilization (47), although it retains EGF binding, tyrosine kinase, and autophosphorylation of other residues and ability to mediate mitogenesis at a reduced level (39).

The use of the binding assay described here can be expanded to evaluate other SH2–tyrosine kinase interactions. It should be possible to quantitatively characterize these interactions, to determine whether different SH2 domains compete for a single receptor on similar or different regions, whether phosphorylation of the SH2 protein can change its affinity for tyrosine kinases, and whether different receptors bind with different affinities to single SH2 domains. Such studies should help in our understanding of the molecular basis for the pleiotropic actions of growth factors.

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