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SH2 Domains Exhibit High-Affinity Binding to Tyrosine-Phosphorylated Peptides Yet Also Exhibit Rapid Dissociation and Exchange

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***src* homology 2 (SH2) domains of intracellular signaling molecules such as phospholipase C-γ and phosphatidylinositol 3′-kinase-associated protein p85 represent recognition motifs for specific phosphotyrosine-containing regions on activated growth factor receptors. The binding of SH2 domains to activated growth factor receptors controls the interaction with signaling molecules and the regulation of their activities. In this report, we describe the kinetic parameters and binding affinities of SH2 domains of p85 toward short phosphotyrosine-containing peptides with the amino acid sequence motif YMXM, derived from a major insulin receptor substrate, IRS-1, by using real time biospecific interaction analysis (BIAcore). Associations were specific and of very high affinity, with dissociation constants of 0.3 to 3 nM, between phosphopeptides and the two separate SH2 domains contained within p85. Nonphosphorylated peptides showed no measurable binding, and the interactions were specific for the primary sequence very close to the phosphotyrosine residue. Moreover, the interactions between phosphopeptides and SH2 domains of other signaling molecules were of much lower affinity. Interestingly, the binding of the SH2 domains to the tyrosine-phosphorylated peptides was of high affinity as a result of a very high on rate, of 3×10^7 to 40×10^7/M/s; at the same time, the rate of dissociation, of 0.11 to 0.19/s, was rapid, allowing for rapid exchange of associating proteins with the tyrosine phosphorylation sites.**

Activation of receptors with tyrosine kinase activity by growth factors promotes tyrosine autophosphorylation and associations with proteins containing *src* homology 2 (SH2) domains (3, 9, 19, 22, 39, 43), such as phospholipase C-γ (PLC-γ) (24, 26, 44), GTPase-activating protein (GAP) of *ras* (1, 16, 23, 29), phosphatidylinositol (PI) 3′-kinase-associated p85 (6, 13–15, 32, 41), and GRB2 (21). Specific regions containing phosphorylated tyrosine within platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) receptors which are responsible for binding to SH2 domains of signaling molecules have been identified (13–15, 23, 28, 37). The functional significance of these interactions is not fully understood; however, the ubiquity of the interactions suggests that they serve to mediate phosphorylation events crucial for the control of activity of signaling molecules by both receptor-linked and cytoplasmic tyrosine kinases (3, 4, 9–12, 17, 20, 29–31, 36). Indeed, it has been recently shown that FGF-induced PI hydrolysis is eliminated by a point mutation of the FGF receptor at Y-766, which prevents the association with and tyrosine phosphorylation of PLC-γ (27, 28, 33). Similarly, elimination of Y-740 and Y-751 of the PDGF receptor abolishes the binding of p85 and stimulation of PI 3′-kinase activity (13). Important in defining functional significance will be quantitative measures of the kinetics and strength of these interactions.

We have used a new technique for real-time kinetics and affinity measurements, BIAcore (7, 12), to quantitate interactions between the SH2 domains of p85 and the sequence motif YMXM or YXXM. p85 contains one SH3 domain and two SH2 domains (6, 32, 41), one near the carboxy terminus and one nearer the amino terminus of the protein (Fig. 1), both of which can interact with certain tyrosine-phosphorylated receptors and oncogenic proteins (11, 18, 25). Phosphopeptides have been shown qualitatively to bind to SH2-containing proteins (5). This tyrosine phosphorylation motif was originally identified within the kinase insert region of the PDGF receptor as mediating the binding of that receptor to p85 (13–15). The specific tyrosine-phosphorylated sequences that we have used are derived from a recently identified insulin receptor substrate, IRS-1 (42). IRS-1 is a major substrate both in vivo and in vitro of the insulin receptor kinase (35). It contains several possible tyrosine phosphorylation sites, including six sites with a motif of YMXM and four with a motif of YXXM. Further, peptides containing the YMXM sequences are excellent substrates for the insulin receptor (40). It has recently been shown that tyrosine-phosphorylated IRS-1 strongly associates with PI 3′-kinase via the SH2 domains of p85 and that phosphorylated YMXM peptides compete for this interaction (2). Further, phosphorylated IRS-1 associates with p85 in cells and activates PI 3′-kinase in vitro (2). Hence, in insulin-stimulated cells, phosphorylation of at least some of the YMXM sequences within IRS-1 results in association between IRS-1 and p85 and subsequent activation of the catalytic subunit of PI 3′-kinase. In this report, we show that the SH2 domains of p85 bind to tyrosine-phosphorylated YMXM peptides with dissociation constants of 0.3 to 3 nM. Nonphosphorylated peptides showed no measurable binding. The high-affinity

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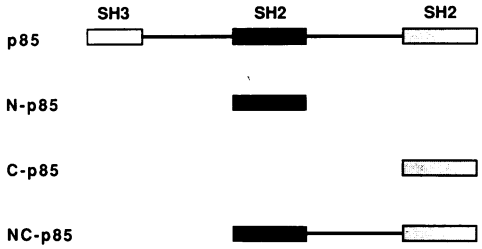

FIG. 1. Structures of p85-GST fusion proteins. GST fusion proteins of various regions of p85 were produced in *E. coli* and purified by affinity chromatography with glutathione-agarose beads as previously described (11). Shown are the amino-terminal SH2 domain of p-85 (N-p85), the carboxy terminal SH2 domain of p85 (C-p85), and both SH2 domains and intervening sequence of p85 (NC-p85). The GST region of fusion proteins is not presented.

binding is due to a very high on rate, yet a rapid rate of dissociation allows exchange of associating protein with the tyrosine-phosphorylated peptides.

MATERIALS AND METHODS

SH2 domain expression and isolation. The SH2 domains of human p85 were expressed as glutathione *S*-transferase (GST) fusion proteins as described previously (11). The protein expression by transformed *Escherichia coli* HB101 were induced with 1 mM isopropylthiogalactopyranoside (IPTG). The fusion proteins were purified by affinity chromatography on glutathione-agarose beads (Sigma) followed by elution with 15 mM reduced glutathione. Dithiothreitol (10 mM) was included in the lysis buffer and elution buffer. Protein concentration was determined spectrophotometrically by analyzing the A_{280}. The fragments expressed are as follows: the N-terminal SH2 of p85, N-p85 (amino acids 321 to 440), the C-terminal SH2 of p85, C-p85 (amino acids 614 to 724), and both SH2 domains of p85, including the intervening region (amino acids 321 to 724).

Peptide synthesis. An Fmoc-based strategy for sequential peptide synthesis was used in conjunction with standard side chain-protecting groups as described previously (2). N^{α}-Fmoc-*O*-(*O*,*O*-dimethoxyphosphoryl)-L-tyrosine [Fmoc-Tyr ($OP(PCH_3)_2$] was used for incorporation of phosphotyrosine (2). Peptides were purified by ether precipitation, gel filtration, and preparative reverse-phase high-pressure liquid chromatography (HPLC). Analytical HPLC demonstrated that the purified products were a single component; amino acid analysis (ABI 420) and results obtained from plasma desorption mass spectrometry (ABI BioIon) were as expected.

Cross-linking of peptides. Carboxyl groups of the matrix were activated with 50 mM *N*-hydroxysuccinimide and 200 mM *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide for 3 min, and then 1 mg of pY-628 per ml in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5) with 1 M NaCl was injected onto the matrix for 4 min with a flow rate of 5 μl/min. All peptides were immobilized by this procedure, except that the concentrations of pY-727 and Y-727 were increased to 5 mg/ml during the reaction. After binding and elution for each curve, 10 mM HCl with 500 mM NaCl was injected to dissociate remaining bound proteins, to regenerate the surface for another round of binding.

RESULTS AND DISCUSSION

Our goal in this study was to measure the kinetic parameters and equilibrium dissociation constants for the binding of SH2 domains of p85 to tyrosine-phosphorylated IRS-1 peptides. Two short peptides containing Tyr-628 and Tyr-727, respectively (see Table 2), were immobilized onto a carboxymethyl-dextran polymer attached to a gold-coated surface within a flow chamber (7, 12). Solutions containing proteins with SH2 domains (Fig. 1) were flowed through the chamber, and the amount of protein associated with the immobilized molecules was assessed by detection of a surface plasmon resonance signal (7, 12), which under the conditions used reports changes in the index of refraction (due to concentration of the protein) very near the gold-coated surface. It has been shown before that the resonance signal scales directly with the relative concentration of protein accumulated within the matrix. The peptides were made with phosphotyrosine and attached protecting groups incorporated during synthesis and were checked by reverse-phase HPLC to demonstrate purity.

Figure 2 displays a sample of the real-time measurements of binding of the amino-terminal SH2 domain of p85 (N-p85, expressed as a fusion protein with GST [11]) to immobilized pY-628. Individual injection curves are shown in overlay format in Fig. 2; the bar at the bottom shows when the SH2 protein was injected into the flow chamber. Following the injection period, elution buffer was flowed through the chamber. Upon injection, N-p85 bound rapidly to steady-state levels (at the higher concentrations). After injection, N-p85 dissociated slowly from the matrix of phosphopeptide binding sites. At low concentrations of N-p85, longer injection times were used in similar experiments to be sure that steady-state binding was reached. The steady-state level bound at each concentration was analyzed in reciprocal format similar to Scatchard analysis (38) for all of the data recorded. An example of this analysis for binding of N-p85 is shown in the insert of Fig. 2A. The dissociation constants estimated by curve fitting for these data are presented in Table 1. The highest affinity found was for C-p85 binding to immobilized pY-628, with a dissociation constant of 0.34 nM. The affinity of N-p85 for pY-727 was lower, with a dissociation constant of 3.40 nM. For any of the SH2-containing proteins, no binding to unphosphorylated peptides was seen, as shown for one case in Fig. 2C. In addition, the GST protein alone up to a concentration of 1 mM showed no binding to any of the peptides tested (data not shown).

The interactions between SH2 domains and tyrosine-phosphorylated peptides were highly specific. Both SH2 domains of p85 bound to IRS-1 tyrosine-phosphorylated peptides with high affinity. However, the affinities of SH2 domains of other signaling molecules for the peptides were very low: GAP SH2 and PLC-γ SH2 domains bound with affinities reduced by 500- and 1,000-fold, respectively, relative to the binding of N-p85 to the same peptide (43a). We have recently shown that PLC-γ SH2 domains bind with high affinity (K_D of 1 to 10 nM) to the tyrosine phosphorylation site of the EGF or FGF receptor. However, the affinity of GAP SH2 domains and p85 SH2 domains toward the tyrosine-phosphorylated FGF receptor peptide was comparatively low (43a). Specificity for the interaction was thus demonstrated in both directions, that is, specificity for phosphopeptide sequence and specificity for the SH2 domain.

To determine the relative affinities of several peptides, rather than cross-linking each peptide to the BIAcore dext-

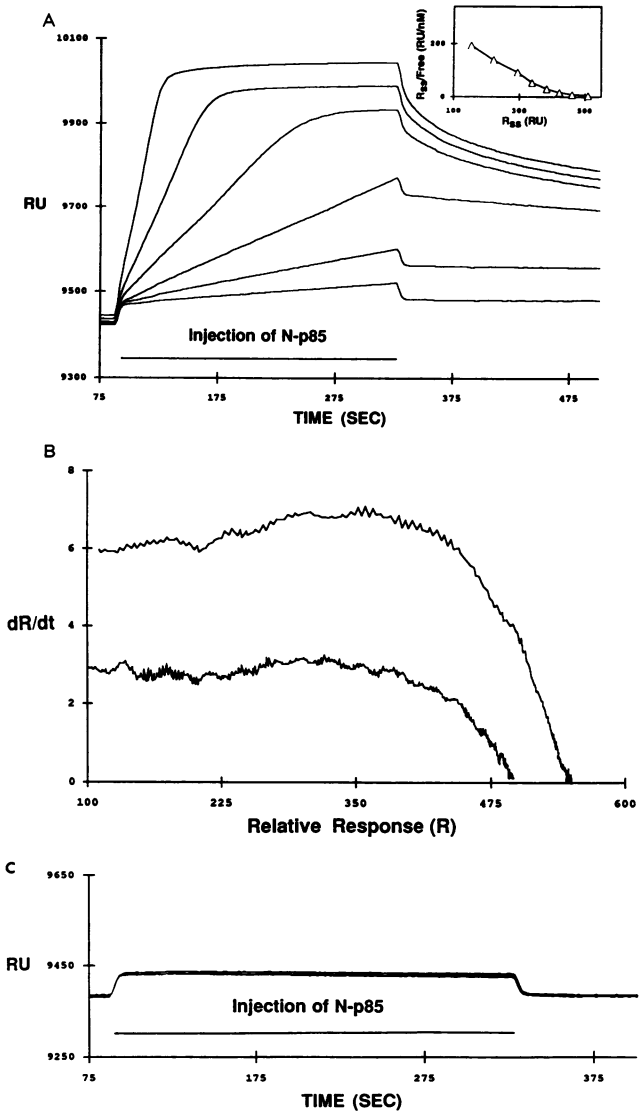

FIG. 2. Raw binding data. Plotted is the resonance signal (R) as a function of time for several concentrations of N-p85 injected into the system (for the time indicated by the bar at the bottom) and then eluted. Either pY-628 (A) or Y-628 (C) had been stably cross-linked to the carboxymethyl-dextran matrix on the gold-coated surface of the flow system (7, 12). Concentrations of N-p85 injected were 3.13, 6.25, 12.5, 25, 50, and 100 nM. Note the small step changes in resonance signal at the start and finish of all injections in panels A and C. These changes represent a small difference in the bulk refractive index of the injected solutions compared with the elution buffer and were subtracted from the binding data. For data analysis, data similar to those for panel A were recorded with longer injection times than shown to ensure that steady state was reached. Steady-state binding values, R_{SS}, were analyzed in Scatchard format (insert). (B) Slope of the resonance signal during the injection of N-p85 as a function of the resonance signal (see equation 2 in the text) for the 25 and 50 nM curves of panel A. RU, resonance units.

ran matrix, we used the various peptides to inhibit the binding of N-p85 and C-p85 proteins to immobilized pY-628 (Fig. 3). In this way, all peptides could be tested under identical conditions. The estimated affinities of the peptides were calculated by comparing the 50% inhibitory concentration values from the inhibition curves relative to the measured affinities of pY-628 for N-p85 or C-p85 (Table 2). In agreement with the dissociation constants given in Table 1, the affinities of pY-628 and pY-727 were very similar and much greater than the affinity of the unphosphorylated peptides. Two additional peptides, pY-658 and pY-608, had similar affinities, and the affinities of unphosphorylated Y-658 and Y-608 were greatly reduced. Smaller peptides derived from the pY-628 sequence competed for the p85 proteins as well. Deletion of the three amino-terminal residues and one carboxy-terminal residue of pY-628 resulted in a significant loss of affinity, of 8-fold for C-p85 and 100-fold for N-p85, indicating that the deleted residues contribute to binding interactions with the SH2 proteins. The shorter sequence was used to test the importance of the first residue after the phosphorylated tyrosine. Glu, Leu, Val, and Ile were nearly equally effective, while other substitutions af-

TABLE 1. Estimated equilibrium dissociation constants and rate constants

Immobilized ligand	Binding protein	K_D (nM)	k_{off} (/s)	k_{on}[a] (10^8/M/s)
pY-628	N-p85	3.27 ± 0.18	0.156	0.48
	C-p85	0.34 ± 0.17	0.149	4.4
	NC-p85	0.75 ± 0.41	0.149	2.0
pY-727	N-p85	3.40 ± 1.21	0.113	0.33
	C-p85	0.90 ± 0.60	0.186	2.1
	NC-p85	0.82 ± 0.40	0.170	2.1

[a] Calculated from the measured parameters as k_{off}/K_D. The k_{on} values could not be determined directly, because the flow of protein into the system limited the rate of association when association rate constant k_{on} was $>10^6$/M/s.

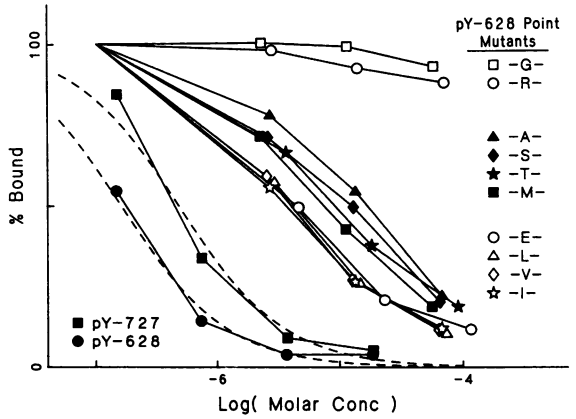

FIG. 3. Competitive binding of peptides. Onto a matrix cross-linked with pY-628 was injected 100 nM C-p85 premixed with various concentrations of different peptides. Injections were at a flow rate of 5 μl/min and lasted for 4 min. Plotted is the relative response at 4 min as a percentage of the maximal response, 75 to 83 resonance units, reached in the absence of competing peptide. The relative response is plotted as a function of peptide concentration for each competing peptide used. This is an inhibition assay done under the conditions of high concentration of probe; the concentration of immobilized pY-628 within the flow chamber matrix was roughly 0.5 μM. Hence, the 50% inhibitory concentration value of each peptide does not reflect the equilibrium dissociation constant directly but is a relative measure of the affinity for the binding protein versus the cross-linked pY-628 probe. The dotted lines for pY-727 and pY-628 show the curves for these data fit to a mass action inhibition expression.

TABLE 2. Relative affinities of peptides

Peptide	Primary structure	Estimated K_D (nM)[a] with:	
		C-p85	N-p85
pY-628	GDGYMPMSPKS	0.340	3.27
Y-628		120	167
pY-727	(K)TGDYMNMSPVG	1.01	2.11
Y-727		800	>300
pY-628-I	YIPMSPKS	8.31	214
pY-628-V	YVPMSPK	8.72	190
pY-628-L	YLPMSPK	9.33	419
pY-628-E	YEPMSPK	10.6	99.2
pY-628-M	YMPMSPK	16.2	386
pY-628-T	YTPMSPK	20.6	304
pY-628-S	YSPMSPK	23.1	>600
pY-628-A	YAPMSPK	31.6	>600
pY-628-R	YRPMSPK	810	>600
pY-628-G	YGPMSPK	1100	>600

[a] Calculated from 50% inhibitory concentration values of inhibition curves (as in Fig. 3) relative to K_D determined for pY-628 binding for C-p85 or for N-p85 (Table 1). Uncertainties for these values as estimated by the fitting procedures were less than 10% of the values.

fected affinity to different extents. Best in this position were bulky hydrophobic groups or glutamate.

BIAcore data can be used directly to study the kinetics of interactions (7, 12). However, the kinetics of binding for the interactions were not well suited for direct analysis. The amount of N-p85 (e.g., Fig. 2) associated with the surface increased linearly with time until binding was near saturation, very unlike a typical approach to equilibrium seen for slower interactions. During binding the resonance signal, R, should behave as follows (34):

$$dR/dt = k_{on}\, C\, (R_{max} - R) - k_{off}\, R \quad (1)$$

where k_{on} and k_{off} are apparent first-order rate constants of association and dissociation, C is the concentration of binding protein injected, R_{max} is the maximum response at saturated binding, R is response as a function of time, that is, occupied binding sites (in units of resonance signal), and $(R_{max} - R)$ is unoccupied binding sites (in units of resonance signal).

The equation after rearranging is as follows:

$$dR/dt = k_{on}\, C\, R_{max} - (k_{on}\, C + k_{off})\, R \quad (2)$$

According to equation 2, a plot of dR/dt against R should be a straight line. In Fig. 2B, dR/dt is plotted as a function of R for two of the binding curves of Fig. 2. The function is biphasic, suggesting that binding is limited by delivery of N-p85 to the surface rather than by association alone. That is, association with the surface is so fast that the concentration of free N-p85 at the surface is lower than the injected concentration; delivery to the surface by flow and diffusion from the region of laminar flow to the surface is relatively slow. In this case, direct measurement of dissociation kinetics is not possible for much the same reason. Before newly dissociated N-p85 can diffuse out of the matrix of binding sites, it rebinds. The problem of transport-limited binding arises only when either the association constant is higher than about 10^6/M/s or the number of sites on the matrix is very high (34).

The true rate of dissociation can be measured by preventing rebinding. This is easy to do in most cases, by adding a competing ligand during the dissociation experiment (Fig. 4), much the same as measuring dissociation of radiolabeled ligand in a standard binding experiment in the presence of

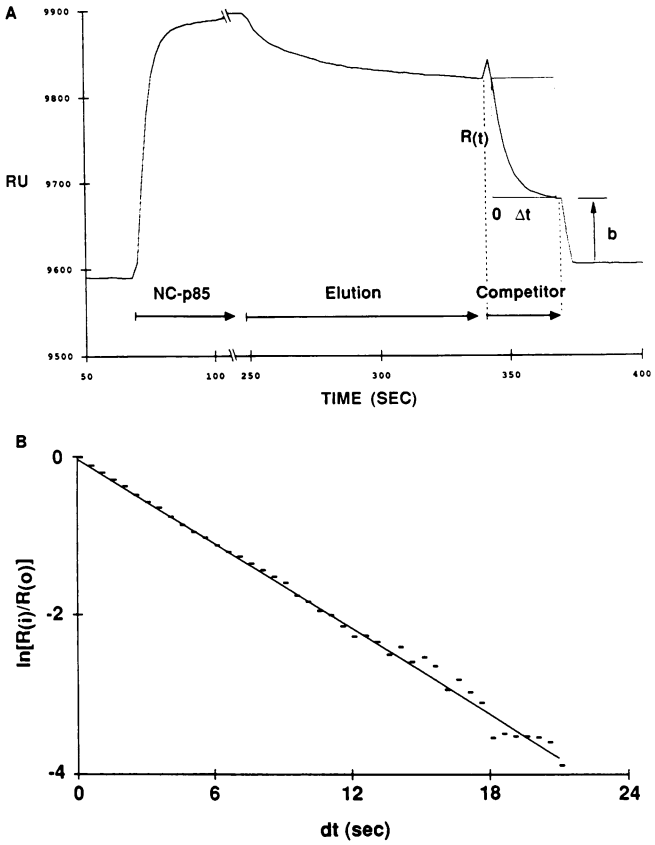

FIG. 4. Dissociation of SH2 domain in the presence of competing peptide. At a flow rate of 10 μl/min, 100 nM NC-p85 was injected onto a matrix cross-linked with pY-628 for 3 min, and then elution was begun. During elution, 100 μM pY-628 was injected for 30 s. The boxed section, beginning 5 s after the start of injection of competing ligand, was well fit as a single exponential decay, $R(t)=R(0)\, e^{-kt}$. These data are plotted in logarithmic scale in panel B. In panel A, the value of b represents the difference in resonance unit (RU) signal between elution buffer with and without the competing peptide, due to the bulk refractive index. The value dropped to the initial signal within 5 s of the end of the injection, reflecting the time required to change the buffer across the surface of the resonance chip at the flow rate used. The results were the same for competition with 100 μM pY-727 or with 5 mM phenylphosphate. Unphosphorylated peptides or phosphorylated peptides with low affinity for C-p85 did not affect the dissociation rate at a concentration of 100 μM.

excess unlabeled ligand. Following binding of NC-p85 to pY-727 immobilized on the matrix, either free pY-628 (100 μM) or free pY-727 (100 μM) was injected. These peptides were then available to bind to newly dissociated NC-p85 and reduce its rebinding to the matrix. Under these conditions, NC-p85 dissociated rapidly. The same results were seen for binding to pY-628. Injection of nonphosphorylated Y-628 or Y-727 up to a concentration of 100 μM had very little effect. Hence, despite the fact that the interactions are of high affinity, the SH2-phosphotyrosine peptide interactions showed rapid dissociation and ligand exchange.

The apparent first-order rate constants of dissociation under conditions of added competing ligand, k_{off}, are listed in Table 1. For all interactions, the rate constants ranged from 0.11 to 0.19/s. It should be noted that these are very fast rates of dissociation for interactions of such high affinity. Also listed are the apparent first-order rate constants of

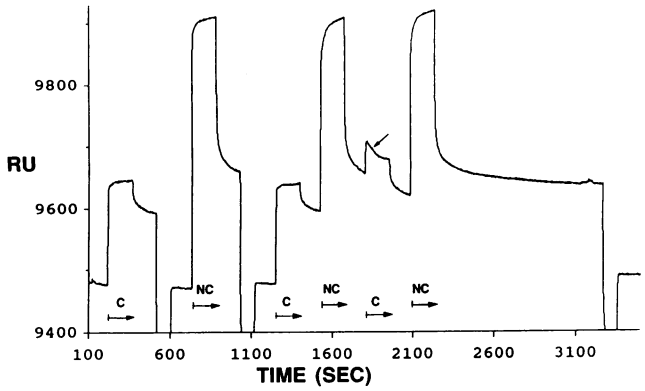

FIG. 5. Exchange of C-p85 and NC-p85 on a pY-628 surface. Onto a surface with immobilized pY-628 peptide were injected 100 nM C-p85 (C) and 100 nM NC-p85 (NC) alternately as shown. This concentration was more than 100-fold higher than the K_D value for each protein (Table 1) so that the surface was saturated rapidly. Following the first injections of each, the surface was stripped of SH2 protein by treatment with 10 mM HCl. Because NC-p85 is larger than C-p85, the resonance signal was considerably higher. After the second injection of C-p85, the surface was not stripped. Instead NC-p85 was injected while the surface was nearly saturated with C-p85. If C-p85 had remained bound during this second injection of NC-p85, then binding of NC-p85 would have been greatly reduced; however, the same level of resonance signal was reached as for the first injection. Hence, NC-p85 rapidly displaced C-p85. C-p85 was then injected without stripping the surface. Again the injected SH2 protein, C-p85, displaced the previously bound protein, NC-p85, although not efficiently as had NC-p85. Note that during the third injection of C-p85, instead of a positive slope showing increasing binding to the surface, a more negative slope was seen than before injection (small arrow), reflecting enhanced dissociation of NC-p85 and replacement with C-p85. Following the third injection of NC-p85, the elution was continued for a longer time to demonstrate the slow dissociation from the surface when no competing ligand was added to block rebinding. At the end of the data curve, the surface was stripped with 100 μM pY-727 and returned to the same level as at the start of the experiment. RU, resonance units.

association, k_{on}, calculated by dividing the rate constants of dissociation by the equilibrium dissociation constants, K_D, determined by Scatchard analysis of steady-state binding. The precisions of the calculated on rates are lower than the precisions of the off rates, which were determined by direct measurements. The rate constants of association ranged from 3×10^7 to 40×10^7/M/s. These rates are among the fastest known for receptor-ligand interactions. Interleukin-2 and formylmethionylleucylphenylalanine, for example, bind to their respective cell surface receptors with a k_{on} of 3×10^7/M/s (8, 45), while the larger ligand EGF binds to its soluble receptor with a k_{on} of 10^5/M/s (46). Hence, phosphorylated IRS-1 protein binds with very high affinity and a very high rate of association to the SH2 domains of p85, the regulatory subunit of PI 3′-kinase.

Rapid dissociation in the presence of competing ligand suggests that the SH2-containing proteins can rapidly displace each other from tyrosine-phosphorylated binding sites. This is directly demonstrated in Fig. 5, in which saturating concentrations of C-p85 and NC-p85 were alternately injected onto a surface with immobilized pY-628. Since NC-p85 is considerably larger, it gives a higher resonance signal. When NC-p85 was injected, the same high resonance signal was rapidly reached whether it was injected onto an empty surface (first injection of NC-p85) or onto a surface saturated with C-p85 (second injection of NC-p85). Hence, despite the fact that the SH2 interactions are of high affinity, different SH2-containing proteins can rapidly exchange.

We have measured the kinetics and affinities for a purified system with short phosphopeptide fragments immobilized on a dextran polymer and free proteins containing SH2 domains within the BIAcore apparatus. We note that the binding parameters determined may not be identical to those for the native molecules within cells. In particular, attachment of a short peptide to a matrix may affect the structure and affinity of the peptide toward a binding protein. Further, a short peptide in isolated form may bind differently from the same peptide sequence within the larger natural protein. In an additional series of similar experiments, however, we have found that the phosphorylated intracellular domain of the EGF receptor binds with the same kinetics to SH2 proteins derived from PLC-γ as do short phosphopeptides isolated from the receptor (43a). In addition, if the binding of phosphopeptides to SH2 proteins was multimeric, then the affinities determined could have been incorrect because of avidity effects. However, multimeric interactions were unlikely, since changing the amount of peptide cross-linked to the surface over a 10-fold range had little effect on measured affinity constants (data not shown).

The quantitative data presented support the notion that the IRS-1 protein is important to signal transduction initiated by insulin binding to the insulin receptor (2, 40, 42). In addition, our study represents an attempt to understand the organization behind the complexity of SH2 interactions in quantitative terms. The ability to quantitate the kinetic parameters and dissociation constants and hence the exact specificities for all possible SH2-phosphotyrosine interactions will be very important. The features that we have seen from the quantitation of interactions between p85 and the IRS-1 phosphotyrosine sequences are as follows. Interactions are specific in several ways: very specific for phosphorylated as opposed to unphosphorylated tyrosine sequences, specific for the primary sequence very close to the phosphotyrosine, and specific for the SH2 domains of different cellular proteins. Further, the interactions are very high in affinity. Inside the cell where local concentrations are often high, such a high-affinity interaction would be essentially irreversible. Interestingly, our data suggest that the nature of the interactions lends itself to very specifically controlled reversibility. Despite the high affinity, off rates are very high, so that proteins binding to the phosphotyrosine sequences, including presumably SH2 domain-containing proteins and tyrosine phosphatases, can rapidly exchange. Coupled with this, affinity is greatly reduced for unphosphorylated peptides. Hence, despite high affinity, the interactions could be controlled rapidly and quantitatively by phosphorylation and dephosphorylation. It will be important in these regards to quantitate the interactions between a wide range of phosphotyrosine peptides and of tyrosine-phosphorylated receptor domains with SH2-containing proteins both to determine the hierarchy of affinities and to determine whether the features that we have observed are generalized features for these signaling interactions.

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S.F. and M.Z. contributed equally to this report.

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