Physical and Functional Interactions between SH2 and SH3 Domains of the Src Family Protein Tyrosine Kinase p59^{fyn}

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The Src family protein tyrosine kinases participate in signalling through cell surface receptors that lack intrinsic tyrosine kinase domains. All nine members of this family possess adjacent Src homology (SH2 and SH3) domains, both of which are essential for repression of the enzymatic activity. The repression is mediated by binding between the SH2 domain and a C-terminal phosphotyrosine, and the SH3 domain is required for this interaction. However, the biochemical basis of functional SH2-SH3 interaction is unclear. Here, we demonstrate that when the SH2 and SH3 domains of p59^{fyn} (Fyn) were present as adjacent domains in a single protein, binding of phosphotyrosyl peptides and proteins to the SH2 domain was enhanced, whereas binding of a subset of cellular polypeptide ligands to the SH3 domain was decreased. An interdomain communication was further revealed by occupancy with domain-specific peptide ligands: occupancy of the SH3 domain with a proline-rich peptide enhanced phosphotyrosine binding to the linked SH2 domain, and occupancy of the SH2 domain with phosphotyrosyl peptides enhanced binding of certain SH3-specific cellular polypeptides. Second, we demonstrate a direct binding between purified SH2 and SH3 domains of Fyn and Lck Src family kinases. Heterologous binding between SH2 and SH3 domains of closely related members of the Src family, namely, Fyn, Lck, and Src, was also observed. In contrast, Grb2, Crk, Abl, p85 phosphatidylinositol 3-kinase, and GTPase-activating protein SH2 domains showed lower or no binding to Fyn or Lck SH3 domains. SH2-SH3 binding did not require an intact phosphotyrosine binding pocket on the SH2 domain; however, perturbations of the SH2 domain induced by specific high-affinity phosphotyrosyl peptide binding abrogated binding of the SH3 domain. SH3-SH2 binding was observed in the presence of proline-rich peptides or when a point mutation (W119K) was introduced in the putative ligand-binding pouch of the Fyn SH3 domain, although these treatments completely abolished the binding to p85 phosphatidylinositol 3-kinase and other SH3-specific polypeptides. These biochemical SH2-SH3 interactions suggest novel mechanisms of regulating the enzymatic activity of Src kinases and their interactions with other proteins.

While the growth factor receptors possess intrinsic tyrosine kinase domains (74), a number of key cell surface receptors, including the lymphocyte antigen receptors, signal through noncovalently associated Src family tyrosine kinases (2, 13). For example, the unique N-terminal (NT) domain of p59^{fyn} (Fyn) physically interacts with the CD3 (ϵ and γ) and ζ chains of the T-cell antigen receptor (TCR) (61, 72) and components of other hematopoietic cell receptors (2). Similarly, the unique N terminus of p56^{lck} (Lck) interacts with CD4 and CD8 coreceptors (60, 76). The p60^{src} (Src) and Fyn also associate with activated receptor tyrosine kinases such as the platelet-derived growth factor (PDGF) receptor (34), and microinjection of anti-Src/Fyn antibodies or of DNA constructs encoding dominant-negative proteins reduced the mitogenic response of fibroblasts to PDGF (73).

A physiological role for the interaction of Fyn with components of the TCR was suggested by increased responsiveness of T cells to TCR engagement when Fyn was overexpressed and increased or decreased TCR signalling in thymocytes of trans-

genic mice expressing activated or dominant-negative Fyn, respectively (11, 14). Direct evidence for a role of Fyn was provided by gene disruption experiments, which resulted in defective signalling in mature thymocytes (1, 68). Use of an alternative copy of exon 7 results in a brain-specific form of Fyn (12). While the membrane receptors with which Fyn associates in the nervous system have just begun to be identified (75), Fyn^{-/-} knockout mice exhibit specific neurologic deficits such as impaired long-term potentiation, spatial learning, and hippocampal development (25), impaired ability to initiate suckling reflex (80), and defective myelination (75).

The interaction of Lck with the cytoplasmic tails of the CD4 and CD8 coreceptors is critical for CD4- and CD8-dependent enhancement of antigen stimulation in T cells (23, 60, 76). In addition, Lck plays a critical physiological role in early T-cell development, as demonstrated by expression of Lck transgenes and genetic knockout studies in mouse (41, 53).

All nine members of the Src family tyrosine kinases possess adjacent SH3 and SH2 domains in the same relative positions between the unique NT and conserved catalytic domains (Fig. 1A) (2, 12, 13, 63, 72). While the role of SH2 domains to bind phosphotyrosine (pY)-containing peptide motifs and to promote the assembly of activated signalling complexes is now well established (51), the functions of the SH3 domain are just beginning to be elucidated. The SH3 domains bind to proline-

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FIG. 1. Binding of TCR-induced phosphotyrosyl proteins to the Fyn SH2 domain and influence of the neighboring NT and SH3 domains on binding. (A) Schematic representation and nomenclature of GST fusion proteins in relation to full-length Fyn protein (top).

rich peptide sequences, suggesting that they too serve as protein-protein interaction modules (9, 59). Biochemical and genetic experiments have demonstrated that SH3 domains of the growth factor receptor-binding protein Grb2 bind to guanine nucleotide exchange protein Sos, thus coupling cell surface receptors to Ras signalling pathway (40). In addition, SH3 domains have been shown to play a role in binding between the components of other signalling pathways (20, 22, 24, 36, 55, 57, 71, 79). Nuclear magnetic resonance and crystal structural analyses of isolated SH2 (3, 18, 50, 78) and SH3 (4, 33, 43, 46, 81) domains have defined their respective ligand-binding surfaces, supporting the modular nature of these domains which was suggested by their independent existence in many proteins (51).

The SH3 and SH2 domains of Src family kinases are essential to maintain the repressed state of these enzymes, as deletion of either domain leads to constitutive activation of enzymatic activity and uncovers the transforming potential of these proto-oncogenes (2, 13, 27, 42, 48, 62, 70, 77). In addition, certain point mutations within the SH3 or SH2 domain can also activate the kinase activity and transforming ability (15, 47). Notably, Lck protein with an SH3 domain deletion was unable to mediate antigen-specific triggering in T cells, despite enhanced tyrosine kinase activity (5, 77). An activated phenotype is also induced in Src family kinases by mutation or deletion of the conserved pY located near the C terminus, and it is now widely accepted that negative regulation requires its binding to SH2 domain (2, 13, 28, 35, 42, 45, 48, 70). The mechanism of negative regulation by the SH3 domain is unclear. However, recent studies show that the SH3 domain of c-Src is required for the negative regulatory interaction between the SH2 domain and C-terminal pY, suggesting that the SH2 and SH3 domains may interact to achieve repression (42, 48, 70). A functional SH2-SH3 interaction is further suggested by the requirement for both domains for stable association of activated Src with its 110- and 68-kDa substrates in transformed chicken fibroblasts (31, 71). However, the biochemical basis of an interaction between the SH3 and SH2 domains is unknown. Here, we demonstrate a direct physical interaction between SH3 and SH2 domains of Fyn and Lck. Strong heterologous SH3-SH2 binding was observed between Src family members but not with other proteins. We also show that in fusion proteins containing both the SH2 and SH3 domains, occupancy of each domain modulates the ligand binding through the other domain. The cross-talk between SH3 and SH2 protein-protein interaction modules may provide a novel mechanism of regulating the activity of Src family

Amino acid (A.A.) numbers are on the right. (B) Specific binding of Fyn SH2 to activation-induced phosphotyrosyl proteins and abrogation of binding by the R176K mutation in the βB5 residue of the SH2 domain. Jurkat cells were incubated for 2 min at 37°C at 10⁸/ml in medium with a 1:200 dilution of anti-CD3 (SPV-T3b) (+) or a control anti-CD8 MAb (OKT8) (−, in panel C only), followed by lysis and binding as described in Materials and Methods. Bound polypeptides were detected by anti-pY immunoblotting with MAb 4G10 followed by ¹²⁵I-protein A. Whole cell lysates, 0.75 million to 1 million cells. Binding reactions included 50 μg of fusion proteins and lysate from 30 × 10⁶ cells; 10.5% gels were used. ζ chain and ZAP-70 are indicated. The residual signal at about 38 kDa (lane 4) is due to nonspecific binding to fusion protein. (C) Adjacent NT and SH3 domains increase SH2-mediated phosphotyrosyl protein binding. Arrowheads point to SH3-binding p120 and p38/p40, whose binding was enhanced in SH3SH2 and NTSH3SH2 binding reactions, and to ZAP-70, whose binding did not increase.

tyrosine kinases and formation of SH2- and SH3-dependent signalling complexes.

MATERIALS AND METHODS

Peptides. The phosphotyrosyl peptide including tyrosine 324 of the hamster medium-sized tumor antigen (EPQpYEEI PIYL or pYEEI; the SH2-binding motif is underlined) (66) and its unphosphorylated analog (YEEI) were synthesized and purified by high-pressure liquid chromatography as described previously (16). The proline-rich peptides p85 SH3-N (phosphatidylinositol [PI] 3-kinase p85 α amino acids [aa] 83 to 101; ISPPTPKPRPPRPLPVAPG), p85 SH3-C (PI 3-kinase p85 α aa 299 to 317; NERQPAPALPPKPPKPTTV) (49), SOS-N20 (human son-of-sevenless aa 1143 to 1162; GTDEVVPPPPVPPRRPESA) (8), and DYNAMIN (rat brain dynamin aa 786 to 806; PAVPPARPGSRGPAGPPAG) (4, 24) were synthesized in an Applied Biosystems peptide synthesizer.

Generation of GST fusion constructs. Generation of Fyn (12, 63) and Lck (54) glutathione S-transferase (GST) fusion constructs in pGEX2T.K (30) by PCR has been described previously (58). These fusion proteins are schematically represented in Fig. 1A. The Fyn SH2 R176K mutant was derived by PCR using a mutagenic primer with AAG instead of CGC at nucleotides 1105 to 1107 and cloned into pGEX2T.K vector. The Fyn SH3 W119K mutant was similarly derived by PCR using mutagenic primers with AAG instead of TGG at nucleotides 355 to 357. The presence of only the engineered mutations was confirmed by sequencing. pGEX2T constructs of p85 PI 3-kinase N- and C-terminal SH2 domains have been described elsewhere (66). GST constructs expressing Src (52) and Grb2 (69) SH2 domains were provided by Gerald Gish and Tony Pawson, and those expressing murine Abl, Crk, and N-terminal GTPase-activating protein SH2 domains (37, 39) were provided by Bruce Mayer.

Fusion proteins were affinity purified on glutathione-Sepharose beads (Pharmacia) from the Triton TX-100 (TX-100)-soluble fraction of isopropyl thiogalactopyranoside (IPTG)-induced *Escherichia coli* DH5 α or NB42 (30, 65), quantitated by the Bradford assay (bovine gamma globulin standard; Bio-Rad), and assessed for purity (more than 95%) and lack of degradation on Coomassie blue gels.

Metabolic labelling with [³⁵S]methionine and [³⁵S]cysteine. Cells (5×10^6 /ml) were labelled for 4 to 5 h in methionine- and cysteine-free RPMI 1640 with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 5% dialyzed fetal bovine serum, and 250 μ Ci of [³⁵S]methionine and [³⁵S]cysteine (EXPRESS; New England Nuclear) per ml. The cells were washed in phosphate-buffered saline and lysed in cold lysis buffer at 5×10^6 /ml. Lysis buffer contained 0.5% TX-100 (Pierce or Fluka), 50 mM Tris (pH 7.5) (at room temperature), 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 mM sodium fluoride.

Activation of Jurkat cells and anti-pY immunoblotting. Jurkat T cells were washed and resuspended in RPMI with 20 mM HEPES and 0.2 mM sodium orthovanadate at 10^8 /ml, preincubated for 2 min at 37°C, and then incubated with either control (anti-CD8 antibody OKT8; American Type Culture Collection) or activating (anti-CD3 ϵ antibody SPV-T3b; from Hergen Spits) antibody (1:200 ascites fluid) for 2 min. Cells were lysed at 5×10^7 per ml in cold lysis buffer (see above) and then subjected to binding to fusion proteins (see below) and anti-pY blotting with antibody 4G10 as described previously (58). ¹²⁵I-labelled protein A (Amersham) was used for detec-

Binding of cellular polypeptides to GST fusion proteins. Purified GST fusion proteins noncovalently coupled to glutathione-Sepharose beads were rocked with cell lysate for 1 h at 4°C and washed six times in lysis buffer. For ³⁵S-labelled lysate binding, lysate from 10^6 cell equivalents was incubated with 25 μ g of fusion protein. For anti-pY blots, lysates from 3×10^7 to 4×10^7 cell equivalents were incubated with 50 μ g of fusion proteins. For peptide competition experiments, fusion proteins on beads were preincubated with peptides for 30 min. All samples were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels under reducing conditions. ³⁵S-labelled samples were visualized by fluorography, using dimethyl sulfoxide-2,5-diphenyloxazole (New England Nuclear).

Binding of Fyn fusion proteins to p85 PI 3-kinase expressed in insect cells. Lysates of *Spodoptera frugiperda* Sf9 cells expressing the p85 subunit of PI 3-kinase (1/10 of a 60-mm-diameter plate in 250 μ l) (32) were incubated with fusion proteins (25 μ g) for 30 min, washed six times, and subjected to Western blotting (immunoblotting) with a p85 C-terminus-specific antibody (1:1,000; Transduction Laboratories).

Binding of fusion proteins to phosphopeptides by using a BIAcore instrument and phosphopeptide library selection were performed as described previously (52, 66).

SH3-SH2 binding. A consensus protein kinase A phosphorylation site engineered in the pGEX2T.K vector immediately after thrombin cleavage site was used for labelling (30). Fifty to 100 μ g of purified GST fusion proteins noncovalently attached to beads was incubated in 30 μ l of buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 12 mM MgCl₂, 1 mM dithiothreitol, 20 μ Ci of [γ -³²P]ATP (New England Nuclear), and 10 U of purified catalytic subunit of protein kinase A (heart muscle kinase; Sigma). After 30 min at 4°C, the reaction was stopped with 1 ml of stop buffer (10 mM sodium phosphate [pH 8], 10 mM sodium pyrophosphate, 10 mM EDTA, 1 mg of bovine serum albumin per ml). After washing, the labelled SH2 or SH3 fragment was cleaved with thrombin (Sigma) (65), the beads with attached GST and uncleaved protein were removed, and the supernatant was diluted in lysis buffer.

Between 10^5 and 2.5×10^5 cpm of ³²P-labelled protein (without the GST portion) was incubated in a 200- to 500- μ l volume with 10 to 25 μ g of unlabelled bead-immobilized fusion proteins. After 1 to 2 h of rocking at 4°C, beads were washed and bound material was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography.

For all peptide inhibition experiments, the bead-immobilized fusion proteins and soluble proteins or cellular lysates were preincubated with the indicated concentrations of peptides for 30 to 45 min and then incubated together.

RESULTS

Recent studies have demonstrated that the SH2 and SH3 domains of signalling proteins mediate protein-protein interactions by binding to their respective ligands, the phosphotyrosyl and proline-rich peptide motifs (4, 18, 43, 46, 51, 59, 66, 78, 81). Both the SH2 and SH3 domains are required for repression of the Src family kinases (13, 42, 48, 70), suggesting a functional interaction between these modules. We investigated the possibility that the functional interaction between these domains involves their influence on each other's ligand binding. For this purpose, we expressed the Fyn SH2 and SH3 domains in bacteria as GST fusion proteins and purified them on glutathione-Sepharose (Fig. 1A). Purified GST fusion proteins noncovalently immobilized on glutathione-Sepharose beads were used to assess binding to either defined ligands (phosphopeptides or baculovirus-expressed p85 subunit of the

PI 3-kinase) or binding polypeptides present in lysates of the human T-cell line Jurkat. Earlier studies have demonstrated that SH2 and SH3 domains expressed in bacteria fold correctly and can be used to examine domain-specific ligand binding (4, 18, 43, 46, 51, 59, 66, 78, 81).

Specific phosphotyrosyl protein binding to the Fyn SH2 domain. To assess the phosphotyrosyl protein binding to GST fusion proteins, we induced tyrosine phosphorylation of cellular proteins in Jurkat cells by stimulation with the anti-CD3 monoclonal antibody (MAb) SPV-T3b, a treatment known to phosphorylate several known (such as TCR ζ , ζ -associated ZAP-70, and phospholipase C γ 1) as well as unidentified cellular proteins (7, 29). As expected, stimulation of Jurkat cells with the anti-CD3 MAb for 2 min induced the tyrosine phosphorylation of a number of proteins whose sizes were similar to those described by others (Fig. 1B, lane 1, and data not shown) (7, 29). Anti-pY blotting of anti-TCR ζ immunoprecipitates demonstrated the expected phosphorylation of the TCR ζ chain and its activation-dependent association with ZAP-70 (data not shown) and confirmed the identity of the corresponding comigrating species observed in whole cell lysates (Fig. 1B, lane 1) (7).

Anti-pY blotting of binding reactions carried out with fusion proteins showed that Fyn SH2 specifically bound to several anti-CD3-induced phosphotyrosyl proteins (Fig. 1B, lane 3); in contrast, the GST control showed no detectable binding (lane 2). Relatively low levels of phosphotyrosyl proteins were seen in Fyn SH2 binding reactions carried out with lysates of unstimulated cells (data not shown). Binding of cellular phosphotyrosyl proteins to Fyn SH2 fusion protein was inhibited in a concentration-dependent manner by the high-affinity phosphotyrosyl peptide pYEEI but not the corresponding nonphosphorylated peptide YEEI (data not shown) (52, 66). Importantly, a conservative single point mutation (R176K) in the phosphotyrosine contact residue β B5 of Fyn SH2 (18) resulted in nearly complete loss of phosphotyrosyl protein binding (Fig. 1B; compare lanes 3 and 4). The residual signal at 38 to 40 kDa is partly due to nonspecific binding to the fusion protein itself. Analogous mutation in the Abl SH2 domain was shown to abrogate pY binding without altering the protein folding (39). Together, these results established a pY-dependent binding of Fyn SH2 fusion protein to cellular phosphotyrosyl proteins.

Adjacent NT and SH3 domains enhance Fyn SH2-mediated ligand binding. To assess if the presence of adjacent SH3 or NT plus SH3 domains influences binding mediated by the SH2 domain, we examined the binding of longer fusion proteins of Fyn to cellular phosphotyrosyl proteins. The SH2 fusion protein showed the expected binding to a number of phosphotyrosyl proteins (Fig. 1C, lane 4) compared with GST alone (lane 3). In contrast, the SH3 fusion protein bound only to a 120-kDa phosphotyrosyl protein (p120; lane 5), an interaction mediated by the SH3 ligand-binding surface as shown by inhibition of this binding by SH3-specific proline-rich peptides but not by the pYEEI phosphopeptide (58). Both the SH3SH2 and the NTSH3SH2 proteins bound to the array of phosphotyrosyl proteins observed with the SH2 domain alone (Fig. 1C; compare lanes 6 and 7 with lane 4). While the increased binding of p120 to the SH3SH2 fusion protein (lane 6) was expected (as this protein binds directly to both domains) (58), appreciably higher binding to proteins of 40 and 38 kDa (arrowheads in Fig. 1C) and to ζ (bracket) was noticed compared with the SH2 fusion protein (lane 4). The NTSH3SH2 fusion protein (lane 7) showed a markedly increased binding to most phosphotyrosyl polypeptides. However, binding of the NTSH3SH2 fusion protein to the 68-kDa protein (which comigrates with and presumably is ZAP-70)

TABLE 1. Relative affinities of phosphotyrosyl peptides for Fyn fusion proteins as determined by competition assay using a BIAcore instrument^a

Peptide ^b	Sequence	ED ₅₀ (μ M)		
		SH2	SH3SH2	NTSH3SH2
hmt pY324	EPQpYEEIPIYL	7.2	4	0.94
Lck pY505	TEGQpYQPQP(A ^c)	560	151	78
Fyn pY531	EPQpYQPGNL	600	263	66
PDGFR pY740	DGGpYMDMSKDE	156	106	38

^a The conditions for this competition assay have been previously described (52). The interassay variation was <10%.

^b Peptide names indicate the protein of origin, and the number designation is the position of the tyrosine in the amino acid sequence. Y and residues +1 to +3 of the binding motif are in boldface. Fyn pY531 is identical to Src pY527. hmt, hamster polyomavirus middle-sized tumor antigen. PDGFR, PDGF receptor.

^c Added to the sequence for peptide synthesis requirements.

was lower than its binding to the SH2 fusion protein (lane 4), indicating that increased binding to other polypeptides was not due to better accessibility of the SH2 domain as a result of its increased distance from the GST fusion partner or an increase in the bioactive fraction of the fusion protein preparations, both of which would be expected to equally increase binding to various cellular polypeptides. The binding of phosphotyrosyl proteins to the NTSH3 fusion protein (lane 8) was similar to that seen with the SH3 fusion protein (lane 5), suggesting that increased binding seen with the NTSH3SH2 fusion protein was mediated by the SH2 domain. This conclusion was supported by the specific competition of phosphotyrosyl proteins binding to NTSH3SH2 by the pYEEI phosphopeptide (see Fig. 3B, lanes 3 to 6). Thus, the presence of the SH3 domain in GST fusion proteins of Fyn, in particular when the NT domain was also present, increased the binding of cellular phosphotyrosyl polypeptides to SH2 domain.

To further characterize the increased ability of longer fusion proteins of Fyn to bind to phosphotyrosyl proteins, we determined their affinity for phosphotyrosyl peptide motifs. For this purpose, we quantitated the binding of Fyn fusion proteins to phosphotyrosyl peptides by using surface plasmon resonance measurements on a BIAcore instrument (52). The pYEEI motif present in hamster middle-sized tumor antigen (hmt pY324) was previously shown to bind to Src family SH2 domains with high affinity (52, 66). Therefore, we immobilized this phosphopeptide on the BIAcore sensor chip and measured the concentrations of four phosphopeptides that inhibited the binding of fusion proteins to the matrix-immobilized peptide by 50% (ED₅₀); ED₅₀ is inversely related to affinity. As expected, pYEEI (hmt pY324) itself effectively competed for binding to SH2, reflecting a high affinity (ED₅₀, 7.2 μ M; Table 1). The binding of Lck pY505 and Src pY527/Fyn pY531 carboxyl-tail peptides to SH2 was expectedly poor (ED₅₀s, 560 and 600 μ M, respectively), whereas the binding of PDGF receptor pY740 was intermediate (ED₅₀ of 156 μ M) (52, 66). Notably, the phosphopeptides bound to SH3SH2 and NTSH3SH2 fusion proteins with higher affinities, as shown by a decrease in the ED₅₀ values (Table 1). For example, the ED₅₀ values for the hmt pY324 peptide were 1.8- and 7.6-fold lower, respectively, with SH3SH2 and NTSH3SH2 fusion proteins compared with SH2. A similar pattern was observed with all peptides, although an increase in the affinity of the SH3SH2 fusion protein was more noticeable with low-affinity peptides (Lck pY505 and Fyn pY531). These results show that the presence of adjacent SH3 or NT and SH3 domains increases the affinity of the SH2 domain for phosphotyrosyl peptide motifs.

TABLE 2. Recognition specificities of Fyn fusion proteins determined by selection of phosphotyrosyl peptide motifs from a peptide library

Position	Protein	Relative preference of amino acids ^a								
		E	Q	N	T	Y	I	V	M	F
pY+1	SH2	1.6	1.7	—	1.5	1.5	—	1.5	—	—
	SH3SH2	2.1	2	—	1.7	1.7	—	1.7	—	—
	NTSH3SH2	2.3	—	—	2	2	—	—	—	—
pY+2	SH2	1.7	1.5	1.5	—	—	—	—	—	—
	SH3SH2	2	1.7	1.7	—	—	—	—	—	—
	NTSH3SH2	2	—	—	—	—	—	—	—	—
pY+3	SH2	—	—	—	—	1.4	—	1.7	—	1.3
	SH3SH2	—	—	—	—	—	1.6	2	1.7	1.4
	NTSH3SH2	—	—	—	—	—	2.3	1.5	1.7	1.4

^a The enrichment values of the amino acids selected by the indicated Fyn fusion proteins at positions +1, +2, and +3 C terminal to the pY residue of the phosphopeptide mixture. The highest value at each position is in boldface. These values were calculated by comparison with control (GST) beads as described previously (66). —, no preference.

To determine if the presence of adjacent SH3 or NT plus SH3 domains also affected the selectivity of the SH2 domain for its ligands, we analyzed the phosphotyrosyl peptide motifs selected by different fusion proteins from a phosphotyrosyl peptide library with degenerate amino acid residues at positions +1 to +3 with respect to pY (66) (Table 2). The Fyn SH2 domain showed an expected preference for the hydrophilic residues at positions +1 and +2 and for a hydrophobic residue at position +3 relative to pY. Significantly, the presence of an adjacent SH3 (SH3SH2) or NT and SH3 domains (NTSH3SH2) in the fusion proteins narrowed the range of amino acids observed at positions +1 to +3 and increased the preference for EEI residues at these positions. Thus, three independent assays revealed a significant positive influence of the adjacent SH3 and NT domains on the SH2-mediated binding to phosphotyrosyl peptide motifs.

The SH3 domain is capable of binding to the p85 subunit of PI 3-kinase when expressed next to NT and SH2 domains. While the phosphotyrosyl protein and peptide binding assays described above provided direct assays for SH2-mediated binding, the ligand-binding properties of SH3 domains and possible influence of the neighboring domains are not well characterized. In particular, it was important to determine whether the small SH3 domain expressed next to larger NT and SH2 domains was able to interact with known SH3-specific ligands. Previous work has demonstrated an association of the p85 subunit of PI 3-kinase with Fyn tyrosine kinase in vivo and direct binding of p85 to Fyn SH3 domain in vitro (57). In addition, proline-rich sequences are present in p85 PI 3-kinase and corresponding peptides block the in vitro binding of p85 with the Fyn SH3 fusion protein (32). Therefore, to assess SH3-specific binding of Fyn fusion proteins, we incubated bead-immobilized fusion proteins with TX-100 lysates of insect cells infected with a p85 PI 3-kinase baculovirus expression vector (32). Bound p85 was visualized by Western blotting with a specific anti-p85 antibody.

Baculovirus-expressed p85 showed prominent binding to the Fyn SH3 fusion protein (Fig. 2A, lanes 2 and 7) but not to GST alone (lane 1). When the p85-derived proline-rich peptide p85 SH3-N (aa 83 to 101) (49) was included during incubation, a concentration-dependent inhibition of p85 binding was observed (lanes 8 to 10), confirming earlier results (32). Importantly, a single point mutation (W119K) in one of the four conserved aromatic residues that line the putative ligand-

binding pouch on the Fyn SH3 domain (46) led to a complete abrogation of binding to p85 protein (Fig. 2B; compare lanes 2 and 3). A small but reproducible binding of p85 protein to bead-immobilized GST-Fyn SH2 was observed (Fig. 2A, lane 5). This binding was phosphotyrosine independent, as comparable binding was seen with the R176K SH2 mutant (lane 6) and may represent binding of Fyn SH2 to the p85 SH3 domain (see below for SH2-SH3 binding). Importantly, fusion proteins that contained the SH2 (SH3SH2; Fig. 2A, lane 3) or NT and SH2 (NTSH3SH2; Fig. 2A, lane 4) domains retained the ability to bind to p85 protein. p85 binding to SH3SH2 and NTSH3SH2 fusion proteins was somewhat better, perhaps reflecting cumulative SH3- and SH2-mediated binding. Thus, the Fyn SH3 domain expressed in the context of adjacent NT and SH2 domains is capable of interacting with a specific binding protein, indicating that its ligand-binding surface is accessible.

Specific binding of the Fyn SH3 domain to cellular polypeptides. To assess the potential influences of adjacent domains on SH3-mediated ligand binding, we first characterized the ability of the Fyn SH3 fusion protein to specifically interact with cellular polypeptides. For this purpose, we carried out binding experiments between glutathione-Sepharose-immobilized fusion proteins of Fyn and TX-100 lysates of ³⁵S-labelled Jurkat cells and visualized the specific binding polypeptides by SDS-PAGE followed by fluorography. As shown in Fig. 2C, a number of cellular polypeptides were observed in SH3 binding reaction (lane 2) but not in the GST control (lane 1). The profiles of specific binding proteins observed with SH3 domains of the related kinases Lck and Src were similar, although the relative binding of individual polypeptides to different SH3 domains differed (data not shown). The Fyn SH3-binding proteins included the p85 subunit of the PI 3-kinase (data not shown), confirming recent results (36, 55, 57).

The binding of cellular polypeptides to the Fyn SH3 fusion protein was abrogated in a concentration-dependent manner by the proline-rich peptide p85 SH3-N (p85 PI 3-kinase aa 83 to 101) (Fig. 2C; compare lanes 3 to 6 with lane 2) at concentrations similar to those that blocked the SH3 interaction with p85 PI 3-kinase (compare with Fig. 2A, lanes 8 to 10). The inhibition with p85 SH3-N was nearly complete at 200 μ M and detectable even at 2 μ M. Three additional proline-rich peptides (PI 3-kinase p85 α aa 299 to 317, human son-of-sevenless aa 1143 to 1162, and rat brain dynamin aa 786 to 806) (4, 8, 24, 49) also inhibited the binding of cellular polypeptides to Fyn SH3; however, 5- to 10-fold higher concentrations were required for comparable inhibition, further confirming the specificity of proline-rich peptides (data not shown). Phosphotyrosyl (pYEEI, which binds to SH2; lanes 7 to 10) or nonphosphorylated (YEEI; lanes 11 to 14) hmT Y324 peptide did not inhibit binding of cellular polypeptides to the SH3 domain, although the pYEEI peptide (but not the YEEI peptide) did inhibit binding of cellular polypeptides specific for the SH2 domain (data not shown).

The specificity of Fyn SH3 binding to cellular polypeptides present in ³⁵S-labelled Jurkat cell lysates was further demonstrated by an essentially complete abrogation of this binding by the W119K point mutation in the ligand-binding pouch of the SH3 domain (Fig. 2D; compare lanes 2 and 3).

Neighboring NT and SH2 domains decrease binding to a subset of SH3-specific polypeptides. To assess the influence of NT and SH2 domains on SH3-mediated binding to cellular polypeptides, we examined the profiles of cellular polypeptides that bound to SH3 versus either SH2 or the multidomain fusion proteins (NTSH3, SH3SH2, or NTSH3SH2). Interestingly, a number of polypeptides interacted with the SH2 fusion

FIG. 2. Binding of baculovirus-expressed p85 PI 3-kinase and cellular polypeptides to the Fyn SH3 domain and influence of neighboring NT and SH2 domains on binding. (A) Binding of baculovirus-expressed PI 3-kinase p85 to GST Fyn fusion proteins and inhibition by proline-rich peptide p85 SH3-N. Twenty-five micrograms of each fusion protein (top) coated on glutathione-Sepharose beads was rocked with baculovirus PI 3-kinase p85 protein (from 1/10 of a 60-mm-diameter plate) for 45 min, and bound p85 protein (arrowhead) was detected by Western blotting with an anti-p85 antibody. Lanes 7 to 10 show binding to Fyn SH3 fusion protein in the presence of the indicated concentrations of proline-rich peptide p85 SH3-N (p85 aa 83 to 101). Lane 11 represents direct blotting of the insect cell lysate (half of that used for binding). (B) Abrogation of baculovirus p85 PI 3-kinase binding by the W119K mutation in the Fyn SH3 domain. The W119K mutation in the putative proline-rich peptide-binding pouch was generated by PCR; 25 μ g of wild-type or W119K mutant SH3 fusion protein coated on glutathione-Sepharose beads was used to bind to baculovirus p85 as in panel A. Note that binding of p85 to mutant SH3 is similar to background binding to GST. (C) Binding of specific cellular polypeptides to Fyn SH3 and concentration-dependent inhibition of binding by proline-rich peptide P85 SH3-N (lanes 3 to 6) but not by phosphorylated (pYEEI; lanes 7 to 10) or unphosphorylated (YEEI; lanes 11 to 14) hmt Y324 peptide. Lysates of 35 S-labelled Jurkat cells were incubated with GST fusion proteins noncovalently immobilized on glutathione-Sepharose beads, and bound proteins were visualized by fluorography. Peptide concentrations were as follows: 2 mM (lanes 3, 7, and 11), 200 μ M (lanes 4, 8, and 12), 20 μ M (lanes 5, 9, and 13), and 2 μ M (lanes 6, 10, and 14). (D) Specificity of cellular polypeptide binding to Fyn SH3 fusion protein revealed by the W119K mutation. Binding to 35 S-labelled cellular polypeptides was carried out as for panel C. Note that the Fyn SH3 W119K fusion protein shows only background binding similar to that seen with GST. (E) Effect of neighboring NT and SH2 domains on binding of 35 S-labelled cellular polypeptides of Jurkat cells to Fyn SH3. Note the SH3-specific (filled arrowheads) and SH2-specific (open arrowheads) cellular polypeptides that show less or no binding to Fyn SH3SH2 and NTSH3SH2 proteins. (F) Phosphotyrosine-independent binding of cellular polypeptides to the Fyn SH2 fusion protein. Binding to lysates of 35 S-labelled Jurkat cells was carried out as for panel C. The data shown were part of the experiment shown in panel D, which shows the common GST control. Note that the binding of most polypeptides is unaffected by the R176K mutation, which abolishes pY-dependent binding (see Fig. 1B).

protein although the cells had not been stimulated to trigger tyrosine phosphorylation of cellular proteins (Fig. 2E, lane 2). The SH2-binding polypeptides were, however, distinct from those observed with the SH3 domain fusion protein (lane 3). For example, polypeptides of 32, 37, 39, 50, 56, 85, 125, 130, and 190 kDa bound to the SH2 domain (lane 2), while polypeptides of 36, 48, 54, 58, 60, 61, 65, 75, 82, 95, 100, 115, 128, 145, 150, and 165 kDa bound to the SH3 domain (lane 3). A comparison of SH3-binding polypeptides with those bound to SH3SH2 (lane 4) and NTSH3SH2 fusion proteins (lane 5) showed a decrease or loss of binding to several SH3-specific polypeptides (arrowheads on the right). However, the loss of binding to SH3-specific polypeptides was selective, as the binding of several specific polypeptides (e.g., at 65 and 50 kDa) was unaltered. Thus, the presence of adjacent NT and SH2 domains within the fusion proteins had a marked negative influence on the binding of cellular polypeptides specific for the SH3 domain.

The majority of SH2-specific polypeptides detected in ³⁵S-labelled cell lysates were not tyrosine phosphorylated and are able to bind to the R176K Fyn SH2 mutant (Fig. 2F), which fails to bind pY (Fig. 1B). However, the binding of these polypeptides to the SH2 domain was sensitive to phosphopeptide-induced structural perturbations in the SH2 domain, as shown by specific inhibition of their binding by phosphotyrosyl peptides (see Fig. 3A, lanes 15 and 16). Comparison of multidomain fusion proteins with SH2 showed a decrease or loss of binding to several of these SH2-specific polypeptides (Fig. 2E; compare lanes 4 and 5 with lane 2; arrowheads on the left). Thus, while the pY-binding pocket of the SH2 domain was accessible in the presence of adjacent NT and SH3 domains (see above), binding of the SH2 domain to a number of nonphosphotyrosyl polypeptides was reduced or abolished. It is possible that the region on the SH2 domain that mediates binding to these nonphosphotyrosyl proteins overlaps with the region involved in its interaction with the SH3 domain.

Binding experiments using ³⁵S-labelled lysates also revealed a putative NT-specific 68-kDa polypeptide which bound to the NTSH3 but not to the SH3 fusion protein (Fig. 2E; compare lanes 3 and 6). This polypeptide was not observed in NTSH3SH2 fusion protein binding reactions (compare lanes 5 and 6), suggesting that the neighboring SH2 domain may also influence the interaction of polypeptides with the NT domain.

Together, these results provide strong *in vitro* evidence for an influence of the adjacent noncatalytic SH2, SH3, and NT domains of Fyn in regulating ligand binding specific for each domain. Changes in binding to SH2-specific polypeptides are also observed in multidomain fusion proteins of Lck (our unpublished data and reference 63a). Thus, interdomain interactions may be a general feature of Src family kinases. Although the identities of most SH3-specific and SH2-specific polypeptides observed in our binding assays have not been determined, competition with domain-specific peptides and the influence of a point mutation in the ligand-binding pouch of the SH3 domain demonstrated the specificity of binding.

SH2 domain occupancy reverses the inhibitory effect of the SH2 domain on SH3-mediated ligand binding. To further characterize the potential significance of the interaction between SH2 and SH3 domains revealed by the experiments described above, we examined whether these interactions were influenced by occupancy of SH2 and SH3 domains by their specific ligands. As shown above, the presence of an unoccupied SH2 domain in SH3SH2 and NTSH3SH2 fusion proteins reduced the binding of the SH3 domain to several cellular polypeptide ligands; phosphopeptide-induced structural changes might be anticipated to restore binding of these polypeptides

to the SH3SH2 fusion protein. As shown in Fig. 3A, binding of the SH3-specific p100 and p60 to the SH3SH2 fusion protein was reduced compared with binding to the SH3 fusion protein (compare lanes 2 and 6; arrowheads). The phosphorylated (compare lane 6 with lanes 7 to 11) but not the nonphosphorylated (compare lanes 6 and 12) hmT Y324 peptide induced an increase in binding of p100 and p60; the increase, albeit modest, was reproducible. The effect of SH2 occupancy on p100 and p60 was selective, as binding to other SH3-specific polypeptides (e.g., p65; arrowhead) did not change, while binding of SH2-specific p32 was inhibited in a concentration-dependent manner (arrowhead). Notably, binding of p65 to SH3SH2 fusion protein was substantially more resistant to proline-rich peptide inhibition than was its binding to SH3 (compare lanes 5 and 13). Concurrent addition of phosphotyrosyl hmT Y324 clearly enhanced the proline-rich peptide competition (compare lanes 13 and 14; arrowhead), indicating that SH2 occupancy increased binding of the proline-rich peptide. The alternate possibility that p65 binds less well to the SH3 domain when a phosphopeptide is included during binding appears unlikely since the phosphopeptide alone did not alter p65 binding to SH3SH2 (lanes 7 to 11). Thus, the SH2 domain occupancy specifically increased the binding of cellular polypeptides and a peptide ligand to a linked SH3 domain, consistent with a biochemical communication between the SH3 and SH2 domains within the SH3SH2 fusion protein.

SH3 domain occupancy enhances the phosphotyrosyl protein binding to a linked SH2 domain. As demonstrated above, the presence of NT and SH3 domains enhanced the affinity of the linked SH2 domain. In view of the effects of SH2 occupancy on SH3-mediated binding, we examined whether the SH3 occupancy influences the binding of phosphotyrosyl motifs to the SH2 domain. Addition of SH3-specific peptide p85 SH3-N induced an increase in cellular phosphotyrosyl protein binding to the NTSH3SH2 fusion protein (Fig. 3B; compare lane 2 with lanes 7 to 10); the enhancement was difficult to detect with SH3SH2 fusion proteins (data not shown), perhaps reflecting the stabilizing effect of NT on SH3-SH2 interaction. Phosphotyrosyl peptide inhibition demonstrated that basal (lanes 3 to 6) as well as proline-rich peptide-enhanced (lanes 11 and 12) binding was mediated through the SH2 domain (except p120, which binds to both domains) (58). BIAcore binding experiments showed that addition of the proline-rich p85 SH3-N peptide enhanced the binding of phosphotyrosyl peptide hmT pY324 to the Fyn NTSH3SH2 fusion protein up to threefold (data not shown). Thus, when both domains are present in a single molecule, occupancy of the SH3 domain enhances the binding of ligands to the SH2 domain. The effect of the proline-rich peptide was observed at relatively high concentrations. A possible reason for this is a lower binding of proline-rich peptides to the SH3 domain when a neighboring SH2 domain is present; this is clearly suggested by incomplete competition of SH3-specific p65 binding to the SH3SH2 fusion protein (Fig. 3A, lane 13), which is in contrast to a complete inhibition of binding of the same polypeptide to the SH3 fusion protein (lane 5).

Physical interaction between purified SH3 and SH2 domains. The experiments described above demonstrated that adjacent SH2 and SH3 domains within fusion proteins modify each other's ligand binding and can communicate the effects of ligand occupancy with each other. Since both SH2 and SH3 domains serve as protein-protein interaction modules, we wished to determine if these domains can interact with each other biochemically. To assess direct binding between the SH3 and SH2 domains, we radiolabelled the fusion proteins with [γ -³²P]ATP by using an engineered protein kinase A site,

FIG. 3. Ligand-dependent communication between SH3 and SH2 domains. (A) SH2 domain occupancy increases binding of certain cellular polypeptides to a linked SH3 domain in the Fyn SH3SH2 fusion protein. Beads coated with the indicated fusion proteins (25 μ g) were preincubated for 30 min with different concentrations of peptides (shown at the top) and then allowed to bind to lysates of 35 S-labelled Jurkat cells for 1 h as in Fig. 2C. Note that SH2 domain occupancy with a phosphopeptide (pYEEI) increases the binding of p100 and p60 (arrowheads) to the linked SH3 domain in the SH3SH2 fusion protein (compare lanes 7 to 11 with lanes 6 and 12). Binding of other polypeptides, such as p65 (arrowhead), did not increase. Also note that addition of pYEEI enhances the ability of proline-rich peptide to outcompete the SH3-binding p65 (lane 13 versus 14). (B) SH3 domain occupancy with a proline-rich peptide increases cellular phosphotyrosyl protein binding to a linked SH2 domain in the NTSH3SH2 fusion protein. Beads coated with 50 μ g of the indicated fusion proteins were preincubated with different concentrations of phosphotyrosyl (pYEEI) or proline-rich (p85 SH3-N, p85 aa 83 to 101) peptide and then incubated with unlabelled cell lysates (50×10^6 cell equivalents) of anti-CD3-stimulated Jurkat cells. Bound proteins were detected by anti-pY immunoblotting with MAb 4G10. Lanes 11 and 12 show binding reactions carried out in the presence of indicated concentrations of both peptides. Proline peptide-induced increase in phosphotyrosyl protein binding is noticeable at 100 and 500 μ M concentrations (lanes 8 and 7 compared with lane 2). Note pYEEI peptide inhibition of both the basal (lanes 3 to 6) and proline peptide-enhanced (lanes 11 and 12) phosphotyrosyl protein binding.

cleaved the GST portion with thrombin, and removed it by binding to glutathione-Sepharose (30). The soluble 32 P-labelled SH2 or SH3 domain (after removal of the GST portion) was incubated with unlabelled GST fusion proteins noncovalently immobilized on glutathione-Sepharose beads, and specifically bound proteins were detected by autoradiography of SDS-polyacrylamide gels. To detect relatively tight binding, we performed the assay in 0.5% TX-100, which is known to disrupt weaker interactions such as that between TCR and Fyn (61).

The soluble Fyn 32 P-SH2 domain showed specific binding to SH3 and NTSH3 fusion proteins immobilized on beads (Fig. 4A, top, lanes 3 and 4). In contrast, binding of 32 P-SH2 to unlabelled SH2 (lane 2) was similar to background binding to GST alone (lane 1). In a reciprocal experiment, 32 P-SH3

showed specific binding to immobilized SH2 (Fig. 4A, bottom, lane 2), whereas its binding to SH3 or NTSH3 was similar to the background level (lanes 3 and 4). Soluble Fyn 32 P-NTSH3 also showed specific binding to SH2 but not to SH3 fusion protein beads (data not shown). Thus, purified SH2 and SH3 domains of Fyn can directly bind to each other in vitro. As lower backgrounds were observed with 32 P-SH3, subsequent experiments were performed with this domain as the labelled partner.

The SH3 and SH2 domains of the closely related Src family kinase Lck (54) also bound to each other (Fig. 4B, bottom, lane 5). In addition, heterologous binding of Fyn 32 P-SH3 to Lck SH2 (Fig. 4B, top, lane 5) and of Lck 32 P-SH3 to Fyn SH2 (Fig. 4B, bottom, lane 2) was observed. Furthermore, both the Fyn and Lck 32 P-SH3 domains bound to immobilized Fyn^T SH2

FIG. 4. Direct in vitro binding between SH3 and SH2 domains of Fyn and effects of peptide ligands. The fusion proteins were labelled on the engineered protein kinase A site with [γ - 32 P]ATP and thrombin cleaved, and isolated labelled SH2 and SH3 were separated from GST and the uncleaved fusion protein by using glutathione-Sepharose. 32 P-labelled proteins were incubated in lysis buffer with fusion proteins (25 μ g) noncovalently immobilized on glutathione-Sepharose, washed three times, and resolved by SDS-PAGE (15% gel). Arrows indicate the mobility of 32 P-labelled proteins. (A) Specific binding between Fyn SH2 and SH3 domains. Soluble 32 P-SH2 (top, arrow) or 32 P-SH3 (bottom, arrow) was incubated with bead-immobilized fusion proteins indicated at the top. The numbers below each panel represent relative increases (arbitrary units) in signal compared with GST, as determined with a PhosphorImager (Molecular Dynamics). The spots near the edges are from excluded lanes and should be ignored. (B) SH3-SH2 binding is observed with both Fyn and Lck. Note that Fyn and Lck 32 P-SH3 bind to both Fyn and Lck SH2 proteins on beads but neither binds to Grb2 SH2. Also note comparable SH3 binding of wild-type and R176K mutant Fyn SH2. (C) Specificity of SH2-SH3 binding. Note that all Src family SH2 domains (Fyn, Lck, and Src) bind to both Fyn and Lck 32 P-SH3 proteins. Other SH2 domains do not show binding with the exception of p85 C-terminal SH2 (lane 6). (D) SH3-SH2 binding is not abolished by the W119K mutation in Fyn SH3 domain. Wild-type (top) and W119K mutant (bottom) Fyn SH3 fusion proteins were 32 P labelled as for panel A, and equal counts (2.5×10^5 cpm) of each labelled SH3 peptide were allowed to bind to bead-immobilized fusion proteins (25 μ g) shown at the top. Direct loadings of 250, 500, 1,000, and 2,000 cpm of each labelled SH3 protein (lanes 5 to 8) show comparable labelling. Note that Fyn SH3 (W119K) binding to Fyn SH2 is lower than wild-type SH3 binding but still quite substantial. (E) Inhibition of SH3-SH2 binding by SH2 occupancy. Fyn 32 P-SH3 was incubated with the indicated fusion protein beads with or without (–) the indicated peptide, and bound material was visualized by autoradiography. Note dose-dependent and complete inhibition with pYEEI, whereas unphosphorylated peptide YEEI or proline-rich peptide p85 SH3-N has no effect. (F) SH3 binding to the SH2 domain is independent of the pY-binding pocket. Note comparable binding of Fyn 32 P-SH3 to the wild type (lane 16) and the R176K mutant (lane 8), which does not bind to phosphotyrosyl proteins. Also note that the inhibitory effect of the pYEEI peptide on SH3 binding is not observed with R176K mutant SH2, demonstrating the specificity of peptide inhibition.

(lane 8). Fyn^T SH2 is identical to Fyn^B SH2 (simply referred to as Fyn here) except at 7 of 14 C-terminal residues (12, 63). Under identical conditions, Fyn and Lck 32 P-SH3 binding to unrelated Grb2 SH2 was undetectable (lane 7), demonstrating the specificity of SH3-SH2 binding. The specificity was further confirmed by using SH2 domains from a number of additional proteins (Fig. 4C). This experiment revealed that heterologous binding of Fyn and Lck 32 P-SH3 to Src SH2 was readily

demonstrable, whereas little or no binding was observed with immobilized SH2 domains of other proteins with the exception of the C-terminal SH2 domain of p85 PI 3-kinase, which showed appreciable binding to the Fyn SH2 fusion protein (lane 6). A markedly lower binding of 32 P-p85 SH3 to Fyn, Lck, and Src SH2 domains was observed, whereas p85 SH2 fusion proteins showed no binding to p85 SH3 (data not shown). These experiments are consistent with a preferential binding

between SH3 and SH2 domains of Src family kinases. The differences in binding of Fyn SH3 to Lck SH2 in different experiments (compare Fig. 4B and C) are likely to be due to differences in the biological activities of fusion protein preparations; however, the binding was reproducibly observed.

SH3 binding is not mediated by pY-binding pocket of the SH2 domain. To gain further insight into the mechanism of binding between Fyn SH3 and SH2 domains, we examined whether this binding involved SH2 recognition of pYs on the SH3 domain. This was unlikely, as the bacterially expressed proteins are not tyrosine phosphorylated and the ^{32}P -labelling was carried out with a true serine/threonine kinase (cyclic AMP-dependent protein kinase). However, to formally rule out a role for pY-binding pocket, we assessed binding of ^{32}P -labelled Fyn and Lck SH3 to the R176K mutant of the Fyn SH2 domain, which fails to bind pY (Fig. 1E). The patterns of binding of ^{32}P -SH3 to wild-type and R176K mutant SH2 fusion proteins were comparable (Fig. 4B, lanes 2 and 3; Fig. 4F, lanes 8 and 16). Two additional SH2 domain mutants (S186P and R190C in β -strand C) which are partially defective in pY binding (20a) also showed ^{32}P -SH3 binding comparable to that of wild-type SH2 (data not shown). Finally, we have also observed binding between SH3 and SH2 domains when an unlabelled SH3SH2 fusion protein without a GST piece was incubated with bead-immobilized Fyn SH2 and the bound SH3SH2 was detected with an SH3-specific antibody (20b). Together, these results indicate that the SH3 binding to SH2 domain is independent of pY binding and suggest that the SH3-binding region on the SH2 domain may be distinct from the pY-binding pocket.

The W119K mutation in the putative ligand-binding pouch of the SH3 domain does not abolish SH3-SH2 binding. To assess the possible role of the putative ligand-binding pouch on the surface of the SH3 domain in SH3-SH2 binding, we examined binding between Fyn SH2 and the SH3 W119K mutant. As shown above, this mutation completely abrogated the binding to a defined SH3 ligand (p85 PI 3-kinase) (Fig. 2B) as well as to cellular binding polypeptides (Fig. 2D). As seen in Fig. 4D, a substantial although somewhat reduced binding of the Fyn SH3 W119K mutant to the Fyn SH2 fusion protein was observed (lane 2; compare upper and lower panels). Direct loading of ^{32}P -labelled SH3 proteins (lanes 5 to 8) showed that labelling patterns of the two proteins were comparable. While these results do not rule out an important role of the SH3 ligand-binding pouch in SH3-SH2 binding, they suggest that this binding may significantly differ from proline-rich peptide binding to SH3 (also see below).

SH2 domain occupancy disrupts SH3-SH2 binding. To address the potential biological implications of a physical interaction between SH3 and SH2 domains, we examined whether SH3-SH2 binding was influenced by ligand occupancy. Although the effect of ligands on SH3 domain structure has not been examined, a phosphopeptide-induced conformational change has been demonstrated for the PI 3-kinase p85 SH2 domain (64). Specific binding between ^{32}P -SH3 and bead-immobilized SH2 was not substantially affected by proline-rich peptide p85 SH3-N (Fig. 4E, lanes 3 to 5) at concentrations that completely inhibited binding of Fyn SH3 to cellular polypeptides (Fig. 2C) or to baculovirus-expressed p85 PI 3-kinase (Fig. 2A). A similar lack of effect was observed with three additional proline-rich peptides (PI 3-kinase p85 α aa 299 to 317, human son-of-sevenless aa 1143 to 1162, and rat brain dynamin aa 786 to 806) (4, 8, 24, 49). These results are consistent with the ability of the Fyn SH3 W119K mutant to bind to SH2 (Fig. 4D). In contrast to the lack of effect with proline-rich peptides, the phosphotyrosyl (pYEEI; Fig. 4E,

lanes 6 to 10; Fig. 4F, lanes 10 to 15) but not the nonphosphorylated (YEEI; Fig. 4E, lanes 11 to 15; Fig. 4F, lane 17) hMT Y324 peptide inhibited the SH3-SH2 binding at low micromolar concentrations. The effect of the pYEEI peptide required an intact pY-binding pocket, since peptide inhibition was not observed when the Fyn-SH2 R176K (β B5) mutant was used for binding to ^{32}P -SH3 (Fig. 4F, lanes 2 to 7). Thus, while SH2-SH3 binding is independent of the pY-binding pocket, this binding is exquisitely sensitive to perturbations induced by SH2 domain occupancy.

DISCUSSION

In this study, we have carried out *in vitro* binding analyses with purified fusion proteins to define two novel aspects of interaction between SH2 and SH3 domains of Fyn, a prototype Src family tyrosine kinase with well-established roles in T lymphocytes (1, 11, 14, 61, 68, 72) and in neuronal functions (25, 75, 80). First, we demonstrate that when SH3 and SH2 domains are expressed in their natural context as adjacent domains of a fusion protein, they markedly influence each other's ligand-binding abilities. Moreover, these domains appear to communicate with each other such that the occupancy of the SH2 domain by a specific phosphotyrosyl peptide enhanced the ligand binding to a linked SH3, whereas occupancy of the SH3 domain with a specific proline-rich peptide enhanced the SH2 ligand binding. Second, we demonstrate a direct *in vitro* binding between purified SH3 and SH2 domains of the Src family tyrosine kinases Fyn and Lck. Although SH2-SH3 binding was pY independent, it was highly sensitive to structural perturbations of the SH2 domain induced by phosphotyrosyl peptide binding. Moreover, SH3-SH2 binding was resistant to SH3-specific proline-rich peptide inhibition and could still be observed with an SH3 mutant that completely lacked the binding to SH3-specific ligands.

Bacterially expressed SH2 and SH3 domains have been widely used to examine their ligand-binding properties *in vitro* (9, 38, 66), and nuclear magnetic resonance and crystal structural analyses of these domains following purification from bacteria have established the correct folding of these domains, in some cases even when examined in linkage with fusion partners (3, 4, 18, 33, 43, 50, 78, 81). Recent crystal structural analysis of the SH3SH2 fragment of Lck protein directly established the correct folding of both domains within a single molecule (17). Thus, it is unlikely that findings reported here are an artifact of incorrectly folded fusion proteins. In addition, we used a number of binding assays to show that our fusion proteins retained appropriate ligand-binding activity. For example, the Fyn SH2 domain showed expected phosphotyrosyl protein and peptide binding, and a high-affinity phosphotyrosyl peptide selectively inhibited this binding. In addition, a conservative R $\rightarrow$ K mutation in the β B5 pY contact residue of the SH2 domain (18, 39, 78) abolished its binding activity. Similarly, the SH3 fusion protein showed expected binding to recombinant p85 subunit of the PI 3-kinase, and this interaction was inhibited by proline-rich peptides (32, 36, 55). Furthermore, a single point mutation (W119K) in one of the aromatic residues that line the putative ligand-binding pouch on the SH3 domain (17, 46) completely abrogated PI 3-kinase p85 binding. The ability of the multidomain fusion proteins (SH3SH2 and NTSH3SH2) to bind to phosphotyrosyl proteins and peptides (SH2 ligands) and to baculovirus-expressed p85 subunit of the PI 3-kinase (an SH3-binding protein) demonstrated that both the SH2 and SH3 domains in these proteins were active.

The SH2 and SH3 domains are independent structural

modules that exist either alone or together in a number of proteins apparently independent of the context (51). However, these domains are found in the same relative position N terminal to the catalytic domain and C terminal to the unique region of all nine members of the Src family of protein tyrosine kinases (2, 12, 13, 63, 72). The relative positions of these domains are conserved during evolution (26, 67), suggesting that their locations are functionally significant. Indeed, a functional interaction between SH2 and SH3 domains of Src family kinases has been suggested by the similarity of activated phenotypes induced by mutations within each domain or by their deletions (2, 13, 15, 27, 42, 47, 48, 62, 70, 77). Moreover, the SH3 domain appears to be essential for the interaction of the SH2 with C-terminal tyrosine-phosphorylated tail that mediates negative regulation of Src family kinases (42, 48, 70).

The studies presented here demonstrate that adjacent SH2 and SH3 domains of a Src family kinase Fyn when expressed within fusion proteins do not behave independently but rather modulate each other's ligand binding. For example, the presence of NT and SH3 domains enhanced the binding of SH2 domain to most phosphotyrosyl proteins induced in the Jurkat T-cell line by anti-TCR stimulation. However, binding to one of the polypeptides that comigrates with ZAP-70 did not increase, indicating that enhancement was selective. Competition binding assays with phosphopeptides demonstrated an increase in the affinity of the SH2 domain when the SH3 or NT plus SH3 domains were present in fusion proteins. Finally, the selectivity of the SH2 domain for the pYEEI motif was enhanced by the presence of adjacent NT and SH3 domains.

In contrast to SH2 domains, the binding properties of SH3 domains have been less well characterized. To assess the effect of neighboring domains on SH3-mediated binding, we used lysate binding assays to identify a number of cellular polypeptides that specifically bind to Fyn SH3 (as well as to Src and Lck SH3; data not shown). Binding of these polypeptides was inhibited by several previously identified SH3-specific proline-rich peptides. Moreover, binding to these polypeptides was abolished by the W119K mutation in the putative SH3 ligand-binding pouch, further substantiating the specificity of this interaction. Interestingly, a marked decrease in binding to a subset of these polypeptides was observed when fusion proteins included neighboring NT and SH2 domains. The reduction in binding was selective since all fusion proteins (SH3, SH3SH2, and NTSH3SH2) bound equally to other proteins in cell lysates (e.g., p65) and to baculovirus-expressed p85 protein (small increase is likely to be due to low SH2-mediated pY-independent binding; Fig. 2A). Since the presence of NT alone (in NTSH3) did not reduce binding to SH3-specific polypeptides, whereas a reduction was noticeable with SH2 alone (in SH3SH2), we believe that the decrease reflects primarily an influence of the SH2 domain, with NT providing a stabilizing influence on SH3-SH2 interaction. However, a direct interaction between NT and SH2 cannot be definitively ruled out. Interestingly, an N-terminal 31-aa extension in the c-Crk protein was recently shown to enhance the binding of cellular phosphotyrosyl proteins to its SH2 domain (19), and analyses of the Lck protein suggest an influence of sequences within the unique NT region on SH2-mediated binding (63a).

In addition to a mutual influence on ligand binding ability, the SH2 and SH3 domains appear to communicate the state of their occupancy to each other. Notably, occupancy of the SH2 domain in the SH3-SH2 fusion protein allowed restoration of binding to two SH3-specific polypeptides (p100 and p60 in Fig. 3A) whose binding was decreased by the presence of an unoccupied SH2 domain. Thus, in *in vitro* experiments, SH3-mediated binding can be regulated by the occupancy of a

linked SH2 domain. Conversely, the SH3 domain occupancy induced a modest increase in the SH2-dependent binding to phosphotyrosyl proteins. These results indicate that SH3 and SH2 domains expressed as adjacent domains of a fusion protein are in communication, and ligand binding modulates their interaction. The nature of structural changes in the SH3SH2 or NTSH3SH2 proteins that mediate the effects of ligand occupancy, for example, conformational changes, remains to be determined. Phosphotyrosyl peptide-induced conformational change has been demonstrated for PI 3-kinase p85 SH2 domain (64).

The ligand-dependent communication between adjacent SH2 and SH3 domains of Src family proteins can be expected to play a role in signalling provided that these domains can simultaneously interact with their respective ligands without steric hindrance from bound polypeptides, particularly since these domains are rather small. The recently determined crystal structure of the Lck SH3SH2 protein (17) showed the ligand-binding regions of covalently linked SH2 and SH3 domains to be distant, consistent with their ability to independently contact their ligands. We have recently identified a novel cellular polypeptide (named p120) which shows a proline peptide-dependent binding to SH3 domains and a phosphotyrosyl peptide-dependent binding to SH2 domains of Fyn and Lck (58). Significantly, this polypeptide is capable of simultaneously binding to adjacent SH3 and SH2 domains in the Fyn NTSH3SH2 fusion protein *in vitro*, binds to Fyn and Lck proteins *in vivo*, and is rapidly tyrosine phosphorylated upon TCR stimulation. Previous studies have also demonstrated that stable *in vivo* association of tyrosine-phosphorylated Src substrates p110 and p68 requires both the SH2 and SH3 domains of v-Src protein (31, 71).

The studies presented here also demonstrate a novel direct binding between the two Src homology domains of Src family kinases. Binding between the SH3 and SH2 domains exhibited selectivity, as a number of non-Src family SH2 domains, which were fully active in pY binding (not shown), failed to bind to Fyn or Lck SH3. However, heterologous binding between SH2 and SH3 domains of three different Src family kinases (Fyn, Lck and Src) was readily demonstrable. Moreover, only minimal binding of SH2 domains of Src kinases to the p85 PI 3-kinase SH3 domain was observed (unpublished data). Thus, it is likely that conserved structural features within the Src family SH2 and SH3 domains contribute to binding between these modules. Indeed, the crystal structure of the Lck SH3SH2 protein has demonstrated binding between the SH2 and SH3 domains, resulting in a head-to-tail dimer, and has revealed several Src-family-specific conserved residues to be involved in SH3-SH2 binding (17).

The binding between Fyn SH3 and SH2 domains does not appear to be due to the presence of pYs on the SH3 domain. Bacteria lack tyrosine kinases, and anti-pY blotting did not show specific tyrosine phosphorylation on fusion proteins. Furthermore, binding of the R176K SH2 mutant to the SH3 domain was comparable to that of the wild-type SH2 domain. Moreover, *in vitro* phosphorylation of fusion proteins with protein kinase A was not responsible for SH2-SH3 binding, since binding was also observed with unphosphorylated proteins when an anti-SH3 antibody was used for detection (20b). The Lck SH3SH2 crystal structure revealed an interaction of the putative proline-rich peptide-binding region of the SH3 domain with the SH2 domain. Several proline-rich peptides which specifically inhibited the binding of the SH3 domain to its protein ligands failed to inhibit binding between SH3 and SH2 domains. Furthermore, while the W119K mutation in the putative proline peptide-binding pouch of Fyn SH3 completely

abolished its *in vitro* binding to p85 PI 3-kinase, a substantial degree of SH2 binding was observed with this mutant. Since the crystal structure of a ligand-occupied SH3 domain is currently unavailable, a precise interpretation of these results is difficult at present. It is possible that the SH2 contact site and the ligand-binding region of the SH3 domain only partially overlap. Moreover, binding between isolated SH2 and SH3 domains may differ in some respects from that observed with the SH3SH2 protein. Further mutagenesis and structural analyses will be required to resolve these possibilities.

Although the pY-binding pocket was not required for binding between SH2 and SH3 domains, binding was exquisitely sensitive to occupancy with an SH2-specific peptide. The sensitivity of SH2-SH3 binding to ligand-induced perturbations strongly supports the likelihood that this interaction is important in the signalling function of Src family kinases. Binding between SH2 and SH3 domains could mediate either an intra- or an intermolecular interaction. We were able to show binding of purified Fyn SH3SH2 to bead-immobilized Fyn SH2 and of purified SH3 to bead-immobilized Fyn SH3SH2 fusion proteins (data not shown). Thus, both the SH2 and SH3 domains of the SH3SH2 fusion protein appear to be available for SH2-SH3 binding. This result is consistent with the crystal structure of the Lck SH3SH2 fragment, which showed binding of SH3 and SH2 domains across a dimer (17). In light of these results, we favor the idea that modulation of the ligand-binding properties of SH2 and SH3 domains when expressed as linked domains of a single molecule (potentially an intramolecular interaction) and the direct binding between SH3 and SH2 domains (potentially an intermolecular interaction) reflect two distinct SH2-SH3 interactions. However, these two types of interactions may be related and, as suggested below, may cooperatively regulate the Src kinases.

While we have examined only the interactions of noncatalytic domains of the Src family kinases (all fusion proteins with a kinase domain were insoluble and could not be tested), our results can be integrated with previous mutational analyses (2, 13, 15, 27, 42, 47, 48, 62, 70, 77) and recent crystal structural studies of Lck SH3SH2 (17) to suggest a model of how physical and functional interactions between the SH3 and SH2 domains may both promote basal repression and provide a mechanism to assemble activation-dependent signalling complexes. Eck et al. have shown that the physical interaction between the Lck SH2 and SH3 domains creates a more suitable binding groove for the tail peptide (17). We demonstrate that adjacent NT and SH3 domains enhance the affinity of the SH2 domain. Since the predominant phosphotyrosyl motif available under basal conditions is the tyrosine-phosphorylated C tail, the two mechanisms should favor the repressed state. Inhibition of the physical interaction between SH2 and SH3 domains by structural perturbations, presumably due to signals received from associated receptors and mimicked in our experiments by phosphopeptides, would open up the protein and allow it to exhibit its kinase activity. This hypothesis is fully consistent with mutational analyses that have demonstrated that the SH3, SH2, and intact C-tail pY are essential to maintain a repressed state of Src family kinases (2, 13, 15, 27, 28, 35, 42, 45, 47, 48, 62, 70, 77).

The decreased binding of the SH3 domain to some of its cellular ligands as a result of functional (as seen here) or physical interaction (17) with the SH2 domain is likely to prevent complex formation with downstream signalling proteins when the kinase is inactive. Following activation, release of the SH3 domain from the SH2 domain would make it available for binding to its ligands; this binding may be further enhanced by the occupancy of the adjacent SH2 domain by

newly generated high-affinity phosphotyrosyl motifs (such as seen here with p100 and p60; Fig. 3A). Binding of the SH3 domain to its cellular targets would then establish activation-dependent contacts and allow transmission of signals to downstream effectors. This mechanism should ensure that no signals are transduced in the absence of receptor activation. Interestingly, binding of the SH3 domains to GTPase dynamin (24) or PI 3-kinase (56) and binding of phosphopeptide or phosphorylated insulin receptor substrate 1 to the SH2 domain of PI 3-kinase activated these enzymes (6, 44). Consistent with a role for SH3-SH2 interaction in regulating the assembly of signalling complexes, stimulation of platelets with thrombin induced a redistribution of activated c-Src protein to detergent-insoluble cytoskeletal complexes (10); oncogenically activated Src protein shows an SH2-dependent constitutive localization to such complexes (21).

In conclusion, we have demonstrated two novel functional and physical interactions between the SH2 and SH3 domains of Src family kinases *in vitro*. Further *in vivo* analyses should help assess the biological role of these interactions in the regulation of Src kinases.

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