SH2 Domain Proteins as High-Affinity Receptor Tyrosine Kinase Substrates†

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ABSTRACT: Activation of a growth factor receptor tyrosine kinase (RTK) is accompanied by a rapid autophosphorylation of the receptor on tyrosine residues. Receptor activation has been shown to promote the association of signal-transducing proteins containing SH2 domains (second domain of src homology). These receptor-associated proteins can, in turn, be phosphorylated by the RTK, an event which presumably regulates their activities. It has been suggested that SH2 domains in signal-transducing proteins target these proteins as substrates of the activated RTK. To test this hypothesis, recombinant proteins were generated that contained tyrosine phosphorylation sites of the erbB3 receptor and/or the SH2 domain of c-src. Incorporation of the SH2 domain led to a decrease in K_M and an increase in V_{max} for the substrate. The K_M determined for one chimeric SH2/erbB3 substrate was among the lowest reported for epidermal growth factor RTK substrates. Experiments with a truncated kinase lacking C-terminal autophosphorylation sites indicated that the reduction in K_M for these substrates was mediated by interactions between the substrate SH2 domain and phosphotyrosine residues of the RTK. These interactions could also inhibit RTK activity. These results demonstrate that the SH2 domain can effectively target substrates to a RTK and that SH2 domain proteins can regulate RTK activity.

A variety of polypeptide growth factor receptors that possess intrinsic protein tyrosine kinase activity have now been characterized. These include the receptors for epidermal growth factor (EGF[1]), platelet-derived growth factor (PDGF) and insulin, and also certain protooncogene products, such as c-kit (the stem cell factor receptor) and c-fms (the macrophage colony-stimulating factor receptor) (Cadena & Gill, 1992; Yarden & Ullrich, 1988). The signal transduction pathways activated by these receptors are diverse and involve other protein kinases and enzymes responsible for second messenger generation. Some of these signal-transducing proteins physically interact with the activated growth factor receptors to form signal transduction complexes (signal recognition particles) in the cytoplasmic membrane (Cantley *et al.*, 1991; Ullrich & Schlessinger, 1990; Williams, 1989).

Frequent among the components of these signal transduction complexes are proteins that contain a conserved protein structural domain originally identified in the src family of nonreceptor tyrosine kinases (Koch *et al.*, 1989, 1991; Pawson & Gish, 1992). SH2 domains (second domain of src homology) have been identified in phospholipase C-γ (PLC-γ) isoforms (Stahl *et al.*, 1988), the 85-kDa noncatalytic subunit of phosphatidylinositol (PI) 3-kinase (Escobedo *et al.*, 1991; Otsu *et al.*, 1991; Skolnik *et al.*, 1991), the ras GTPase activating protein (ras GAP) (Trahey *et al.*, 1988; Vogel *et al.*, 1988), the c-src protein tyrosine kinase (Koch *et al.*, 1989), and recently in the growth factor receptor-bound protein 2 (GRB2) (Lowenstein *et al.*, 1992). Each of these proteins has been shown to physically associate with growth factor receptors [for reviews, see Carpenter (1992), Schlessinger & Ullrich (1992)]. SH2 domains have also been identified in the v-crk (Mayer *et al.*, 1988), v-akt (Bellacosa *et al.*, 1991), and vav (Bustelo *et al.*, 1992) oncoproteins, each of which may also be involved in tyrosine kinase signal transduction.

SH2 domain proteins bind with high affinity to the phosphorylated substrates of receptor tyrosine kinases (RTKs) (Anderson *et al.*, 1990; Margolis *et al.*, 1990; Moran *et al.*, 1990; Sun *et al.*, 1991; Yamamoto *et al.*, 1992). These substrates include the growth factor receptors themselves, which when activated become phosphorylated on specialized tyrosine autophosphorylation sites. The interactions between phosphorylated receptors and SH2 domain proteins are apparently critical for the formation of signal transduction complexes. It has also been proposed that these interactions facilitate the phosphorylation of SH2 domain proteins by RTKs (Rhee, 1991). In particular, the activating phosphorylations of PLC-γ by the EGF RTK are apparently promoted by the interactions between the SH2 domains of PLC-γ and the phosphorylated EGF receptor [Rotin *et al.*, 1992a; Sorkin *et al.*, 1992; but see also Vega *et al.* (1992)].

In the present study, recombinant proteins containing an SH2 domain fused to tyrosine kinase substrates were used to examine the effects of tyrosine kinase/SH2 domain interactions on substrate phosphorylation reactions. In general, the K_M characterizing tyrosine kinase substrate interactions was markedly reduced for substrates incorporating the SH2 domain. The recombinant SH2 domain-containing substrates exhibited K_M values among the lowest reported for substrates of the EGF RTK. These results indicate that incorporated SH2 domains can effectively target substrates to activated RTKs. In addition, an inhibitory effect of SH2 domain proteins on substrate phosphorylation was observed, which could be an important means of regulating RTK activity *in vivo*.

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[1] Abbreviations: EGF, epidermal growth factor, PDGF, platelet-derived growth factor; PLC, phospholipase C; PI, phosphatidylinositol; GAP, GTPase activating protein; SH2, second domain of src homology; RTK, receptor tyrosine kinase; TKD, recombinant EGF receptor tyrosine kinase domain; GST, glutathione S-transferase; MBP, *E. coli* maltose binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; f_I, fractional inhibition occurring at saturating inhibitor concentration: K_I, inhibition constant.

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EXPERIMENTAL PROCEDURES

Materials. ATP sodium salt, reduced glutathione, glutathione-agarose (G-4510), human thrombin (T-6884), and immunological reagents were purchased from Sigma Chemical Company. DEAE-Sephacel was a product of Pharmacia Fine Chemicals, and [γ-^{32}P]ATP (50 Ci/mmol) was acquired from Du Pont New England Nuclear. pMAL expression vectors and cross-linked amylose resin were purchased from New England Biolabs, and pGEX-1N and –3X vectors (Smith & Johnson, 1988) from Amrad (Victoria, Australia). The derivative pGEX-KG (Guan & Dixon, 1991) was kindly provided by Dr. Robert Deschenes. Human *erb*B3 cDNA (Plowman *et al.*, 1990) was provided by Dr. Greg Plowman. The chicken c-*src* cDNA was derived from pM5HHB5 (Kmiecik & Shalloway, 1987).

Expression and Purification of GST and MBP Fusion Proteins. Various peptides found within the sequences of the erbB3 receptor and the chicken c-src tyrosine kinase were expressed as fusion proteins in *Escherichia coli.* 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 (Smith & Johnson, 1988) or cross-linked amylose (Guan *et al.*, 1987; Kellerman & Ferenci, 1982), respectively. Appropriate restriction fragments of cDNA coding for the specific peptides were cloned into either a pGEX-family vector (Guan & Dixon, 1991; Smith & Johnson, 1988) to generate a GST fusion protein or a pMAL vector (Guan *et al.*, 1987) to produce an MBP fusion protein. The constructions were designed to place cDNA in frame with the GST and MBP coding sequences. Standard methods for the subcloning of cDNA fragments were employed (Sambrook *et al.*, 1989), and the fidelity of the constructions was verified by restriction mapping.

GST fusion proteins were purified by glutathione-agarose affinity chromatography (Smith & Johnson, 1988) as previously described (Koland *et al.*, 1990). These fusion proteins were eluted in 5 mM reduced glutathione in Buffer A [20 mM HEPES/Na, 50 mM NaCl, 10% (vol/vol) glycerol, pH 7.4]. MBP fusion proteins were purified on a cross-linked amylose matrix (Kellerman & Ferenci, 1982) by an identical protocol, except that these fusion proteins are eluted with 10 mM maltose in Buffer A. When necessary, free glutathione or maltose was removed from the purified fusion proteins by extensive dialysis against Buffer A at 4 °C. Each fusion protein showed a mobility in SDS-PAGE consistent with its predicted molecular weight. Depending upon the nature of the fused sequence, the yield of purified fusion protein varied greatly (1–200 mg from a 750-mL culture). In some cases, proteolytic degradation of the purified fusion proteins was evident, but the preparations were otherwise homogeneous. Protein concentration was estimated by using the Bradford assay (Bradford, 1976).

The isolated SH2 domain of c-src was generated by thrombin cleavage of the GST-SH2 fusion protein. GST-SH2 (600 μg) was digested with thrombin (8 μg, 18 units) for 15 min at room temperature in a 50 mM Tris/HCl, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% β-mercaptoethanol, pH 8.0 buffer (Smith & Johnson, 1988). The products were resolved by Superose FPLC (Pharmacia) in Buffer A at 4 °C. Fractions containing the 14-kDa SH2 domain were pooled and concentrated with a Centricon 10 microconcentrator (Amicon).

Expression of Active EGF Receptor Tyrosine Kinase Domain Forms. Recombinant full-length and truncated EGF receptor tyrosine kinase domain (TKD) forms (see Figure 1C) were expressed in the baculovirus/insect cell system.

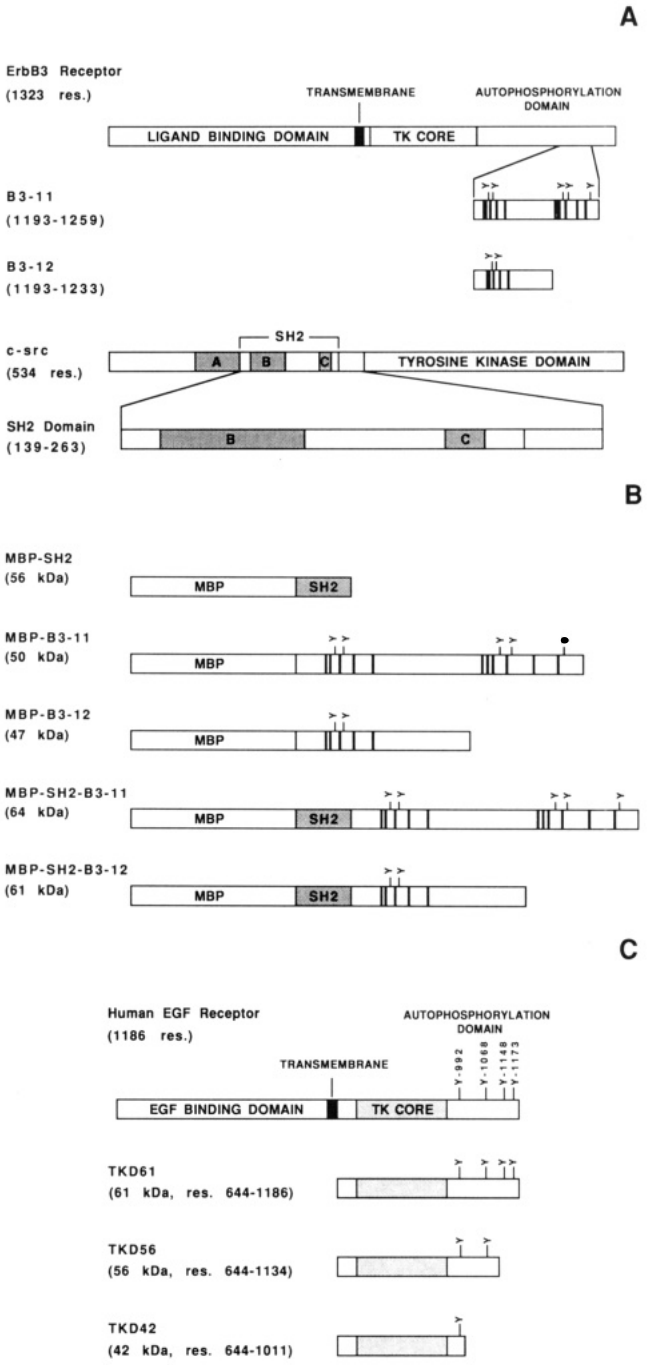

FIGURE 1: Structures of recombinant peptide substrates and EGF receptor tyrosine kinase domains. (A) Schematic structures of the c-src tyrosine kinase and the putative receptor/tyrosine kinase encoded by the *erb*B3 cDNA. The location of the predicted C-terminal autophosphorylation domain of the erbB3 protein is shown. Some of the candidate tyrosine autophosphorylation sites within this domain are identified, and black bars indicate the positions of acidic residues. In the c-src structure, the locations of the SH2 domain and the B and C subdomains are indicated. (B) Recombinant substrates expressed as maltose binding protein (MBP) fusion proteins. Restriction fragments of the *erb*B3 and c-*src* cDNAs were subcloned into the pMal bacterial expression vector to yield plasmids encoding the indicated fusion proteins. (C) The tyrosine kinase domain (TKD) of the EGF receptor was expressed in the baculovirus/insect system. Two C-terminally truncated forms were also generated, which lacked some of the primary sites of tyrosine residue autophosphorylation as indicated.

Specific restriction fragments of human EGF receptor cDNA were subcloned into either the pAcYM1 (Matsuura *et al.*, 1987) or pAcYMP1 (Guy *et al.*, 1992) baculovirus transfer vector. The CsCl-purified plasmid transfer vectors were cotransfected with wild-type *Autographa californica* (bac-

ulovirus) genomic DNA into cultured *Spodoptera frugiperda* (Sf21) cells by the calcium phosphate method (Summers & Smith, 1987). Recombinant baculovirus was isolated by a dilution/DNA hybridization method (Fung *et al.*, 1988) and plaque purified (Summers & Smith, 1987). Clones expressing high levels of recombinant TKD were identified by Western immunoblotting and tyrosine kinase activity assays (Koland & Cerione, 1988).

For large scale preparation of the recombinant proteins, Sf21 cells were grown in spinner flask culture (125 mL) to a density of 2×10^6 cells/mL and infected with recombinant virus (~5 pfu/cell) (Summers & Smith, 1987). At 48 h after infection, cells were harvested and washed gently in 20 mL of lysis buffer (50 mM Tris/HCl, 10 mM benzamidine, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) supplemented with 250 mM sucrose. The cells were resuspended in 10 mL of sucrose-free lysis buffer and homogenized. The homogenate was clarified by centrifugation for 20 min at 80 000*g* and diluted with an equal volume of column buffer [20 mM HEPES/Na, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% (v/v) glycerol, pH 7.4]. The solution was applied to a 5-mL DEAE-Sephacel column, which was eluted with a 50-mL linear gradient of 50–350 mM NaCl in column buffer. Peak fractions were identified by a tyrosine kinase activity assay (Koland & Cerione, 1988) and pooled. The partially purified TKD forms (~10% pure, typically 5 mg protein/2.5×10^8 cells) were supplemented with glycerol to 35% (v/v) and stored at −20 °C. All purification steps were done at 4 °C or on ice. Phosphoamino acid analysis (Kamps, 1991) of various phosphorylated substrates indicated that these preparations were essentially free of protein serine and threonine kinase activity.[2]

Substrate Phosphorylation Kinetics. The phosphorylation of recombinant protein substrates containing erbB3 and SH2 domain sequences was assayed by incubating one of the autophosphorylated EGF receptor TKD forms in the presence of $MnCl_2$, [γ^{32}P]ATP, and varying concentrations of the recombinant substrates. The erbB3 substrates were present both as chimeric peptides MBP-SH2-B3-11 and −12 and as the parent fusion proteins MBP-B3-11 and −12. To begin the assay, an aliquot of the TKD protein (10 μL, ~5 μg) was allowed to autophosphorylate in the presence of excess unlabeled ATP (143 μM) for 15 min at room temperature in assay buffer [20 mM HEPES/Na, 10 mM $MnCl_2$, 50 mM NaCl, 0.1% Triton X-100, 10% (v/v) glycerol, pH 7.4]. Following this prephosphorylation, 15 μL of [γ^{32}P]ATP (2 mCi/mL, 50 Ci/mmol) was added to the sample (50 μL total volume). Aliquots (5 μL) of this mixture were then immediately added to solutions of the peptide substrate in assay buffer, and the 36-μL reaction mixtures were incubated for 15 min at room temperature [final concentrations: 15 μM ATP (~10^4 cpm/pmol), 10 mM $MnCl_2$, 0–10 μM peptide]. The reactions were then stopped by the addition of 14-μL aliquots of 5-times-concentrated SDS-PAGE sample buffer. The ^{32}P-labeled phosphoproteins were resolved by SDS-PAGE (Laemmli, 1970), identified by autoradiography, and quantified by scintillation counting of the bands excised from dried gels in Ready Gel scintillation cocktail (Beckman).

Kinetic constants were determined by the fitting of rate equations with a nonlinear least-squares minimization algorithm (Nelder & Mead, 1965). Whereas the phosphorylation data obtained with the MBP-B3-11 and −12 peptides could be well fit by a simple hyperbolic equation, data obtained with the SH2 domain-containing substrates MBP-SH2-B3-11 and −12 were better fit by eq 2 (see Results), which contained a substrate inhibition term and two additional parameters. These parameters (f_I and K_I) were determined by independent inhibition experiments (see Figure 4 and Table I), so that only V_{max} and K_M were adjusted in the fitting of eq 2. The equations and best-fit parameters were used to generate the theoretical curves that are shown in the figures.

V_{max}/K_M ratios (see Table II) were obtained from the slopes of the velocity versus substrate concentration profiles near zero substrate concentration, as determined by linear regression. These ratios allowed a model-independent comparison of the substrate efficiency.

Table I: Inhibition of EGF Receptor TKD Forms by the MBP-SH2 Protein[a]

kinase	f_I	K_I (μM)
TKD61	0.74	1.5
TKD56	0.85	1.4
TKD42	0.85	0.35

[a] The activity of each recombinant EGF receptor TKD form was assayed with MBP-B3-11 (1.0 μM) as the peptide substrate and varying quantities of the inhibitory MBP-SH2 protein (0–10 μM) added (see Figure 4 and Experimental Procedures). The inhibition data were fit by eq 1 with a nonlinear least-squares algorithm, and the best-fit parameters obtained are given.

RESULTS

Interaction of SH2 Domain-Containing Proteins with the EGF Receptor Tyrosine Kinase Domain. The SH2 domain of c-src and protein tyrosine kinase substrates incorporating this SH2 domain were expressed in *E. coli* as either maltose binding proteins (MBP) or glutathione S-transferase (GST) fusion proteins (see Figures 1A and B). Fusion of peptide sequences downstream of either MBP or GST sequences enabled the rapid purification of the recombinant proteins by affinity chromatography. To observe the effect of tyrosine kinase autophosphorylation on the phosphorylation of substrates, three distinct EGF receptor TKD forms were expressed in the baculovirus/insect cell system (see Figure 1C). Whereas the TKD61 protein possessed the authentic C-terminus of the EGF receptor, the TKD56 and TKD42 proteins had C-terminal truncations that progressively reduced the capacity of the TKD to autophosphorylate. The autophosphorylation of the TKD42 protein was roughly 5% of that observed with the TKD61 protein.[2]

As shown by an affinity matrix assay, the MBP-SH2 and GST-SH2 fusion proteins each strongly associated with the phosphorylated TKD61 protein (see Figure 2). The TKD did not associate with the MBP or GST carrier proteins. A previous quantitative analysis (Sierke *et al.*, 1993) indicated that the interaction of the TKD61 protein and the SH2 domain occurs with a dissociation constant of approximately 0.3 μM. And, in concord with other investigations (Anderson *et al.*, 1990; Margolis *et al.*, 1990), the interaction of the SH2 domain with the TKD is potentiated by the phosphorylation of tyrosine residues on the TKD (Sierke *et al.*, 1993).

Phosphorylation of Recombinant Substrates by the EGF Receptor TKD. A survey indicated that the documented K_M values for substrates phosphorylated by the EGF RTK range from 0.1 μM to well over 1 mM, with the lowest value being reported for progesterone receptor subunit phosphorylation (Ghosh-Dastidar *et al.*, 1984). Given the submicromolar affinity of the recombinant c-src SH2 domain for the phosphorylated EGF receptor TKD, it was considered that a high-affinity substrate might be generated by fusion of a suitable phosphorylation site sequence to the SH2 domain.

[2] S. L. Sierke and J. G. Koland, unpublished results.

Table II: Kinetic Constants Characterizing the Phosphorylation of Recombinant Substrates by Distinct EGF Receptor TKD Forms[a]

kinase	substrate	K_M (μM)	V_{max} (nmol·min^{-1}·mg^{-1})	$\frac{(V_{max}/K_M)_{+SH2}}{(V_{max}/K_M)_{-SH2}}$[b]
TKD61	MBP-B3-11	7.2 ± 0.9	0.66 ± 0.07	
	MBP-SH2-B3-11	1.2 ± 0.04	2.3 ± 0.2	14.0 ± 2.0
	MBP-B3-12	30 ± 5.5	0.87 ± 0.12	
	MBP-SH2-B3-12	7.2 ± 2.2	3.1 ± 0.4	8.2 ± 2.2
TKD56	MBP-B3-11	7.2 ± 1.1	0.81 ± 0.17	
	MBP-SH2-B3-11	1.8 ± 0.4	4.1 ± 1.1	16.1 ± 1.8
	MBP-B3-12	28 ± 15	1.1 ± 0.5	
	MBP-SH2-B3-12	20 ± 12	9.3 ± 3.1	6.9 ± 2.4
TKD42	MBP-B3-11	1.4 ± 0.1	0.059 ± 0.003	
	MBP-SH2-B3-11	6.2 ± 1.8	0.53 ± 0.13	0.93 ± 0.09
	MBP-B3-12	4.8 ± 0.7	0.042 ± 0.005	
	MBP-SH2-B3-12	16 ± 1.0	0.46 ± 0.10	0.79 ± 0.08

[a] The activity of each recombinant EGF receptor TKD form was assayed in the presence of varying concentrations (0–10 μM) of the indicated peptide substrates (see Figure 3). The initial velocity data for substrates containing an SH2 domain were fit by eq 2 with the parameters f_I and K_I held constant at the values given in Table I. Data for substrates without an SH2 domain were fit by the hyperbolic rate equation. Best-fit values for the variable parameters K_M and V_{max} were obtained by nonlinear least-squares minimization, and the ranges of duplicate analyses are indicated. V_{max} values for different TKD forms should not be compared, as the TKD content of the preparations varied. [b] V_{max}/K_M value for an SH2 domain-containing substrate divided by that of the parent substrate. Model-independent V_{max}/K_M values were obtained directly as the initial slopes of the velocity versus substrate concentration plots and hence differ somewhat from those calculated as the aggregate of individual rate constant values. Details are given in Experimental Procedures.

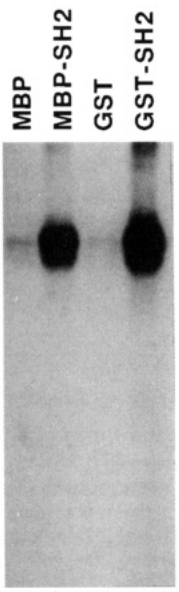

FIGURE 2: Specific binding of TKD61 to MBP-SH2 and GST-SH2. Partially purified recombinant TKD61 protein (3 μg) was prephosphorylated in the presence of 10 mM $MnCl_2$ and 10 μM [γ^{32}P]ATP and then allowed to interact with 5 μg of either MBP-SH2 or GST-SH2 protein in the presence of 25 mM EDTA (100 μL final volume). The MBP-SH2 and GST-SH2 proteins were precipitated by the addition of cross-linked amylose resin or glutathione-agarose, respectively, and the precipitates were subjected to SDS-PAGE and autoradiography. In control experiments, the MBP and GST carrier proteins were substituted for MBP-SH2 and GST-SH2 as indicated. This binding assay has been previously described in detail (Sierke *et al.*, 1993).

Previously, C-terminal sequences of the erbB3 protein were expressed as recombinant proteins and shown to be substrates for the EGF RTK.[2] The *erbB3* gene product is a putative RTK closely related in structure to the EGF receptor and the v-*erb*B and c-*erb*B2/*neu* oncogene products (Kraus *et al.*, 1989; Plowman *et al.*, 1990). The unique C-terminus of the erbB3 protein is replete with candidate tyrosine phosphorylation sites, some of which are indicated in Figure 1A. As shown in Figure 1B, these C-terminal sequences of the erbB3 protein were expressed in the MBP-B3-11 and –12 fusion proteins. To examine the effects of the incorporation of a SH2 domain into tyrosine kinase substrates, the erbB3 sequences were fused downstream of the c-src SH2 domain in the MBP-SH2-B3-11 and –12 fusion proteins (see Figure 1B). The MBP protein itself was not a substrate for the EGF RTK.[2]

Figure 3 compares the MBP-B3-11 and MBP-SH2-B3-11 proteins as substrates for the distinct TKD forms. In these experiments, each of the TKD species was allowed to autophosphorylate in the presence of unlabeled ATP prior to the addition of the peptide substrate and [γ-^{32}P]ATP. In this manner, the capacity of substrates to interact with TKD forms that were phosphorylated to differing extents could be examined. When the TKD61 and TKD56 kinases were assayed (Figure 3A and B), the incorporation of the SH2 domain into the MBP-B3-11 substrate clearly augmented the phosphorylation of the substrate. This effect of the SH2 domain was not apparent when the TKD42 kinase was assayed (Figure 3C). Whereas the MBP-B3-11 phosphorylation data could be fit by simple hyperbolic curves (see below), the MBP-SH2-B3-11 phosphorylation kinetics were not hyperbolic. It appeared that insertion of the SH2 domain into the MBP-B3-11 substrate significantly enhanced phosphorylation at low substrate concentrations but introduced an inhibitory effect at high substrate concentrations. Qualitatively similar data were obtained for the phosphorylation of the MBP-B3-12 and MBP-SH2-B3-12 substrate pair by the three TKD forms.

Additional experiments showed that the GST-SH2 fusion protein was also a substrate for the EGF RTK, although it was phosphorylated at a much lower rate than any of the erbB3-derived peptides (data not shown). This substrate exhibited K_M values of 0.8, 0.7, and 2.3 μM when phosphorylated by TKD61, TKD56 and TKD42, respectively. The K_M for GST-SH2 was therefore significantly higher with TKD42, the TKD form with the lowest capacity for autophosphorylation.

Inhibition of EGF Receptor TKD Activity by an SH2 Domain Protein. Given the inhibitory effect seen at high concentrations of the substrates that contained the SH2 domain, it was considered that SH2 domain proteins might, in general, function as inhibitors of RTK activity. The availability of the MBP-SH2 and MBP-B3-11 proteins allowed a direct demonstration of this inhibitory effect. Inhibition of the three TKD proteins was characterized by assaying MBP-B3-11 phosphorylation activity as a function of MBP-SH2

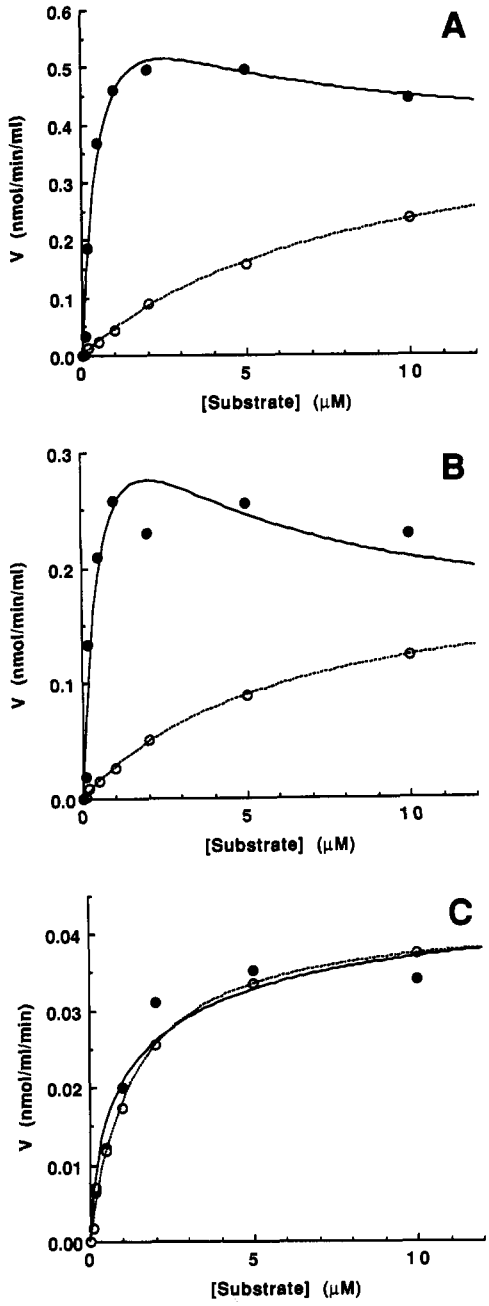

FIGURE 3: Phosphorylation of recombinant substrates by EGF receptor TKD forms. Samples of EGF receptor TKD, either the TKD61 (A), TKD56 (B), or TKD42 (C) protein, were allowed to autophosphorylate in the presence of excess unlabeled ATP. After the addition of [γ^{32}P]ATP, aliquots of the kinase sample were added to solutions of a recombinant substrate protein, either MBP-B3-11 (○) or MBP-SH2-B3-11 (●), at varying concentrations. After a 15-min incubation at room temperature, SDS-PAGE sample buffer was added to quench the phosphorylation reaction. The phosphorylated substrate was then resolved by electrophoresis and quantified by scintillation counting. The data were modeled by use of eq 2 or the simple hyperbolic equation as described in the text, and the best-fit theoretical curves generated using these equations and the parameters of Tables I and II are shown. Details are given in Experimental Procedures.

concentration. Figure 4 shows the inhibition of the TKD56 kinase by the MBP-SH2 protein. The inhibition data obtained were fit by the inverse hyperbolic function

$$V = V_0\left[1 - f_I\frac{[S]}{K_I + [S]}\right] \quad (1)$$

in which S indicates the inhibitory MBP-SH2 protein, V_0 is the kinase activity measured in the absence of the inhibitor,

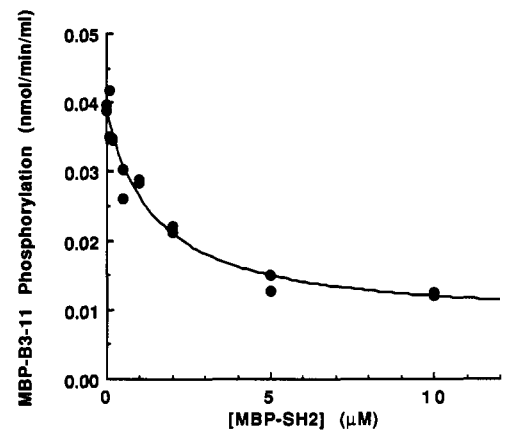

FIGURE 4: Inhibition of EGF receptor TKD activity by the MBP-SH2 protein. The phosphorylation of the MBP-B3-11 substrate (1.0 μM concentration) by each of the three TKD forms was assayed in the presence of varying concentrations of the MBP-SH2 protein (0–10 μM), exactly as described in the legend of Figure 3. The inhibition data were fit by eq 1, and the best-fit parameters are given in Table I. Representative data for the TKD56 protein are shown. Also shown is a threoretical curve generated with eq 1 and best-fit parameters. Details are given in the text.

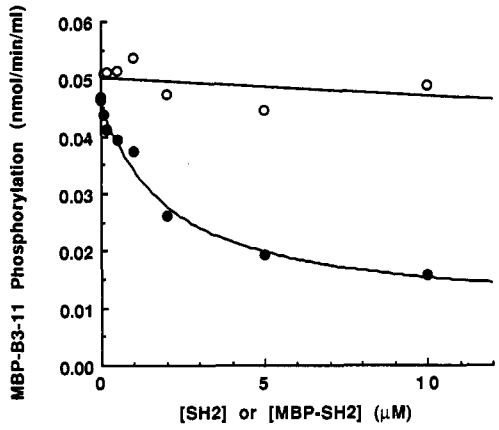

FIGURE 5: Comparison of MBP-SH2 and the isolated SH2 domain as inhibitors of the EGF receptor TKD. The isolated SH2 domain of c-src was generated by thrombin cleavage of a recombinant fusion protein. In an experiment conducted exactly as described in the legend of Figure 4, the isolated SH2 domain (○) and MBP-SH2 (●) were compared as inhibitors of the MBP-B3-11 phosphorylation activity of TKD61.

f_I is the fractional inhibition observed at a saturating concentration of MBP-SH2, and K_I is a constant characterizing the concentration dependence of the inhibitory interaction. The best-fit parameter values obtained by fitting this equation to the inhibition data for the three TKD forms are given in Table I.

To examine the role of the MBP carrier protein in this inhibition phenomenon, the experiment was repeated with the isolated SH2 domain, which was generated by thrombin cleavage of the recombinant GST-SH2 fusion protein. Figure 5 compares the MBP-SH2 protein and the isolated SH2 domain as inhibitors of the MBP-B3-11 phosphorylation activity of TKD61. Clearly the isolated SH2 domain was not an effective inhibitor of the TKD61 activity. This indicated that the MBP domain was involved in the inhibitory interaction of the MBP-SH2 fusion protein with the TKD. The MBP protein itself had no effect on TKD activity (data not shown).

Kinetic Analysis of the Substrate Phosphorylation Data. With the assumption that the inhibitory effects of MBP-SH2-B3-11 on the substrate phosphorylation reaction were identical to those of MBP-SH2, the MBP-SH2-B3-11 phosphorylation data (see Figure 3) could be modeled by an equation with two

fixed and two adjustable parameters:

$$V = V_{max}\frac{[S]}{K_M + [S]}\left[1 - f_I\frac{[S]}{K_I + [S]}\right] \quad (2)$$

The parameters f_I and K_I were held fixed during the fitting at the values determined by the independent inhibition experiments (see Table I). The V_{max} and K_M values were determined by the fitting and indicated the maximal velocity and Michaelis–Menten constant for the substrate that would have been observed in the absence of the substrate inhibition effect. The MBP-SH2-B3-11 phosphorylation data were well fit by eq 2, and the parameter values obtained by the nonlinear least-squares fitting program are given in Table II. Also given are the V_{max} and K_M values for the phosphorylation of the MBP-B3-11 substrate, which obeyed Michaelis–Menten kinetics. Theoretical curves generated by use of eq 2 and the determined parameters are shown in the panels of Figure 3. Very similar data were obtained with the MBP-B3-12 and MBP-SH2-B3-12 substrate pair, and the corresponding best-fit parameter values are also presented in Table II.

The K_M and V_{max} values for the SH2 domain substrates given in Table II should be considered tentative, as they are dependent upon assumptions about the mechanism of substrate inhibition implicit in eq 2 that have not been rigorously validated. A more straightforward, model-independent means of substrate comparison was to examine the V_{max}/K_M ratios derived directly from the slopes of the velocity versus substrate concentration profiles at low substrate levels (see Table II). In the cases of the TKD61 and TKD56 enzymes, incorporation of the SH2 domain into the two substrates MBP-B3-11 and MBP-B3-12 led to enhancements in V_{max}/K_M of approximately 15- and 7-fold, respectively. Obviously the SH2 domain-containing substrates were more efficiently phosphorylated at low concentrations by these TKD forms. In contrast, the V_{max}/K_M ratios were little changed in the case of the TKD42 enzyme, which indicated a role for TKD autophosphorylation in the observed enhancement of V_{max}/K_M.

DISCUSSION

The steady-state kinetic properties of recombinant peptide substrates of the EGF RTK were examined in this study. Each of these peptides contained C-terminal sequences of the erbB3 protein that are putative autophosphorylation sites, and two peptides also incorporated the SH2 domain of the c-src tyrosine kinase (see Figures 1A and B). These recombinant proteins were shown to be strongly phosphorylated by the EGF RTK with K_M values in the micromolar range (see Figure 3 and Table II). To determine how the phosphorylation of the SH2 domain-containing substrates of the EGF RTK could be modulated by SH2 domain-receptor phosphotyrosine residue interactions, three distinct TKD forms differing in their capacity to autophosphorylate were tested. For example, the trucated TKD42 protein (see Figure 1C) lacked the three major tyrosine autophosphorylation sites identified in the native receptor but did contain a demonstrated site of autophosphorylation, Tyr_{992} (Walton *et al.*, 1990). Recently, the phosphorylation of this residue has been shown to promote the high-affinity binding of the SH2 domain protein PLC-γ to the EGF receptor (Rotin *et al.*, 1992b; Vega *et al.*, 1992).

As shown in Figure 3, the incorporation of an SH2 domain into the MBP-B3-11 substrate markedly enhanced the phosphorylation of the substrate by both the TKD61 and TKD56 proteins. This enhancement was effected by a decrease of approximately 5-fold in the substrate K_M, as well as an increase in V_{max} and a corresponding ~15-fold increase in V_{max}/K_M. In contrast, the incorporation of an SH2 domain had little effect on the V_{max}/K_M ratio of this protein as a substrate for TKD42. Hence, it appeared that the enhancement of phosphorylation of the SH2 domain-containing substrate was a result of physical interactions between the substrate SH2 domain and phosphorylated C-terminal tyrosine residues present only in the TKD61 and TKD56 proteins. Consistent with this mechanism is the observation that the SH2 domain binds to the phosphorylated EGF receptor TKD with a dissociation constant of approximately 0.3 μM (Sierke *et al.*, 1993), a value not greatly lower than the K_M values documented here for those substrates of the TKD56 and TKD61 kinases that contained a SH2 domain. The MBP-SH2-B3-11 protein had the lowest K_M value of substrates for the C-terminally complete TKD61 kinase. This K_M value (1.2 μM) is among the lowest reported for substrates of the EGF RTK.

The presence of an SH2 domain in a substrate increased the V_{max} for phosphorylation by each TKD form, although this enhancement tended to be counterbalanced by an inhibitory effect seen at high concentrations of the SH2 domain-containing substrates. The magnitude of this substrate inhibition was estimated by examination of the MBP-SH2 protein as an inhibitor of MBP-B3-11 phosphorylation. The MBP-SH2 protein was shown to be an inhibitor of each of the TKD forms, although the inhibition constant and the maximal extent of inhibition seen at saturating MBP-SH2 concentrations depended upon the TKD analyzed (see Table I). In contrast, the isolated SH2 domain did not significantly inhibit TKD activity (Figure 5). These results indicated that the observed inhibition by MBP-SH2 involved the MBP domain of the fusion protein, perhaps as a steric factor. It should be noted that many of the SH2 domain proteins known to interact with RTK proteins *in vivo* (e.g., p85, PLC-γ, and ras GAP) are actually larger than the MBP-SH2 fusion protein and could certainly exert similar steric effects.

As the MBP protein itself was not an inhibitor, the inhibition mechanism appeared to require interactions between the SH2 domain and the TKD proteins. Each of the TKDs has been shown to autophosphorylate under the conditions of these experiments, and each of the phosphorylated TKD proteins binds with high affinity to the c-src SH2 domain (Sierke *et al.*, 1993). The inhibition by the SH2 domain-containing proteins could then have been due to an interaction with a specific phosphorylated tyrosine residue present in all three of the TKD proteins, i.e., Tyr_{992}. Interaction of SH2 domain-containing substrates with a C-terminal phosphorylation site not present in the TKD42 protein (e.g., Tyr_{1068}, Tyr_{1148}, or Tyr_{1173}) was apparently responsible for the reduction of substrate K_M seen with the TKD56 and TKD61 kinases. It is therefore possible that, although autophosphorylation of the EGF receptor at one site can activate phosphorylation of SH2 domain-containing substrates, autophosphorylation at another site can potentiate inhibition by SH2 domain proteins. If the SH2 domains found in the known physiological substrates of the EGF receptor can interact selectively with distinct autophosphorylation sites, a novel mechanism for the fine tuning of receptor protein tyrosine kinase activity would be provided. Consistent with this hypothesis are previous studies showing an enhanced *in vivo* phosphorylation of cellular substrates by mutant EGF receptor proteins with truncated C-terminal autophosphorylation domains (Chen *et al.*, 1989; Decker *et al.*, 1992; Walton *et al.*, 1990). However, other results have indicated a reduction of the *in vivo* phosphorylation activity of C-terminally truncated EGF (Sorkin *et al.*, 1992) and erbB2 (Di Fiori *et al.*, 1990) receptor proteins. Future work will be directed at examining this potentially important

mechanism of regulation of protein tyrosine kinase activity in signal transduction complexes.

In summary, when EGF receptor TKD forms that strongly autophosphorylate were assayed, substrates containing an SH2 domain showed V_{max}/K_M values significantly higher than those of the corresponding substrates lacking an SH2 domain. The loss of this effect when the truncated TKD42 protein was assayed indicated that interactions between the SH2 domain and specific C-terminal residues in the TKD were responsible for this enhancement of phosphorylation. The results of this study are entirely consistent with the hypothesis that the presence of SH2 domains in tyrosine kinase substrates can promote their phosphorylation by autophosphorylated receptor proteins.

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