Distinct binding patterns of HS1 to the Src SH2 and SH3 domains reflect possible mechanisms of recruitment and activation of downstream molecules

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Abstract
We previously identified a gene, LckBP1, which encodes a protein that binds to the Lck SH3 domain and is identical to murine HS1. Using unstimulated T lymphocytes, we further demonstrated that Lck binds to HS1 in vivo and that HS1 is tyrosine phosphorylated upon TCR stimulation. In the present report, we analyzed the binding pattern of several src kinases and HS1 in greater detail. The Lck SH3 domain binds to HS1 constitutively, while the Lck SH2 domain associates with HS1 only upon TCR stimulation. A similar binding pattern was observed with Lyn and HS1, but not with Fyn and HS1, in which the Fyn SH2 region associates with HS1 upon TCR stimulation but the Fyn SH3 region does not associate with HS1 regardless of TCR stimulation. Such distinct binding patterns of the src kinase SH2 and SH3 domains to HS1 may represent a mechanism by which src family kinases select substrates and activate particular downstream signaling pathways.

Introduction
The Rous sarcoma virus oncogene, src, has tyrosine kinase domains as well as other unique domains, i.e. src homology modules (SH) 2 and 3 (1). The SH2 regions bind phosphorylated tyrosine residues (2,3) and different SH2 regions have different ligand specificities. Several biological functions have been postulated for the SH2 domains including changes in the localization of proteins involved in signal transduction and induction of conformational changes that alter the catalytic activity of the interacting proteins (4–6). The SH3 region binds to a stretch of ~10 amino acids that contains a proline-rich sequence, e.g. the Abi SH3 domain binds to the proline-rich region of 3BP-1,2 (7,8), the Grb2 SH3 domain binds to Sos proline-rich regions (9,10) and the Crk SH3 domain binds to Abi proline-rich regions (11).

Although the SH2 and SH3 domains have different binding specificities, some signaling proteins bind to both the SH2 and SH3 domains of src. For example, the Lck SH3 and SH2 domains bind to p120 (12); the Fyn SH3 and SH2 domains bind p62/Sam-68 (13), AFAP (14) and p120 (12); and the Src SH3 and SH2 domains bind AFAP (14). The binding pattern of p62/Sam-68 to Fyn might shed light on the mechanisms of such molecular associations through both src SH2 and SH3 regions. A mutant Fyn molecule lacking the SH3 region does not induce phosphorylation of p62/Sam-68 and does not associate with p62/Sam-68 through the src SH2 region, indicating that the initial association through the src SH3 region is required for the phosphorylation of src association molecules. Such phosphorylation is necessary for binding of src SH2 regions.

In hematopoietic cells, several src kinases have been shown to play critical roles in the development and activation of lymphocytes. Lck plays an essential role in mediating the antigen receptor signaling in T lineage cells. The observations that (i) introduction of an intact \textit{lck} gene restored the defective TCR-mediated signaling in a lck mutant human cell line (15) and (ii) injection of anti-Lck antibody into T cells resulted in a block in TCR-mediated signaling (16), suggest a requirement for Lck in TCR-mediated signaling. The SH2 domain of Lck binds to tyrosine phosphorylated CD45 (17), ZAP-70 (18) and p120 (12). Important roles of Lck are further confirmed by Lck-deficient mice. Lck-deficient mice indicate a dramatic reduction in double-positive (CD4⁺CD8⁺) thymocytes and...
hardly detectable single-positive (CD4^+CD8^- CD4^-CD8^+) thymocytes (19). In transgenic mice overexpressing a catalytically inactive version of Lck, T cell differentiation is blocked at the double-negative stage to double-positive stage (20,21). Fyn associates with the TCR complex (22), and overexpression of Fyn in mice increases tyrosine phosphorylated protein and calcium concentrations upon TCR stimulation (23). Fyn-deficient mice show a dramatically decreased calcium response (24,25). The Fyn SH2 domain binds to HS1 (26), and p120^Cbl (27). Fyn associates with the B cell receptor complex (BCR) and is activated upon BCR cross-linking, which in turn results in the activation of phosphatidylinositol-3-kinase (28). The Lyn SH2 domain also binds HS1 (26,29). The use of Lyn knock-out mice has confirmed the critical role of Lyn in the development, activation and tolerance of B cells (30,31).

Previously, we isolated LckBPI as an Lck SH3 binding protein (32) and found that LckBPI is identical to murine HS1 (33), which becomes tyrosine phosphorylated upon TCR stimulation (32,34). HS1 is also a major substrate of Lyn (29). These data suggest that HS1 mediates an antigen receptor signal through Lck in T cells and Lyn in B cells. Although HS1-deficient mice do not exhibit significant defects in T and B cell development and activation, proliferation of T and B cells obtained from these mice is reduced upon antigen receptor stimulation in vivo (34). HS1 also appears to be involved in B and T cell selection (34).

In this report, we analyzed the molecular interaction between several src kinases and HS1 in greater detail. In vitro binding analyses showed that HS1 binds constitutively to the Lck and Lyn SH3 regions and binds to their SH2 regions upon activation. Our analyses also show that HS1 binds to the Fyn SH2 region upon receptor stimulation but does not bind to the Fyn SH3 region regardless of TCR stimulation. Such a binding pattern suggests mechanisms for selection and activation of molecules downstream of src kinases.

**Methods**

**Construction of glutathione S-transferase (GST) fusion proteins**

The GST-LckSH3, GST-LckSH2, GST-LckSH32 and GST-HS1SH3 fusion proteins have been described (32). cDNA fragments of the SH3 and SH2 domains of Fyn/Lyn were generated by PCR from mRNA of B6 mouse. The amplified fragments were digested with NotI and subcloned into the pGEX-4T (Pharmacia, Uppsala, Sweden) vector. The primers used to generate the SH3 and SH2 domains are detailed below.

- **Fyn SH3 domain** [nucleotides 472–669; (35)]: 5’ primer, 5’-CCGCGGCCGCGCATGGAAGTGTGGTTGCACTTCGTTTGTG-3’; reverse primer, 5’-CCCCGCGGCCGGCTTACTCTCTCTCTAGATGGA-3’.

- **Fyn SH2 domain** [nucleotides 470–793; (36)]: 5’ primer, 5’-CCCCGCGGCCGCGCATGGAAGTGTGGTTGCACTTCGTTTGTG-3’; reverse primer, 5’-CCCCGCGGCCGGCTTACTCTCTCTCTAGATGGA-3’.

- **Lyn SH2 domain** [nucleotides 470–793; (36)]: 5’ primer, 5’-CCCCGCGGCCGCGCATGGAAGTGTGGTTGCACTTCGTTTGTG-3’; reverse primer, 5’-CCCCGCGGCCGGCTTACTCTCTCTCTAGATGGA-3’.

- **Lyn SH3 domain** [nucleotides 275–469; (36)]: 5’ primer, 5’-CCCCGCGGCCGCGCATGGAAGTGTGGTTGCACTTCGTTTGTG-3’; reverse primer, 5’-CCCCGCGGCCGGCTTACTCTCTCTCTAGATGGA-3’.

Lyn SH2 domain [nucleotides 470–793; (36)]: 5’ primer, 5’-CCCCGCGGCCGCGCATGGAAGTGTGGTTGCACTTCGTTTGTG-3’; reverse primer, 5’-CCCCGCGGCCGGCTTACTCTCTCTCTAGATGGA-3’.

**Cells and transfection**

T cell hybridoma DO-11.10 cells, kindly provided by Dr lwata (37), and WEHI231 cells were maintained in RPMI 1640 with 10% FCS. Murine NIH 3T3 from the RIKEN cell bank (Ibaraki, Japan) were maintained in DMEM with 10% calf serum. DNA transfection was done by calcium phosphate precipitation, as described by Gorman (38), with a minor modification.

**Antigen receptor stimulation**

T cell hybridoma cells were stimulated by plating on anti-CD3e-coated plastic culture dishes for 10 min. As a negative control, non-stimulated cells were used. After T cell stimulation, plates were washed three times with PBS and cells were lysed directly by the addition of TNE buffer (10 mM Tris–HCl, pH 7.8, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 mM NaF, 2 mM NaN_3, 10 μg/ml aprotinin, 10 μg/ml leupeptin). WEHI231 cells were stimulated with affinity-purified goat antibody to IgM (Jackson Immunoresearch, West Grove, PA) for 3 min. Cells were washed three times with PBS and lysed with TNE buffer.

**GST fusion protein binding assay and Western blot analysis**

Cell lysates (1–2×10^6 cells) were cleared by centrifugation and treated with excess protein A–Sepharose (Pharmacia). The pre-cleared cell lysates were incubated at 4°C overnight with glutathione–Sepharose beads (Pharmacia) bound to 50 μg of GST fusion proteins. Beads were washed five times with TNE buffer, lysed with SDS sample buffer and the resulting solutions were boiled for 10 min. Proteins were separated by SDS–PAGE and transferred to nitrocellulose filters. The filters were blocked with TBST (10 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20) containing 10% BSA (MILES, Kansas, IL) and incubated with anti-murine HS1 mAb. HS1 antibody was detected using the ECL detection system (Amersham, Arlington Heights, IL). Antibodies used include: an anti-HS1 mAb (Sumitomo Denko, Kanagawa, Japan); an anti-p120^Cbl polyclonal antibody (Santa Cruz, Santa Cruz, CA); and an anti-phosphotyrosine mAb, 4G10 (UBI, Lake Placid, NY).

**Results**

**Binding pattern of HS1 to the Lck SH2 and SH3 domains**

We previously observed an in vivo association between Lck and HS1 using unstimulated T cell lines and showed in filter binding assays that the Lck SH3 domain binds to at least two proline-rich regions of murine HS1 (32). To analyze the binding pattern of Lck and HS1 more precisely, we examined the interaction of these molecules using an in vitro binding assay. The Lck SH3, Lck SH2, Lck SH32 and HS1–SH3 domains
were expressed as fusion forms with GST (Fig. 1A). DNA sequencing to confirm each construct, including GST–Fyn and GST–Lyn recombinant constructs (Fig. 1B), revealed several differences in the nucleotide sequences of murine Fyn and Lyn from those previously reported (35,36). The differences in the DNA sequences were further confirmed by independent PCR experiments and sequencing. Such different DNA sequences result in the amino acid substitutions; position 77 isoleucine in the Fyn SH3; position 179 glutamate in Fyn SH3 and position 61 leucine in Lyn SH2 (shown as underlined letters in Fig. 1B). The substituted amino acids of Lyn and Fyn shown here are identical to those of their human counterparts (39,40). Except for these sites, the rest of the DNA sequences were identical to the murine and different from human DNA sequence, excluding the possibility of contaminated human DNA amplification and sequence. The differences might rest in the use of different mouse strains, which may have different DNA sequences. Note that we used the B6 strain of mice for these experiments. SDS–PAGE confirmed the proper size of proteins synthesized by our DNA constructs (Fig. 1C).

In our previous study, we used anti-LckBP1 (identical to anti-murine HS1) antiserum prepared by our investigators (32). In the present study, we used commercially available monoclonal anti-HS1 antibody for all experiments. To confirm the specificity of this antibody for proteins encoded by the gene we isolated as LckBP1, we transfected HS1 cDNA (identical to LckBP1 cDNA) into NIH 3T3 cells and analyzed the cell lysates by Western blot analysis; anti-HS1 antibody specifically recognized an 85 kDa product in the transfected cells (Fig. 2A, lane 2).

Cells of the T cell hybridoma line DO.11-10, with or without TCR stimulation, were lysed and incubated with GST fusion proteins non-covalently bound to glutathione–Sepharose beads. Bound proteins were separated by SDS–PAGE, transferred to nitrocellulose filters and probed with anti-HS1 antibody (Fig. 2B). In the absence of TCR stimulation, Lck SH3 (GST–LckSH3) and Lck SH3–SH2 (GST–LckSH32) bound to HS1 (Fig. 2B, lanes 5 and 9), but Lck SH2 (GST–LckSH2) did not (Fig. 2B, lane 7). Upon TCR stimulation, binding of GST–LckSH3 protein to HS1 was detected (Fig. 2B, lane 6), and a strong association of GST–LckSH2 and GST–LckSH32 proteins with HS1 was observed (Fig. 2B, lanes 8 and 10 respectively), although the band for the latter protein was more intense. This enhanced binding of GST–LckSH32 to HS1 might be explained by the increased number of HS1 molecules associating with Lck upon TCR stimulation. Alternatively, such enhancement might reflect an increased affinity of the SH2 domain for phosphorytosine induced by a structural change in HS1 molecules or the presence of the N-terminal region of Lck. In addition, the amount of HS1 associated with Lck SH3 was decreased upon TCR stimulation (Fig. 2B, lane 6). At least two mechanisms could be considered for this observation. First, the decreased binding of Lck SH3 to HS1 may be due to a structural change of HS1 by the tyrosine phosphorylation upon TCR stimulation. The other possibility is that the tyrosine phosphorylated HS1 preferentially associates with the SH2 domain of Lck. Consequently, HS1 might bind to endogenous Lck or exogenous GST–LckSH2 rather than exogenous GST–LckSH3. The GST protein alone showed no significant binding (Fig. 2B, lanes 1 and 2).

We also analyzed the association between the HS1–SH3 domain and the whole HS1 molecule. Such an association would predict intramolecular binding of two proline-rich regions in HS1 molecules with the SH3 region or dimerization.
found that Lck also binds to HS1 through the Lck SH2 region and Fyn (26) bind to HS1 through the src SH2 region. We although proof of this hypothesis awaits formal in vivo analysis. 

These data indicate that the two proline-rich regions of HS1 do not bind to the SH3 region in HS1. These data also suggest that dimerization of HS1 or intramolecular binding of the two domains in the HS1 molecule rarely occurs in vivo, although proof of this hypothesis awaits formal in vivo analysis.

Binding pattern of HS1 to the SH3 and SH2 domains of Lyn and Fyn
It has been reported that the src family kinases Lyn (29), Blk and Fyn (26) bind to HS1 through the src SH2 region. We found that Lck also binds to HS1 through the Lck SH2 region upon TCR stimulation and through the Lck SH3 domain with or without TCR stimulation (Fig. 2B). A similar binding pattern has been described for Lyn and HS1; Lyn binds to HS1 without BCR stimulation and binds to HS1 at >4-fold higher levels in vivo upon IgM cross-linking (29). Although the precise binding pattern between Lyn and HS1 was not defined in that study, the overall observations on Lck and Lyn suggest that HS1 binds constitutively to src family kinases through the src SH3 region and that the SH2 domain of src family kinases associates with phosphotyrosine in HS1 upon TCR stimulation.

To examine this hypothesis, we tested the in vitro association between other src family SH3 domains and HS1. The SH3 and SH2 domains of Fyn and Lyn were expressed as GST fusion forms (Fig. 1 shows the DNA constructs), and non-covalently bound to glutathione-Sepharose beads. T or B cell lysates with or without receptor stimulation were incubated with GST fusion protein-coupled beads, and proteins bound to the beads were separated by SDS-PAGE, transferred to nitrocellulose filters and probed with anti-HS1 antibody.

Significant binding of GST–Fyn SH3 protein (GST–FynSH3) to HS1 was not observed regardless of TCR stimulation (Fig. 3A, lanes 5 and 6), although binding of HS1 to GST–Fyn SH2 (GST–FynSH2) was greatly enhanced upon TCR stimulation (Fig. 3A, lane 8). As predicted, the GST and the GST–HS1SH3 proteins did not associate with HS1 with or without TCR stimulation (Fig. 3A, lanes 1–4). The lack of HS1 binding to Fyn SH3 might be explained by a defect in the Fyn SH3 DNA construct. To examine this possibility, we tested the binding pattern of Fyn SH3 to other signaling molecules. Based on previous reports that the GST–FynSH3 fusion protein can bind the p120<sup>cW</sup><sup>c</sup> phosphoprotein (12,41), we analyzed the association between GST–FynSH3 and p120<sup>cW</sup><sup>c</sup> in an in vitro binding assay. Cell lysates were immunoprecipitated by GST fusion protein and immunoblotted and probed with anti-phosphotyrosine antibody. The same filter was reprobed with anti-p120<sup>cW</sup><sup>c</sup> antibody. Binding of GST–FynSH3 to a tyrosine phosphorylated 120 kDa molecule (p120) was observed upon TCR stimulation (Fig. 3B, lane 4). The bands detected for p120 were confirmed for p120<sup>cW</sup><sup>c</sup> using anti-p120<sup>cW</sup><sup>c</sup> antibody (Fig. 3B, lanes 7 and 8). Thus Fyn SH3 bound to p120<sup>cW</sup><sup>c</sup>, regardless of TCR stimulation. These results are in agreement with previous observations (12,41) and exclude the possibility that our defective Fyn SH3 DNA construct fails to identify binding of HS1 to Fyn SH3. Although we cannot formally rule out possible in vitro artifact, it is likely that the GST–FynSH3 fusion protein is able to bind to other signaling molecules such as p120<sup>cW</sup><sup>c</sup>, but not to HS1. The binding pattern of HS1 and Fyn differs from that of HS1 and Lck, suggesting that the role of HS1 in binding Lck differs from its role in Fyn binding.

**HS1 binding pattern of Lyn in T and B cells**

We further examined the association between Lyn and HS1 using T and B cell lysates. Cell lysates from the B cell line WEHI231, with or without IgM cross-linking, were analyzed for binding between HS1 and Lyn SH2 using our in vitro binding assay. The GST–Lyn SH3 protein (GST–LynSH3) bound to HS1 regardless of IgM cross-linking (Fig. 4A, lanes 5 and 6). The GST–Lyn SH2 protein (GST–LynSH2) bound to HS1 upon IgM cross-linking as expected, but not
Fig. 3. HS1 (A) and p120c-cbl (B) binding pattern to Fyn. (A) DO.11-10 cells were either unstimulated (-) or stimulated (+) with CD3e and lysed in lysis buffer. Lysates were immunoprecipitated with GST (lanes 1 and 2), GST–HS1SH3 (lanes 3 and 4), GST–FynSH3 (lanes 5 and 6) or GST–FynSH2 (lanes 7 and 8) non-covalently coupled to beads. Bound proteins were immunoblotted with anti-HS1 antibody. (B) Bound proteins on GST- or GST–FynSH3-coupled beads were immunoblotted with anti-phosphotyrosine 4G10 antibody (lanes 1–4) and reprobed with anti-p120c-cbl antibody (lanes 5–8).

Fig. 4. SH3 and SH2 domains of Lyn mediate HS1 binding. (A) WEHI231 cells were either unstimulated (-) or stimulated (+) with anti-IgM antibody and lysed in lysis buffer. Lysates were incubated with GST (lanes 1 and 2), GST–HS1SH3 (lanes 3 and 4), GST–LynSH3 (lanes 5 and 6) or GST–LynSH2 (lanes 7 and 8) non-covalently coupled to beads. Bound proteins were immunoblotted with anti-HS1 antibody. (B) DO.11-10 cells were either unstimulated or stimulated with CD3e and lysed in lysis buffer. Lysates were incubated and immunoblotted as in (A).

Discussion

The src family kinases, Lyn, Blk and Fyn, have been shown to bind HS1 through the src SH2 region (26,29). We previously showed that HS1 binds to the Lck SH3 domain without TCR stimulation (32). To determine whether Lck also binds to HS1 through the Lck SH2 domain, or whether other src kinases bind to HS1 through the src SH3 domain, we analyzed the association pattern between HS1 and these src kinase molecules in vitro.

Lck and Lyn bind to HS1 in vivo without antigen receptor stimulation (29,32), suggesting that HS1 binds constitutively to src family kinases at their SH3 domains. In the present study, we show that Lyn and Lck bind HS1 through the SH3 domain of Lyn. Although the SH3 domains of src kinases have very similar amino acid sequences (Fig. 1B), each SH3 region of this family appears to have different specificity and affinity for their binding proteins.
We also show that upon TCR or BCR stimulation, tyrosine-phosphorylated HS1 binds to src kinases at the src SH2 domain. These observations suggest the following in vivo binding pattern of HS1 to Lyn and Lck: HS1 binds to Lyn and Lck constitutively and, upon receptor stimulation, HS1 might be an immediate substrate for Lyn and Lck. Phosphorylated HS1 might bind more extensively to the Lyn SH2 domain in addition to the SH3 domain. Such an induced stronger association between HS1 and Lyn or between HS1 and Lck might be required for further phosphorylation of HS1. Alternatively, such additional binding at the SH2 region might block the SH2 domain of Lyn or Lck for other signaling molecules to access their SH2 regions. Further in vivo functional studies are needed to answer this question.

Activation of Lck is regulated by intramolecular binding between tyrosine residue (Y505) and the SH2 domain (42). Since HS1 has an SH3 domain and proline-rich regions, it could associate and change tertiary structure for its regulation, as seen in Lck. No association between the SH3 domain and proline-rich regions was detected by in vitro binding assay, indicating that an intramolecular association between the HS1 and proline-rich regions is unlikely in vivo.

The Lyn SH2 and SH3 domains associate with HS1 in T cell lysates as well as in B cell lysates. Lyn is specifically expressed in B lineage, but not in T lineage cells. The Lck SH2 and SH3 domains also associate with HS1 in T (Fig. 2B) and B cell lysates (data not shown). Lck is abundantly expressed in T cells but at significantly lower levels in B cells. The binding affinity of HS1 appears to be much higher for Lyn than for Lck. These data suggest that HS1 associates with Lyn in B cells preferentially because of higher binding affinity of HS1 to Lyn and higher expression of Lyn than of Lck in B cells, while HS1 associates with Lck because of the absence of Lyn in T cells. These observations support the current hypothesis that HS1 transduces signals from Lyn in B cells and from Lck in T cells.

Several cellular proteins, including AFAP-110, HS1, p62/Sam-68 and p120C-cbl, have both potential tyrosine phosphorylation sites and proline-rich SH3 binding regions. These regions bound to the SH2 and SH3 domains of src family kinases respectively (12-14,27,41). Based on such binding potential of these proteins, the following binding pattern of these proteins is hypothesized. Cellular proteins bind to tyrosine kinase at the SH3 region constitutively, and after tyrosine phosphorylation of cellular proteins, they could bind to tyrosine kinase at the SH2 region additionally to strengthen their association.

Our observations indicate that src kinases bind constitutively to downstream signaling molecules through the SH3 domain and bind through the SH2 domain after activation. This mechanism might be generally used for selection and activation of downstream signaling molecules for src kinases.

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Abbreviations
β-gal β-galactosidase

References

BCR B cell receptor
GSt glutathione-S-transferase
LckBP1 Lck binding protein 1
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