SH2 Domains Mediate the Sequential Phosphorylation of HS1 Protein by p72^{syk} and Src-Related Protein Tyrosine Kinases†

Maria Ruzzene, Anna Maria Brunati, Oriano Marin, Arianna Donella-Deana, and Lorenzo A. Pinna*

Dipartimento di Chimica Biologica, Università di Padova, e Centro per le Biomembrane del Consiglio Nazionale delle Ricerche, Via Trieste 75, 35121 Padova, Italy

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ABSTRACT: The protein tyrosine kinase p72^{syk} readily phosphorylates hematopoietic lineage cell-specific protein p50/HS1 with high stoichiometry (up to 4 mol of P_i/mol of protein) and favorable kinetic constants (K_m 77 nM, k_{cat} 0.37 s^{-1}), at sites that display the motif that is specifically recognized by the SH2 domains of Src tyrosine kinases. Such a phosphorylation converts p50/HS1 into a good substrate for c-Fgr, which in contrast is nearly inactive on nonphosphorylated p50/HS1. A phosphopeptide reproducing one of the main p50/HS1 site affected by p72^{syk}, but neither its dephosphorylated derivative nor other phosphopeptides with different structure, blocks the secondary phosphorylation of phospho(p50/HS1) by c-Fgr but not its primary phosphorylation by p72^{syk}. It also prevents the coimmunoprecipitation of phospho(HS1) with c-Fgr by anti-(c-Fgr) antibodies. In contrast the HS1[393−402] phosphopeptide is ineffective on the kinase activity of c-Fgr when tested with peptide substrates, showing that inhibition of p50/HS1 phosphorylation is not exerted at the catalytic site of c-Fgr. The sequential phosphorylation of p50/HS1 as well as its specific blockage by the HS1 phosphopeptide is also observable if c-Fgr is replaced by two other Src-related kinases, namely, Lyn and Fyn, as secondary phosphorylating agents. None of these Src-related kinases, however, can carry out the phosphorylation of p50/HS1 at the sites affected by p72^{syk}, even after prolonged incubation. Our data suggest that sequential phosphorylation might represent a general mechanism by which p72^{syk} and other Syk-related kinases generate substrates for Src-related protein tyrosine kinases. They also show that sequential phosphorylation (requiring the concerted action of a primary and a secondary kinases) cannot be surrogated by "processive" phosphorylation where a single kinase catalyzes both the primary and secondary phosphorylation, although both these modes of multiple phosphorylation are based on interactions between SH2 domains of the kinase and phosphotyrosyl sites of the substrate.

The reversible phosphorylation of seryl, threonyl, and tyrosyl residues of proteins provides a general and widespread mechanism for cell regulation. It has been calculated that a third of all proteins in eukaryotes undergo phosphorylation (Hubbard & Cohen, 1993), while up to 3% of the eukaryotic genome is likely to code for protein kinases, i.e., the enzymes responsible for protein phosphorylation. These figures corroborate the concept that most protein kinases are pleiotropic enzymes, each of them committed to the phosphorylation of numerous protein substrates which in some instances may exceed 100 (e.g., Pinna, 1994). Such a situation, in conjunction with the observation that often an individual protein is phosphorylated by several kinases at distinct residues, makes the problem of protein kinase selectivity a formidable one. In the case of Ser/Thr protein kinases a lot of evidence has been accumulated that the primary structure around the target residue plays a primary role as specificity determinant. The site specificity of protein tyrosine kinases, especially the non-receptor ones, is, with few exceptions, less stringent than that of Ser/Thr protein kinases (Pinna & Donella-Deana, 1994). This feature, in conjunction with the observation that non-receptor protein kinases are very often equipped with modules, the so-called Src homology domains (SH2 and SH3)[1] committed to molecular recognition, support the view that substrate recognition by this class of protein kinases might crucially depend on these domains. The involvement of SH2 domains in substrate recruitment by receptor protein tyrosine kinases is already well documented (Kazlauskas, 1994). The SH2 domain of the non-receptor Abl tyrosine kinase has been recently shown to play a prominent role in the binding and hyperphosphorylation of p130^{CAS}, a model substrate that has multiple potential phosphorylation sites (Mayer et al., 1995). The term "processive phosphorylation" has been coined to describe this mechanism, which implies the preliminary low-efficiency phosphorylation of a tyrosine which, once phosphorylated, promotes high-affinity binding to Abl SH2 domain, followed by the "secondary" phosphorylation of additional sites. Similar examples of processive phosphorylation by Abl and Lyn tyrosine kinases have been described also by Duyster et al. (1995) and Donella-Deana et al. (1996), respectively. On the other hand, we have studied the mechanism of *in vitro* phosphorylation of the hematopoietic lineage cell-specific p50/HS1 protein (Kitamura et al., 1989), a major substrate of non-receptor tyrosine kinases, highly conserved between human and mouse (Kitamura et al., 1995),

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* Address correspondence to this author at the Dipartimento di Chimica Biologica, Università di Padova, Via Trieste 75, 35121 Padova, Italy.

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[1] Abbreviations: SH2, Src homology 2; SH3, Src homology 3; TPK, tyrosine protein kinase; CSK, C-terminal Src kinase; FSO_2BzAdo, *p*-fluorosulfonylbenzoyl 5′-adenosine; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

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readily phosphorylated upon cross-linking of surface IgM and IgD in B cells (Yamanashi et al., 1993; Hata et al., 1993), and of the surface receptor for IgE ($Fc_\epsilon R$) on mast cells (Fukamachi et al., 1994). HS1 protein has been suggested to play a relevant role in signal transduction, since its expression is reduced in mouse B lymphoma cell variants resistant to IgM cross-linking (Benhamou et al., 1994), and the expression of the human HS1 protein in these cell lines can restore a normal sensitivity to apoptosis induced by IgM cross-linking (Fukuda et al., 1995). The importance of HS1 protein for the proliferative responses to antigen receptor cross-linking in splenic B and T cells has been further demonstrated by generating HS1-deficient mice (Taniuchi et al., 1995). We have shown that *in vitro* efficient phosphorylation of p50/HS1 by the Src-related protein tyrosine kinases c-Fgr and Lyn requires the previous phosphorylation of p50/HS1 by the Src-unrelated tyrosine kinase p38/TPKIIB (displaying high similarity with the catalytic domain of $p72^{syk}$) at site(s) ideally suited for interacting with the Src kinase SH2 domains (Brunati et al., 1995a). Such sequential phosphorylation, if proved to occur through SH2 interactions, would be reminiscent of the "processive" phosphorylation, differentiated, however, by the implication of two instead of one tyrosine kinases: a "primary" kinase is committed to the phosphorylation of tyrosyl residue(s) which are recognized by the SH2 domains of the "secondary" one (a Src kinase). This latter can then phosphorylate additional residues. Here we show that indeed previous phosphorylation of p50/HS1 by either $p72^{syk}$ or p38/TPKIIB promotes an SH2-dependent association with several Src-related tyrosine kinases followed by the phosphorylation of new sites. Prolonged direct incubation with the Src kinases alone does not obviate the requirement of p38/TPKIIB or $p72^{syk}$ as priming agents.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP was from Amersham. Antibodies anti-(c-Fgr) (C-1), directed against residues 492−507, were from Santa Cruz Biotechnology. All other proteins and chemicals were from Sigma.

Purification of Tyrosine Kinases. p38/TPKIIB, CSK, c-Fgr, Fyn, $p72^{syk}$, and Lyn were purified from rat spleen, as described by Brunati et al. (1992, 1993, 1995b) and Donella-Deana et al. (1992). All purified tyrosine kinases were free of phosphotyrosine, as judged by lack of reactivity with anti-phosphotyrosine antibodies (monoclonal PY20, from ICN Biochemical).

Peptide Synthesis. The phosphopeptides and their unphosporylated analogues were obtained as described by Donella-Deana et al. (1995).

Purifiction of p50/HS1. p50/HS1 was purified form rat spleen cytosol, as described by Brunati et al. (1995a).

Phosphorylation of p50/HS1. Phosphorylation of p50/HS1 was performed at 30 °C in a final volume of 20 μL, in the presence of 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 10 μM [γ-^{32}P]ATP (specific activity 500−2000 cpm/pmol), and the tyrosine kinase. The amounts of the enzymes used were 5−10 ng of p38/TPKIIB, 10−20 ng of $p72^{syk}$, 50−100 ng of c-Fgr, and 50−100 ng of Lyn. The amount of p50/HS1 was 100 ng, unless indicated otherwise. For the evaluation of ^{32}P incorporated, the reactions were stopped by addition of 2% SDS and the samples were subjected to SDS−11% PAGE, followed by autoradiography. The radioactive band was excised and radioactivity was determined in a liquid scintillator. K_m and V_{max} values were determined by double-reciprocal plots, constructed from initial-rate measurements fitted to the Michaelis−Menten equation. The values reported are the means of at least three separate experiments; the SE value was less than 15%.

Preparation of Phosphorylated p50/HS1. p50/HS1 (2.5 μg) was phosphorylated by $p72^{syk}$ (500 ng) in the presence of 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 20 μM unlabeled ATP for 45 min and purified by MonoQ FPLC, as described by Brunati et al. (1995a). Phosphorylated p50/HS1 (with a stoichiometry of about 4 mol of P_i/mol of protein) was eluted by 0.45 M NaCl, whereas the nonphosphorylated species eluted at 0.40 M NaCl. No evidence was obtained of species with intermediate degrees of phosphorylation.

Phosphopeptide Mapping of p50/HS1. p50/HS1 phosphoradiolabeled by incubation with the indicated tyrosine kinase was resolved by SDS−PAGE, transferred electrophoretically to nitrocellulose filters and localized by autoradiography. Tryptic and V8 peptide mappings were performed as described elsewhere (Brunati et al., 1995a).

Peptide Phosphorylation Assay. c-Fgr activity on an angiotensin II derivative, containing an additional Arg residue at the C-terminus, was assayed at 30 °C in a final volume of 50 μL, in the presence of 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 10 μM [γ-^{32}P]ATP (specific activity 2000 cpm/pmol), 1.5 mM angiotensin, and 100 ng of c-Fgr. The reaction (kept in the linear range with respect to time and enzyme concentration) was stopped after 15 min by spotting the samples on phosphocellulose paper (P-81) and washing with 0.5% phosphoric acid (Glass et al., 1978).

Immunoprecipitation. Approximately 0.3 μg of p50/HS1, phosphorylated by $p72^{syk}$ in the presence of [γ-^{32}P]ATP, was incubated with anti-(c-Fgr) IgG and 0.5 μg of FSO_2BzAdo-treated c-Fgr (Ruzzene et al., 1994), at 0 °C for 3 h. The immunocomplexes were precipitated and analyzed by SDS−11% PAGE and autoradiography, as described by Brunati et al. (1995a).

RESULTS

Previous Phosphorylation by $p72^{syk}$ Is a Prerequisite for P50/HS1 Phosphorylation by Src-Related c-Fgr. $p72^{syk}$ readily phosphorylates p50/HS1, at the same sites also affected by p38/TPKIIB (Brunati et al., 1995a), as judged by superimposable ^{32}P-peptide mappings (not shown). This was expected considering the relatedness of $p72^{syk}$ and p38/TPKIIB [see Brunati et al. (1995a)]. Phosphorylation reaches a plateau after 20 min of incubation with a stoichiometry approaching 4 mol of P_i/mol of protein. The kinetic values for phosphorylation of p50/HS1 by $p72^{syk}$ are $K_m = 77$ nM and $k_{cat} = 0.37$ s^{-1}.

A typical experiment showing the sequential mechanism of p50/HS1 phosphorylation is illustrated in Figure 1. It can be seen that the direct phosphorylation of p50/HS1 by c-Fgr is modest (Figure 1A, lane 2). Previous incubation of p50/HS1 with $p72^{syk}$ and unlabeled ATP/Mg^{2+}, however, converts p50/HS1 into an excellent substrate for c-Fgr (lane 3). The omission of either $p72^{syk}$ or ATP/Mg^{2+} in the preincubation medium abolishes subsequent c-Fgr-catalyzed phosphorylation (lanes 4 and 5). This latter, moreover, is proportional

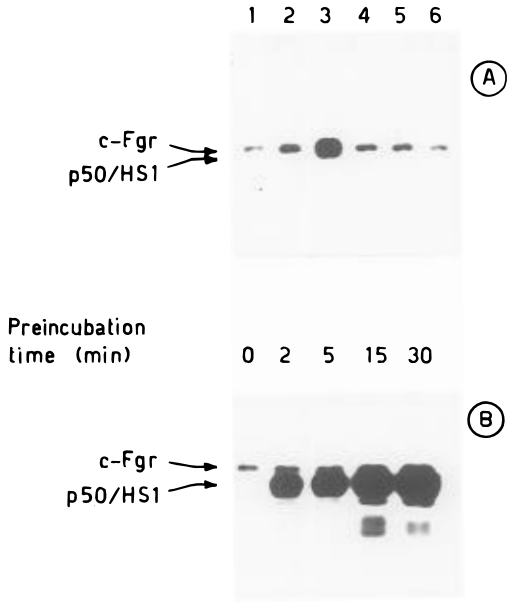

FIGURE 1: Sequential phosphorylation of p50/HS1 initiated by p72^{syk} and accomplished by c-Fgr. (**A**) Purified p50/HS1 was preincubated for 30 min at 30 °C alone (lane 2) or in the presence of p72^{syk} plus unlabeled ATP/$MnCl_2$ (lane 3), unlabeled ATP/$MnCl_2$ without p72^{syk} (lane 4), p72^{syk} without ATP/$MnCl_2$ (lane 5), or c-Fgr plus unlabeled ATP/$MnCl_2$ (lane 6). At the end of preincubation, the samples were heated for 20 min at 60 °C to inactivate p72^{syk}, cooled to 30 °C, and incubated for 20 min with c-Fgr and [γ-^{32}P]-ATP. Lane 1 shows the autophosphorylation of c-Fgr in the absence of p50/HS1. (**B**) Purified p50/HS1 was preincubated with p72^{syk} and 40 μM unlabeled ATP/$MnCl_2$ for the indicated times, before blockage as in panel A. By analogy with a parallel experiment with radioactive ATP, the stoichiometry of primary phosphorylation by p72^{syk} was esimated to be 0.7, 2.0, 3.4, and 3.8 mol of P_i/mol of p50/HS1 after 2, 5, 15, and 30 min of incubation, respectively. All the samples were further incubated for 20 min at 30 °C with c-Fgr and [γ-^{32}P]ATP and radioactive phosphorylations were detected by SDS−PAGE and autoradiography. The autoradiograph is shown. The radioactive phosphate transferred by c-Fgr into p50/HS1 previously phosphorylated by 30 min of incubation with p72^{syk} was estimated to approach 3 mol of P_i/mol of protein by scintillation counting of the excised band. The positions of c-Fgr and p50/HS1 are indicated. Other experimental details are described under Materials and Methods.

to the extent of primary phosphorylation as outlined by the experiment of Figure 1B, showing that the phosphorylation rate by c-Fgr increases by increasing the primary phosphorylation, which is proportional to the time of preincubation with unlabeled ATP/Mg^{2+} and p72^{syk}. This corroborates the concept that only the p50/HS1 molecules phosphorylated by p72^{syk} are susceptible to phosphorylation by c-Fgr. It should be noted that preincubation with unlabeled ATP/Mg^{2+} and either c-Fgr (Figure 1A, lane 6) or two other Src-related protein kinases, Lyn and Fyn (not shown), does not trigger any subsequent phosphorylation by c-Fgr, highlighting the specific requirement of p72^{syk} in order to convert p50/HS1 into a target for c-Fgr.

This point of view is further corroborated by the kinetic experiments of Figure 2, comparing phospho(p50/HS1) (obtained by incubation with p72^{syk} and subsequently separated from unphosphorylated p50/HS1 and p72^{syk} by Mono-Q FPLC) and nonphosphorylated p50/HS1. Even when the concentration of the substrates is increased, unphosphorylated p50/HS1 remains a poor target for c-Fgr, whereas the phosphorylation of phospho(p50/HS1) dramatically increases according to a saturation curve whose sigmoidal shape

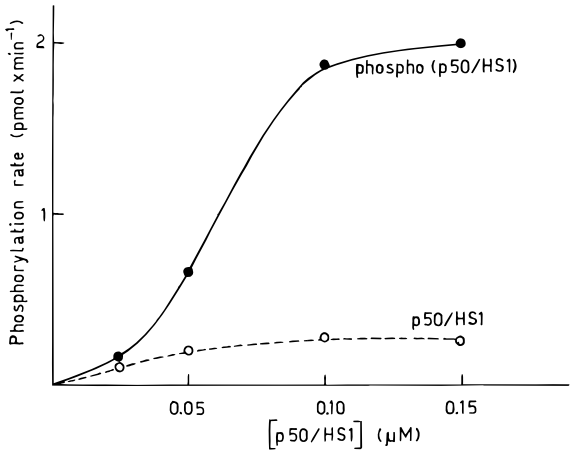

FIGURE 2: Kinetics of phosphorylation of p50/HS1 and phospho-(p50/HS1) by c-Fgr. c-Fgr was incubated at 30 °C for 10 min in the presence of 40 μM [γ-^{32}P]ATP with increasing amounts of either p50/HS1 (○) or p50/HS1 previously phosphorylated by p72^{syk} in the presence of unlabeled ATP and purified by Mono-Q FPLC (●). The radioactivity incorporated into the protein was evaluated by SDS−PAGE of the samples, autoradiography, excision of the band, and scintillation counting. Other experimental details are described under Materials and Methods.

suggests some sort of cooperativity by which the substrate (p50/HS1 phosphorylated by p72^{syk}) exerts a positive allosteric effect on its secondary phosphorylation by c-Fgr.

^{32}P-Peptide mappings showed that, as expected, the p50/HS1 sites affected by p72^{syk} are the same as those which are phosphorylated by Syk-related p38/TPKIIB (not shown). These sites are located in the C-terminal region of p50/HS1 and are distinct from those phosphorylated by c-Fgr (Brunati et al., 1995a).

p72^{syk} Promoted Sequential Phosphorylation of p50/HS1 Is Also Catalyzed by Lyn and Fyn Protein Tyrosine Kinases. c-Fgr is one of the members of the Src family, including nowadays not less than 10 members, sharing the same overall structure and remarkable similarities inside their conserved catalytic, SH2 and SH3 domains (Mustelin et al., 1994). It was interesting, therefore, to check whether the sequential phosphorylation of p50/HS1 initiated by p72^{syk} and p38/TPKIIB might represent a general mechanism for generating substrates for other members of the Src family, besides c-Fgr. Special attention has been given to Lyn and Fyn, since both are implicated in the activation of B cells (Clark et al., 1992) where HS1 protein also is involved, and the former has been reported to phosphorylate HS1 protein in stimulated Daudi cells (Yamanashi et al., 1993). As shown in Figure 3, similar to c-Fgr, neither Lyn nor Fyn appreciably phosphorylates p50/HS1 unless this protein is previously phosphorylated by incubation with unlabeled ATP/Mg^{2+} in the presence of either p38/TPKIIB or p72^{syk}. This finding supports the concept that p72^{syk} and/or related protein tyrosine kinases are committed to the generation of substrates for the Src-related protein tyrosine kinases through a sequential phosphorylation mechanism.

Sequential Phosphorylation of p50/HS1 Is Specifically Prevented by a HS1-Derived Phosphopeptide. It has been already observed (Brunati et al., 1995a) that the rationale for sequential phosphorylation of p50/HS1 could reside in the peculiar structure of the sites affected by p38/TPKIIB, two of which display the motif (Yp−acidic−acidic−hydrophobic) which binds with high affinity to the SH2

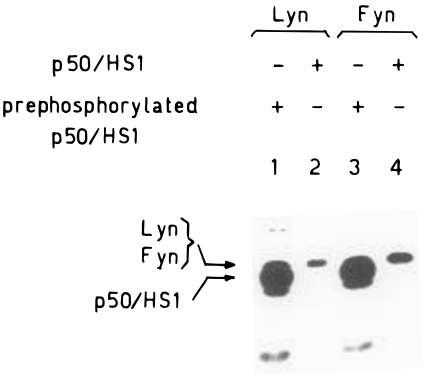

FIGURE 3: Sequential phosphorylation of p50/HS1 initiated by p72^{syk} and accomplished by Lyn and Fyn. p50/HS1 was preincubated for 30 min at 30 °C in the presence (lanes 1 and 3) or in the absence (lanes 2 and 4) of p72^{syk} and unlabeled ATP/$MnCl_2$. Samples were then heated at 60 °C for 20 min to inactivate p72^{syk} and further incubated for 20 min at 30 °C in the presence of [γ-^{32}P]-ATP plus Lyn (lanes 1 and 2) or Fyn (lanes 3 and 4). Radioactive phosphorylations were detected by SDS−PAGE and autoradiography. The positions of the tyrosine kinases and p50/HS1 are indicated. Other experimental details are described under Materials and Methods.

domains of the Src family protein tyrosine kinases (Songyang et al., 1993, 1994). A peptide reproducing one of these sites is indeed an outstanding substrate for p72^{syk}, while it is nearly unaffected by Src-related protein kinases Lyn and c-Fgr, and any modification in the (Y)EEV triplet determining high-affinity binding to the Src SH2 domains is also detrimental for p72^{syk}-catalyzed phosphorylation (Brunati et al., 1995b). The phosphoderivative of this peptide, PEGDYpEEVLE, was therefore synthesized in order to analyze its ability to prevent the sequential phosphorylation of p50/HS1 by competing against the binding of phospho(p50/HS1) to Src kinases. As shown in Figure 4, the HS1 phosphopeptide but not its dephosphorylated derivative, nor a number of phosphopeptides with different structure (not shown), actually prevents, in a dose-dependent manner, the phosphorylation of phospho-(p50/HS1) by c-Fgr (panel A). Similar results were obtained if Lyn or Fyn was used instead of c-Fgr (not shown). The stringent specificity of this inhibition is highlighted by the observation that the same phosphopeptide neither inhibits (but rather stimulates) c-Fgr activity assayed with a peptide substrate not susceptible to sequential phosphorylation (panel B) nor affects the primary phosphorylation of p50/HS1 by p72^{syk}. This latter is conversely inhibited by the nonphosphorylated peptide, presumably through a competitive mechanism at the catalytic site (panel C). In summary, the HS1 phosphopeptide specifically prevents the phosphorylation of *prephosphorylated* p50/HS1 by the Src kinases.

These data in conjunction with the finding that the HS1 phosphopeptide also prevents the coimmunoprecipitation of prephosphorylated p50/HS1 with c-Fgr by anti-(c-Fgr) antibodies (Figure 5), provides the evidence for a pivotal role of SH2-mediated interactions between Src kinases and prephosphorylated p50/HS1 in the sequential mode of p50/HS1 phosphorylation. Consistent with this interpretation, the HS1 phosphopeptide increases the activity of c-Fgr downregulated by CSK (not shown), suggesting that it binds to the same site where Tyr-511 phosphorylated by CSK exerts its inhibitory effect (Superti-Furga et al., 1993; Ruzzene et al., 1994).

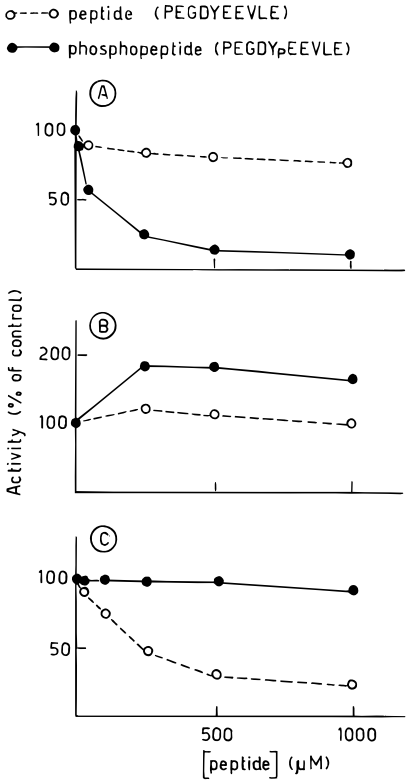

FIGURE 4: Specific blockage of sequential phosphorylation of p50/HS1 by HS1[393−402] phosphopeptide. c-Fgr activity toward p50/HS1 previously phosphorylated by p72^{syk} (**A**) or toward angiotensin II (**B**) was measured in the presence of increasing concentrations of the peptide HS1[393−402], PEGDYEEVLE (○) or its phosphorylated derivative, PEGDYpEEVLE (●). The effect of the same peptides on the primary phosphorylation of p50/HS1 by p72^{syk} is shown in panel **C**. Incubations were carried out for 15 min at 30 °C under conditions described in Materials and Methods. Activity is expressed as percentage of the controls, obtained in the absence of HS1 peptides.

FIGURE 5: Coimmunoprecipitation of c-Fgr and phospho(p50/HS1) is prevented by the phosphopeptide HS1[393−402]. p50/HS1, phosphoradiolabeled by p72^{syk} in the presence of [γ-^{32}P]ATP, was incubated with anti-(c-Fgr) IgG and FSO_2BzAdo-inactivated c-Fgr, with no further additions (lane 1) or in the presence of HS1[393−402] phosphopeptide (lane 2) or its nonphosphorylated derivative (lane 3). The presence of p50/HS1 in the pellets after immunoprecipitation was detected by SDS−PAGE and autoradiography. The peptides, when present, were 1 mM.

DISCUSSION

The work described here provides a mechanistic explanation for the role of SH2 domain of non-receptor tyrosine kinases in mediating the sequential phosphorylation of a multivalent substrate. The basal observation was that the hematopoietic lineage cell-specific protein p50/HS1 can be efficiently phosphorylated by Src-related c-Fgr only after it has been extensively phosphorylated by p38/TPKIIB (Brunati

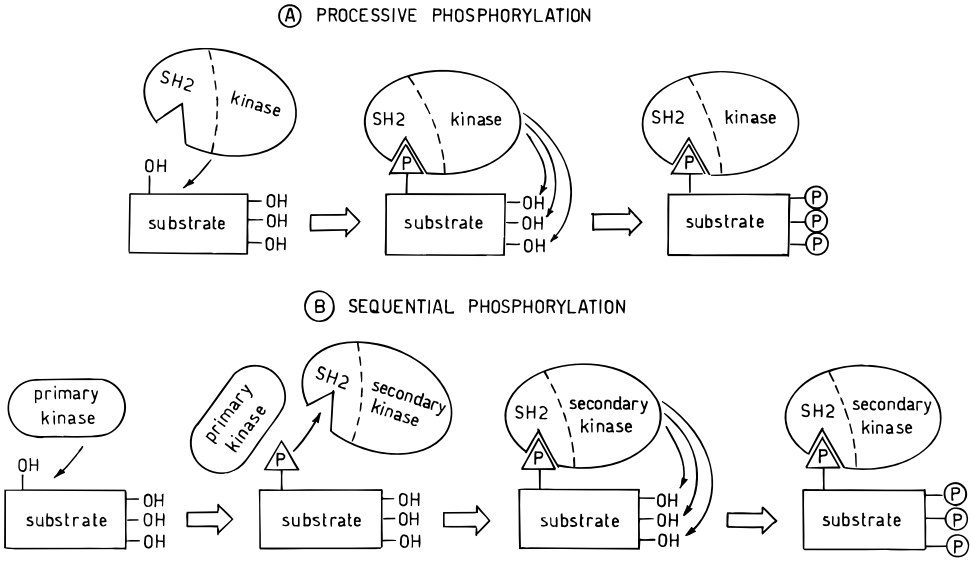

FIGURE 6: Comparison of processive phosphorylation (Mayer et al., 1995; Duyster et al., 1995) with the sequential phosphorylation model described in this paper.

et al., 1995a), a protein tyrosine kinase very similar to the catalytic domain of p72^{syk} (Brunati et al., manuscript in preparation). Here we show that (i) p72^{syk} can surrogate p38/TPKIIB in the initial step of p50/HS1 sequential phosphorylation; (ii) two other members of the Src family, namely, Lyn and Fyn, can replace c-Fgr in the second step of p50/HS1 sequential phosphorylation; (iii) the "secondary" sites phosphorylated by the Src kinases are distinct from the "primary" sites phosphorylated by p72^{syk} and p38/TPKIIB; (iv) prolonged incubation of p50/HS1 with increasing amounts of Src kinases alone does not trigger any stoichiometric phosphorylation comparable to that promoted by the sequential mechanism; (v) a phosphopeptide featured after one of the known phosphorylation sites for p72^{syk} and p38/TPKIIB in p50/HS1 interrupts the sequential phosphorylation after the initial step by preventing the "secondary" phosphorylation of p50/HS1 (previously phosphorylated by the Syk kinases); and (vi) the same peptide also prevents the coimmunoprecipitation of phospho(p50/HS1) with c-Fgr and Lyn while it is ineffective on the "primary" phosphorylation of p50/HS1 by p72^{syk}.

The concept that the HS1 phosphopeptide is acting through specific interaction with the Src kinase SH2 domains is corroborated by a number of observations, including (a) failure of its dephosphorylated derivative as well as a number of phosphopeptides lacking the consensus for Src SH2 domains to interrupt the sequential phosphorylation of p50/HS1; (b) specificity of inhibition of phospho(p50/HS1) phosphorylation, while the phosphorylation of peptide substrates incapable of interacting with Src SH2 domains is not inhibited at all by the HS1 phosphopeptide; (c) capability of the HS1 phosphopeptide to interfere with other properties of Src kinases that depend on the availability of free SH2 domains, like inhibition by CSK. Collectively these observations, in conjunction with the strikingly overlapping consensus sequences for p72^{syk}-mediated phosphorylation and Src SH2 domain recognition (Brunati et al., 1995b), strongly support a mechanism of sequential p50/HS1 phosphorylation as depicted in Figure 6. The possible physiological relevance of such a mechanism is strongly suggested by the findings that HS1 protein is readily phosphorylated upon B cell activation (Yamanashi et al., 1993; Hata et al., 1993) and also represents the major ligand for SH2 domains of the Src family kinases (Baumann et al., 1994). The recurrence, moreover, of phosphoacceptor sites like that of p50/HS1, ideally featured for both phosphorylation by p72^{syk} and binding to Src SH2 domains, but not for direct Src-mediated phosphorylation in a number of proteins (Table 1), suggests that p72^{syk} and/or related kinases may play a general role in generating protein substrates readily phosphorylatable by Src-related kinases. Such a hypothesis is corroborated by the phosphorylation of one such protein, erythrocyte band 3, through a sequential mechanism similar to that of p50/HS1 (Brunati et al., 1995c).

Table 1: Natural Occurrence of Phosphosacceptor Sites Featured for both Efficient Phosphorylation by p72^{syk} and High-Affinity Binding to Src SH2 Domains[a]

sequence	protein	Tyr position
EDEPEGDYEEV	human HS1 protein	(Y397)
EELQDDYEDM	human erythrocyte band 3	(Y8)
ENLEQEEYEDP	human erythrocyte band 3	(Y21)
EEEGKDEYDEV	human erythrocyte band 3	(Y904)
LTSEEYEEL	bovine polyoma large T	(Y8)
ERVDDGDYDDV	rat 50S ribosomal protein L11	(Y151)
AALEKDYEEV	rat tubulin α chain	(Y432)
EEEGEAYEEP	rat B-cell CD19	(Y409)
EEEPQYEEI	hamster middle T antigen	(Y324)
EGDEIYEDL	human Vav oncogene	(Y126)

[a] Proteins containing the motif tyrosine−acidic−acidic−hydrophobic were found with the FASTA and BLAST programs used to search the Swiss Protein data base. A subset of proteins, presenting several acidic residues upstream from the tyrosine, with special reference to one at position −1, were selected.

The sequential phosphorylation model proposed here is reminiscent of but distinct from multiple phosphorylation involving "phosphate-directed" protein kinases, either Ser/Thr-specific (Roach, 1991) or Tyr-specific (Donella-Deana et al., 1993). In this latter case the primary kinase phosphorylates a residue which is directly recognized as specificity determinant by the secondary "phosphate-directed" kinase that will phosphorylate another residue nearby. Roach (1991) coined the term "hierarchical phosphorylation" to indicate this kind of multiple interdependent phosphorylation. In contrast the sequential mechanism dealt with here implies that the primary phosphorylation activates a module that is

recognized by a domain of the secondary kinase which is distinct from the catalytic one (in our case, the SH2 domain of the Src kinases). Considering these substantial differences and to avoid confusion, we propose to apply the term "sequential" phosphorylation to the mechanism described here instead of "hierarchical" phosphorylation, as it was used in a previous paper (Brunati et al., 1995a).

On the other hand, "sequential" phosphorylation is also distinct from the "processive" phosphorylation mediated by SH2 domains as well, but involving just a single protein kinase instead of two (Mayer et al., 1995; Duyster et al., 1995). We propose that distinctive features, such as the presence of an acidic residue at position −1, which optimize phosphorylation by p72^{syk} while hampering Src-mediated phosphorylation (Brunati et al., 1995b; Songyang et al., 1995), may be crucial in determining the implication of two different tyrosine kinases rather than one, in the sequential mechanism of phosphorylation as opposed to the processive one. The diversity between these mechanisms, schematically represented in Figure 6, is highlighted by our observation that the p50/HS1 phosphoacceptor sites affected by p72^{syk} and p38/TPKIIB, whose phosphorylation initiates the whole process, are entirely unaffected by Src kinases even under extreme conditions (high kinase to substrate ratios and prolonged incubation). In other words, the complete phosphorylation of p50/HS1 can be achieved only through the sequential and not the processive mechanism. It should be concluded therefore that these are distinct and independent mechanisms, though both exploit the interaction between the SH2 domain of the kinase and phosphotyrosyl residue(s) of the substrate which is going to be multiply phosphorylated. Both processive and sequential phosphorylations ultimately indicate that the substrate specificity of many non-receptor tyrosine kinases may depend on the binding specificity of their associated SH2 domains. Their *raison d'etre*, however, may be quite distinct; processive phosphorylation looks like a device for amplifying a message by broadening the specificity of phosphorylation; in contrast, sequential phosphorylation displays the feature of a "two keys" mechanism aimed at rendering tighter the control over crucial steps of signaling pathways.

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