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Thrombin-induced Tyrosine Phosphorylation of HS1 in Human Platelets Is Sequentially Catalyzed by Syk and Lyn Tyrosine Kinases and Associated with the Cellular Migration of the Protein*

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Thrombin stimulation of platelets triggers Tyr phosphorylation of several signaling proteins, most of which remain unidentified. In this study, we demonstrate for the first time that hematopoietic lineage cell-specific protein 1 (HS1) undergoes a transient Tyr phosphorylation in human platelets stimulated with thrombin. The protein is synergistically phosphorylated by Syk and Lyn tyrosine kinases according to a sequential phosphorylation mechanism. By means of specific inhibitors (PP2, SU6656, and piceatannol) and phosphopeptide-specific antibodies, as well as by coimmunoprecipitation and binding competition experiments, we show that Syk acts as the primary kinase that phosphorylates HS1 at Tyr^{397} and that Syk phosphorylation is required for HS1 interaction with the Lyn SH2 domain. Upon docking to Syk-phosphorylated HS1, Lyn catalyzes the secondary phosphorylation of the protein at Tyr^{222}. Once the secondary Tyr phosphorylation of HS1 is accomplished the protein dissociates from Lyn and undergoes a dephosphorylation process. HS1 Tyr phosphorylation does not occur when thrombin-induced actin assembly is inhibited by cytochalasin D even under conditions in which Syk and Lyn are still active. Immunofluorescence microscopic analysis shows that the agonist promotes HS1 migration to the plasma membrane and that the inhibition of Lyn-mediated secondary phosphorylation of HS1 abrogates the subcellular translocation of the protein. All together these results indicate that HS1 Tyr phosphorylation catalyzed by Syk and Lyn plays a crucial role in the translocation of the protein to the membrane and is involved in the cytoskeleton rearrangement triggered by thrombin in human platelets.

Platelet activation is accompanied by a remarkable increase in tyrosine phosphorylation of a variety of signaling proteins. The phosphorylation occurs in successive waves suggesting that distinct mechanisms, taking place in temporal order, regulate the protein tyrosine kinase and/or phosphatase activities during the activation process (1–3). The non-receptor tyrosine kinases belonging to Src and Syk/ZAP70 families are among the enzymes implicated in the platelet Tyr-phosphorylating events (1–7). A phosphotyrosine proteomic approach showed that more than 60 proteins are unique in thrombin-activated human platelets when compared with resting platelets, but the identity of most of them remains unknown (8).

Hematopoietic lineage cell-specific protein 1 (HS1)[1] is an intracellular protein expressed exclusively in hematopoietic cells (9) and suggested to play a crucial role in epo-induced differentiation of erythroid cells (10). The protein, which is structurally related to cortactin, contains a variety of structurally significant motifs, including an amino-terminal domain responsible for the binding to the Arp2/3 complex (11), followed by four 37-amino acid repeats that can form a helix-turn-helix structure frequently found in the DNA binding domain of various transcriptional factors (9). The latter region contains: three phosphatidylinositol biphosphate-binding motifs supposed to be involved in F-actin binding (12), a proline-rich region that represents a SH3 (Src homology 3) binding motif and an SH3 domain located at the COOH-terminal extremity. In B-lymphocytes, T-lymphocytes, and mast cells HS1 becomes highly Tyr phosphorylated following receptor engagement (13–15), in parallel with the activation of Syk and Src-related tyrosine kinases (13, 14, 16–19). Direct association of HS1 with Lyn and Lck tyrosine kinases has been described in T-cell and during the erythropoietin-induced differentiation of erythroid cells (10, 19). Evidence that HS1 plays a role in the receptor-mediated apoptosis and proliferative responses was provided by experiments performed with HS1-deficient mice (20). However, the workings of the hemostatic process in HS1-deficient animals was not mentioned either in this (20) or in other reports. Tyr phosphorylation of HS1 is required for B-cell receptor-induced B cell apoptosis (16, 21) and leads to its caspase-mediated cleavage (22). The last process is hindered by HS1 phosphorylation mediated by the Ser/Thr protein kinase CK2 (23).

We have previously demonstrated that recombinant HS1 can be synergistically phosphorylated *in vitro* by Syk and tyrosine kinases belonging to the Src family (24, 25). Syk phosphorylates Tyr^{378} and Tyr^{397} of the protein and generates high affinity binding sites for the Src family SH2 (Src homology 2) domain. This provides the structural requisite for the subsequent association with and the secondary phosphorylation of HS1 by Src-related tyrosine kinases (24, 25). Because the HS1 sequential phosphorylation shown *in vitro* has never been demonstrated in intact cells, we examined both the occurrence and the mechanism of HS1 Tyr phosphorylation in thrombin-stim-

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[1] The abbreviations used are: HS1, hematopoietic lineage cell-specific protein 1; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo-[3,4*d*]pyrimidine; piceatannol, *trans*-3,3′,4,5′-tetrahydroxystilbene; GST, glutathione *S*-transferase; SH2, Src homology 2; SH3, Src homology 3; IP, immunoprecipitates.

ulated human platelets. Moreover, as these particular cells are devoid of nucleus and possess only part of the machinery required for apoptotic cell death, we investigated new potential physiological roles for HS1 Tyr phosphorylation.

Our results demonstrate that HS1 undergoes a transient Tyr phosphorylation upon thrombin-induced stimulation of human platelets. The sequential steps underlying the mechanism of HS1 Tyr phosphorylation, synergistically catalyzed by Syk and Lyn tyrosine kinases, were dissected. Tyr phosphorylation of the protein is suggested to be dependent on actin assembly and involved in HS1 cellular migration to the plasma membrane associated with thrombin-elicited platelet activation.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—[γ-^{32}P]ATP was purchased from Amersham Biosciences. Recombinant Lyn SH2 domain and the cytoplasmic domain of band 3 were expressed and purified as previously described (26). Human plasma α-thrombin (~1000 NIH units per mg of protein) and phosphatase inhibitor mixture were from Sigma. PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo-[3,4*d*]pyrimidine), PP3, SU6656, piceatannol (*trans*-3,3′,4,5′-tetrahydroxystilbene), cytochalasin D, and protease inhibitor mixture were from Calbiochem (Darmstadt, Germany). The peptide cdc2 was synthesized as described elsewhere (27). Other reagents were from Sigma.

Anti-Lyn and anti-Syk polyclonal antibodies, raised against protein residues 44–63 and 257–352, respectively, and goat anti-mouse fluorescein isothiocyanate secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody specific for HS1, raised against the peptide reproducing the sequence from Val^{306} to Ser^{320} of human HS1 was used for immunoprecipitation experiments (24); the monoclonal anti-HS1 antibody used for Western blotting was purchased from BD Biosciences (San Diego, CA). Phosphopeptide-specific polyclonal antibodies HS1(Tyr(P)^{397}) and HS1(Tyr(P)^{222}) were developed in New Zealand White rabbits against phosphopeptides corresponding to the following sequences of human HS1: Cys-APTTA-(P)YKKTTT (amino acids 217–226) and PEGD(P)YEEVLE-Cys (amino acids 393–402). Anti-phosphotyrosine (PY-20) monoclonal antibody was purchased from ICN Biotechnology (Irvine, CA).

Purification of Tyrosine Kinases—The non-receptor tyrosine kinases c-Fgr, Lyn, and Syk were purified from rat spleen as previously described (27–29).

Platelet Isolation and Treatment—Blood was obtained from healthy human donors with their informed consent and according to the Declaration of Helsinki. All donors denied ingestion of drugs that are known to interfere with platelet function for at least 1 week before blood sampling. Platelet-rich plasma and washed platelets were prepared, as previously reported (30), from fresh blood that was immediately mixed with one-sixth volume of acid citrate-dextrose anticoagulant (85 mM sodium citrate, 71 mM citric acid, 111 mM dextrose, pH 6.5) also containing 1 μg/ml prostacyclin, 50 milliunits/ml hirudin, and 20 μg/ml apyrase. Platelet stimulation was performed at 37 °C with 50 milliunits/ml of α-thrombin in the presence of 0.5 mM $CaCl_2$. Unless otherwise indicated, the following compounds were separately added to the cellular suspension for the indicated times before thrombin stimulation: PP2, PP3, and SU6656 (5 μM, 5 min), piceatannol (15 μM, 5 min), cytochalasin D (10 μM, 2 min), pervanadate, prepared by mixing equimolar solutions of hydrogen peroxide and sodium orthovanadate (1 mM, 6 min). Controls for experiments containing inhibitors of protein kinases and phosphatases were routinely performed with the same amount of vehicle used for inhibitor addition.

Anti-HS1, Anti-Syk, Anti-Lyn, and Anti-GST Immunoprecipitations—Control and treated platelets (60×10^6 for each assay) were lysed by suspension (1 h at 4 °C) in 450 μl of buffer A containing 20 mM Tris/HCl, pH 7.5, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 50 mM NaCl, 1 mM sodium orthovanadate, phosphatase inhibitor mixture, and protease inhibitor mixture. After centrifugation ($14{,}000 \times g$ for 30 min), the supernatants were incubated for 5 h at 4 °C with the appropriate antibody bound to protein A-Sepharose. The immunocomplexes were washed three times by centrifugation and resuspension in 50 mM Tris/HCl, pH 7.5, containing protease inhibitor mixture and 1 mM sodium orthovanadate. The pellets were solubilized in 65 μl of SDS-PAGE sample buffer (50 mM Tris/HCl buffer, pH 8.9, containing 5 mM EDTA, 380 mM glycine, 2% SDS, and 1% β-mercaptoethanol), boiled for 5 min at 100 °C, subjected to SDS-PAGE (10% gels), transferred to nitrocellulose membranes, and immunostained with the appropriate antibody.

Tyrosine Kinase Activity Assays of Immunoprecipitates (IP)—Assays of the tyrosine kinase activity of immunocomplexes obtained with anti-Lyn and anti-Syk antibodies, as described above, were performed in 30 μl of incubation mixture containing 50 mM Tris/HCl, pH 7.5, 10 mM $MnCl_2$, 30 μM [γ-^{32}P]ATP (specific activity 0.45 nCi/pmol), 200 μM sodium orthovanadate and either 300 ng of the cytoplasmic domain of band 3 or 200 μM cdc2-(6–20) peptide (26, 27), which served as specific substrates for Syk and Lyn, respectively. After 10 min incubation at 30 °C, phosphorylation of the cytoplasmic domain of band 3 was analyzed by SDS-PAGE followed by autoradiography. In the experiments with the cdc2-(6–20) peptide, the reactions were stopped by spotting 25 μl of the incubation mixture onto P81 phosphocellulose paper, which was then processed as described elsewhere (27).

Subcellular Localization of HS1, Syk, and Lyn—Platelets were sonically disrupted in 1 ml of isotonic buffer containing 50 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 1 mM orthovanadate, and protease inhibitor mixture. Cellular debris was isolated by 10 min centrifugation at $900 \times g$. The supernatant was centrifuged for 1 h at $200{,}000 \times g$ to separate the cytosol from the particulate fraction. The pellet was then incubated for 1 h in buffer A containing 1% Triton X-100 instead of 1% Nonidet P-40. The Triton X-100-soluble fraction was obtained by centrifugation for 1 h at $200{,}000 \times g$. The remaining Triton-resistant material was referred to as the membrane cytoskeletal fraction. All steps were carried out at 4 °C.

Immunolocalization of HS1 by Fluorescence Microscopy—After stimulation with 50 milliunits/ml thrombin for 1 min at room temperature, either with or without pretreatment with 5 μM PP2, platelets were fixed with 2% paraformaldehyde for 5 min at room temperature, washed twice in phosphate-buffered saline (1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, and 2.7 mM KCl, pH 7.4), and settled on glass slides. Platelets were permeabilized in 0.03% saponin-containing phosphate-buffered saline for 15 min at room temperature and then incubated for 1 h at 37 °C with monoclonal anti-HS1 antibody diluted 1/10 in phosphate-buffered saline, supplemented with 1% bovine serum albumin. After two washes with phosphate-buffered saline, HS1 labeling was revealed by incubation with goat anti-mouse fluorescein isothiocyanate secondary antibody for 1 h at 37 °C. Incubation with only the secondary antibody was used as negative control. Coverslips were then mounted in FluorSave Reagent™ (Calbiochem) for the fluorescence microscopic observation of cells that was carried out using a Zeiss Axiovert 100 (Zeiss, Oberkochen, Germany) equipped with a digital CCD videocamera AxioCam Hr (Zeiss). Data were acquired and analyzed by AxioVision software (Zeiss).

Immunostaining—Proteins transferred to nitrocellulose membranes were incubated with the indicated antibody followed by the appropriate biotinylated second antibody and developed using an enhanced chemiluminescent detection system (ECL, Amersham Biosciences). The densitometric analysis of the anti-Tyr(P)-HS1 spots was performed by Image Station 440 (Kodak).

RESULTS

HS1 Undergoes a Transient Tyr Phosphorylation in Human Platelets Stimulated by Thrombin—Stimulation of human platelets with thrombin triggered an increase of protein Tyr phosphorylation and the phosphorylation degree enhanced in the presence of pervanadate, a broad protein tyrosine phosphatase inhibitor (Fig. 1*A*). HS1 is one of the proteins undergoing a transient Tyr phosphorylation, as judged by anti-Tyr(P) immunostaining of anti-HS1 IP obtained from cellular lysates (Fig. 1*B*, *lanes 1–5*). It is noteworthy that, despite the predicted molecular mass of about 54 kDa, the protein was constantly isolated on SDS-PAGE with an apparent molecular mass around 75 kDa, because of the rod-like shape and rigidity of its central region (25). Preliminary experiments, performed with different thrombin concentrations, showed that HS1 Tyr phosphorylation reached the maximum at different stimulation times depending on the agonist concentration: the higher the thrombin concentration the faster the protein Tyr phosphorylation peak attainment. Henceforward we present results obtained upon platelet treatment with 50 milliunits/ml thrombin, which elicited HS1 Tyr phosphorylation that peaked at about 1 min of incubation and gradually declined thereafter. Because the decrease of protein Tyr phosphorylation was not ascribable to lesser amounts of immunoprecipitated HS1 (Fig. 1*B*), the occurrence of a dephosphorylation process, which rapidly fol-

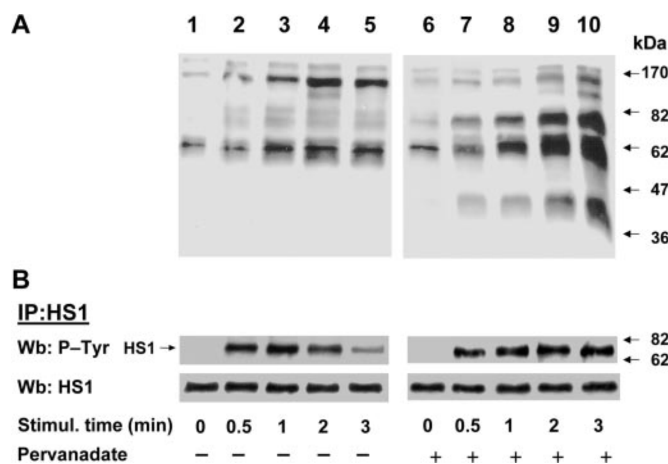

FIG. 1. **Tyr phosphorylation of HS1 in thrombin-stimulated platelets.** Platelets were preincubated in the absence (*lanes 1–5*) or presence of 1 mM sodium pervanadate (*lanes 6–10*) for 6 min and then stimulated with 50 milliunits/ml thrombin for the indicated times. *A*, cells were lysed and aliquots of cell lysates were analyzed by Western blotting with anti-Tyr(P) antibody. *B*, cell lysates were immunoprecipitated with anti-HS1 antibody and the blots of the immunocomplexes were immunostained with anti-Tyr(P) antibody, stripped, and reprobed with anti-HS1 antibody. The molecular mass markers are indicated on the *right*. Technical details are reported under "Experimental Procedures." The figure represents at least six separate experiments. *Wb*, Western blot.

lowed the protein Tyr phosphorylation could be hypothesized. To validate this hypothesis the platelets were stimulated in the presence of the tyrosine phosphatase inhibitor pervanadate. In these conditions the extent of the HS1 Tyr phosphorylation triggered by thrombin persisted throughout the time studied (Fig. 1*B*, *lanes 6–10*).

Syk and Src-related Tyrosine Kinases Are Involved in HS1 Tyr Phosphorylation Triggered by Thrombin—In the attempt to assess whether in platelets Syk and Src kinases were indeed involved in HS1 Tyr phosphorylation, the cells were stimulated with thrombin in the presence of either the Syk inhibitor piceatannol or two structurally unrelated Src kinase inhibitors, PP2 and SU6656 (31–33). Preliminary *in vitro* experiments performed with Syk and Src tyrosine kinases purified from rat spleen (27–29) showed that PP2 and SU6656 were highly selective for Src kinases and did not affect Syk activity, whereas piceatannol inhibited also the Src kinases although with an IC_{50} 4–6-fold higher than that displayed toward Syk (results not shown). On the basis of these findings we tested the action of the three inhibitors in thrombin-stimulated platelets. Fig. 2*A* shows the *in vitro* kinase activity exhibited by anti-Syk IP obtained from platelets stimulated in the absence or presence of kinase inhibitors. Thrombin treatment of human platelets induced a marked, although transient, increase of Syk activity compared with that of resting cells (Fig. 2*A*, *columns 1–3*). As expected, whereas PP2 and SU6656 did not appreciably affect Syk tyrosine kinase (Fig. 2*A*, *columns 4–9*), piceatannol almost completely inhibited this enzyme (Fig. 2*A*, *columns 10–12*).

The Src family consists of at least nine non-receptor tyrosine kinases (Src, Lyn, Fyn, Fgr, Lck, Hck, Yes, Yrk, and Blk), which regulate a wide spectrum of events (34). Among the Src kinases expressed in platelets we focused our attention on Lyn because this tyrosine kinase has been demonstrated both to constitutively associate with HS1 in resting hematopoietic cells (10, 19) and phosphorylate this protein upon B-cell receptor stimulation (13, 16). Immunoprecipitation experiments performed, in parallel with those of Fig. 2*A*, with anti-Lyn antibody showed that while Lyn activity in control platelets was almost undetectable, it was up-regulated (by about 9-fold) after 1 min of thrombin stimulation, and declined by about 25% after a further minute of incubation (Fig. 2*B*, *columns 1–3*). The reduced activities of both Syk and Lyn, detected after 2 min of thrombin stimulation, were expected as a consequence of the translocation of the two enzymes to the cellular cytoskeletal fraction from which the tyrosine kinases could not be immunoprecipitated. Treatment of platelets with either PP2 or SU6656 almost completely counteracted the Lyn activity (Fig. 2*B*, *columns 4–9*), whereas 15 μM piceatannol decreased by about 20% this enzyme activity (Fig. 2*B*, compare *columns 2* and *3* with *columns 11* and *12*). Because piceatannol concentrations higher than 15 μM substantially reduced the Lyn activity also, this inhibitor concentration was used in all following experiments.

To verify the specific contribution of Syk and Lyn in the cellular HS1 Tyr phosphorylation we analyzed this parameter in anti-HS1 IP obtained from platelets stimulated in the presence of the different inhibitors. Fig. 2*C* shows that the peak of HS1 Tyr phosphorylation triggered by thrombin is less in the presence of the Src inhibitor PP2 or SU6656 (compare *lanes 2* with *lanes 5* and *8*). However, in these conditions, the residual HS1 anti-Tyr(P) immunostaining did not further decrease in the following minute (Fig. 2*C*, *lanes 5* and *6* and *8* and *9*). This finding suggests that the Src-catalyzed phosphorylation of HS1 is a requisite for the following protein dephosphorylation, *i.e.* for the decrease of the phosphorylated protein amount observed after 2 min incubation in control conditions (Fig. 2*C*, *lanes 2* and *3*). The finding that PP3, an inactive analogue of PP2, was ineffective not only on Lyn activity but also on HS1 Tyr phosphorylation (not shown) supported the assumption of involvement of Src kinase(s) in the protein phosphorylation. When platelets were stimulated in the presence of piceatannol, which in our conditions inhibited drastically Syk and only partially Lyn (Fig. 2, *A* and *B*), the thrombin-elicited HS1 Tyr phosphorylation was completely abolished (Fig. 2*C*, *lanes 11* and *12*).

To confirm that the HS1 tyrosines phosphorylated in platelets by Syk and Lyn were the same residues previously identified by us in *in vitro* experiments (24, 25), we prepared two antibodies raised against synthetic phosphopeptides, which reproduced the HS1 sequences containing Tyr^{397} ($HS1(Tyr(P)^{397})$) and Tyr^{222} ($HS1(Tyr(P)^{222})$), which are phosphorylated *in vitro* by Syk and Lyn, respectively (24, 25).

HS1 IP obtained, as in Fig. 2, from platelets stimulated either in the absence or presence of the tyrosine kinase inhibitors were analyzed by Western blot with the two phosphopeptide-specific antibodies. These experiments confirmed that HS1 residues Tyr^{397} and Tyr^{222} were indeed phosphorylated in thrombin-stimulated platelets (Fig. 3, *lanes 1–3*) and validated the selective action of the inhibitors used in our experimental conditions. In fact addition of PP2 or SU6656 abrogated only the secondary Lyn-catalyzed phosphorylation of HS1 Tyr^{222} (Fig. 3, *lanes 4–9*), whereas piceatannol abolished the phosphorylation of both residues by inhibiting the primary Syk-catalyzed phosphorylation, which is a prerequisite for the occurrence of the secondary Lyn-catalyzed process (Fig. 3, *lanes 10–12*).

HS1, Once Phosphorylated by Syk, Interacts with Lyn—As mentioned in the Introduction, we have previously demonstrated that recombinant HS1 is phosphorylated *in vitro* according to a sequential mechanism, which implies that the Syk-phosphorylated HS1 residues act as docking sites for the protein binding to the SH2 domain of Lyn that, in turn, catalyzes the secondary phosphorylation of the protein (24, 25). In the previous paragraph we demonstrated that Syk phosphorylation is a prerequisite for the Src-catalyzed phosphorylation of the protein. To verify the occurrence of the interaction between

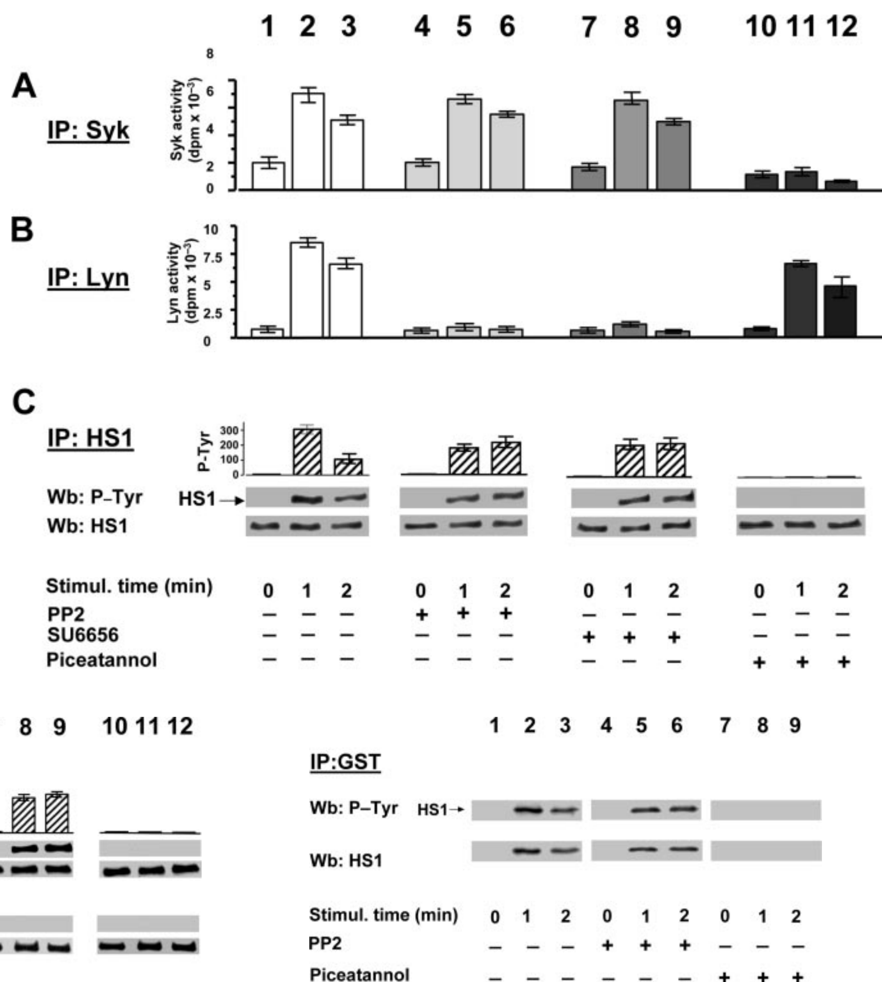

FIG. 2. **HS1 Tyr phosphorylation in platelets stimulated with thrombin either in the absence or presence of tyrosine kinase inhibitors.** Platelets, preincubated in the absence (*lanes 1–3*) or presence of 5 μM PP2 (*lanes 4–6*), 5 μM SU6656 (*lanes 7–9*), or 15 μM piceatannol (*lanes 10–12*) were stimulated with 50 milliunits/ml thrombin for the indicated times. Cellular lysates were then immunoprecipitated with anti-Syk (*A*), anti-Lyn (*B*), or anti-HS1 (*C*) antibodies. Syk activity (*A*) and Lyn activity (*B*) of the immunocomplexes were tested *in vitro* as described under "Experimental Procedures." Reported values are means of four separate experiments with S.E. indicated by *vertical bars*. Anti-HS1 immunocomplexes were analyzed by Western blotting (*Wb*) with anti-Tyr(P) antibody (*C*). Mean of densitometric analysis (arbitrary units) of HS1-Tyr phosphorylation is reported *above* each anti-Tyr(P) spot. The figure represents at least four separate experiments. Statistical significance of differences: *A*, Syk activity in the presence of piceatannol *versus* control $p < 0.01$; *B*, Lyn activity in the presence of PP2 and SU6656 or piceatannol *versus* control $p < 0.001$ and 0.05, respectively.

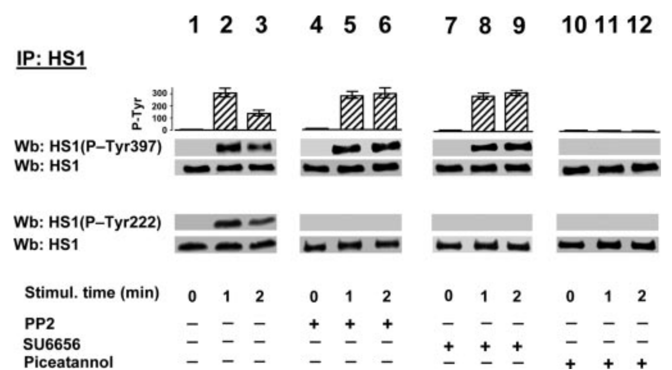

FIG. 3. **Phosphorylation of HS1 Tyr^{222} and Tyr^{397} in thrombin-stimulated platelets.** HS1 was immunoprecipitated from platelet lysates obtained as described in the legend to Fig. 2*C*. Anti-HS1 immunocomplexes were analyzed by Western blotting (*Wb*) with the phosphopeptide-specific antibodies anti-HS1($Tyr(P)^{397}$) or anti-HS1($Tyr(P)^{222}$). Blots were then reprobed with anti-HS1 antibody. Mean of the densitometric values of HS1($Tyr(P)^{397}$) phosphorylation is reported *above* the relative spots. The figure represents four separate experiments.

FIG. 4. **Interaction of Syk-phosphorylated HS1 with GST-SH2 domain.** Platelets, preincubated in the absence (*lanes 1–3*) or presence of either 5 μM PP2 (*lanes 4–6*) or 15 μM piceatannol (*lanes 7–9*), were stimulated with 50 milliunits/ml thrombin for the indicated times. Cellular lysates were immunoprecipitated with anti-GST antibody in the presence of 4 μM recombinant GST/SH2 domain. The blots of the immunocomplexes were immunostained with anti-Tyr(P) antibody, stripped, and reprobed with anti-HS1 antibody. The figure is representative of at least four separate experiments. *Wb*, Western blot.

Syk-phosphorylated HS1 and Lyn, we expressed the Lyn SH2 domain fused to the GST protein and performed competition experiments in thrombin-stimulated platelets. Platelet lysates were immunoprecipitated with anti-GST antibody in the presence of the GST-SH2 domain, which competed with endogenous Lyn for the binding to the primarily Syk-phosphorylated HS1 residues. The anti-GST-(SH2) immunocomplexes were then assayed for the presence of coimmunoprecipitated Tyr-phosphorylated HS1. Indeed, following thrombin-induced Tyr phosphorylation, HS1 interacted and coimmunoprecipitated with the recombinant Lyn SH2 domain (Fig. 4, *lanes 1–3*). The occurrence of the GST-SH2/HS1 interaction was also analyzed in IP obtained from platelets stimulated in the presence of PP2, which abolished the secondary Src-catalyzed phosphorylation of HS1. In these conditions a less but permanent degree of HS1 Tyr phosphorylation was detected in parallel with constant amounts of coimmunoprecipitated HS1 (Fig. 4, *lanes 5* and *6*). In contrast, piceatannol, which prevented the phosphorylation of HS1 (Fig. 2), also counteracted coimmunoprecipitation of the protein with the GST-SH2 domain (Fig. 4, *lanes 8* and *9*). These data confirm that Syk acts as a primary kinase and demonstrates that Syk phosphorylation is a prerequisite for protein binding to Lyn via the enzyme SH2 domain.

The occurrence of an interaction between Syk-phosphorylated HS1 and Lyn was also validated by their coimmunoprecipitation shown in the experiments of Fig. 5, where platelets were stimulated with thrombin for 1 min in the presence of increasing PP2 concentrations. Cells were lysed after stimulation and the lysates were treated with either anti-HS1 or anti-Lyn antibodies and the relative immunoprecipitates were analyzed. As expected, PP2 inhibited in a dose-dependent way the secondary Tyr phosphorylation of HS1 (Fig. 5*A*), whereas it did not affect the primary Syk-catalyzed phosphorylation of the protein as demonstrated by immunostaining with the Syk-specific phosphoantibody HS1($Tyr(P)^{397}$). The finding that the amount of HS1 coimmunoprecipitated with Lyn increased in parallel with the enhancement of the PP2 concentration (Fig. 5*B*) and the predictable increase of the HS1 amount only primarily phosphorylated, suggests that the occurrence of the secondary phosphorylation of HS1 induces the dissociation of the enzyme/protein-substrate complex.

Distinct Binding Patterns of HS1 to Lyn—It has been shown in other resting hematopoietic cells that the SH3 domain of Lyn

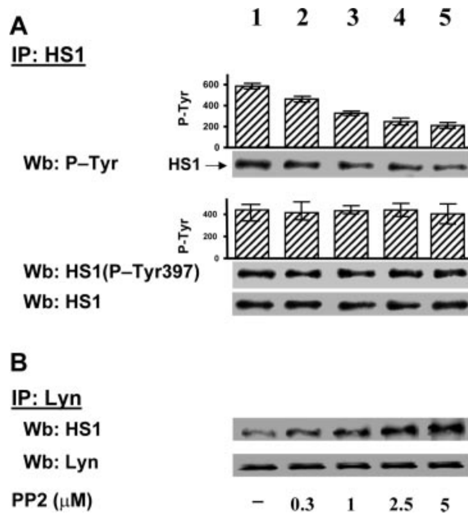

FIG. 5. **Lyn coimmunoprecipitation with Syk-phosphorylated HS1 in thrombin-stimulated platelets.** Platelets were stimulated for 1 min with 50 milliunits/ml thrombin in the absence (*lane 1*) or presence of increasing PP2 concentrations (*lanes 2–5*); they were then lysed and the cellular lysates were immunoprecipitated with either anti-HS1 (*A*) or anti-Lyn (*B*) antibodies. *A*, the blots of anti-HS1 IP were immunostained with either anti-Tyr(P) antibody or anti-HS1(Tyr(P)^{397}) antibody. Blots were then reprobed with anti-HS1 antibody. *B*, the blots of anti-Lyn IP were immunostained with anti-HS1 antibody and reprobed with anti-Lyn antibody. The figure is representative of four separate experiments. *Wb*, Western blot.

is constitutively bound to HS1 (10, 19). We have shown above that the agonist-triggered Syk-mediated phosphorylation of HS1 promotes protein binding to the Lyn SH2 domain (Figs. 4 and 5). To dissect the steps underlying HS1/Lyn interactions, we analyzed the time course of HS1-Lyn complex formation following thrombin-induced stimulation. In resting platelets HS1 was detected in anti-Lyn IP (Fig. 6, *lane 1*). Because, in these conditions, HS1 is not phosphorylated (Fig. 1), the interaction is likely to be ascribed to the enzyme SH3 domain and the cognate Pro-rich region of the protein. In agreement with the above findings (Figs. 4 and 5) indicating the occurrence of an interaction between the Lyn SH2 domain and HS1 Syk-phosphorylated residues, we found that after 1 min of thrombin stimulation the amount of HS1 bound to Lyn was higher than that detected in resting platelets (Fig. 6, *lanes 1* and *2*). The amount of HS1 coimmunoprecipitated with Lyn then decreased (Fig. 6, *lanes 3* and *4*), suggesting that, once the secondary phosphorylation is accomplished, the phosphorylated substrate dissociates from the enzyme. Accordingly PP2, which inhibited the secondary HS1 phosphorylation, prevented detachment of HS1 from Lyn (Fig. 6, compare *lanes 3* and *4* and *7* and *8*, and see also Fig. 5*B*). Parallel sets of experiments directed at evidencing a potential interaction between HS1 and Syk were performed and no coimmunoprecipitation of the protein with this kinase either in resting or stimulated platelets was found (results not shown).

Tyr Phosphorylation of HS1 follows Actin Polymerization—It is well established that thrombin stimulation of platelets triggers a rapid rearrangement of actin cytoskeleton (35) and that Src and Syk tyrosine kinases are implicated in this process (36–38). HS1 contains an F-actin binding domain at the NH_2 terminus, which has been suggested to interact with the actin filaments and regulate their assembly (11, 12). To verify whether HS1 Tyr phosphorylation is involved in the F-actin cross-linking activity and cytoskeleton reorganization, we performed a set of experiments in the presence of the inhibitor of actin polymerization, cytochalasin D. Preliminary experiments in which platelets were preincubated with 10 μM inhibitor for

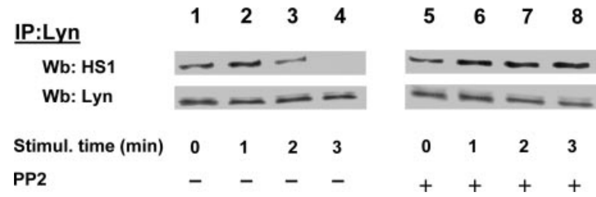

FIG. 6. **Interactions between HS1 and Lyn following thrombin stimulation of platelets.** Platelets were stimulated for the indicated times with 50 milliunits/ml thrombin in the absence (*lanes 1–4*) or presence of 5 μM PP2 (*lanes 5–8*). Cellular lysates were then immunoprecipitated with anti-Lyn antibody and the blots were immunostained with anti-HS1 antibody and reprobed with anti-Lyn antibody. The figure is representative of three separate experiments. *Wb*, Western blot.

different times prior to thrombin stimulation were carried out. Activity and subcellular localization of both Syk and Lyn were analyzed in these conditions (not shown). Preincubation with cytochalasin D for 2 min was then chosen for the following experiments as in these conditions the inhibitor only slightly affected the agonist-induced Syk and Lyn activation (about 21 and 15% of inhibition after 1 min stimulation, respectively) (Fig. 7, *A* and *B*). At variance, translocation of the two enzymes to the Triton X-100-insoluble cytoskeletal fraction, an event that follows the activation of the two tyrosine kinases (35–37), was highly counteracted by cytochalasin D (Fig. 7, *A* and *B*). In these conditions, although the competent tyrosine kinases were active, HS1 Tyr phosphorylation did not occur (Fig. 7*C*).

To investigate subcellular localization of HS1 during platelet activation, the protein was directly visualized by immunolocalization. In resting platelets HS1 fluorescence appeared characteristically punctated and randomly distributed (Fig. 8*A*, *left panel*). After 1 min of thrombin stimulation the fluorescent scattered patches migrated and concentrated at the plasma membrane concomitant with platelet activation (Fig. 8*A*, *right panel*). Fluorescence microscopic analysis of platelets stimulated in the presence of either SU6656 (Fig. 8*B*) or PP2 (Fig. 8*C*) showed that the inhibition of Lyn-catalyzed phosphorylation of HS1 completely prevented the agonist-induced migration of the protein, whereas the presence of PP3, the inactive analogue of PP2, did not affect HS1 migration induced by thrombin (Fig. 8*D*).

DISCUSSION

In this report we demonstrate for the first time that in thrombin-stimulated platelets the protein HS1 undergoes a transient tyrosine phosphorylation sequentially catalyzed by Syk and Lyn tyrosine kinases. Tyr phosphorylation of the protein does not occur when actin assembly is prevented and constitutes a prerequisite for protein migration to the plasma membrane, which takes place in thrombin-stimulated platelets. In particular, our experimental data indicate the below described steps underlying HS1 Tyr phosphorylation/dephosphorylation triggered in platelets by thrombin stimulation.

In resting cells, an aliquot of the protein HS1 is constitutively bound to the tyrosine kinase Lyn by an interaction that, as demonstrated by other authors in other cells (10, 19), is most likely mediated by the enzyme SH3 domain and the cognate Pro-rich region of HS1. This interaction would occur between the non-phosphorylated protein (Figs. 1 and 6) and the Lyn down-regulated conformation present in resting platelets as a result of Csk-mediated phosphorylation of Lyn Tyr^{507}, which gives rise to an intramolecular interaction of the phosphoresidue with the enzymes own SH2 domain (34, 39, 40).

Platelet stimulation with thrombin makes Lyn to adopt an open conformation inducing its activation (34, 39, 40), which occurs concomitantly with Syk activity increase. The agonist

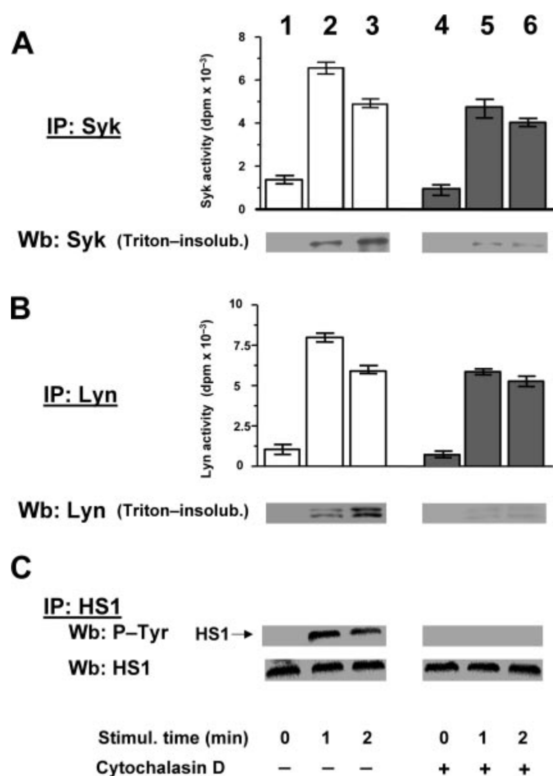

FIG. 7. **Effect of cytochalasin D on Syk and Lyn activities, and on Tyr phosphorylation of HS1.** *A* and *B*, platelets, preincubated in the absence (*lanes 1–3*) or presence of 10 μM cytochalasin D (*lanes 4–6*) for 2 min, were stimulated for the indicated times with 50 milliunits/ml thrombin. Cellular lysates were immunoprecipitated with anti-Syk (*A*) or anti-Lyn (*B*) antibodies. Tyrosine kinase activities of the immunocomplexes were tested *in vitro* as described under "Experimental Procedures." In parallel experiments, platelets were treated with lysis buffer containing 1% Triton X-100, and the detergent-soluble and -insoluble fractions were separated as detailed under "Experimental Procedures." Aliquots of the detergent-insoluble fraction were blotted and immunostained with anti-Syk or anti-Lyn antibodies (*lower part* of *panels A* and *B*, respectively). The figure represents at least four separate experiments with S.E. indicated by *vertical bars*. *C*, cellular lysates were immunoprecipitated with anti-HS1 antibody and the IP were immunostained with anti-Tyr(P) antibody and reprobed with anti-HS1 antibody. The figure represents four different experiments. *Wb*, Western blot.

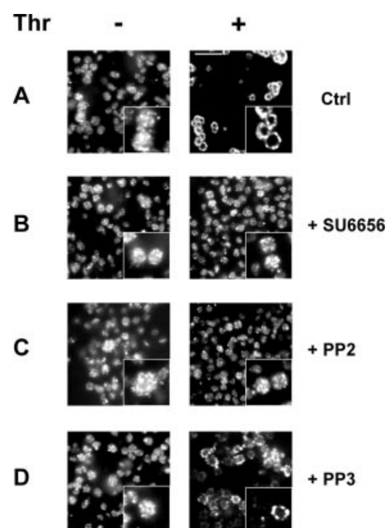

FIG. 8. **Immunolocalization of HS1 in thrombin-stimulated platelets.** Platelets were preincubated for 10 min in the presence of vehicle (*A*), 5 μM SU6656 (*B*), 5 μM PP2 (*C*), or 5 μM PP3 (*D*). Cells were then incubated for 1 min without (*left panels*) or with 50 milliunits/ml thrombin (Thr) (*right panels*). Platelet preparation and fluorescence microscopy analysis were performed as detailed under "Experimental Procedures." The figure is representative of four separate experiments.

also promotes actin assembly, which is associated with HS1 Tyr phosphorylation (Figs. 7 and 8).

Syk acts as the primary kinase toward HS1, as protein Tyr phosphorylation did not occur in cells stimulated in the presence of the Syk inhibitor piceatannol. Syk phosphorylates HS1 Tyr^{397} as demonstrated by immunoblotting with the phosphopeptide-specific antibody (Fig. 3). Because of the high similarity of the sequences containing Tyr^{378} and Tyr^{397} (7 of 10 amino acids), the anti-HS1($Tyr(P)^{397}$) antibody recognized *in vitro* both Syk-phosphorylated residues (results not shown), thus we could not discriminate whether only Tyr^{397} or both tyrosines are also in platelet targets of Syk (24). Syk-phosphorylated residue(s) act as docking site(s) for the SH2 domain of Lyn. This interaction is proven by the coimmunoprecipitation of Syk-phosphorylated HS1 with Lyn, which increases in platelets stimulated in the presence of increasing amounts of the Src inhibitor PP2 (Fig. 5*B*). The occurrence of binding of the Lyn SH2 domain with the protein substrate is also supported by coimmunoprecipitation of Syk-phosphorylated HS1 obtained with recombinant Lyn SH2 domain added to lysates of platelets stimulated in the presence of PP2 (Fig. 4). Although SH2 and SH3 domains have been shown to display different ligand specificity (41), some signaling proteins have been demonstrated to interact with both SH2 and SH3 domains of Src family tyrosine kinases (19, 42, 43). Here we show that the constitutive binding of HS1 to Lyn is not sufficient to induce the Lyn-mediated protein Tyr phosphorylation in resting or thrombin-stimulated platelets. In fact, active Lyn cannot phosphorylate HS1 until the protein is primed by Syk (Fig. 2*C*), which triggers the interaction between Syk-phosphorylated HS1 and the Lyn SH2 domain. Once docked to the Lyn SH2 domain, the protein is recognized as a substrate by the catalytic domain of the Src-related kinase and secondarily phosphorylated at Tyr^{222} (Fig. 3).

The concerted action of the non-receptor tyrosine kinases belonging to the Src and Syk/ZAP70 families is a general theme in hematopoietic cell signal transduction. However, most of the observations to date demonstrate that signal transduction by antigen receptors initiates with the activation of Src family tyrosine kinases, which triggers the recruitment and activation of tyrosine kinases belonging to the Syk/ZAP70 family, which in turn phosphorylate various other proteins (reviewed in Ref. 44). In contrast, HS1 Tyr phosphorylation associated to platelet activation represents one case in which Syk activation is an event independent of Src kinase activity. Indeed, we have demonstrated that Syk phosphorylation of HS1 takes place also when the Src activity is abrogated by selective inhibitors and that this primary phosphorylation is a prerequisite for the following protein phosphorylation mediated by Lyn (Figs. 2 and 3).

We have elsewhere demonstrated a sequential mechanism, mediated by Syk and Lyn, in Tyr phosphorylation of the transmembrane protein band 3 triggered in human erythrocytes by oxidative or osmotic stresses (26). At variance with other protein-tyrosine kinases, Syk displays remarkable site specificity and phosphorylates tyrosine residues located in sequences that must contain both the requirements for its specific recognition and the motif for binding to Src SH2 domain (45). The presence in other proteins of phosphoacceptor sites like those of HS1 and band 3 that, once specifically phosphorylated by Syk, may become binding sites for Src kinases (24), suggests that the protein sequential phosphorylation might be a rather general mechanism in the signaling pathways mediated by Syk and Src-related tyrosine kinases. This might be the case of the Tyr

phosphorylation of cortactin triggered in platelets by thrombopoietin and thrombin, where both Syk and Src tyrosine kinases have been suggested to be involved (46).

The secondary phosphorylation of HS1 seems to be a crucial step in the protein-mediated events of the signaling cascades. In fact the conformational modification of the protein structure likely induced by its secondary phosphorylation weakens the interactions with Lyn domains and causes dissociation of the protein-enzyme complex (Fig. 6). Moreover, whereas HS1 appears coalescent in patches randomly distributed in resting platelets, in activated platelets the protein migrates toward the plasma membrane in parallel with the accomplishment of its secondary phosphorylation and reorganization of the cytoskeleton network (Fig. 8). This conclusion comes from the finding that the inhibition of the Lyn-catalyzed HS1 phosphorylation produced by two structurally unrelated inhibitors hindered the cellular migration of the protein. Accordingly HS1 cell translocation mediated by Tyr phosphorylation has been recently demonstrated in B cells, where the protein migrates into the lipid rafts microdomain in response to B-cell receptor cross-linking (17).

After Lyn phosphorylation, HS1 would become a substrate of protein-tyrosine phosphatase(s), which would rapidly reduce the Tyr phosphorylation extent of the protein. This hypothesis is supported by the experiments performed with the tyrosine phosphatase inhibitor pervanadate (Fig. 1) and by the finding that HS1 only primarily phosphorylated does not undergo dephosphorylation (Figs. 2*C* and 3). Therefore, when Lyn is inhibited, Syk-phosphorylated HS1 is recruited by the Lyn SH2 domain and can neither migrate toward the plasma membrane nor be dephosphorylated by tyrosine phosphatase(s).

The present data highlight the sequential mechanism of HS1 Tyr phosphorylation/dephosphorylation occurring in thrombin-stimulated platelets and provide pieces of evidence that protein Tyr phosphorylation is dependent on the functional actin assembly and involved in the platelet cytoskeletal rearrangements triggered by the agonist. Accordingly, it has been suggested that HS1 is involved in the morphological change of cells during apoptosis (22) and found that platelets from Lyn-deficient mice do not undergo secretion-dependent irreversible aggregation induced by the submaximal thrombin concentration (47).

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