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Interactions between SH2 Domains and Tyrosine-Phosphorylated Platelet-Derived Growth Factor β-Receptor Sequences: Analysis of Kinetic Parameters by a Novel Biosensor-Based Approach

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The interaction between SH2 domains and phosphotyrosine-containing sequences was examined by real-time measurements of kinetic parameters. The SH2 domains of the p85 subunit of the phosphatidylinositol 3-kinase as well as of other signaling molecules were expressed in bacteria as glutathione *S*-transferase fusion proteins. Phosphotyrosine-containing peptides, corresponding to two autophosphorylation sites on the human platelet-derived growth factor β-receptor that are responsible for phosphatidylinositol 3-kinase binding, were synthesized and used as capturing molecules, immobilized on a biosensor surface. The association and dissociation rate constants for binding to both sites were determined for intact p85 and the recombinant SH2 domains. High association rates were found to be coupled to very fast dissociation rates for all interactions studied. A binding specificity was observed for the two SH2 domains of p85, with the N-terminal SH2 binding with high affinity to the Tyr-751 site but not to the Tyr-740 site, and the C-terminal SH2 interacting strongly with both sites. This approach should be generally applicable to the study of the specificity inherent in the assembly of signaling complexes by activated protein-tyrosine kinase receptors.

The interaction of growth factors with receptor protein-tyrosine kinases at the cell surface leads to a variety of intracellular events that culminate in DNA synthesis and cell growth. The first such event is receptor autophosphorylation on tyrosine residues, which creates specific high-affinity binding sites for intracellular molecules involved in signal transduction and generation of second messengers (4, 48). One of these proteins, which has been shown to form complexes with most of the receptors studied (3–5, 7, 24, 41, 49), is the phosphatidylinositol (PI) 3-kinase, an enzyme that phosphorylates the inositol ring of PI, PI 4-phosphate, and PI 4,5-bisphosphate at the D-3 position, resulting in the generation of putative second messengers (2, 6). The structure of this enzyme has recently been shown to be heterodimeric, consisting of a regulatory 85-kDa subunit, which mediates the interaction with activated receptors (8, 13, 15, 34, 38, 45), and a catalytic 110-kDa subunit (14, 38).

Other enzymes found in receptor complexes include phospholipase C-γ (PLCγ), a key enzyme in the generation of the intracellular messengers Ca^{2+} and inositol triphosphate (IP_3) (28, 37); GTPase-activating protein (GAP), which converts the active, GTP-bound form of $p21^{ras}$ to its inactive, GDP-bound form (19, 25); members of the *src* family of cytoplasmic tyrosine kinases, which have been implicated in normal and tumorigenic cell growth (29); and finally the serine/threonine kinase Raf, which is a substrate, although it is not yet clear whether it complexes directly with growth factor receptors (36). A specificity is observed in these interactions, so that distinct receptors associate with subsets of the signaling proteins. For example, the colony-stimulating factor-1 receptor interacts with the PI 3-kinase and GAP but not with PLCγ (42, 49). Only the platelet-derived growth factor (PDGF) β-receptor associates with all the above enzymes and is the best-characterized example of complex formation (4).

The mechanism by which receptor complexes are assembled has been investigated in detail. Two components are required, tyrosine-phosphorylated sequences on the receptors and the presence of SH2 (*src* homology 2) domains on the substrates. The latter are conserved sequences of approximately 100 amino acids and have been found in all molecules that interact with receptors except Raf (27, 40). They are independently folding domains which confer common recognition properties on the proteins in which they are found. In the absence of phosphotyrosine, the same receptor sequences do not interact at all with the SH2 domains.

Several autophosphorylation sites have been mapped on growth factor receptors, and site-directed mutagenesis as well as other studies have attempted to define the sequences that interact specifically with PI-3 kinase, PLCγ, GAP, and others. The PDGF receptor is the best-studied example in this respect. It has been shown that Tyr-740 and Tyr-751 (all numbers are for the human PDGF β-receptor) are sites of interaction with the PI 3-kinase (10, 21). Site-directed mutagenesis of either tyrosine to a phenylalanine results in partial loss of PI 3-kinase binding, whereas mutation of both tyrosines abolishes binding completely, an event which, at least in one cell type, leads to loss of response to the mitogenic action of PDGF (10). The site of interaction with GAP has been shown to reside at Tyr-771; mutation to a phenylalanine, however, while abolishing GAP binding, does not result in loss of the mitogenic response (10). Tyrosines 1009 and 1021 at the C terminus of the human

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PDGF β-receptor have recently been shown to be involved in the interaction with PLCγ (44). The site of pp60$^{c\text{-}src}$ binding has not yet been established, although it should be expected to be distinct from those for other signaling molecules in order to provide specificity for binding. On the colony-stimulating factor-1 receptor, Tyr-721, which is equivalent to Tyr-751 of the PDGF receptor, has been shown to be involved in PI 3-kinase binding (43), and the same enzyme is bound by Tyr-315 on polyomavirus middle T antigen (47). Finally, Tyr-766 on the fibroblast growth factor receptor is thought to mediate binding of this molecule to PLCγ (35).

In order to investigate the specificity of these interactions, it is important to be able to accurately measure their kinetic parameters. Several assays for SH2 domain binding have been developed, including the interaction of biotinylated glutathione *S*-transferase (GST)-SH2 probes with nitrocellulose-immobilized proteins (32), the use of agarose-coupled phosphopeptides (12), competition by phosphopeptides for binding to immobilized receptors (10), a filter assay with ^{125}I-labeled GST-SH2 domain fusion proteins (50), and an assay based on the binding of ^{35}S-labeled GST-SH2 domains to immunoprecipitated receptors (51). Although these methods have been very useful in identifying SH2 domain-binding proteins and in competition studies, they do not provide accurate determination of kinetic parameters, such as association and dissociation constants, although most have suggested that these interactions have affinities in the nanomolar range. We report here the use of a biosensor approach, in which binding between two interacting molecules is measured in real time and estimation of kinetic parameters is easy to achieve in the absence of radiolabeling. This method has been used to study the relative affinities of several SH2 domains and intact proteins with phosphopeptides corresponding to autophosphorylation sites on the PDGF receptor. The results demonstrate that SH2 domains and short, tyrosine-phosphorylated receptor sequences contain sufficient information to determine the specificity that is inherent in receptor-signaling protein interactions.

MATERIALS AND METHODS

Purification of GST-SH2 domain fusion proteins. Regions corresponding to the SH2 domains in human GAP (amino acids 178 to 277), bovine PI 3-kinase p85α subunit (amino acids 312 to 444 and 612 to 722), and bovine PLCγ1 (amino acids 545 to 659, 663 to 759, and 545 to 759) were isolated by the polymerase chain reaction and cloned into the pGEX-2 bacterial expression vector (46). For isolation of the GST fusion proteins, a 100-ml overnight culture was used to inoculate 2 liters of LB medium containing ampicillin (100 μg/ml). The culture was incubated with vigorous aeration at 37°C until an A_{600} of 0.7 was reached. Isopropylthiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated for a further 4 h. Cells were collected by centrifugation and resuspended in 12 ml of 20 mM sodium phosphate (pH 7.3)–150 mM NaCl (PBS) containing 1% Triton X-100. Cells were lysed by two 20-s sonication bursts. The cell debris was pelleted by centrifugation at 5,000 × *g* for 10 min at 4°C. Affinity chromatography with 1 ml of 50% (vol/vol) glutathione-agarose resin (Sigma) in PBS was used to purify the protein from the clarified lysate. Contaminating proteins were removed by washing the resin three times with 50 ml of PBS containing 1% Triton X-100 and then three times with 50 ml of PBS. Fusion proteins were eluted with 7 ml of 50 mM glutathione in 100 mM ammonium bicarbonate, pH 8.5. The buffer was changed to 50 mM Tris-HCl (pH 8.0)–50 mM NaCl–1 mM dithiothreitol (DTT) on NAP 25 columns (Pharmacia), and the protein was concentrated with a Centricon 30 microconcentrator device (Amicon). Protein purity was assessed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE).

Heterodimers of GST and GST-SH2 were prepared by mixing approximately equal amounts of GST and GST-SH2 homodimers in 5 mM sodium phosphate (pH 7.0)–1 mM glutathione–2 mM DTT (buffer A) and adding solid guanidinium hydrochloride to a final concentration of 6 M. After a 30-min incubation at room temperature, the mixture was dialyzed successively against 10 volumes of 3, 1.5, 0.5, and 0 M guanidinium-HCl in buffer A for approximately 3 h each, followed by dialysis against buffer B (20 mM Tris [pH 8.2], 2 mM DTT, 10% glycerol). The solution was loaded onto a Mono Q anion-exchange column on a SMART chromatography system, and proteins were eluted with a gradient of NaCl in buffer B (0 to 0.3 M over 30 min; flow rate, 0.1 ml/min). Under these conditions, GST–GST-SH2 heterodimers eluted between GST-GST and GST-SH2 homodimers, as determined by SDS-PAGE and mass analysis.

Intact p85α was purified after expression in insect cells with baculovirus vectors by a series of chromatography steps, as described elsewhere (13). All proteins used in biospecific interaction analysis experiments with the BIAcore instrument (Pharmacia) were passed through a Pharmacia desalting column on a SMART chromatography system in order to achieve buffer exchange to the BIAcore running buffer (see below).

Amino acid analysis for the accurate determination of concentrations for all the proteins used was performed on an Applied Biosystems 420A analyzer.

Preparation of biotinylated and phosphorylated peptides. The 17-mer (DMSKDESVDYVPMLDMK) and 9-mer (SVD YVPMLD) Y751 peptides and the 12-mer Y740 peptide (GE SDGGYMDMSK) were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and phosphorylated on their tyrosine residues by using epidermal growth factor-stimulated plasma membranes from A431 cells, as described elsewhere (12). The 9-mer phosphopeptide was also produced synthetically by using Fmoc phosphotyrosine, and the same method was used to obtain a 17-mer Y740P phosphopeptide (SLTGESDGGYMDMSKDE). The phosphorylated peptides were further purified by reversed-phase high-pressure liquid chromatography (HPLC). After drying down and resuspension in 100 μl of 0.1 M phosphate buffer, pH 7.8, approximately 10 μg of phosphopeptide was incubated with a 10-fold molar excess of *N*-hydroxysuccinimide biotin (Pierce) for 1.5 h at room temperature; Tris buffer was added to 0.2 M, and incubation was continued for 30 min. The same procedure was used to biotinylate nonphosphorylated peptides, which were used as controls. The biotinylated peptides were separated by reversed-phase HPLC and identified by their increased molecular weight by time-of-flight laser desorption mass analysis (Lasermat).

Analysis of interactions with the BIAcore instrument. Details of the construction of the instrument and the procedure for obtaining kinetic constants have been described elsewhere (17, 18, 20). The running buffer used in all experiments consisted of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesul-fonic acid, pH 7.4), 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, and 4 mM DTT. Avidin (Boehringer) at 50 μg/ml in 20 mM sodium acetate buffer, pH 4.0, was immobilized on the sensor chip surface, activated

with a 1:1 mixture of *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pharmacia). Excess reactive groups were blocked with ethanolamine (1.0 M). The biotinylated peptides were injected over the avidin at a flow rate of 5 μl/min. Any nonspecifically bound material was removed with a short pulse (4 μl) of 0.1% SDS. GST-SH2 domain fusion proteins were injected over the surface at 5 μl/min at a constant temperature of 25°C, followed by free buffer flow or injection of an excess of phosphorylated, nonbiotinylated peptide. Any material remaining bound to the surface was removed with a 4-μl pulse of 0.1% SDS, which brought the signal to the background level.

The basic method for the estimation of association and dissociation rate constants by the BIAcore has been described before (20). The signal observed when an interaction takes place is measured in resonance units, which correspond to the amount of bound protein. The rate of change of the signal is described by the equation:

$$\frac{dR_A}{dt} = k_{ass} \times C \times (R_{max} - R_A) - k_{diss} \times R_A \quad (1)$$

where k_{ass} is the association rate constant, k_{diss} is the dissociation rate constant, C is the protein concentration, R_{max} is the maximum binding capacity, and R_A is the amount of protein bound to immobilized capturing molecules. Equation 1 can be rearranged as:

$$\frac{dR_A}{dt} = k_{ass} \times R_{max} \times C - (k_{ass} \times C + k_{diss}) \times R_A \quad (2)$$

Since there is negligible dissociation of immobilized ligand and the bulk solution refractive index remains constant (provided that measurements start 10 to 20 s after injection), equation 2 becomes:

$$\frac{dR}{dt} = \text{constant} - (k_{ass} \times C + k_{diss}) \times R \quad (3)$$

where R is the measured relative response. A series of concentrations of the protein are injected, and the system software calculates the slopes, k_s, of dR/dt-versus-R plots. By plotting these slope values against the concentration of protein, rate constants can be determined from the equation:

$$k_s = k_{ass} \times C + k_{diss} \quad (4)$$

Dissociation rate constants can also be measured in free buffer flow after the end of the protein injection or in the presence of excess competing peptide to prevent any rebinding on the surface. In this case, first-order kinetics are described by the equation:

$$\ln(R_{A1}/R_{An}) = k_{diss} \times t \quad (5)$$

where R_{A1} is the bound ligand at time t_1, R_{An} is the bound ligand at time t_n, and t equals $t_n - t_1$.

For kinetic measurements, at least six different concentrations were injected. The upper limit of this range was dictated by the need to avoid steady-state binding occurring very soon after the injection of protein. This would limit the number of points at which dR/dt can be measured, since this value approaches zero at steady state. Dissociation rate constants in buffer flow were calculated from experiments in which maximum protein binding was obtained in order to prevent as much as possible the rebinding of material on the surface. Alternatively, a competing, nonbiotinylated peptide was injected at 20 μM immediately following the protein injection. The statistical evaluation of kinetic parameters was done by comparing independent experiments, using different batches of desalted proteins and different sensor surfaces, because the results obtained from duplicate runs performed under the same conditions were identical.

RESULTS AND DISCUSSION

Expression and purification of SH2 domains. The polymerase chain reaction was used to isolate and clone into the pGEX-2 bacterial expression vector the following SH2 domains: (i) N-terminal SH2 domain of bovine p85α; (ii) C-terminal SH2 of p85α; (iii) N-terminal SH2 domain of human GAP; (iv) N-terminal SH2 domain of bovine PLCγ1; (v) C-terminal SH2 domain of bovine PLCγ1; and (vi) combined N- and C-terminal SH2 domains of PLCγ. Use of the pGEX-2 vector results in the formation of fusion proteins of the domains with part of the GST. Although the fusion proteins can then be cleaved with thrombin to remove GST, the intact fusion proteins were used in this study in order to obtain a better signal in the real-time interaction measurements (see below). SH2 domains are independently folding entities whose conformation is unlikely to be influenced by the neighboring GST domain. Since the fusion proteins consistently gave much better signals, allowing more accurate estimations of kinetic parameters, they were preferred to the cleaved domains for all the experiments reported here. Analysis of the molecular weights of the fusion proteins by gel filtration revealed that they all migrated as dimers, including GST alone. As this could affect the measurement of kinetic parameters and in particular the k_{diss} values, we also used a denaturation-renaturation procedure (see Materials and Methods) to produce a GST–GST-SH2 heterodimer preparation of the p85 N-SH2, which was large enough to give a good-quality signal in our assay but contained only one binding site per molecule. Apart from recombinant SH2 domains, the intact p85α subunit of the PI 3-kinase, containing two SH2 domains, was also used in this study. This was expressed in insect cells with baculovirus vectors and purified to homogeneity (13).

BIAcore method. The phenomenon of surface plasmon resonance has been used for the development of a novel method for the study of interactions between macromolecules (9, 17, 18). This system provides a reliable and reproducible method for the estimation of kinetic parameters. Binding is measured in real time under accurately controlled conditions, such as temperature and flow rate. Moreover, there is no need for radiolabeling of one of the components, and it is not necessary to devise methods for separation of bound and unbound ligand, which invariably requires a certain amount of time and may thus compromise the accurate measurement of kinetic parameters. One of the interacting molecules is immobilized on a dextran layer, which is in turn mounted on a gold surface (16, 31). A monochromatic wedge of light is shone on this surface at a particular angle, resulting in total deflection. Because of surface plasmon resonance, however, one component of the light, termed the evanescent wave, interacts with the electrons on the gold surface, resulting in a dip in the intensity of the reflected light. The angle at which this dip is observed (resonance angle) changes with the refractive index of the medium, which in turn relates to the mass concentration very close to the gold surface. The resonance angle is

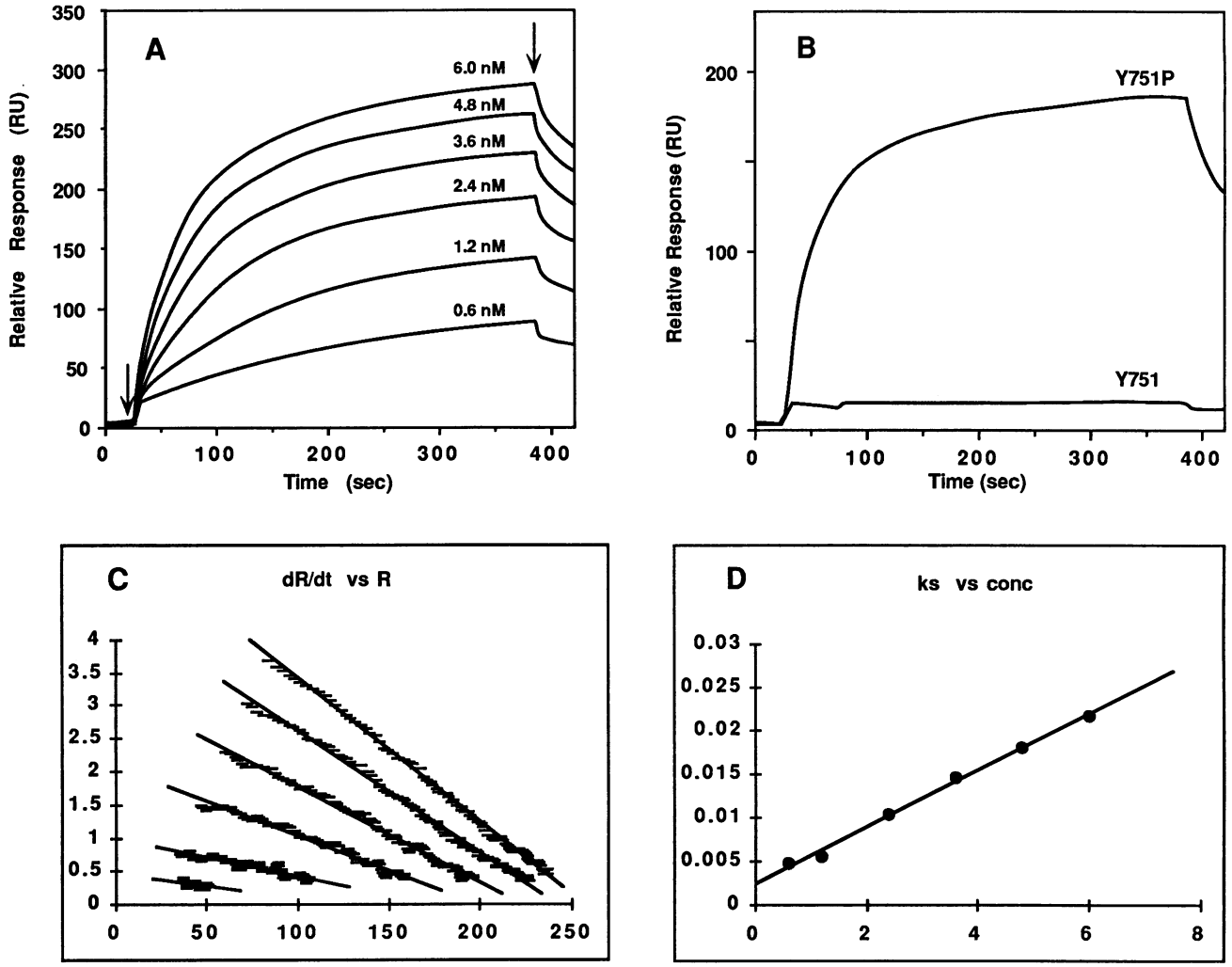

FIG. 1. (A) Interaction of p85α N-SH2 with the phosphopeptide Y751P. Increasing amounts of SH2 domain were injected over avidin-immobilized, biotinylated and phosphorylated peptide. The arrows indicate the beginning and end of the injections. (B) Specificity of binding to phosphorylated and nonphosphorylated immobilized peptide. The same amount of p85α was injected over the two surfaces, and the resulting sensorgrams are superimposed. (C) *dR/dt*-versus-*R* plots of the interactions shown in panel A. The slope (k_s) of each line was plotted against the concentration of injected protein (D) in order to obtain the k_{ass} value.

measured by the instrument and converted to a plot of resonance units (RU) versus time. The higher the RU value, the greater the amount of protein at the dextran matrix. This plot is called a sensorgram, and representative examples are shown in Fig. 1A. The presence of compounds different from those of the running buffer results in significant "bulk effects" being measured by the BIAcore instrument, which may distort the initial phase of the association and dissociation curves. Therefore, all fusion proteins were passed through a desalting column equilibrated in BIAcore running buffer in order to achieve buffer exchange.

Estimation of kinetic parameters requires the repetitive injection of a range of protein concentrations over the immobilized capturing molecule. In order to be ready for a subsequent injection, the biosensor surface has to be regenerated at the end of each cycle so that any noncovalently bound protein will be removed. For this reason, we decided to use the tyrosine-phosphorylated peptides as capturing molecules, as they are not affected by relatively harsh conditions, such as injection of SDS solutions. However, the direct covalent coupling of phosphopeptides to the dextran matrix gave poor results, presumably because these small molecules were not exposed enough to interact with the injected SH2 domains. Therefore, the biotin-avidin interaction principle was used to place a long "spacer" between the phosphopeptides and the matrix. Avidin was covalently immobilized on the matrix as described in Materials and Methods, and then a biotinylated phosphopeptide solution was injected. The high affinity of the biotin-avidin interaction resulted in no significant dissociation of peptide during the course of a typical experiment. This was confirmed by comparing the binding of an antiphosphotyrosine antiserum to the matrix at the beginning and the end of an experiment involving multiple injection and regeneration cycles (data not shown). Biotinylation of the phosphopeptides was performed with *N*-hydroxysuccinimide-biotin, resulting in the covalent attachment of biotin molecules at free amino groups on the peptide. Analysis of the biotinylation mixture by reversed-phase HPLC revealed several peaks representing various degrees of biotinylation, since both amino termini

TABLE 1. Association and dissociation rate constants of the interactions between SH2 domain-containing proteins and phosphopeptides Y751P (17-mer) and Y740P (12-mer)[a]

Peptide	Y740P			Y751P		
	k_{ass} (10^5 M^{-1} s^{-1})	k_{diss} (s^{-1})	K_d (M^{-1})	k_{ass} (10^5 M^{-1} s^{-1})	k_{diss} (s^{-1})	K_d (M^{-1})
p85	19.3 ± 5.8	0.100 ± 0.003	1.93×10^7	92.4 ± 2.7	0.127 ± 0.006	7.28×10^7
p85 N-SH2	0.14 ± 0.04	0.095 ± 0.010	1.47×10^5	33.4 ± 2.0	0.141 ± 0.006	2.37×10^7
p85 C-SH2	15.9 ± 4.3	0.102 ± 0.026	1.56×10^7	16.9 ± 3.3	0.098 ± 0.004	1.72×10^7
PLC C-SH2	1.16 ± 0.03	0.045 ± 0.006	2.58×10^6	16.4 ± 3.4	0.049 ± 0.009	3.35×10^7
PLC N + C	1.51 ± 0.36	0.034 ± 0.002	4.44×10^6	12.0 ± 3.3	0.045 ± 0.001	2.67×10^7
GAP N-SH2	2.06 ± 0.94	0.039 ± 0.008	5.28×10^6	0.40 ± 0.05	0.054 ± 0.007	7.41×10^5

[a] The k_{diss} values were measured in the presence of 20 μM competing nonbiotinylated phosphopeptide. The equilibrium dissociation constants (K_d) were obtained by dividing k_{ass} by k_{diss}.

and lysine groups could be labeled. Mass analysis was used to identify the number of biotin molecules attached, and the peak corresponding to one biotin molecule per peptide was used for subsequent interactions.

No evidence for nonspecific binding was observed for all the interactions tested in this work. In experiments with biotinylated, nonphosphorylated peptides, there was no detectable binding of any of the SH2 domain-containing proteins at concentrations of up to 3 μM (Fig. 1B and data not shown). This result confirms the absolute necessity for the presence of phosphotyrosine in order for this interaction to occur. Furthermore, it demonstrates the very hydrophilic nature of the matrix on which the interactions take place, which results in undetectable nonspecific binding.

Measurement of kinetic parameters. Association and dissociation rate constants were calculated by repetitive injections of increasing concentrations of protein over immobilized phosphopeptides, as described in Materials and Methods (equation 4) (Fig. 1A). *dR/dt* values were obtained after the initial fast change in the bulk solution refractive index, observed immediately after the protein injection, and continued until the interaction approached steady-state binding. During this time period, the *dR/dt*-versus-*R* plots were linear (Fig. 1C), indicating first-order kinetics and no mass transport limitations, allowing accurate estimation of association rate constants (Fig. 1D and Table 1). A recent report has also used the BIAcore biosensor to study the interaction of GST-SH2 domains with phosphopeptides from the IRS1 insulin receptor substrate (11). In this case, the peptides were coupled directly to the biosensor surface. The association curves indicated mass transport limited kinetics, which did not allow the direct measurement of k_{ass} values. These were inferred from Scatchard analysis of steady-state binding values and direct measurements of the dissociation rates (11). We have found that the K_d values obtained from steady-state binding values correlate roughly with those calculated by direct measurement of k_{ass} and k_{diss} but are not as accurate or reproducible.

The values obtained for k_{diss} from equation 4 were often unreliable, as reported previously (20), presumably because of the very high association rate, which would make the $k_{ass} \times C$ values dominate over the k_{diss} term. It was therefore attempted to measure k_{diss} values from the dissociation of bound protein in buffer flow. However, none of the interactions studied in this report appeared to involve first-order kinetics when their dissociation rates were measured in this way. By nonlinear regression analysis, two components could be resolved, which in most cases gave dissociation rate constant values on the order of 10^{-2} s^{-1} and 10^{-4} s^{-1}, respectively. There could be several explanations for this observation, including true second-order kinetics, negative cooperativity effects, or protein rebinding to the immobilized capturing molecule. Although the measurements were done in free buffer flow, this may not necessarily fulfil the "infinite dilution" prerequisite for measurement of dissociation rates (30), especially since the observed association rates were high.

We therefore repeated the dissociation measurements after injection of an excess of phosphorylated peptide (nonbiotinylated) in order to prevent rebinding. Our initial attempts were compromised, since approximately 60 to 100 s elapse between the end of the protein injection and the beginning of the peptide injection, and a high proportion of the bound material dissociates during this time. A modification of the BIAcore software was therefore used to allow the competing peptide to be injected from a second loop in the microfluidic cartridge immediately after the end of the protein injection. As shown in Fig. 2A, increasing amounts of competing phosphopeptide had a dramatic effect on the dissociation rate, with up to 90% of the bound protein dissociating within 10 to 20 s. During this period, first-order kinetics were apparent (correlation coefficient, $R > 0.97$). The k_{diss} values calculated in this way were approximately 10-fold higher than the faster of the rates obtained in buffer flow. Moreover, they were similar for all domains tested, indicating that it is mainly the association rate which determines the affinity of these interactions (Table 1). Similar very fast dissociation rates have been reported for the interaction of SH2 domains with IRS1 phosphopeptides (11).

Using gel filtration, we have found that all GST fusion proteins are dimeric (data not shown). As this could artificially decrease dissociation rates, we tested the binding of a heterodimeric GST–GST-SH2 domain to Y751P. As shown in Fig. 2B and C, there was no significant difference in either the association or the dissociation rates compared with homodimers, in the presence or absence of competing phosphopeptide. This would suggest that the homodimers adopt a conformation that, in our experimental system, does not allow the simultaneous binding of the two domains within a dimer to immobilized peptides. Although only the p85 N-SH2 was tested in this way, it is reasonable to assume that, because of their highly conserved structure, all SH2 domains would behave in the same way.

The observation of high association rates and fast dissociation rates has interesting implications for the regulation of interactions involving SH2 domains and receptor autophosphorylation sites. Rapid on/off interactions would allow other phosphorylation sites, for example, within the SH2

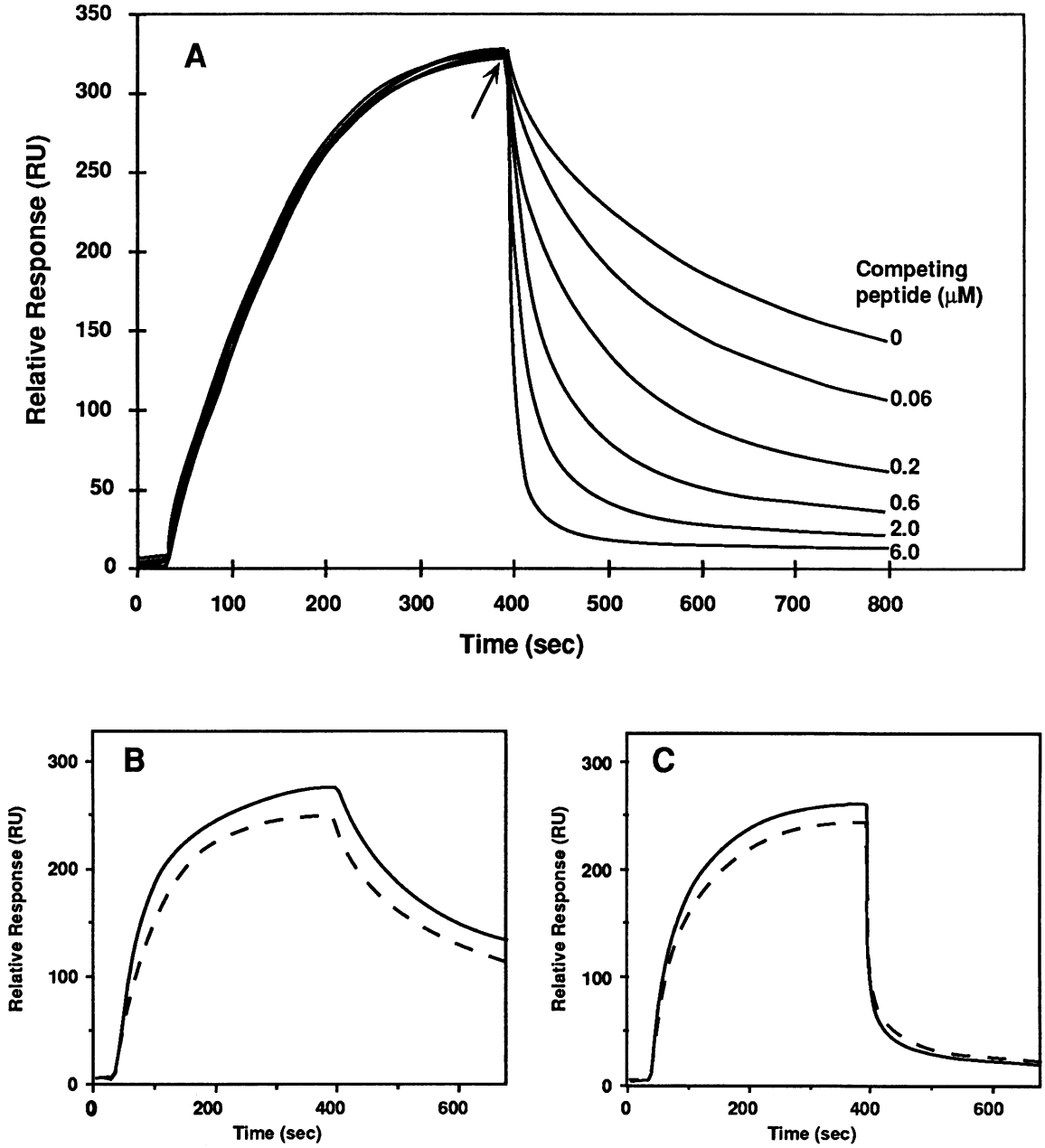

FIG. 2. (A) Dissociation of p85α N-SH2 domain from immobilized phosphopeptide Y751P in the presence of increasing concentrations of nonbiotinylated phosphopeptide. The arrow indicates the end of the SH2 domain injection and beginning of the peptide injection. (B and C) Comparison of the binding of GST-SH2 homodimers (continuous lines) and GST–GST-SH2 heterodimers (broken lines) to immobilized Y751P peptide. Dissociation was done in buffer flow (B) or in the presence of competing phosphopeptide (C).

domain-containing molecules themselves, to interact with the SH2 domains in both intra- and intermolecular ways and thus allow interactions with downstream molecules, deactivation, or other regulatory effects.

Comparison of the binding of the two SH2 domains of p85α to phosphopeptides Y751P and Y740P. Recent site-directed mutagenesis studies have clearly established that both Tyr-751 and Tyr-740 in the kinase insert region of the human PDGF β-receptor contribute to its association with the PI 3-kinase, and a model for this interaction in which the two SH2 domains of p85α each interact with one of these phosphorylation sites, resulting in a high-affinity complex, has been proposed (21). Indeed, mutation of either tyrosine to a phenylalanine reduces the amount of PI 3-kinase bound to the PDGF β-receptor, whereas mutation of both abolishes binding. The availability of both SH2 domains as recombinant fusion proteins and of both the Y751P and Y740P phosphopeptides prompted us to test this model and, in particular, to investigate whether the SH2 domains display a specificity for one or the other of the phosphorylation sites. The association rate constants for the interaction of the N- and C-SH2 domains with the two phosphopeptides were measured with the BIAcore. As shown in Fig. 3, while the C-SH2 did not display any specificity for the two peptides

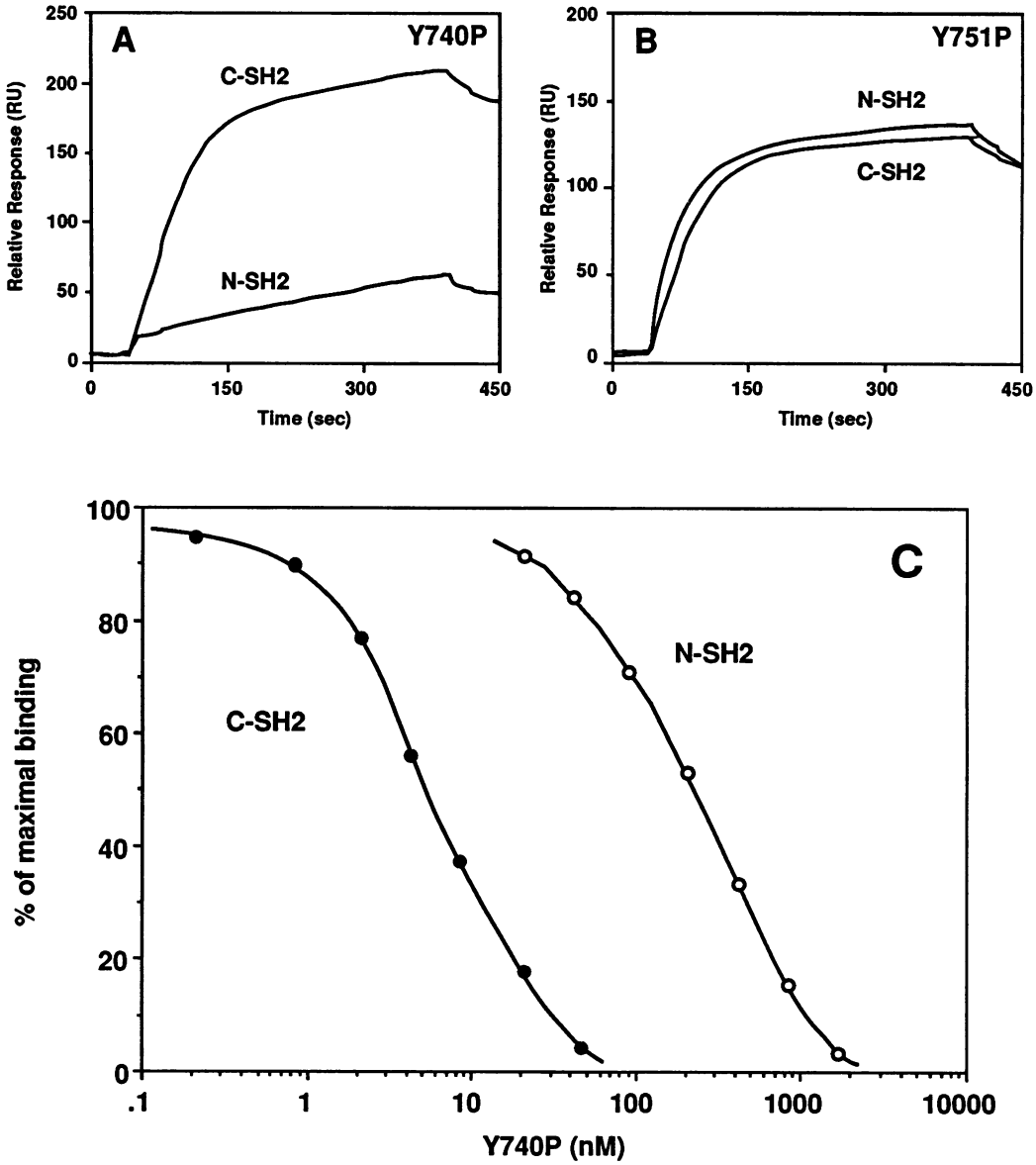

FIG. 3. (A and B) Comparison of the binding of equal concentrations of p85α N-SH2 and C-SH2 on phosphopeptides 12-mer Y740P (A) and 17-mer Y751P (B). (C) Inhibition of SH2 domain binding to immobilized Y751P (17-mer) by increasing concentrations of Y740P (17-mer). Steady-state binding values were measured for the two SH2 domains and expressed as a percentage of the maximal response, which was obtained in the absence of competing peptide.

and interacted with both with a high association rate, the N-SH2 bound with approximately 100-fold-higher affinity to the Y751P than to the Y740P phosphopeptide (Table 1). The apparent k_{diss} values were similar for all the combinations of SH2 domain and phosphopeptide tested, suggesting that the overall affinity reflected the differences in the association rates.

In order to make sure that the differences observed were not due to the different lengths of the peptides compared (17-mer Y751P versus 12-mer Y740P), we synthesized a 17-mer Y740P peptide with the phosphotyrosine spaced from both termini in the same way as in the 17-mer Y751P. Biotinylation of this peptide resulted in the addition of a biotin molecule not only at the N terminus but also at a lysine residue located 5 amino acids downstream from the phosphotyrosine (as determined by mass analysis; data not shown), resulting in loss of the peptide's binding capacity. We therefore used the nonbiotinylated peptide in a competition assay, in which the N- and C-SH2 domains of p85 were injected over immobilized Y751P in the presence of increasing concentrations of 17-mer Y740P. As shown in Fig. 3C, Y740P was effective in inhibiting binding of the C-SH2 at much lower concentrations than required to inhibit binding of the N-SH2. This result shows that the low affinity of the N-SH2 for Y740P is intrinsic to the peptide sequence. Moreover, it is an independent confirmation of the differences observed by direct measurement of the kinetic constants. These data suggest that the high-affinity interaction of the intact p85 protein with the autophosphorylated PDGF β-receptor may be mediated by the binding of the N-terminal

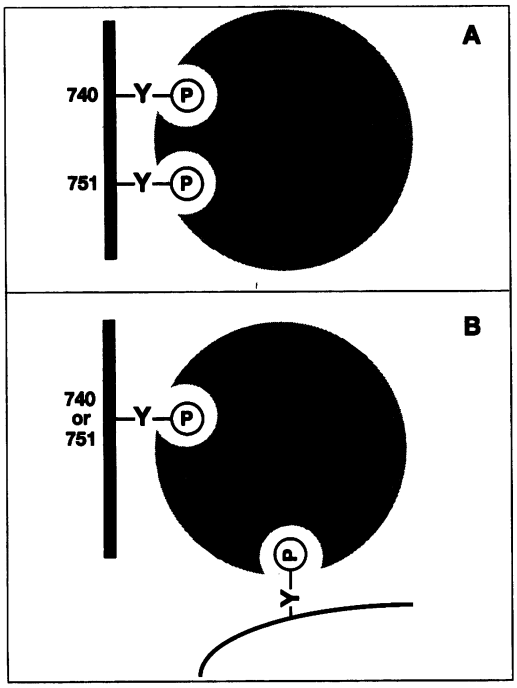

FIG. 4. Two possible models for the interaction of p85α with the PDGF β-receptor. (A) High-affinity binding is mediated by the interaction of the N-SH2 with Y751 and of the C-SH2 with Y740. (B) Binding to either Y740 or Y751 is mediated by the C-SH2, whereas the N-SH2 interacts with tyrosine-phosphorylated sequences on other proteins or the p85α itself.

SH2 to the Tyr-751 site and the C-terminal SH2 to the Tyr-740 site (see model in Fig. 4A). If this model is correct, we should expect p85α to have higher affinity towards a peptide containing both phosphorylated tyrosines. We are trying to overcome the difficulties in synthesizing such a peptide in order to address this question.

It is interesting in this respect that Klippel et al. (26) have compared the binding of the N- and C-terminal SH2 domains of the murine p85 to intact, phosphorylated PDGF receptor immobilized as an immunoprecipitate on Sepharose beads and have reported that the C-SH2 binds with approximately 100-fold-higher affinity than the N-SH2. This finding, as well as the ability of the C-SH2 but not the N-SH2 to completely block the interaction of p85 with the PDGF receptor, may be consistent with our results, since the C-SH2 binds with high affinity to both phosphorylated sites. Although the simplest possible model interpreting our data would be the coordinate binding of the two p85 SH2 domains to the two autophosphorylation sites (Fig. 4A), other models can also be put forward, including, as suggested recently (22), the possibility that the N-SH2 of p85 interacts with phosphotyrosines either on p85 itself (resulting from phosphorylation by the associated receptor) or on other molecules (Fig. 4B). A potential site, just C-terminal to the N-SH2, has been identified recently (39) by being similar in sequence to the residues surrounding Tyr-751. It has not been determined yet, however, whether this site indeed becomes phosphorylated.

When the binding of the intact p85α protein to the two peptides was compared, a four- to fivefold-higher association rate was observed for Y751P. Although this would be consistent with the fact that both SH2 domains interact with this peptide, it is not clear whether one p85α molecule can associate at the same time with two peptide molecules immobilized on the sensor surface. In fact, if that were the case, we would have expected to see a larger difference in the dissociation rates, which in fact were very similar.

The p85 N-SH2 fusion protein used in these experiments contains amino acid extensions N- and C-terminal to the SH2 domain proper, as defined by sequence similarity. We have also constructed a fusion protein that corresponds to the exact borders of the SH2 domain (27). This molecule displayed approximately a 10-fold-reduced affinity for the 17-mer peptide Y751P (k_{ass} = 1.5 × 10^5 M^{-1} s^{-1}; k_{diss} = 0.113 s^{-1}), indicating that the N- and/or C-terminal extensions are necessary for correct folding and the display of high-affinity binding.

An interesting observation was made regarding the binding characteristics of the C-terminal SH2 domain of p85α. Initial experiments were done in buffer without DTT, and it was found that, contrary to the N-SH2 as well as all the other SH2 domains, the C-SH2 did not display binding to any of the phosphopeptides tested. Upon addition of DTT, however, the binding capacity of the C-SH2 domain was restored, and the high association rates shown in Table 1 were observed. No cysteines are present in the N-SH2, whereas three are found in the C-SH2. Interestingly, they are present C-terminal to the highly conserved motif FLVR, which has been shown to be important in phosphotyrosine binding (33). It is highly probable that, during expression in bacteria, a disulfide bond is formed close to the binding site, resulting in the loss of the SH2 domain's ability to recognize phosphotyrosine.

Binding of GAP and PLCγ SH2 domains. Although the binding sites for GAP and PLCγ on the PDGF β-receptor are distinct from those for PI 3-kinase, we decided to test the binding of SH2 domains from these molecules to Y751P and Y740P in order to establish whether they retain a specificity for binding. The N-SH2 domain of GAP displayed a measurable affinity for Y740P (k_{ass} = 2 × 10^5 M^{-1} s^{-1}), 10-fold lower than seen with p85 and its N-SH2. The affinity for Y751P was even lower (k_{ass} = 4 × 10^4 M^{-1} s^{-1}). It seems, therefore, that neither of these two phosphotyrosines would be expected to act as a high-affinity site for GAP binding to the PDGF β-receptor, as has been demonstrated previously by peptide competition experiments (10). It is worth noting, however, that one study has shown that mutation of Tyr-751 to Phe resulted in a decrease, but not abolishment, of in vivo GAP association to the PDGF β-receptor, suggesting the possibility of an additional interaction through this site (25). It should be expected that the GAP SH2 domain will display a higher affinity (comparable to that of the p85α SH2 domains) for a phosphopeptide corresponding to the sequence surrounding Tyr-771 of the PDGF receptor. These experiments are in progress.

The N-terminal SH2 domain of PLCγ did not display any measurable binding to any of the peptides tested. It has previously been shown that this domain is able to form a tight complex with the intact PDGF β-receptor (1). It appears, therefore, that a high degree of specificity may be displayed by this domain, which would be expected to bind with high affinity to a phosphopeptide corresponding to the PLCγ binding site. In contrast, the C-terminal SH2 of PLCγ, as well as a fragment containing both the N- and C-SH2 domains, bound with high affinity to Y751P and with an approximately 10-fold-reduced affinity to Y740P. It is not clear what the significance of this finding is for PLCγ binding. One possibility is that specificity is provided by the N-terminal SH2, whereas the C-terminal SH2 is used for

interactions with other molecules or with phosphorylation sites on PLCγ itself. Alternatively, it could be argued that the C-SH2 of PLCγ loses its specificity when expressed as a recombinant domain.

Binding to short and long Y751P peptides. We compared the binding of SH2 domains to two different peptides surrounding Tyr-751, the 17-mer peptide mentioned above and a shorter, 9-mer peptide. It was found that the affinity of all SH2 domains was much lower for binding to the short peptide than to the long one. This was particularly noted for the intact p85 and its N-SH2, whose affinity could not be determined accurately ($k_{ass} << 1 \times 10^4\ M^{-1}\ s^{-1}$), although binding could be observed at high concentrations. The association rates of the C-terminal SH2 domains of p85α and PLCγ were reduced approximately 10-fold and were still measurable (approx. $10^5\ M^{-1}\ s^{-1}$). There are two possible interpretations for the observed differences between the two peptides. One is that the extra sequence is required for correct recognition and high-affinity binding; although the minimal requirement for binding has been shown to be 4 amino acids after the phosphotyrosine (7, 10), these experiments have not been quantitative, and so it is not clear whether high-affinity binding requires more residues. An alternative explanation is that the shorter peptide, having its N terminus closer to the phosphotyrosine, is not properly presented at the biosensor surface, i.e., the spacer arm provided by these N-terminal amino acids and the attached biotin is not long enough to expose the peptide at a suitable distance from the matrix-bound avidin. This would suggest that our solid-phase assay, which requires immobilization of the peptide, is only suitable for relatively long peptides.

Our results indicate that by using a quantitative method for the measurement of kinetic parameters, a better understanding of interactions involving SH2 domains and receptor autophosphorylation sites can be obtained. In this study, it has been demonstrated that recombinant SH2 domains and tyrosine-phosphorylated peptides from the PDGF β-receptor may retain the binding specificity of intact proteins and therefore provide a model for the assembly of receptor complexes.

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