Molecular Features Underlying the Sequential Phosphorylation of HS1 Protein and Its Association with c-Fgr Protein-tyrosine Kinase*

(Received for publication, September 28, 1998, and in revised form, December 8, 1998)

Anna Maria Brunati, Arianna Donella-Deana, Peter James‡, Manfredo Quadroni§, Antonella Contri, Oriano Marin, and Lorenzo A. Pinna¶

From the Dipartimento di Chimica Biologica, Centro di Studio delle Biomembrane del Consiglio Nazionale delle Ricerche and Centro Ricerca Interdipartimentale Biotecnologie Innovative, University of Padova, 35121 Padova, Italy, ‡Protein Chemistry Laboratory, Swiss Federal Institute of Technology Zentrum, 8090 Zurich, Switzerland, and §Biomedical Research Centre, University of British Columbia, V6T1Z3 Vancouver, British Columbia, Canada

The hematopoietic lineage cell-specific protein HS1 was shown to undergo a process of sequential phosphorylation both in vitro and in vivo, which is synergistically mediated by Syk and Src family protein-tyrosine kinases and essential for B cell antigen receptor-mediated apoptosis. We have now identified tyrosine 222 as the HS1 residue phosphorylated by the Src family protein kinases c-Fgr and Lyn, and we show that a truncated form of HS1 (HS1–208-401) lacking the N-terminal putative DNA binding region and the C-terminal Src homology 3 (SH3) domain is still able to undergo all the steps of sequential phosphorylation as efficiently as full-length HS1. We also show that a stable association of phospho-HS1 with c-Fgr through its SH2 domain requires previous autophosphorylation of the kinase and is prevented by subsequent phosphorylation of Tyr-222. Kinetic studies with HS1 and its truncated forms previously phosphorylated by Syk and with a peptide substrate reproducing the sequence around tyrosine 222 support the view that efficient phosphorylation of HS1 by Src family protein kinases entirely relies on TyrP-SH2 domain interaction with negligible, if any, contribution of local specificity determinants. Our data indicate that the proline-rich region of HS1 bordered by tyrosyl residues affected by Syk and Src family kinases represents a functional domain designed to undergo a process of sequential phosphorylation.

HS1 is an intracellular protein with expression that is limited to hematopoietic and lymphoid cells (1). The gene sequence shows that is composed of 486 amino acids with a predicted *M* of ~54 kDa. In contrast, HS1 has been constantly isolated as a protein with an apparent *M* of ~75 kDa, as judged from SDS-PAGE, giving rise to the suggestion that it might be heavily affected by post-translational modifications, e.g. glycosylation (1). The amino acid sequence of HS1 contains a variety of structurally significant motifs, including an N-terminal region responsible for the binding of mitochondrial protein HS1-associated protein X-1 (2), followed by four 37-amino acids repeats that can form a helix-turn-helix structure frequently found in the DNA binding domain of various transcriptional factors (1); this region contains three putative phosphatidylinositol 4,5-bisphosphate binding motifs and has been suggested to be involved in F-actin binding (3), a proline-rich region localized to the C-terminal central moiety, which may represent an SH3 binding motif and is ending with a stretch of proline-glutamate repeats, and an SH3 domain located at the C-terminal extremity (see schematic representation of HS1 in Fig. 1).

Evidence that HS1 plays a role in the receptor-mediated apoptosis and proliferative responses was provided by the analysis of HS1-deficient mice (4) and WEH1–231 B lymphoma cells (5–7). The observation that HS1 is readily tyrosine phosphorylated after B cell antigen receptor cross-linking, in parallel with activation of Src family kinases (8, 9), is also consistent with a role for HS1 in signal transduction. In vitro studies revealed that HS1 is not a substrate for the Src-related kinases c-Fgr, Lyn, and Fyn unless it is previously phosphorylated by the Src-unrelated protein-tyrosine kinase Syk (10, 11). Syk-mediated phosphorylation affects tyrosyl residues located just upstream from the SH3 domain (notably Tyr-378 and Tyr-397) and generates high affinity binding sites for Src family SH2 domains (12). This provides the structural basis for subsequent association with and secondary phosphorylation by c-Fgr, Lyn, and Fyn (11).

The physiological relevance of this sequential mode of HS1 phosphorylation described in vitro has been recently corroborated by the observation that in intact hematopoietic cells protein-tyrosine kinases Syk and Lyn synergistically phosphorylate HS1. Furthermore, mutation of Tyr-378 and Tyr-397 to Phe has shown that they are the residues critical for B cell antigen receptor-induced phosphorylation, and their mutation rendered the cell insensitive to apoptotic stimuli (6, 7).

These findings prompted us to undertake a study aimed at unraveling the structural features that underlie the sequential mode of HS1 phosphorylation. Here we show that a minimum core of HS1, including the primary and secondary sites of phosphorylation at its C- and N-terminal edges, respectively, but lacking the whole SH3 domain, the HS1-associated protein X-1 (HAX-1) binding motif, and the putative helix-turn-helix
region, is still able to undergo the entire process of sequential phosphorylation as efficiently as full-length HS1. We also show that the molecular mass of HS1 is not altered by post-translational modifications, consistent with the concept that abnormally high $M_c$ rather reflects marked asymmetry and rigidity of the protein.

**EXPERIMENTAL PROCEDURES**

**Materials** — $[^{32}P]ATP$ was from Amersham Pharmacia Biotech. Other chemicals were from Sigma. Recombinant c-Fgr SH2 domain fused with GST was expressed as described previously (13). Anti-GST and anti-His antibodies were from Amersham Pharmacia Biotech. Anti-c-Fgr was purchased from Santa Cruz Biotechnology. The phosphopeptide PEGDyPeeVLE was synthesized as detailed elsewhere (11).

**Protein-Tyrosine Kinases** — Syk, c-Fgr, and Lyn were purified from rat spleen as described previously (14–16).

**Peptides** — The synthesis of peptides NEMEAPTAYKKTTP and KG-GRLRPLPLPPPG was performed using an ABI 431-A automated peptide synthesizer (Applied Biosystems) equipped with 9-fluorenlymethylthoxycarbonyl chemistry according a protocol detailed elsewhere (17). A 2-chlorotrityl resin (Novabiochem), preloaded with C-terminal proline, was used as solid support for the synthesis of NEMEAPTAYKKTTP. The crude peptides were purified by high performance liquid chromatography on a Prep Nova-Pak HR C18 preparative reverse phase column, 6 μm, 25 × 10 mm (Waters). Analytical high performance liquid chromatography and matrix-assisted laser desorption and ionization time of flight mass spectrometry analysis of the purified peptides showed the correctly predicted mass and a purity of 90%.

**Construction of Recombinant Forms of HS1** — HS1 sequences encoding amino acids 1–486 and 208–486, respectively, were amplified by polymerase chain reaction from a human wild type HS1 cDNA (kindly provided by T. Watanabe) (1) using $Pfu$ polymerase (Stratagene), and cloned as BamHI-PstI fragments into the same sites of pTrcHis vector (Invitrogen). Vectors containing the HS1 inserts were cleaved with BamHI and $XhoI$ situated at position 401 of the HS1 sequence. The HS1 fragments encoding amino acids 1–401 and 208–401 were cloned again into pTrcHis. Recombinant proteins were purified as described previously (10). The His tag was cleaved by enterokinase Max (Invitrogen), and the digested proteins were purified by nickel-nitrotriacetic acid-agarose affinity chromatography (Qiagen).

**Preparation of Recombinant Forms of HS1 Primarily Phosphorylated by Syk** — Recombinant proteins (20 μg) were phosphorylated by p38α (0.5 μg) at 30 °C in the presence of 50 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, and 20 μM unlabeled ATP for 30 min. Phosphorylated forms were separated from unphosphorylated proteins, p38α, ATP, and the other reagents by glutathione-Sepharose column (300 μl) coupled to recombinant GST fused with the c-Fgr SH2 domain. The phosphorylated proteins, bound to the column, were eluted with 20 mM phosphotyrosine and detected by immunostaining with anti-phosphotyrosine antibody.

**Phosphorylation of the Recombinant Unphosphorylated or Syk-Phosphorylated Forms of HS1** — Phosphorylation of the recombinant forms of HS1 was performed at 30 °C in 30 μl of incubation mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 5 mM MnCl$_2$, 20 μM unlabeled ATP (specific radioactivity, 1000–2000 cpm/pmol), and the indicated amount of recombinant protein and tyrosine kinase. The reactions were stopped at the indicated time by addition of 2% SDS, and the samples were subjected to 10% SDS-PAGE. The degree of protein phosphorylation was evaluated either by analysis on a Packard Instant Imager or by autoradiography and counting of the identified radiolabeled bands.

**Peptide Phosphorylation** — Peptide NEMEAPTAYKKTTP was phosphorylated in the above-described basal buffer supplemented with the indicated amount of enzyme. The reaction was terminated by addition of 1 ml of 1 N HCl and processed as described elsewhere (18).

Kinetic parameters were measured by 3-min incubation in the presence of 13 and 18 nM c-Fgr and Lyn, respectively. $K_m$ and $V_{max}$ values were determined by double reciprocal plots, constructed from initial rate measurements fitted to the Michaelis-Menten equation.

**32P-Labeled Peptide Mapping of HS1** — HS1 (0.4 μg) sequentially phosphorylated by Syk in the presence of unlabeled ATP and then by c-Fgr in the presence of $[^{32}P]ATP$, as described previously, was submitted to SDS-PAGE. The gel was transferred electrophoretically to a nitrocellulose filter, and phosphorylated HS1 was localized by autoradiography. $[^{32}P]Labeled$ HS1 was excited and tryptically digested. The resulting peptides were separated by two-dimensional thin layer cellulose electrophoresis at pH 8.9, followed by ascending chromatography as described previously (19).

**Gel Filtration on Superdex 200** — The Superdex 200 column mounted on an Amersham Pharmacia Biotech fast performance liquid chroma-
Sequential phosphorylation of recombinant HS1 and its truncated forms initiated by Syk and accomplished by c-Fgr. HS1 recombinant forms (200 nM) unphosphorylated (A) or previously phosphorylated by \( \gamma^32P\)ATP for 5 min at 30 °C either in the presence of c-Fgr (A and B, lanes 1–4) or \( \gamma^32P\)ATP (A, lanes 5–8), A, lanes 1 and 5, and B, lane 1, HS1; A, lanes 2 and 6, and B, lane 2, HS1(Δ1–207); A, lanes 3 and 7, and B, lane 3, HS1(Δ402–486); A, lanes 4 and 8, and B, lane 4, HS1(Δ1–207/402–486). The samples were subjected to SDS-PAGE and the radioactive bands were evidenced by Instant Imager. The position of autoradiolabeled c-Fgr is indicated. Experimental conditions are detailed under “Experimental Procedures.” Results are representative of four separate experiments.

Fig. 3. Sequential phosphorylation of recombinant HS1 and its truncated forms initiated by Syk and accomplished by c-Fgr. HS1 recombinant forms (200 nM) unphosphorylated (A) or previously phosphorylated by \( \gamma^32P\)ATP for 5 min at 30 °C either in the presence of c-Fgr (A and B, lanes 1–4) or \( \gamma^32P\)ATP (A, lanes 5–8). A, lanes 1 and 5, and B, lane 1, HS1; A, lanes 2 and 6, and B, lane 2, HS1(Δ1–207); A, lanes 3 and 7, and B, lane 3, HS1(Δ402–486); A, lanes 4 and 8, and B, lane 4, HS1(Δ1–207/402–486). The samples were subjected to SDS-PAGE and the radioactive bands were evidenced by Instant Imager. The position of autoradiolabeled c-Fgr is indicated. Experimental conditions are detailed under “Experimental Procedures.” Results are representative of four separate experiments.
The hypothesis that the inhibitory effect of the SH2 domain was attributable to its association with phospho-HS1, thus preventing the binding of c-Fgr, was confirmed by documenting the formation of complexes between phospho-HS1 and GST-SH2 domain. This was done by two procedures: (i) gel filtration of phospho-HS1 with an efficiency that correlates to their affinity for the SH2 domains of Src kinases. Later it was corroborated by competition experiments, which showed that phosphopeptides can prevent the secondary phosphorylation of phospho-HS1 with an efficiency that correlates to their affinity for the SH2 domains of Src kinases. This concept has been now further validated by using recombinant SH2 domain of c-Fgr as potential antagonist of secondary phosphorylation of phospho-HS1 (and its truncated derivatives) by c-Fgr. As shown in Fig. 5, the phosphoradiolabelling of HS1 (previously phosphorylated by Syk with unlabeled ATP) is abolished either by adding a phosphopeptide reproducing the main site of primary phosphorylation, in agreement with previous data (11), or by adding the recombinant GST-SH2 fusion protein in the medium in which secondary phosphorylation was performed. Similar results were obtained using truncated forms of HS1 instead of the full-length protein (data not shown).

Formation of Complexes between Phospho-HS1 and Auto-phosphorylated c-Fgr—The implication of SH2 domains of Src tyrosine kinases in the secondary phosphorylation of primarily phosphorylated HS1 was first suggested by the striking similarity between the consensus sequences recognized by Syk (i.e., the agent of primary phosphorylation; Ref. 18) and, once phosphorylated, by the SH2 domains of Src kinases (12). Later it was corroborated by competition experiments, which showed that phosphopeptides can prevent the secondary phosphorylation of phospho-HS1 with an efficiency that correlates to their affinity for the SH2 domains of Src kinases (11). This concept has been now further validated by using recombinant SH2 domain of c-Fgr as potential antagonist of secondary phosphorylation of phospho-HS1 (and its truncated derivatives) by c-Fgr. As shown in Fig. 5, the phosphoradiolabelling of HS1 (previously phosphorylated by Syk with unlabeled ATP) is abolished either by adding a phosphopeptide reproducing the main site of primary phosphorylation, in agreement with previous data (11), or by adding the recombinant GST-SH2 fusion protein in the medium in which secondary phosphorylation was performed. Similar results were obtained using truncated forms of HS1 instead of the full-length protein (data not shown).

The hypothesis that the inhibitory effect of the SH2 domain was attributable to its association with phospho-HS1, thus preventing the binding of c-Fgr, was confirmed by documenting the formation of complexes between phospho-HS1 and GST-SH2 fusion protein. This was done by two procedures: (i) gel

---

**Chromatography**

FIG. 4. $^{32}$P tryptic peptide map of HS1 secondarily radiolabeled by c-Fgr. HS1 was radiolabeled by c-Fgr after a previous phosphorylation catalyzed by p38$^{\alpha}$ in the presence of unlabeled ATP. $^{32}$P-Labeled HS1 tryptic peptides were obtained and separated by two-dimensional thin layer cellulose chromatography as described under "Experimental Procedures." Radioactivity was measured by an Instant Imager. The arrow marks the origin.

---

**Fig. 5. Specific blockage of HS1 sequential phosphorylation by either c-Fgr SH2 domain or HS1(393–402) phosphopeptide.** c-Fgr activity toward HS1 primarily phosphorylated by p38$^{\alpha}$ with unlabeled ATP was measured in the presence of [$^{32}$P]ATP and increasing concentrations of either the recombinant proteins GST (■), GST-SH2 domain (○) (A) or the peptides PEGDYEEVL (▲), PEGDYEEVL (△), and KGGRLRLPLPLLPPG (○) (B). Incubations were carried out for 5 min under conditions described under "Experimental Procedures." The samples were submitted to 10% SDS-PAGE, and the radioactivity incorporated into HS1 was evaluated either by analysis on a Packard Instant Imager or by autoradiography and counting of the identified radiolabeled bands. Activity is expressed as percentage of the controls obtained in the absence of effectors. Results are means of four separate experiments performed with two different preparations of phospho-HS1. All calculated S.E. values are <10%.
HS1 plus GST-SH2 domain (B), HS1 primarily phosphorylated by p38\textsuperscript{\textalpha\textk} plus GST-SH2 domain (C), unphosphorylated c-Fgr (D), HS1 primarily phosphorylated by p38\textsuperscript{\textalpha\textk} plus unphosphorylated c-Fgr and plus 10 mM EDTA (E), autophosphorylated c-Fgr (F), HS1 primarily phosphorylated by p38\textsuperscript{\textalpha\textk} plus c-Fgr previously autophosphorylated for 10 min, plus 10 mM EDTA (to prevent secondary phosphorylation of HS1) (G), and HS1 primarily phosphorylated by p38\textsuperscript{\textalpha\textk} incubated with c-Fgr in a phosphorylation medium for 10 min (H). The amounts of c-Fgr, GST-SH2 domain, and HS1 were 1.5, 2.0, and 5.5 \mu g, respectively. 50-\mu l aliquots of the fractions eluted from the column, as indicated, were submitted to SDS-PAGE and immunostained against either anti-GST (A–C) or anti-c-Fgr (D–H). Arrows denote the elution positions of GST-SH2 domain (SH2) and c-Fgr.

The gel filtration approach was also used to monitor the formation of complexes between phospho-HS1 and the whole c-Fgr kinase (rather than its SH2 domain alone). As shown in Fig. 6D, c-Fgr alone peaks around fraction 26. Preincubation with phospho-HS1 plus \( [\text{\textsuperscript{32}P}]\text{ATP and Mg}^{2+} \) does not significantly change the elution volume of c-Fgr, which is resolved from phosphoradiolabeled HS1 (Fig. 6H), consistent with the concept that after the sequential phosphorylation is completed, HS1 and c-Fgr dissociate from each other. Surprisingly, no association between phospho-HS1 and c-Fgr could be detected even in the absence of ATP (and after addition of EDTA) to prevent the occurrence of secondary phosphorylation (Fig. 6E). It should be concluded therefore that unlike GST-SH2, the SH2 domain incorporated into c-Fgr is unable to bind phospho-HS1. If, however, c-Fgr is preincubated alone with ATP and Mg\textsuperscript{2+} and subsequently added to phospho-HS1 in the presence of EDTA (to prevent secondary phosphorylation of HS1), it coelutes, together with HS1, earlier than c-Fgr alone (Fig. 6G), revealing that the expected c-Fgr-phospho-HS1 complex has been formed. These data show that the formation of a stable complex between HS1 and c-Fgr requires not only primary phosphorylation of HS1 at its SH2 binding sites but also autophosphorylation of c-Fgr. The complex between phospho-HS1 and autophosphorylated c-Fgr (through its SH2 domain), moreover, readily dissociates with completion of secondary phosphorylation of HS1 (Fig. 6F).

**Kinetic Constants for Secondary Phosphorylation of HS1 and Its Truncated Derivatives**—The use of GST-SH2-glutathione-Sepharose (see Fig. 7) made it possible to recover sufficient amounts of phospho-HS1 derivatives free of nonphosphorylated HS1 to be used as substrate in kinetic experiments with Src kinases.

As shown in Table I, full-length phospho-HS1 displays quite a low \( K_m \) value, \( \sim 150 \text{ nM} \), using either c-Fgr or Lyn as secondary phosphorylating agent, consistent with the physiological occurrence of this process (7). The truncated forms of phospho-HS1 display similar phosphorylation efficiencies, corroborating the concept that the N-terminal HS1-associated protein X-1 binding and repeat domains and the C-terminal SH3 domain are not required for optimal sequential phosphorylation of HS1.

The high affinity of phospho-HS1 for Src kinases is likely to reflect its binding to the SH2 domain of the kinase rather than purely catalytic interactions. The sequence of the phosphoacceptor site (Tyr-222) is in fact markedly divergent from the optimal sequences selected by c-Fgr and Lyn in an oriented peptide library (20), and it lacks, in particular, the crucial hydrophobic residue at position n-1, which is important for site recognition by all Src kinases (20, 21). Indeed unphosphorylated HS1 and its truncated forms are not appreciably phosphorylated by c-Fgr (Fig. 3A, lanes 1–4). Moreover, a peptide reproducing the Tyr-222 site resulted in a negligible substrate for both c-Fgr and Lyn, displaying \( K_m \) values of \( \sim 8 \text{ ms} \) (Table I).

**DISCUSSION**

The hematopoietic lineage cell-specific protein HS1 has been shown to be implicated in receptor-mediated apoptotic and proliferative responses (6, 7). After stimulation of B lymphocytes by the antigen receptors, HS1 becomes rapidly phosphorylated on tyrosyl residues in parallel with the activation of Src.
family kinases (8). In vitro studies, however, showed that HS1 is active early after B cell stimulation through mechanisms that require three conditions to be fulfilled: (i) priming phosphorylation of p72-src or its truncated catalytic domain, p38MAPK, at sites that bind the SH2 domain of the Src kinases (11). This kind of sequential mode of phosphorylation appears to operate also in vivo, because a synergism between Syk and Src-related Lyn protein kinase-mediated phosphorylation of HS1 has been reported in hematopoietic cells lines, in which it is required for both nuclear translocation of HS1 and B cell apoptosis (7). It is important therefore to unravel the structural features underlying the sequential phosphorylation of HS1. Initial experiments have shown that the abnormal high apparent Mr observed with SDS-PAGE (75 instead of 54 kDa according to amino acid composition) of HS1 as well as of its N- and C-terminally truncated derivatives is not attributable to post-translational addition of bulky components, e.g. glycosylation or ubiquitinylation. Mass spectrometry measurements are consistent with the theoretical molecular mass, suggesting that abnormally high Mr on SDS-PAGE reflects the properties of a very asymmetrical and extended molecule. The observation that abnormal Mr is also displayed by the doubly truncated form of HS1, HS1Δ1–207/402–486, supports the view that rod-like shape and rigidity are features of its central region, mostly consisting of a proline-rich domain.

Interestingly, such a truncated core of HS1, encompassing residues 208–401 and lacking the whole N-terminal putative helix-turn-helix repeat region and the C-terminal SH3 domain, is still able to undergo all the steps of sequential phosphorylation as efficiently as full-length HS1. Primary phosphorylation sites affected by Syk are located at the end of this core (Tyr-378 and Tyr-397), whereas the site of secondary phosphorylation is close to the N-terminal end of the core (Tyr-222). This site is not affected at all by Syk, and its phosphorylation by c-Fgr and other Src family kinases is absolutely dependent on the previous phosphorylation of the primary sites. Phospho-HS1 (and its truncated forms) are outstanding substrates for Src kinases, with Km values in the nanomolar range. In contrast, unphosphorylated HS1 and its truncated derivatives and a pentadecapeptide reproducing the phosphoacceptor site (Tyr-222) are not phosphorylated to any appreciable extent. Clearly, therefore, high efficiency Tyr-222 phosphorylation is not dictated by local specificity determinants but by conformational features. These rely primarily on the interaction between phospho-HS1 and the SH2 domain of c-Fgr, because the addition of either HS1 phosphopeptide or the SH2 domain of c-Fgr prevents the phosphorylation of Tyr-222.

The formation of stable complexes between HS1 and c-Fgr requires three conditions to be fulfilled: (i) priming phosphorylation of HS1 by Syk to generate the docking sites for the SH2 domain of c-Fgr, (ii) autophosphorylation of c-Fgr, and (iii) lack of phosphate in the secondary site of HS1 (Tyr-222). Although the structural basis of the first feature is in some way self-evident, because it is well established that SH2 domains bind tyrosine only in its phosphorylated form (22), the other two points deserve some comments.

The ability of phospho-HS1 to form stable complexes with the SH2 domain of c-Fgr is proven unambiguously by the experiments with recombinant SH2 (see Fig. 6C); therefore, the failure of phospho-HS1 to bind full-length native c-Fgr came as a surprise. This would imply that either the SH2 domain of c-Fgr is already occupied by phosphotyrosine, notably the C-terminal one (Tyr-511) known to down-regulate Src kinases by intramolecular interaction with SH2 domain (23, 24), or it has a conformation that makes it inaccessible to phospho-HS1. The first possibility was ruled out by the absence of phosphotyrosine in the c-Fgr preparations used in our experiments, as judged by failure to immunoreact with anti-phosphotyrosine serum (data not shown). It has to be assumed therefore that the overall conformation of c-Fgr makes its SH2 domain inaccessible to phosphotyrosyl residues of other proteins. Apparently such a structural hindrance is overcome by autophosphorylation of c-Fgr: after preincubation with ATP and Mg2+, in fact, the complex with phospho-HS1 is readily formed. That this is caused by autophosphorylation is confirmed by effect of EDTA, which prevents both autophosphorylation and association with phospho-HS1 of c-Fgr preincubated with ATP and Mg2+ (see Fig. 6D). It has to be concluded therefore that autophosphorylation (occurring at Tyr-400) has conformational consequences resulting in a better exposure of the SH2 domain.

The observation that once Tyr-222 is phosphorylated the HS1-Fgr complex dissociated would be a trivial one if this were just a regular enzyme-substrate interaction, in which the final product is obviously released from the enzyme. In our case, however, the main interaction does not occur at the catalytic site but far away, because it involves the SH2 domain of the kinase and the primary phosphorylation site(s) of HS1 (Tyr-378 and Tyr-397). It has to be assumed therefore that this interaction is weakened by phosphorylation of Tyr-222, through an allosteric mechanism that ultimately causes the detachment of fully phosphorylated HS1 from c-Fgr. This is a nice example of a mechanism by which TyrP-SH2 interactions can be overcome without the intervention of a protein-tyrosine phosphatase that dephosphorylates phosphotyrosine.

In summary, as shown schematically in Fig. 8, the sequential phosphorylation of HS1 is under the concerted control of a variety of factors impinging on both the substrate and the kinases. First p72-src must be activated. Although the precise regulation of p72-src is still unclear, it is known that it becomes active early after B cell stimulation through mechanisms that imply the interaction of its two SH2 domains with the so-called immunoreceptor tyrosine-based activation motif phosphorylated motifs of membrane proteins (25, 26) and/or direct phosphorylation of p72-src itself (27–29). Active Syk initiates HS1

---

**TABLE I**

**Kinetic constants of phospho-HS1 and its truncated forms for c-Fgr and Lyn protein tyrosine kinases**

| Substrate | c-Fgr | Lyn |
|-----------|-------|-----|
| $K_{cat}$ | $K_m$ | Efficiency | $K_{cat}$ | $K_m$ | Efficiency |
| min$^{-1}$ | $\mu M$ | $K_{cat} \times K_m$ | min$^{-1}$ | $\mu M$ | $K_{cat} \times K_m$ |
| Phospho-HS1 | 2.7 | 0.13 | 20.7 | 2.25 | 0.15 | 15 |
| Phospho-HS1 (Δ1–207) | 2.5 | 0.17 | 14.7 | 1.95 | 0.18 | 11 |
| Phospho-HS1 (Δ402–486) | 3.6 | 0.25 | 14.4 | 2.40 | 0.24 | 10 |
| Phospho-HS1 (Δ1–207/402–486) | 4.2 | 0.09 | 46.6 | 2.10 | 0.10 | 21 |
| NEMEAPTIAKKTP | 0.13 | 7.5 $\times 10^3$ | 17 $\times 10^{-6}$ | 0.17 | 8.2 $\times 10^3$ | 20 $\times 10^{-6}$ |
phosphorylation by acting on its primary sites (notably Tyr-378 and Tyr-397). Most likely this primary phosphorylation takes place by simple recognition of consensus sequences specified by several acidic residues surrounding the tyrosyl residues. This point of view is supported by the observation that short peptides reproducing the Tyr-397 site are readily phosphorylated by either \( \text{pTyr}^{397} \) and \( \text{pTyr}^{385} \) (lacking both the SH2 domains) with very favorable kinetic constants (18), suggesting they include most of the structural elements required for binding to the kinase. Primary phosphorylation of HS1 generates the SH2 binding motif(s) that are a prerequisite for the third step, i.e. recruitment of phospho-HS1 by an Src kinase. Interestingly, however, as discussed above, this step also requires, at least in the case of c-Fgr, previous autophosphorylation of the Src kinase, which in its unphosphorylated form is unable to bind phospho-HS1. This binding, as demonstrated here and in previous work (11), is a necessary condition for the fourth step (secondary phosphorylation) to take place: accordingly a synthetic peptide reproducing the sequence around Tyr-222 but obviously lacking the SH2 domain is not appreciably phosphorylated by c-Fgr (or by another Src kinase, Lyn), displaying a \( K_m \) value of 7.5 mM.

A minimum core region of HS1 spanning between the SH2 domain anchoring sites (Tyr-378 and Tyr-378) and the secondary phosphorylation site (Tyr-222) and including the whole “proline-rich domain” (with its C-terminal tail also termed the “proline-glutamate repeats”; Ref. 1) and the putative nuclear localization sequence represents a necessary and also a sufficient condition for the process of sequential phosphorylation to occur. This core is also responsible for the abnormally high apparent \( M_c \) of HS1 on SDS-PAGE and, even more, on gel filtration, suggesting that the proline-rich region has an asymmetric, rod-shaped conformation.

Murine LckBP1 protein has been suggested to be the murine counterpart of human HS1 (30). Interestingly, the tyrosyl residues implicated in sequential phosphorylation of HS1 are also conserved in LckBP1, corroborating the notion that ability to undergo sequential phosphorylation represents a crucial feature of this class of hematopoietic proteins. In contrast, this may not be the case of cortactins, another class of proteins sharing a number of features with HS1 and LckBP1, in which the proline-rich domain is shorter and the tyrosyl residues implicated in sequential phosphorylation are not conserved (30).

Although the proline-rich region of LckBP1 has been shown to bind the SH3 domain of Lck kinase (30), our data would rule out that such an interaction might play a crucial role in the phosphorylation of HS1 by c-Fgr or Lyn. First, in fact, we could not detect any phosphorylation-independent association of HS1 with c-Fgr. Second, the prolyl-rich peptide RGGRLRPLPPG known to interact with Src SH3 domains (31) failed to affect the phosphorylation of phospho-HS1 at its Tyr-222 site by either c-Fgr (Fig. 5B) or Lyn (data not shown).

Acknowledgment—We are grateful to Dr. T. Watanabe for generously providing the human HS1 cDNA.

REFERENCES

1. Kitamura, D., Kanelco, H., Miyagoe, Y., Ariyasu, T., and Watanabe, T. (1989) Nucleic Acids Res. 17, 9367–9379
2. Suzuki, Y., Demoliere, C., Kitamura, D., Takeshita, H., Deuschel, U., and Watanabe, T. (1997) J. Immunol. 158, 2736–2744
3. He, H., Watanabe, T., Zhan, X., Huang, C., Schuuring, E., Fukami, K., Takenaka, T., Kumar, C. C., Simpson, R. J., and Maruta, H. (1998) Mol. Cell. Biol. 18, 3829–3837
4. Tanisaki, I., Kitamura, K., Maekawa, Y., Fukuda, T., Kishi, H., and Watanabe, T. (1995) EMBO J. 14, 3664–3678
5. Benhamou, L. E., Watanabe, T., Kitamura, D., Cazeneve, P. A., and Sarthou, P. (1994) Eur. J. Immunol. 24, 1995–1999
6. Fukuda, T., Kitamura, D., Tanisaki, I., Maekawa, Y., Benhamou, L. E., Sarthou, P., and Watanabe, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7302–7306
7. Yamanashi, Y., Fukushima, T., Nishizumi, H., Inazu, T., Ishiwa, Y., Kitamura, D., Iida, T., Yamanohara, T., and Yamamoto, T. (1997) J. Exp. Med. 185, 1387–1392
8. Yamanashi, Y., Okada, M., Semba, T., Yamori, T., Umemori, H., Tsumasawa, S., Toyoshima, K., Kitamura, D., Watanabe, T., and Yamamoto, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3631–3635
9. Hata, D., Nakamura, T., Kawakami, T., Kawakami, Y., Herren, B., and Mayumi, M. (1993) Immunol. Lett. 60, 65–71
10. Brunati, A. M., Rizzuene, M., James, P., Guerra, B., and Pinna, L. A. (1995) Eur. J. Biochem. 229, 164–170
11. Rizzuene, M., Brunati, A. M., Marin, O., Donella-Deana, A., and Pinna, L. A. (1996) Biochemistry 35, 5327–5332
12. Songyang, Z., Shoelson, S. E., McGlade, J., Olivier, P., Pawson, T., Bustin, X. R., Barbacid, M., Sahai, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R. A., and Cantley, L. C. (1994) Mol. Cell. Biol. 14, 2777–2785
13. Brunati, A. M., Pinna, L. A., Bergantino, E., Rizzuene, M., Cirri, F., Ramponi, G., and Donella-Deana, A. (1998) Biochem. Biophys. Res. Commun. 243, 700–705
14. Brunati, A. M., James, P., Guerra, B., Rizzuene, M., Donella-Deana, A., and Pinna, L. A. (1996) Eur. J. Biochem. 240, 490–497
15. Brunati, A. M., James, P., Donella-Deana, A., Matsukawa, B., Robbins, K. C., and Pinna, L. A. (1993) Eur. J. Biochem. 216, 323–327
16. Donella-Deana, A., James, P., Staudenmann, W., Cesaro, L., Marin, O.,...
Brunati, A. M., Ruzzene, M., and Pinna, L. A. (1996) *Eur. J. Biochem.* **235**, 18–25
17. Marin, O., Meggio, F., Sarno, S., and Pinna, L. A. (1997) *Biochemistry* **36**, 7192–7198
18. Brunati, A. M., Donella-Deana, A., Ruzzene, M., Marin, O., and Pinna, L. A. (1995) *FEBS Lett.* **367**, 149–152
19. Boyle, W. J., Van der Geer, P., and Hunter, T. (1991) *Methods Enzymol.* **201**, 111–149
20. Ruzzene, M., Songyang, Z., Marin, O., Donella-Deana, A., Brunati, A. M., Guerra, B., and Pinna, L. A. (1997) *Eur. J. Biochem.* **246**, 433–439
21. Songyang, Z., and Cantley L. C. (1995) *Trends Biochem. Sci.* **20**, 470–475
22. Pawson, T., and Gish, G. D. (1992) *Cell* **71**, 359–362
23. Sicheri, F., Moarefi, I., and Kuriyan, J. (1997) *Nature* **385**, 602–609
24. Xu, W., Harrison, S. C., and Eck, M. J. (1997) *Nature* **385**, 595–602
25. Shiue, L., Zoller, M. J., and Brugge, J. S. (1995) *J. Biol. Chem.* **270**, 10498–10502
26. Boyle, W. B., Rollhardt, A. L., Chao, H.-G., Matsueda, G. R., and Bolen, J. B. (1995) *J. Biol. Chem.* **270**, 11590–11594
27. Chu, D. H., Spits, H., Peyron, J. F., Rowley, R. B., Bolen, J. B., and Weiss, A. (1996) *EMBO J.* **15**, 6251–6261
28. Williams, S., Couture, C., Gilman, J., Jascul, T., Deckert, M., Altman, A., and Mustelin, T. (1997) *Eur. J. Biochem.* **245**, 84–89
29. Kurosaki, T. (1997) *Curr. Opin. Immunol.* **9**, 309–318
30. Takemoto, Y., Furuta, M., Li, X.-K., Strong-Sparks, W.-J., and Hascimoto, Y. (1995) *EMBO J.* **14**, 3403–3414
31. Weng, Z., Thomas, S. M., Rickles, R. J., Taylor, J. A., Brauer, A. W., Seidel-Dogan, C., Michael, W. M., Dreyfuss, G., and Brugge, J. S. (1994) *Mol. Cell. Biol.* **14**, 4509–4521