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STRUCTURAL BASIS OF INTERACTIONS BETWEEN EPIDERMAL GROWTH FACTOR RECEPTOR AND SH2 DOMAIN PROTEINS

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The structural basis of the interactions between the activated epidermal growth factor (EGF) receptor and SH2 domain proteins was investigated. The c-src SH2 domain (second domain of src homology) was expressed as a recombinant fusion protein, and an *in vitro* assay was developed to monitor EGF receptor/SH2 domain interactions. EGF receptor tyrosine kinase domain (TKD) forms expressed in the baculovirus/insect cell system were shown to bind to the SH2 domain when phosphorylated. These TKD/SH2 domain interactions were characterized by dissociation constants of 60-320 nM. Deletion analysis indicated that the entire SH2 domain was required for recognition of the phosphorylated TKD. The binding of a highly truncated TKD protein to the SH2 domain suggested that the sites recognized by the SH2 domain included the EGF receptor autophosphorylation site, tyr_{992}. A phosphorylated EGF receptor peptide containing tyr_{992} was also shown to interact with the SH2 domain. This residue may therefore mediate interactions between the EGF receptor and tyrosine kinases in the src family.

A variety of growth factor receptors with intrinsic protein tyrosine kinase activity have now been characterized. Numerous studies have shown that these receptors physically interact with intracellular proteins to form signal transduction complexes in the plasma membrane (**1-3**). The proteins associated with the receptor in these complexes are believed to be responsible for second messenger generation, or otherwise involved in the propagation of mitogenic signals. An important goal in understanding the signal transduction mechanisms of receptor tyrosine kinases is to determine how specific protein sequence elements direct the interactions between the protein constituents of their signal transduction complexes.

Some of the known growth factor receptor-associated proteins contain domains of conserved amino acid sequence originally identified in nonreceptor tyrosine kinases of the src family. The second domain of src homology (SH2) [see (**4**) and Fig. 2A], which includes the B and C

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Abbreviations: PI, phosphatidylinositol; GAP, GTPase activating protein; GRB2, growth factor receptor bound protein 2; SH2, second domain of src homology; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IGF-1, insulin-like growth factor 1; TKD, recombinant EGF receptor tyrosine kinase domain; GST, glutathione S-transferase; MBP, *E. coli* maltose binding protein; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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domains identified by Stahl *et al.* (**5**), is found within the sequences of phospholipase C-γ1, the 85 kDa subunit of phosphatidylinositol (PI) 3-kinase, the ras GTPase activating protein (GAP), GRB2 (growth factor receptor bound protein 2) and the vav oncoprotein. The observation that SH2 domains can independently interact with activated growth factor receptors demonstrates an important theme in signal transduction [for reviews see (**3**,**6**,**7**)]. The isolated SH2 domains of phospholipase C-γ1, PI 3-kinase, GAP and v-src have each been shown to associate with either the EGF, PDGF, insulin or IGF-1 receptors *in vitro* (**8**-**11**). The interaction of growth factor receptors with SH2 domain proteins *in vivo* requires the prior activation of the receptor. Apparently, it is the autophosphorylated form of the growth factor receptor which interacts with the SH2 domain. A recently published X-ray crystallographic structure of a complex between a phosphotyrosine-containing peptide and the SH2 domain of v-src indicates that SH2 domain residues make several critical contacts with the phosphotyrosine residue of the bound peptide (**12**).

We describe here a study of the interaction between the phosphorylated EGF receptor tyrosine kinase domain (TKD) and the SH2 domain of the c-src. The intact SH2 domain or subsequences of this domain were expressed as recombinant fusion proteins and purified by affinity chromatography. The affinity chromatography matrices were further exploited as a convenient means of precipitating the recombinant SH2 domain proteins and any associated phosphoproteins. By this assay it was also possible to estimate the affinity of the interactions between the phosphorylated proteins and the SH2 domain.

Recombinant EGF receptor tyrosine kinase domain (TKD) forms were expressed with the baculovirus/insect cell system, and the interactions of these proteins with the SH2 domain were studied. Various truncated SH2 domain proteins were also used to determine what elements of structure were necessary for binding to the phosphorylated TKD. Finally, a recombinant TKD was used to phosphorylate an EGF receptor-derived peptide on tyrosine residues, so that the potential association of this phosphoprotein with the SH2 domain of c-src could be tested.

MATERIALS AND METHODS

Materials. ATP sodium salt, reduced glutathione, glutathione-agarose (G-4510), and immunological reagents were purchased from Sigma Chemical Company. [γ^{32}P]ATP (50 Ci/mmol) was acquired from Du Pont New England Nuclear. The chicken c-*src* cDNA was derived from pM5HHB5 (**13**).

Expression and purification of recombinant proteins. The expression of recombinant full-length and truncated EGF receptor tyrosine kinase domain (TKD) forms (see Fig. 3A) in the baculovirus/insect cell system will be described elsewhere (S. Sierke and J. Koland, manuscript in preparation). The TKD proteins were partially purified by DEAE-Sephacel chromatography, and the enzyme was stored in 20 mM HEPES/Na, 1 mM dithiothreitol, ~150 mM NaCl, 35% (v/v) glycerol, pH 7.4 at -20°C. The preparations were shown to be devoid of protein serine and threonine kinase activity.

Peptides found within the sequences of the EGF receptor and the chicken c-src tyrosine kinase were expressed as fusion proteins in *Escherichia coli* (S. Sierke and J. Koland, manuscript in preparation). Fusion of the peptide sequences C-terminal to either glutathione S-transferase (GST) or maltose binding protein (MBP) sequences enabled the rapid purification of these peptides by affinity chromatography on glutathione-agarose (**14**) or cross-linked amylose (**15**,**16**), respectively. The fusion proteins were eluted either in 5 mM reduced glutathione or 10 mM maltose in Buffer A [20 mM HEPES/Na, 50 mM NaCl, 10% (v/v) glycerol, pH 7.4]. Free glutathione or maltose was removed from the purified fusion proteins by extensive dialysis against Buffer A at 4°C. Protein concentration was estimated by the Bradford assay (**17**).

Affinity matrix assay for protein-protein interactions. The binding of autophosphorylated TKD forms and phosphorylated tyrosine kinase substrates to the GST-SH2 or MBP-SH2 protein was assayed by precipitating the SH2 fusion protein with the appropriate affinity matrix. To begin the assay, proteins were phosphorylated for 15 min at room temperature in 50 µl of Buffer A supplemented with 0.1% Triton X-100, 10 mM $MnCl_2$, and 10 µM [γ^{32}P]ATP (~8,000 cpm/pmol). Phosphorylation was stopped by the addition of 5 µl of 0.5 M EDTA/Na, pH 7.4, and the GST-SH2 or MBP-SH2 protein (5 µg unless otherwise noted) was added in 50 µl of Buffer A. After a 15 min room temperature incubation, a 50 µl volume of a 1:1 suspension of glutathione-agarose or cross-linked amylose in Buffer A was added, and the suspension was rocked for 30 min at 4°C. The suspension was centrifuged for 45 sec at 10,000 g and the supernatant removed. The affinity matrix was washed in 400 µl of ice-cold Buffer A and collected again by centrifugation. The matrix and associated proteins were suspended in gel sample buffer, and analyzed by SDS-PAGE and autoradiography or immunoblotting (see below). Samples of the supernatant were also analyzed. In control experiments an equal mass of carrier protein (GST or MBP) was added in place of the SH2 domain fusion protein. In other control experiments, either 5 mM reduced glutathione or 10 mM maltose was added prior to the incubation with the affinity matrix, in order to demonstrate the specificity of the precipitation. The inclusion of 0.1% Triton X-100 in the original incubation was found to greatly reduce the nonspecific association of phosphorylated TKD with the affinity matrix.

This assay was also used to estimate the affinities of the interactions between various phosphorylated proteins and the SH2 domain. Here, the amount of the phosphorylated peptide associated with the GST-SH2 protein in the glutathione-agarose pellet was determined as a function of the concentration of GST-SH2 (see Fig. 4). It was assumed that the quantity of phosphorylated protein found in the pellet was equal to the quantity of GST-SH2 bound to the phosphoprotein (a one-to-one stoichiometry), so that a plot of bound phosphoprotein versus GST-SH2 concentration was equivalent to a binding isotherm characterizing the interaction between the two proteins. A nonlinear least squares analysis of these data was used to determine the dissociation constant (K_d) for the interaction. A similar analysis has been used to determine the affinity of interactions between DNA binding proteins and labeled DNA molecules by nitrocellulose filter binding assays [cf. (**18**)].

Electrophoresis methods. SDS-PAGE (**19**) was used to confirm the identity and purity of fusion proteins, and to resolve phosphorylated proteins in the affinity matrix precipitation assay. After electrophoresis, gels were stained with Coomassie Blue R-250 and dried. ^{32}P-Labeled proteins were detected by autoradiography (4-24 h exposures) with Kodak AR-5 film. Standard methods for Western immunoblotting were used to detect unlabeled proteins in SDS gels (**20**). Anti-EGF receptor mouse monoclonal antibody 291-3A (**21**) was used at a dilution of 1/500 in TBS plus 1% bovine serum albumin. Immune complexes were detected with alkaline phosphatase-conjugated second antibodies and color reagents.

RESULTS

An affinity matrix precipitation assay for EGF receptor/SH2 domain interactions. The SH2 domain of the chicken c-src protein includes amino acid residues 137 to 241 (**4**). With the use of available restriction sites in c-*src* cDNA, the sequence of amino acid residues from 139 to 263 was expressed in both glutathione S-transferase (GST) and maltose binding protein (MBP) fusion proteins (see Fig. 2A and *Materials and Methods*). GST and MBP carrier proteins with no fused sequences were also prepared for use in control experiments. To test the potential interaction of GST-SH2 protein with the phosphorylated EGF receptor, a 61 kDa EGF receptor tyrosine kinase domain form (TKD61, see Fig. 3A) generated in the baculovirus/insect cell expression system was employed. The TKD61 protein was first allowed to autophosphorylate in the presence of $MnCl_2$ and [γ^{32}P]ATP. The phosphorylated protein was then incubated with GST-SH2 in the presence of EDTA, which was added to inhibit further phosphorylations. The GST-SH2 protein and any associated TKD61 were then precipitated with the glutathione-agarose matrix (see *Materials and Methods*). Phosphorylated TKD61 associated with GST-SH2 was detected by SDS-PAGE and

autoradiography (see Fig. 1A). A substantial fraction of the phosphorylated TKD61 protein was found in the precipitate.

The sequence-specificity of the indicated TKD61/SH2 domain interaction was demonstrated by substitution of the GST carrier protein for GST-SH2. The carrier protein did not bind a significant quantity of the TKD61 protein (see Fig. 1A). Excess reduced glutathione introduced prior to the glutathione-agarose precipitation step also blocked the precipitation. In another experiment, the TKD61 protein was shown to bind to MBP-SH2, which was precipitated with a cross-linked amylose matrix (data not shown). These experiments indicated that the precipitation of TKD61 was due to specific interactions with the sequence of the GST-SH2 protein that was derived from the c-src SH2 domain.

In these binding experiments the phosphorylated TKD61 protein with incorporated $^{32}P_i$ was detected by autoradiography. When the TKD61 protein was alternatively detected by immunoblotting with an anti-EGF receptor antibody, the dependence of the TKD/SH2 domain interaction upon TKD autophosphorylation could be assessed. Figure 1B shows that when TKD61 was preincubated in the presence or absence of unlabeled ATP, the protein only associated with GST-SH2 when phosphorylated. The diffuse nature of the band in the immunoblot reflects the distinct phosphorylation states of the TKD61 protein, which can be phosphorylated at multiple sites.

Interactions of the EGF receptor with truncated SH2 domains. In an attempt to determine a minimal structural element which recognized the phosphorylated EGF receptor TKD61, a variety

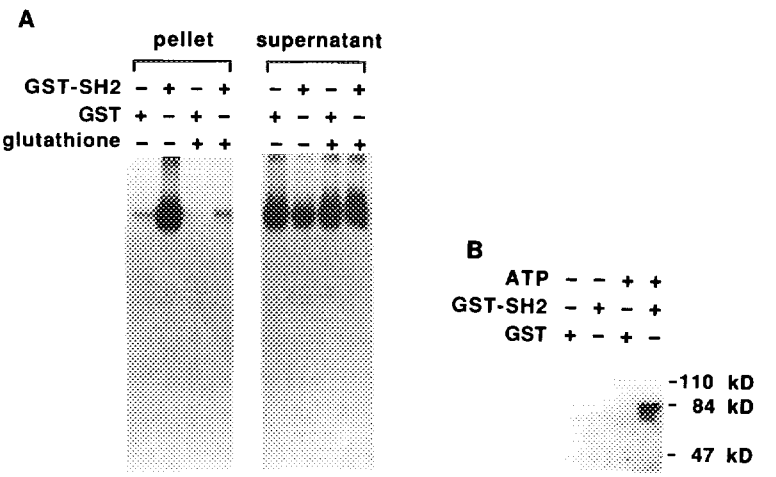

Figure 1. *Affinity matrix assay for phosphoprotein/SH2 domain interactions.* **A.** Specific binding of TKD61 to GST-SH2. Partially purified recombinant TKD61 protein (3 μg) was prephosphorylated in the presence of 10 mM $MnCl_2$ and 10 μM [γ^{32}P]ATP and then allowed to interact with 5 μg of added GST or GST-SH2 protein in the presence of 25 mM EDTA (100 μl final volume). The GST and GST-SH2 proteins were precipitated by the addition of glutathione-agarose, and fractions of the precipitates (total sample) and supernatants (1/4 total sample) were subjected to SDS-PAGE and autoradiography. In a control experiment 5 mM reduced glutathione was added prior to the glutathione-agarose precipitation. **B.** The dependence of the TKD61/SH2 interaction upon TKD autophosphorylation could be observed when the TKD was detected by immunoblotting with an anti-EGF receptor antibody. The experiment was performed exactly as described in **A**, except that 6 μg of TKD61 protein was added, and unlabeled ATP (100 μM) was substituted for [γ^{32}P]ATP. Where indicated, no ATP was included in the preincubation. Details are given in *Materials and Methods*.

of truncated c-src SH2 domains were expressed as GST fusion proteins (see Fig. 2A). Distinct proteins contained the intact B or C domains described by Stahl *et al.* (**5**), or portions of the B domain. None of these truncated SH2 domains bound the phosphorylated TKD61 at levels significantly above the GST control (see Fig. 2B). These results indicated that the bulk of the SH2 domain sequence was required for the structural integrity of the high affinity binding site for phosphotyrosine-containing proteins.

Identification of EGF receptor sequences involved in SH2 domain recognition. Autophosphorylation of the activated EGF receptor can occur on any of several C-terminal tyrosine residues (see *Discussion*). To determine if specific tyrosine phosphorylation sites were involved in the interaction of the EGF receptor with the SH2 domain of c-src, we analyzed the binding of three recombinant EGF receptor TKD forms to GST-SH2 (see Fig. 3A). Whereas TKD61 had a full-length C-terminus, the TKD56 protein lacked two sites of tyrosine autophosphorylation, tyr_{1173} and tyr_{1148}. This protein did contain at least two other sites of autophosphorylation, tyr_{1068} and tyr_{1086}. The highly truncated TKD42 protein lacked all of these sites, but contained tyr_{992}, which has recently been demonstrated to an autophosphorylation site (**22**).

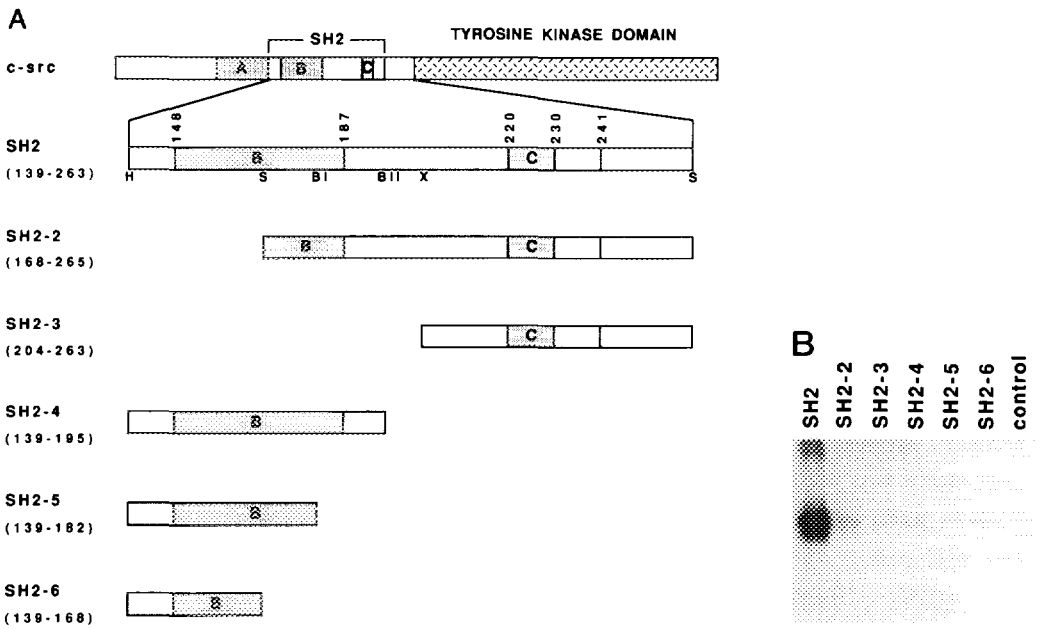

Figure 2. *Binding of TKD61 to subsequences of the c-src SH2 domain.* Truncated SH2 domains were expressed as bacterial fusion proteins, and the affinity matrix assay was used to assess the ability of these proteins to bind phosphorylated TKD61. **A.** Sequences of the c-src SH2 domain expressed as bacterial fusion proteins. Restriction fragments of chicken c-*src* cDNA were cloned in frame with the glutathione S-transferase (GST) coding sequence of plasmid expression vectors. The amino acid residues of c-src incorporated into each of the coded GST fusion proteins GST-SH2 through GST-SH2-6 are indicated in the figure. The full-length SH2 domain sequence was also expressed as a maltose binding protein (MBP) fusion protein (MBP-SH2). Restriction sites used are abbreviated as: H (HhaI), S (SmaI), BI (BanI), BII (BanII), and X (XhoII). **B.** Binding of phosphorylated TKD61 to full-length and truncated SH2 domains. An experiment performed exactly as described in Fig. 1A, except that 2 μg samples of the distinct GST-SH2 fusion proteins described in *A* were used as indicated. Only pellet fractions are shown.

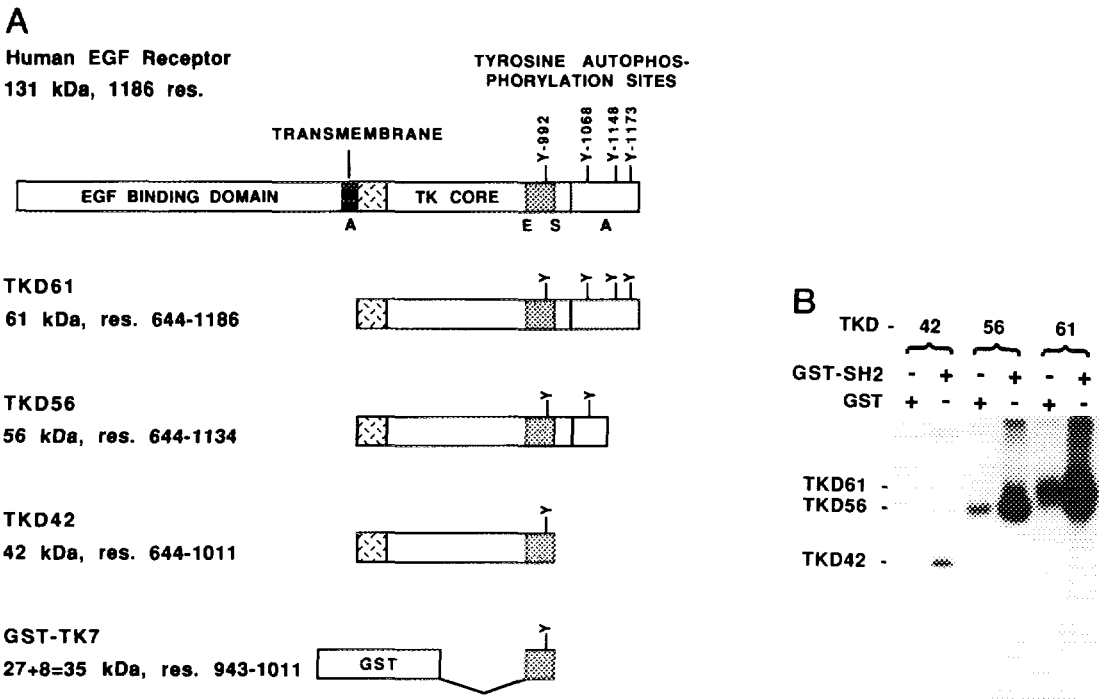

Figure 3. *Binding of full-length and truncated EGF receptor tyrosine kinase domain (TKD) proteins to the SH2 domain of c-src.* **A.** Structures of the TKD forms expressed with recombinant baculovirus vectors. The N-terminus of each TKD form was met$_{644}$ of the intact receptor sequence. Also shown is the structure of the GST-TK7 fusion protein (**21**) containing residues 943 to 1011 of the EGF receptor sequence. Restriction sites used in the constructions are abbreviated as: A (ApaI), E (EcoRI), and S (SacI). **B.** Binding of the TKD42, -56 and -61 proteins to the SH2 domain of c-src. An experiment performed identically to that of Fig. 1A, except that the three distinct TKD proteins were used as indicated. Although the extents of autophosphorylation of the different TKD forms varied, a similar fraction of each autophosphorylated TKD was bound to GST-SH2. Only the pellet fractions are shown.

Each of the TKD forms tested was shown to autophosphorylate, and each associated with the GST-SH2 protein (see Fig. 3B). Although the phosphorylation of TKD42 was significantly weaker than that of the TKD56 and TKD61 proteins, a similar fraction of each phosphorylated TKD form was bound to the SH2 domain (data not shown). As the phosphorylation of the TKD42 protein probably occurred on tyr$_{992}$ (see *Discussion*), it appeared that phosphorylation of this residue was sufficient to effect an interaction of the TKD with the SH2 domain.

Affinity of the interaction between the TKD and the c-src SH2 domain. The binding assay described was also used to estimate the affinities of the interactions between the phosphorylated TKD forms and the SH2 domain of c-src (see *Materials and Methods*). Figure 4 shows the fraction of phosphorylated TKD61 bound to the SH2 domain as a function of the GST-SH2 concentration and the theoretical binding curve that best fit these data. The dissociation constants determined for the three TKD forms were 64 ± 36 nM (TKD42), 277 ± 63 nM (TKD56), and 324 ± 94 nM (TKD61). In these experiments, the fraction of phosphorylated TKD bound to the SH2 domain at saturation was less than one, with values of 0.12 (TKD42), 0.35 (TKD56), and 0.37 (TKD61) seen in typical experiments. It is possible that some of the bound TKD was released

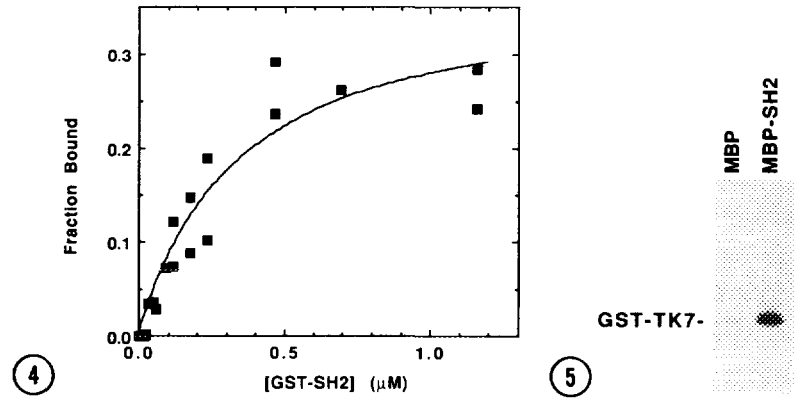

Figure 4. *Estimating the affinity of the TKD61/SH2 interaction.* The affinity matrix assay was used to estimate the dissociation constants for TKD/SH2 interactions. These experiments were performed identically to that shown in Fig. 1A, except that the concentration of GST-SH2 added was varied from 0 to 10 μM. Here the amount of bound TKD61, determined by scintillation counting of gel slices, is plotted as a function of GST-SH2 concentration. Data from two separate experiments are included. Also shown is the hyperbolic curve that best fit the data (K_d=324 nM).

Figure 5. *Binding of a specific EGF receptor phosphopeptide to the SH2 domain of c-src.* Residues 943-1011 of the EGF receptor were expressed as a fusion protein (GST-TK7, see Fig. 3A). This protein was prephosphorylated by the TKD42 kinase in the presence of 10 mM $MnCl_2$ and 10 μM [γ^{32}P]ATP. The phosphorylated fusion protein was isolated from the TKD42 protein and excess radionucleotide by glutathione-agarose affinity chromatography and incubated with the c-src SH2 domain, expressed here as a MBP fusion protein (5 μg in a 100 μl final volume). The MBP-SH2 protein (or MBP in control) was precipitated with cross-linked amylose resin, and the associated phosphoprotein was detected by SDS-PAGE and autoradiography.

from the SH2 domain during washes of the glutathione-agarose pellet, although attempts were made to minimize this effect.

Interaction of a specific EGF receptor phosphopeptide with the c-src SH2 domain. Certain peptide sequences of the EGF receptor have been previously expressed in *E. coli* as GST fusion proteins (**21**). The GST-TK7 fusion protein contains residues 943-1011 of the native EGF receptor (see Fig. 3A), which includes a highly acidic sequence and is part of the CaIn domain described by Chen *et al.* (**23**). The known site of tyrosine autophosphorylation, tyr_{992}, is incorporated in this EGF receptor sequence. This sequence also includes tyr_{954} and tyr_{974}, but these residues have never been demonstrated to be phosphorylated by the EGF receptor kinase.

The GST-TK7 peptide was phosphorylated on tyrosine residues by the TKD42 enzyme, and then separated from the TKD42 enzyme and excess radionucleotide by glutathione-agarose affinity chromatography. Control experiments showed that the GST carrier protein was not phosphorylated by the EGF receptor tyrosine kinase (**21**). The ability of the phosphorylated GST-TK7 protein to interact with the SH2 domain of c-src, here expressed as an MBP fusion protein, could then be directly assessed. Figure 5 demonstrates the specific binding of this EGF receptor-derived protein to the c-src SH2 domain. Note that the entire TK7 sequence including the tyr_{992} autophosphorylation site is found in the truncated TKD42 protein, which also bound to the SH2 domain in its phosphorylated form (see Fig. 3).

DISCUSSION

The present study was an attempt to further define the sequence elements responsible for the interactions between activated growth factor receptors and SH2 domain proteins. To address this issue, we produced a variety of recombinant fusion proteins that incorporated either specific segments of the c-src SH2 domain (see Fig. 2A), or tyrosine phosphorylation sites of the EGF receptor (see Fig. 3A). Affinity matrices provided a rapid means of precipitating the GST-SH2 and MBP-SH2 fusion proteins, so that the association of phosphorylated proteins with the recombinant SH2 domain could be readily detected. The specific binding of a 61 kDa EGF receptor tyrosine kinase domain (TKD61) to a c-src sequence (res. 139-263) containing the intact SH2 domain was clearly demonstrated by this assay. Furthermore, the phosphorylation of the TKD61 protein was shown to be necessary for binding to the SH2 domain (see Fig. 1).

The affinity matrix assay was further exploited in the measurement of the dissociation constants that characterized the interactions between the phosphorylated EGF receptor TKD forms and the SH2 domain of c-src. These constants ranged from 60 to 320 nM, which indicated that the recombinant proteins displayed the same high affinity interactions suggested by numerous qualitative investigations. In a recent quantitative study (**24**), the phosphorylated EGF receptor was shown to bind to the SH2 domains of ras GAP and PI 3-kinase with dissociation constants of 18 nM and 6 nM, respectively. The affinities observed in the present study of EGF receptor/c-src SH2 domain interactions are significantly lower. However, in contrast to the c-src protein, ras GAP and PI 3-kinase each contain tandem copies of the SH2 domain.

Hidaka *et al.* (**25**) have recently demonstrated that an eight amino acid peptide (P1) from the phospholipase C-γ SH2 domain (corresponding to c-src residues 170-177) can bind the phosphorylated EGF receptor. We made numerous attempts to find a subsequence of the c-src SH2 domain that would effectively bind the phosphorylated EGF receptor TKD. Although the proteins GST-SH2-2, -4 and -5 (see Fig. 2A) each contained the c-src sequence corresponding to the P1 peptide, none of the fusion proteins other than the full-length SH2 domain protein (GST-SH2) bound significant quantities of TKD61 (see Fig. 2B). The present data suggest that the entire SH2 domain sequence is necessary for high affinity recognition of phosphotyrosine-containing substrates. This conclusion is consistent with the recently determined X-ray crystallographic structure of a c-src SH2 domain/phosphopeptide complex (**12**), in which highly conserved residues throughout the SH2 domain primary structure are found in close proximity to the bound phosphotyrosine residue.

The receptor for EGF, like other growth factor receptor tyrosine kinases, contains multiple autophosphorylation sites (see Fig. 3A). Whereas the role of autophosphorylation in mitogenic signal transduction remains controversial, it does appear that these phosphorylations are required for the regulation of at least a subset of the biochemical pathways activated by the receptor (**23**). Autophosphorylation of the activated receptor *in vivo* occurs predominantly on tyr_{1173}. The *in vitro* autophosphorylation of the receptor involves also tyr_{1068} and tyr_{1148} (**26**). Recently, tyr_{1086} has also been shown to become phosphorylated *in vitro* (**27**). Truncated receptors lacking these four sites have been expressed in cultured cells by gene transfection, and become phosphorylated

instead on tyr_{992} (**22**). Related experiments in which individual C-terminal residues have been eliminated by site-directed mutagenesis indicate that when phosphorylation sites near the C-terminus are eliminated, sites nearer to the tyrosine kinase core become phosphorylated, and can compensate for the C-terminal sites in signal transduction functions (**28-30**).

In order to determine which of the several phosphorylation sites of the EGF receptor TKD were responsible for its interaction with the SH2 domain of c-src, we generated two C-terminally truncated TKD forms, TKD42 and TKD56, with the baculovirus system (see Fig. 3A). These truncated forms both lacked the primary site of *in vivo* phosphorylation, tyr_{1173}. The TKD56 protein did contain tyr_{992}, tyr_{1068} and tyr_{1086}. TKD42 contained only one known site of phosphorylation, tyr_{992}. This site is of interest because it is located in a receptor subdomain proposed to be involved in calcium mobilization and receptor internalization (**23**).

As shown in Fig. 3B, each TKD form was found to autophosphorylate. Although the TKD42 form was weakly phosphorylated in comparison to the TKD56 and TKD61 proteins, a similar fraction of each phosphorylated TKD was bound to the SH2 domain (see Fig. 3B). Apparently, a phosphorylation site present in all three TKD forms could be recognized by the SH2 domain. Although the TKD42 protein contained several tyrosine residues N-terminal to tyr_{992}, it is likely that phosphorylation occurred exclusively on this residue. Margolis and coworkers (**27**) have shown that all *in vitro* sites of tyrosine phosphorylation are C-terminal to residue 922. Furthermore, a transfected receptor truncated at residue 991 does not autophosphorylate (**22**).

Additional evidence that the phosphorylation of tyr_{992} could promote association of the EGF receptor TKD with the SH2 domain was obtained. A short subsequence of the receptor (residues 943-1011) was incorporated in the GST-TK7 protein [see Fig. 3A and (**21**)] and then phosphorylated with the TKD42 kinase. The phosphorylated GST-TK7 protein was shown to bind to the SH2 domain (see Fig. 5). The evidence presented here therefore suggests that the phosphorylation of tyr_{992} in the EGF receptor TKD is sufficient to promote association with the SH2 domain of c-src. Previous studies have indicated that phosphorylated tyr_{992} is involved in the high-affinity binding of phospholipase C-γ to the EGF receptor [(**31**,**32**), but see also (**33**)].

In conclusion, the interactions between the phosphorylated EGF receptor TKD and the SH2 domain of c-src have been characterized *in vitro*. The dissociation constants for these interactions were found to be in the range of 60-320 nM. None of several truncated SH2 domain species generated were found to bind the phosphorylated TKD. A small peptide (GST-TK7) derived from the EGF receptor C-terminus interacted with the SH2 domain when phosphorylated. The tyr_{992} phosphorylation site found within this peptide could be a mediator of interactions between the EGF receptor and src-family tyrosine kinases *in vivo*.

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