PTB Domain Binding to Signaling Proteins Through a Sequence Motif Containing Phosphotyrosine

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Src homology 2 (SH2) domains mediate assembly of signaling complexes by binding specifically to tyrosine-phosphorylated proteins. A phosphotyrosine binding (PTB) domain has been identified which also binds specifically to tyrosine-phosphorylated targets, but is structurally different from SH2 domains. Expression cloning was used to identify targets of PTB domains. PTB domains bound to phosphotyrosine within a sequence motif, asparagine-X_1-X_2-phosphotyrosine (where X represents any amino acid), that is found in many signaling proteins and is not recognized by SH2 domains. Mutational studies indicated that high affinity binding of PTB domains may require a specific conformation of the motif.

Growth factors and oncogenes activate tyrosine kinases and generate tyrosine-phosphorylated proteins during signaling. SH2 domains bind specifically to phosphotyrosine and adjacent residues and thereby mediate assembly of tyrosine-phosphorylated proteins into signaling complexes (*1–6*). The PTB domain was originally identified as a 186-residue segment of the signaling protein Shc, which binds specifically to the tyrosine-phosphorylated form of an unidentified 145-kD protein in response to many growth factors, but is structurally dissimilar to members of the SH2 domain family (*7*). To determine the targets to which PTB domains bind, we needed a method of screening a library of tyrosine phosphorylated proteins. Standard methods were used to express proteins from a bacteriophage λ gt11 complementary DNA (cDNA) library and immobilize them on filters (*8*). The proteins on filters were then phosphorylated in vitro with recombinant platelet-derived growth factor (PDGF) receptor tyrosine kinase, washed, and incubated with ^{32}P-labeled PTB domain protein derived from Shc as a probe (*9*). The probe was detected by autoradiography. A clone was identified that bound the PTB domain probe only when subjected to phosphorylation conditions before hybridization (*10*). In principle, modifications of this method with other kinases might be generally applicable to expression cloning of proteins involved in phosphorylation-dependent protein-protein interactions.

The clone identified corresponded to amino acids 1086 to 1255 of c-ErbB2 or c-neu, a receptor tyrosine kinase proto-oncogene product. This region of c-ErbB2 contains seven tyrosines, five of which are autophosphorylation sites (*11–13*). To verify that the PTB domain bound to c-ErbB2 that had been autophosphorylated in vivo, PTB domain was incubated with lysate from SKBR3 human breast carcinoma cells, which contain overexpressed and autophosphorylated c-ErbB2. The ErbB2 protein from these cells specifically associated with a glutathionine-S-transferase (GST)–PTB domain fusion protein, but not with a GST fusion protein containing residues 1 to 45 of Shc, which lie outside of the PTB domain (*7*) (Fig. 1A). Dephosphorylation of the c-ErbB2 from SKBR3 cells with tyrosine-specific phosphatases completely eliminated binding to the PTB domain (*10*). Therefore, the PTB domain appeared to specifically associate in vitro with the tyrosine-phosphorylated form of c-ErbB2. The c-ErbB2 protein also associated with Shc in vivo (Fig. 1A) through a mechanism that required phosphorylation of c-ErbB2 at the five autophosphorylation sites (*14*). Therefore, c-ErbB2 is a candidate for a target of the PTB domain in vivo.

Peptides derived from the c-ErbB2 sequence were synthesized, and phosphotyrosine was substituted for each of the seven tyrosines mentioned above. The peptides were tested for their ability to compete with the PTB domain for binding to c-ErbB2 from SKBR3 lysates (Table 1). Peptides pY1221-pY1222, pY1196, and pY1248 blocked PTB domain binding to c-ErbB2. The amounts required to inhibit binding by 50% (IC_{50}'s) were 50 nM, 1 μM, and 1 μM, respectively (Fig. 1B). Phosphopeptides pY1112, pY1127, and pY1139 were ineffec-

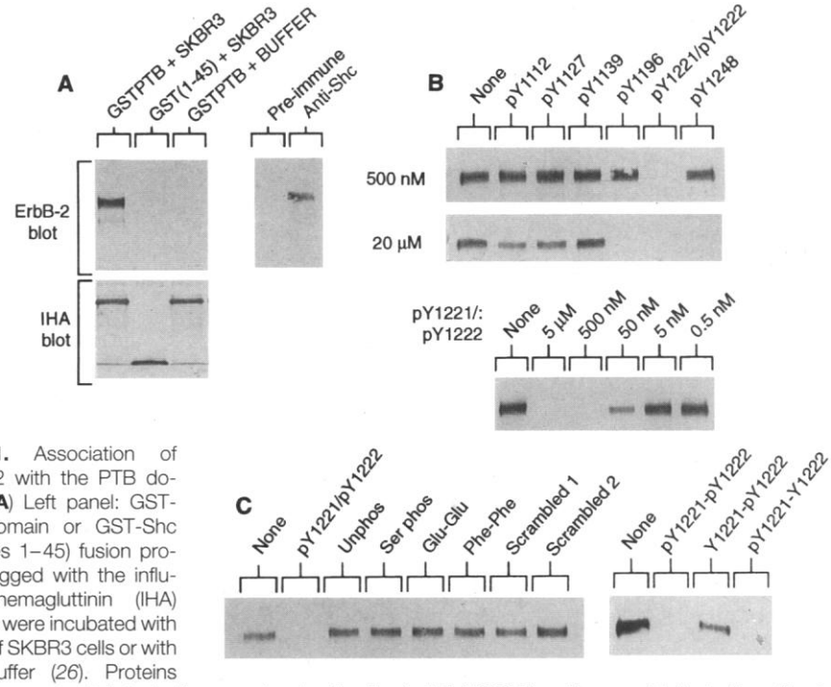

Fig. 1. Association of c-ErbB2 with the PTB domain. (**A**) Left panel: GST-PTB domain or GST-Shc (residues 1–45) fusion proteins tagged with the influenza hemagluttinin (IHA) epitope were incubated with lysate of SKBR3 cells or with lysis buffer (*26*). Proteins were immunoprecipitated with monoclonal antibodies to IHA (12CA5) and immunoblotted with antibodies to c-ErbB2 (top panel) or with 12CA5 (bottom panel). Right panel: Proteins from lysate of SKBR3 cells were immunoprecipitated with pre-immune serum or antiserum to Shc (anti-Shc) and immunoblotted with antibodies to c-ErbB2. (**B** and **C**) IHA-tagged GST-PTB domain was incubated with SKBR3 lysate as in (A) in the presence or absence of the indicated peptides, immunoprecipitated with 12CA5, and immunoblotted with antibodies to c-ErbB2. Equal amounts of GST-PTB domain protein were immunoprecipitated as determined by immunoblotting with 12CA5 (*10*). Peptide sequences are given in Table 1. Peptides (500 nM or 20 μM) were used as indicated in (B), upper panel. The indicated concentrations of pY1221-pY1222 were tested in (B), lower panel. Concentration of peptides in (C) was 300 nM.

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tive. Unphosphorylated peptides or peptides in which phosphotyrosine was substituted with phosphoserine or glutamic acid did not compete for binding (Fig. 1C). Phosphorylated peptide pY1221-pY1222 that had been dephosphorylated with tyrosine-specific phosphatases also was ineffective (*10*). Thus, the PTB domain specifically recognized phosphotyrosine.

Because not all phosphotyrosine-containing peptides were recognized by the PTB domain, the presence of phosphotyrosine alone was not the only determinant of effective competition. The PTB domain appeared to bind preferentially to phosphotyrosine at position 1222 (Fig. 1C and Table 1). Scrambled peptides that contained phosphotyrosines within a rearranged primary sequence failed to compete for binding (Fig. 1C). These data demonstrated that binding requires not only phosphotyrosine, but also the presence of a specific amino acid sequence.

To determine which residues in peptide Y1221-pY1222 were important for binding to the PTB domain, a series of peptides containing point mutations were tested in this assay (Table 2). The most important residue for binding to PTB domain was Asn^{1219}; even conservative substitutions of this residue completely eliminated the ability of the peptide to compete for binding to PTB domain. Replacement of Asp^{1218} impaired peptide binding activity, although this residue was not absolutely required for competition. Replacement of Trp^{1223} with Phe was tolerated, although an Ala substitution was not. This suggested that large hydrophobic or aromatic residues at this position confer higher affinity. Although mutation of other residues had smaller effects on activity, all mutations caused some loss of apparent affinity.

To demonstrate directly that the phosphopeptides bind to the PTB domain, biotinylated peptides were incubated with the PTB domain, and the PTB domain was immunoprecipitated. The presence of bound peptide was detected with streptavidin-coupled alkaline phosphatase. The PTB domain bound directly to phosphorylated peptide pY1221-pY1222, but not to the unphosphorylated peptide (Fig. 2). The PTB domain did not bind to phosphorylated peptides containing conservative point mutations at Asn^{1219}. The SH2 domain of Shc did not bind phosphorylated peptide pY1221-pY1222.

The in vivo Shc binding sites on the nerve growth factor receptor (Trk), polyoma middle T antigen, ErbB3, and the epidermal growth factor (EGF) receptor contain N-P-X-phosphotyrosine motifs (where N represents asparagine, P represents proline, and X represents any amino acid) (Table 1) (*15–18*). However, the sequences surrounding N-P-X-Y in these proteins are poor targets for the SH2 domain of Shc (*19*), especially considering that the specificity of SH2 domain binding sites is determined primarily by residues COOH-terminal to the phosphotyrosine (*3, 5, 6, 19–22*). Our data indicate that these residues constitute a recognition sequence for the PTB domain of Shc and that the Shc SH2 domain does not bind to the N-X_1-X_2-pY motif (Fig. 2). This interaction appears to be important because mutation of the N-P-X-pY motif in middle T antigen, yields a molecule that no longer binds Shc in vivo and fails to cause transformation (*15*). Similarly, the N-X_1-X_2-pY motifs in

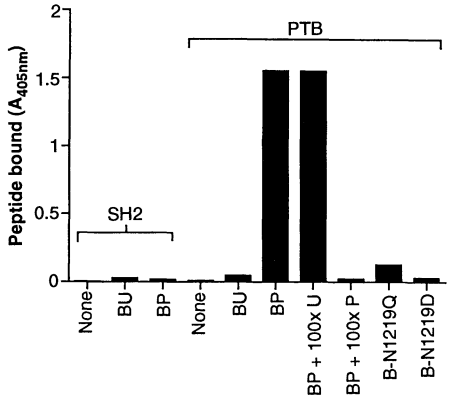

Fig. 2. Direct binding of phosphopeptides to the PTB domain. IHA-tagged PTB domain was incubated with biotinylated pY1221-pY1222 (BP), unphosphorylated peptide (BU), or pY1221-pY1222 containing mutations at Asn1219 (B-N1219Q and B-N1219D). The PTB domain was immunoprecipitated with antibodies to IHA, washed, and assayed for bound peptide with streptavidin-coupled alkaline phosphatase (*27*). Nonbiotinylated peptides (either phosphorylated, P, or not, U) were used at 100 times the concentration of the biotinylated peptides as a competitor to demonstrate specific binding. A representative experiment is shown.

Table 1. Competition by peptides for c-ErbB2 binding to the PTB domain. The numbers in the peptide names refer to tyrosines in the c-ErbB2 sequence. Phosphotyrosine and phosphoserine, are denoted pY and pS, respectively and shown in bold type. Approximate amounts of peptide required to inhibit binding of cErbB2 to the PTB domain by 50% (IC_{50}), if determined, are shown. Shc binding sites for middle T antigen, Trk, EGF receptor, and erbB3 are included for comparison. The asparagine (N) residue required for PTB binding is shown in bold type. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Peptide name	Sequence	IC_{50}
pY1221-pY1222	PAFSPAFD**N**L (**pY**) (**pY**) WDQNSSEQG	50nM
Y1221-pY1222	AFD**N**LY (**pY**) WDQNS	30nM
pY1196	AFGGAVE**N**PE (**pY**) LAPRAGTASQ	1μM
pY1248	EGTPTAE**N**PE (**pY**) LGLDVPV	1μM
pY1139	APLACSPQPE (**pY**) VNQPEVRPQS	>100μM
pY1112	SPHDLSPLQR (**pY**) SEDPTLPL	100μM
pY1127	TLPLPPETDG (**pY**) VAPLACSPQ	>100μM
Middle T Ag	LLS**N**PT (**pY**) SVMR	
ErbB3	AFD**N**PD (**pY**) WHSRLF	
Trk	IE**N**PQ (**pY**) FSDA	
EGF receptor	SLD**N**PD (**pY**) QQDFF	
Consensus	xxx**N**xx (**pY**) xxxxx	
Unphos	PAFSPAFD**N**L**YY**WDQNSSEQG	>30μM
Ser phos	PAFSPAFD**N**L (**pS**) (**pS**) WDQNSSEQG	>30μM
Glu-Glu	PAFSPAFD**N**L**EE**WDQNSSEQG	
Phe-Phe	PAFSPAFD**N**L**FF**WDQNSSEQG	
pY1221-Y1222	AFD**N**L (**pY**) YWDQNS	1μM
Scrambled 1	DSWDQNQLFS (**pY**) (**pY**) SFAPEGPAN	>30μM
Scrambled 2	DSW (**pY**) SQNQLFDSFAPEG (**pY**) PAN	

Table 2. Effect of single amino acid substitutions on binding of phosphopeptides to the PTB domain. Peptides were synthesized in which the indicated amino acid of peptide Y1221-pY1222 (ErbB2 residue) was replaced with the indicated amino acid (substitution). Peptides were tested for ability to inhibit binding of PTB domain to c-ErbB2 as described (Fig. 1). Residues are numbered according to their position in c-ErbB2. Inhibition of binding is quantified as +++ (IC_{50}, 50 to 500 nM), ++ (IC_{50}, 500 nm to 5 μM), + (IC_{50}, 5 μM to 50 μM), and − (IC_{50} > 50 μM).

ErbB2 residue	Substitution	Inhibition
F1217	A	++
D1218	A	+
	S	+
N1219	A	−
	Q	−
	D	−
L1220	A	++
Y1221	A	++
	F	++
W1223	A	−
	F	++
D1224	A	+++
Q1225	A	++
	N	++
	D	++
N1226	A	++
S1227	A	++

ErbB2 that bind the PTB domain in vitro are autophosphorylation sites in vivo and are involved in ErbB2-mediated transformation (*11–13*, *23*).

Our data indicate that PTB domains recognize tyrosine-phosphorylated targets through a different mechanism than do SH2 domains. PTB domains bind to phosphotyrosine within a motif containing an essential asparagine NH_2-terminal to the phosphotyrosine, whereas SH2 domains recognize residues COOH-terminal to the phosphotyrosine (*3*, *5*, *6*, *19–22*). The SH2 and PTB domains of Shc do not recognize the same sequence motifs or phosphorylated proteins (Fig. 2) (*7*). Further, the apparent affinity of the Shc PTB domain for peptide pY1221-pY1222 is as much as 100 times greater than that of a typical SH2 domain–peptide interaction in similar assays (*6*). Finally, multiple residues on both sides of the phosphotyrosine in the PTB domain binding site contribute to overall affinity. Thus, unlike SH2 domains, the PTB domain may make physical contacts with many residues in the phosphorylated peptide, or a specific conformation adopted by the phosphorylated peptide may contribute to binding. The former possibility is unlikely, given the variability of these residues outside the N-X_1-X_2-pY consensus in peptides that bind the PTB domain (Table 1). N-P-X-Y motifs adopt tight reverse-turn conformations which are dependent on the asparagine and tyrosine residues for structural stability and which are important for the function of these motifs as internalization signals (*24*, *25*). The asparagine and tyrosine side chains are closely juxtaposed in these structures. Thus, PTB domain binding to tyrosine-phosphorylated targets appears to require the motif N-X_1-X_2-pY within a specific secondary structure and to provide a mechanism for regulated protein-protein interactions during signaling by growth factors and oncogenes.

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9. Sf9 cells expressing residues 526 to 1067 of the mouse PDGF receptor cytoplasmic domain encoded in a recombinant baculovirus were prepared and lysed as described (*7*). [M. D. Summers, and G. E. Smith, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures* (Texas Agricultural Station, College Station, TX, 1987)]. Plaques (1.1×10^6) of an oligo(dT)–primed Balb/c 3T3 fibroblast cDNA λ gt11 library were plated and transferred to isopropyl-β-D-thiogalactopyranoside (IPTG)–impregnated polyvinylidene difluoride (PVDF) filters using standard techniques (*8*). The filters were incubated in TBSTM [20 mM tris-HCl (pH 7.4), 137 mM NaCl, 10 mM $MgCl_2$ and 0.1% Triton X-100] containing bovine serum albumin (5%) and then incubated in TBSTM containing one-fifth volume of lysate from cells expressing the PDGF receptor cytoplasmic domain, 250 μM adenosine triphosphate, and 1 mM sodium orthovanadate at room temperature for 30 min. The filters were washed and incubated with ^{32}P-labeled GST-PTB domain fusion protein as described (*7*).
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26. Influenza hemagluttinin–tagged GST fusion proteins were expressed from recombinant baculovirus in Sf9 cells (*7*). Sf9 cells or confluent SKBR3 cells were lysed in 2× hybridization buffer (*7*) containing protease inhibitors and 1 mM sodium orthovanadate. Approximately 100 ng of GST-PTB domain was incubated with 1 μg of total SKBR3 lysate protein in 1× hybridization buffer for 30 min at 4°C. Proteins were immunoprecipitated with 12CA5 (2 μg) and protein A–Sepharose, and the sedimented material was washed three to five times before immunoblot analysis with antibodies to c-Neu. The PTB domain was incubated with the indicated concentrations of peptide for 30 min at 4°C before it was added to the SKBR3 cell lysate. In experiments involving serine-phosphorylated peptides, 1 μM okadaic acid and 1 mM EGTA were included in the buffers. Peptides were synthesized as described (*6*) and purified by high-performance liquid chromatography (HPLC).
27. Peptides were biotinylated during synthesis and purified by HPLC. GST-PTB domain or GST-Shc SH2 domain (*7*) proteins (100 ng) were incubated in 1× hybridization buffer with 500 nM biotinylated peptide for 1 hour at 4°C, immunoprecipitated as in Fig. 1, and washed once. The sedimented material was incubated with 0.25 units of streptavidin–alkaline phosphatase for 5 min at 4°C. The sedimented material was washed twice more, incubated for 3 min at room temperature with *p*-nitrophenol phosphate (1 mg/ml) in 100 mM glycine (pH 10.1), 1 mM $ZnCl_2$, and 1 mM $MgCl_2$, and absorbance at 405 nm was measured.

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Inducible Gene Expression in Trypanosomes Mediated by a Prokaryotic Repressor

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An inducible expression system was developed for the protozoan parasite *Trypanosoma brucei*. Transgenic trypanosomes expressing the tetracycline repressor of *Escherichia coli* exhibited inducer (tetracycline)-dependent expression of chromosomally integrated reporter genes under the control of a procyclic acidic repetitive protein (PARP) promoter bearing a *tet* operator. Reporter expression could be controlled over a range of four orders of magnitude in response to tetracycline concentration, a degree of regulation that exceeds those exhibited by other eukaryotic repression-based systems. The tet repressor–controlled PARP promoter should be a valuable tool for the study of trypanosome biochemistry, pathogenicity, and cell and molecular biology.

The medical importance of *Trypanosoma brucei* and its numerous departures from higher eukaryotic strategies for genomic organization and regulation [reviewed in (*1*)] have established it as a singular model system among the lower eukaryotes. The unorthodox biology of this parasite has both increased the intrinsic interest of the group and suggested potential targets for chemotherapy. The ability to gain insight from the evolutionary perspective that this system offers on higher eukaryotic biology, or to evaluate candidate drug targets, depends critically on an ability to address functional questions about in vivo roles of specific gene products. Whereas DNA-mediated stable transformation of trypanosomatids was accomplished several years ago (*2*), the study of genes essential for parasite growth has remained problematic in the absence of a means to control gene expression exogenously.

We report the development of an inducible expression system for *T. brucei* that

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