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Relocation of Syk to the actin filament network and subsequent association with Fak

Syk のアクチン細胞骨格への再分布と、続く Fak との会合

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Abbreviations: PTK, protein tyrosine kinase; BCR, B cell antigen receptor; SH2, src homology region 2; CD16, FcγRIII; PLC, phospholipase C; MAPK, mitogen-activated protein kinase.

Enzyme: Protein-tyrosine kinase (EC 2.7.1.112)

SUMMARY

Previous studies demonstrated that Syk protein tyrosine kinase (Syk) is activated by thrombin in platelets. To elucidate the function of Syk in platelets, we have biochemically examined the intracellular location of Syk and the molecules associated with Syk, following platelet activation. In human platelets, thrombin induces the relocation of Syk to the cytoskeletal fraction presumably via Syk tyrosine phosphorylation. Relocated Syk is associated with the actin filament network, and the early phase (10-90 s) of this association can be partially inhibited by the pretreatment of platelets with cytochalasin D, an inhibitor of actin polymerization. Upon thrombin stimulation, Syk becomes associated with Fak as demonstrated by co-immunoprecipitation. The association of both kinases can be inhibited by pretreatment of platelets with cytochalasin D. Interestingly, reconstitutional experiments, using COS cells transfected with various porcine Syk mutants, revealed that the kinase domain, but not the kinase activity, of Syk is required for the association of Syk with the actin filament network. Taken together, these findings suggest that thrombin-induced association of Syk with Fak correlates with the state of actin polymerization, and may play an important role in platelet activation.

INTRODUCTION

Thrombin activates platelets by cleaving its cell surface seven transmembrane spanning receptors, and thereby triggers intracellular signal transduction, including phosphoinositide hydrolysis, mobilization of intracellular calcium and protein tyrosine phosphorylation [1-4]. Multiple non-receptor protein-tyrosine kinases (PTKs), Src-family PTKs, focal adhesion kinase (Fak) and Syk, are involved in platelet activation [5].

Syk was originally identified in spleen, and is expressed exclusively in cells of hematopoietic lineages, in particular B lymphocytes and platelets [6, 7]. Both Syk and Lyn PTKs regulate B cell antigen receptor (BCR)-mediated signal transduction, including the tyrosine phosphorylation of proteins, an increase in phosphatidylinositol hydrolysis, and mobilization of cytoplasmic free calcium [8]. Engagement of the BCR results in the activation of Syk, via two src homology region 2 (SH2) domains, by its association with immune receptor, tyrosine-based, activation motifs (D/E)XXYXXL(X)$_{6-8}$YXXL), which are located in the cytoplasmic region of lymphocyte antigen receptors [9-11]. Subsequently, Syk is activated by autophosphorylation and Lyn-dependent phosphorylation [10, 12]. It has been reported that clustering of chimeric transmembrane PTKs bearing a CD16 (FcγRIII) extracellular domain and Syk kinase intracellular domain (CD16:7:Syk) induces tyrosine phosphorylation of cellular proteins, including phospholipase Cγ (PLCγ), and triggers calcium mobilization [13]. In this respect, it is important to note that PLCγ is directly associated with, and phosphorylated by, Syk following BCR activation [14].

Syk is not only phosphorylated and activated by antigen receptor stimulation in B cells, but is by thrombin in platelets [15, 16]. During the activation process, protein tyrosine phosphorylation occurs in successive waves, and activation of Syk is regulated by both initial integrin-independent and subsequent integrin-dependent mechanisms [5, 16]. Activated Syk relocates to a cytoskeletal complex, which correlates with the two phases of actin polymerization and platelet aggregation [17]. Since the thrombin receptor does not possess an immune receptor, tyrosine based, activation motif, thrombin-induced activation of Syk remains unclear.

Fak is tyrosine phosphorylated and activated upon association with integrin engagement, and may participate in the formation of focal adhesion complexes, which are co-localized with actin binding proteins [18]. These protein complexes play an important role in modulating cell adhesion and shape change. The cytoplasmic tails of β integrins interact directly with the N-terminal sequence of Fak. Both this N-terminal sequence and the C-terminal focal adhesion targeting sequence, are required for integrin mediated Fak activation [18, 19]. Integrin dependent activation of Fak results in autophosphorylation of the kinase, which is

recognized by the SH2 domain of Src or its related kinases, creating a further association site via tyrosine phosphorylation. Fibronectin-induced Fak phosphorylation creates an SH2 binding site for an adapter protein, Grb2, which may link integrin association to the Ras/MAPK signal transduction cascade [20].

In this report, we present evidence that thrombin induces association of Syk with Fak in human platelets. Interestingly, the relocation of Syk and its association with Fak was dependent upon the state of actin polymerization. Furthermore, the kinase domain, but not the kinase activity of ectopically expressed Syk is required for the association of the kinase with the actin filament network in COS cells.

EXPERIMENTAL PROCEDURES

Cells, Antibodies and Reagents. Platelets were isolated by differential centrifugation from venous blood obtained from healthy volunteers, which were finally suspended in modified Tyrode-Hepes buffer (135 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 mg/ml BSA, 3.3 mM NaH_2PO_4, and 20 mM Hepes/NaOH, pH 7.4) containing 0.1 mM $CaCl_2$ prior to platelet activation by 0.1 U/ml thrombin [15, 21]. In some experiments, platelets were preincubated with 20 μM cytochalasin D or 200 μg/ml of Arg-Gly-Asp-Ser peptide for 15 min at 37°C prior to thrombin stimulation. COS cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% (vol./vol.) fetal calf serum (FCS). Plasmid DNA (15 μg each DNA/100-mm dish) were transfected into COS cells using the calcium phosphate precipitation method [22].

The anti-phosphotyrosine mAb 4G10 was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit polyclonal anti-Fak serum BC3 was kindly provided by Dr. J. T. Parsons (University of Virginia), and hFak1 was kindly provided by Dr. J. E. Maguire (University of Chicago) [23]. The anti-human Syk mAb 101 was purchased from Wako Pure Chemicals (Osaka, Japan) [24]. Rabbit polyclonal anti-human Syk antibody was purchased from Santa Cruz (Santa Cruz, CA). Rabbit polyclonal anti-porcine Syk antibody was produced by immunizing rabbits with keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to a partial sequence of the linker region of porcine Syk [6]. Human thrombin and Arg-Gly-Asp-Ser peptide were purchased from Sigma (St. Louis, MO).

Isolation of the Cytoskeletal Fraction. The extraction of the cytoskeletal fraction was carried out as previously described [17, 24]. Platelets were treated with 0.1 U/ml of thrombin for the indicated times, and lysed by adding 1 volume of CSK buffer (100 mM Tris/HCl, pH 7.4, 20 mM EGTA, 2 mM Na_3VO_4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$), and 2 % (vol./vol.) Triton X-100). After incubation on ice for 10 min, the cytoskeletal fraction was pelleted by centrifugation at 12,000 × g for 10 min at 4°C, then washed once with 1:2 dilution of CSK buffer. The triton-soluble fraction was from the resulting supernatant.

Immunoblot Analysis. For immunoblot analysis, equal amount of proteins or immunoprecipitates from whole cell lysates, actin filament network, or subfractions of platelets were separated by 8% SDS-PAGE, and transferred to polyvinylidene difluoride membrane filters. After blocking with TBS buffer (10 mM Tris/HCl, pH 8.0, and 150 mM NaCl) with 5% BSA, membrane filters were incubated with primary antibodies in TBS buffer for 1 h at room temperature. Membranes were developed with goat anti-mouse or rabbit IgG mAb conjugated to horseradish peroxidase,

using enhanced chemiluminescence (ECL). Reprobing was performed according to the Manufacturer's instructions.

Immunoprecipitation. Equal amount of proteins from COS cells, platelets, or platelets subfractions were solubilized in lysis buffer (40 mM Tris/HCl, pH 7.3, 100 mM NaCl, 100 mM sodium fluoride, 10 mM EDTA, 40 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 2 μg/ml of aprotinin, 2 μg/ml of leupeptin, 1 mM $PhMeSO_2F$, 0.2 % (mass/vol.) SDS, and 1% (vol./vol.) Triton X-100) [25]. The lysates were centrifuged to remove insoluble materials, and resulting supernatants were immunoprecipitated with the respective antibodies and protein A-Sepharose for 2 h at 4°C. Each immunoprecipitate was washed five times with lysis buffer, solubilized in Laemmli sample buffer, then separated on 8% SDS-PAGE.

Isolation of the Actin Filament Network, Polymerized Actin and Actin binding Proteins. Preparation of actin filament network was carried out as described [25]. Protein from the cytoskeletal fraction was solubilized in 1 ml of KI buffer (0.6 M KI, 100 mM Pipes, pH 6.5, 100 mM KCl, 100 μM Na_3VO_4, 10 μg/ml of leupeptin, and 1 mM $PhMeSO_2F$) for 20 min at 4°C, and centrifuged at 40,000 × g for 20 min at 4°C. The supernatants were dialyzed against the polymerization buffer (10 mM Pipes, pH 6.8, 1 mM EGTA, 2 mM $MgCl_2$, 100 μM Na_3VO_4, 10 μg/ml of leupeptin, and 1 mM $PhMeSO_2F$) at 4°C for 3 h, then centrifuged at 12,000 × g for 20 min at 4°C. The resulting pellets were resuspended in 50 mM Tris/HCl, pH 7.4, and used for following analysis. Densitometric analysis was carried out using Macintosh NIH Image software.

DNA Construction. For the construction of the porcine Syk expression vector (pEF-porcine Syk), the cDNA fragment for porcine Syk [6] was cloned into the EcoRI-cleaved polylinker region of the pEF expression vector [22]. The cDNA fragment for the kinase-negative porcine Syk ([Arg395]Syk) which contains a point mutation within the ATP binding site (Lys395 to Arg395) of porcine Syk was created by the polymerase chain reaction [12]. The cDNA fragment of the kinase domain-deleted porcine Syk (des-(350-628)-Syk) was constructed using a synthetic oligonucleotide which inserted a thymine, resulting in a frame shift mutation, thus causing termination of the protein at Glu349. The cDNA fragment of the tandem SH2 domain deleted porcine Syk (des-(10-272)-Syk) was created by using oligonucleotides, resulting in the deletion from Phe10 to Ala272. The cDNA fragments of [Arg395]Syk, des-(350-628)-Syk and des-(10-272)-Syk were cloned into the polylinker region of the pEF expression vector, respectively.

RESULTS

Thrombin treatment induces the tyrosine phosphorylation, and relocation of a form of Syk which is conformationally different, compared to control, in human platelets.

Activation of human platelets by thrombin results in the relocation of Syk, which has a retarded mobility on SDS-PAGE as compared to control, to the cytoskeletal fraction (Fig. 1A). Syk isolated from the cytoskeletal fraction appears to have a slightly higher molecular mass than Syk isolated from the Triton-soluble fraction. It has been previously reported that thrombin induces tyrosine phosphorylation of Syk [16]. To clarify the level of Syk tyrosine phosphorylation, Syk was immunoprecipitated from equal amounts of protein from both Triton-soluble and cytoskeletal fractions of activated platelets, using an anti-Syk mAb. Immunoblot analysis indicated that Syk found in the cytoskeletal fraction was tyrosine phosphorylated (Fig. 1B). In addition, anti-Syk immunoprecipitates contained a 76 kDa protein, which was recognized by both anti-phosphotyrosine and anti-Syk mAbs (Fig. 1B). This could be an alternative form of Syk present in platelets, however, the identity of this protein is unclear. Further investigation will be required for this molecule.

Thrombin induces the association of Syk with the actin filament network.

Cytoskeletons are composed of actin filaments, intermediate filaments, and microtubules. A selective extraction procedure was used to obtain the actin filament network, which containing polymerized actin and actin-binding proteins. Following Thrombin stimulation, there is a significant increase in the level of F-actin in this fraction [25]. Equal amounts of protein from the actin filament network were probed for Syk, at the indicated time points, following thrombin stimulation. Thrombin induced a 5-6 fold increase in the amount of Syk in this fraction, as measured by densitometry (Fig. 2A). This increase in the amount of Syk present in this fraction persisted for up to 300 s (data not shown). In addition, Syk detected within this fraction was tyrosine phosphorylated (data not shown). Pretreatment of platelets with cytochalasin D, an inhibitor of actin polymerization, resulted in the inhibition of Syk association with the actin filaments, particularly at early time points (10-90 s) (Fig. 2A and B). In contrast, inhibition of this molecular association was not observed at a later time point (300 s) (data not shown). We previously reported that the relocation of Syk to cytoskeleton was regulated by actin polymerization in early phase after thrombin stimulation, and by integrin signaling in later phase, respectively [17]. Therefore, relocation of Syk to actin filament network under cytochalasin D pretreatment might be mediated by integrin $\alpha_{IIb}\beta_3$ signaling pathway (Fig. 2A and B).

Thrombin induces association of Syk with Fak in human platelets.

Thrombin induces the relocation of non-receptor PTKs, in addition to Syk, to the cytoskeleton [26]. Syk was detected in anti-Fak immunoprecipitates from thrombin-activated platelets, indicating that Syk becomes associated with Fak upon thrombin stimulation (Fig. 3A). This was observed at 90 s following thrombin stimulation, reaching a maximum at 300 s (Fig. 3A). Since translocation of Syk to the cytoskeleton was kinetically more rapid [17] (Fig. 2A), this data indicated that Syk translocated to the actin filament network and was then subsequently associated with Fak. Consistent with the fact that the majority of Fak redistributes to the cytoskeletal fraction upon stimulation [25], the association of both kinases was observed in the cytoskeletal fraction, but not in the Triton-soluble fraction (Fig. 3B).

To determine whether this association correlates with the reorganization of the cytoskeleton, we examined the effect of cytochalasin D upon this molecular association. Pretreatment of platelets with cytochalasin D inhibited the thrombin-induced association of Syk with Fak (Fig. 3C). Pretreatment of platelets with cytochalasin D could appeare to correlate amount of tyrosine phosphorylated Syk, which bound to Fak, to the inhibition of actin polymerization (Fig. 1) [16].

Fak is activated and tyrosine phosphorylated upon integrin $\alpha_{IIb}\beta_3$ engagement in platelets, and is involved in focal adhesions, by tethering actin stress fibers to the cytoplasmic face of the plasma membrane [18, 21]. Inhibition of integrin $\alpha_{IIb}\beta_3$-mediated signaling by addition of an Arg-Gly-Asp-Ser peptide, blocked the tyrosine phosphorylation of Fak, whereas the co-immunoprecipitation of Syk with Fak was only slightly blocked (Fig. 3C). This data indicated that, only phosphorylation of Syk was necessary for the association of Syk with Fak, however it was not the only factor involved for complete binding.

Anti-phosphotyrosine immunoblot analysis of anti-Fak immunoprecipitates, revealed that 120, 76 and 72 kDa proteins were tyrosine phosphorylated upon thrombin stimulation (Fig. 3C). The 72 and 120 kDa proteins appear to be tyrosine phosphorylated Syk and Fak, respectively, although the identity of the 76 kDa protein is not clear (Fig. 1B).

The kinase domain, but not the kinase activity of Syk is required for the association of Syk with the actin filament network.

Syk possesses two SH2 domains and a kinase domain at its N- and C-terminal regions, respectively [6]. The kinase activity and tyrosine phosphorylation of Syk are necessary for association of the kinase with PLCγ in the BCR signaling pathway [14]. In addition, the tandem SH2

domains of Syk are required for the association with immune receptor, tyrosine-based, activation motifs on several antigen receptors [10, 12, 27].

Since platelets are not suitable for mutation analysis, we utilised COS cells to identify which domain of Syk is required for association with the actin filament network. A series of porcine Syk expression vectors ([Arg395]Syk, des-(350-628)-Syk and des-(10-272)-Syk) were constructed and expressed transiently in COS cells (Fig. 4A). The expression levels of these mutants were comparable as assessed by anti-Syk immunoblot analysis (Fig. 4B, left). Anti-Syk immunoprecipitates from wild type porcine Syk and Syk des-(10-272)-Syk were tyrosine phosphorylated, as assessed by antiphosphotyrosine immunoblotting, and exhibited kinase activity, presumably due to their intrinsic kinase activity, as assessed by an *in vitro* kinase assay (data not shown). Using COS cells expressing wild type or the respective mutant Syk proteins, we investigated the possible interaction of various Syk proteins with the endogenous actin filament network. Anti-Syk immunoblot analysis revealed that wild type Syk, [Arg395]Syk, and des-(10-272)-Syk were capable of associating with the actin filament network, whereas des-(350-628)-Syk did not (Fig. 4B, right). These results indicate that the kinase domain, but not the tandem SH2 domains of Syk is required for the association with actin filament network. Furthermore, it became evident that the kinase activity of Syk is not required for this association.

DISCUSSION

In this report we provide evidence that thrombin induces the association of Syk with Fak in human platelets. Syk and Fak are non-receptor PTKs, which functionally couple with membrane receptors relaying signals from the extracellullar ligand to cytoplasmic molecules and the cytoskeleton [5]. Syk and a highly related T cell PTK, ZAP-70, contain two SH2 domains essential for recruitment to the BCR and the T cell receptor (TCR), respectively, in an immune receptor, tyrosine-based, activation motifs-dependent fashion [11, 28].

Conformational changes in Syk can be induced by tyrosine phosphorylation of the kinase or by binding of the phosphorylated immune receptor, tyrosine-based, activation motif peptide derived from FcεRIγ [29]. Tyrosine phosphorylated Syk was detected predominately in the cytoskeletal fraction of activated platelets (Fig. 1), although the mechanism of Syk activation is still unclear. Since the thrombin receptor doesn't contain an immune receptor, tyrosine-based, activation motif, other molecules containing this motif may participate in thrombin-induced Syk activating pathway.

In platelets, Syk kinase activity increases within 10 s following thrombin stimulation [15, 16]. Relocation of Syk to the actin cytoskeleton may result in an intracellularly localised, increased concentration of Syk, which induces the aggregation of Syk in platelets, as proposed by the experiments using a CD16:7:Syk chimeric molecule [13]. It is interesting, that the results from COS cells indicated that the association of Syk with the actin filament network was not dependent upon the Syk SH2 domains. Even in the absence of intrinsic kinase activity, Syk associated with the actin filament network in COS cells (Fig. 4). Although it can be argued that a different cell line was utilised, the findings from COS cells proposed the mechanism whereby Syk could bind to the actin cytoskeleton via its kinase domain, and be regulated by the dynamic mobilization of the actin cytoskeleton in platelets (Fig. 2).

Cytochalasin D partially inhibited the association of Syk with Fak (Fig. 3). Since cytochalasin D pretreatment inhibited tyrosine phosphorylation of Syk in platelets [16], it may reflect an involvement of actin polymerization in Syk activation. Consistent with this idea, pretreatment of platelets with cytochalasin D has a marked affect on the amount of activated Syk associated with Fak (Fig. 3).

Fak is activated by integrin association at a late phase of platelet activation [21]. In contrast, thrombin induces the activation of Syk [15] and relocation of Syk to the actin filament network at an early phase of platelet activation (Fig. 2). Association of Syk with Fak was not observed 10 s after thrombin stimulation (data not shown). In the immune receptor, tyrosine-based, activation motifs-containing FcγR signaling

pathway, Syk is required for this motif-dependent actin assembly [30]. Taken together, Syk could be involved in the reorganization of the actin cytoskeleton resulting in the subsequent association with translocated Fak, indicating a role(s) of Syk in linking the actin filament system to focal contact-like areas.

There have been several reports of a functional association of ZAP-70 with Fak or its related kinase. In T cells, an alternative form of Fak, called FakB, was immunologically identified and was shown to associate with ZAP-70 in T cells [31]. Tyrosine phosphorylation and enzymatic activation of FakB are induced upon activation of the TCR and the BCR [31]. Moreover, a chemokine induces the activation of Fak, which is stably complexed with ZAP-70 in human T cells [32]. Immunoblot analysis and kinase assays revealed that ZAP-70 as well as the focal adhesion protein, paxillin, were found in anti-Fak immunoprecipitates [32]. In this respect, our findings indicate that the association of the Syk/ZAP-70 family PTKs with Fak, is not restricted to lymphocytes and may play an important role in integrating the cellular signals in a wide range of hematopoietic cells (Fig. 3).

This is the first report which demonstrates the association of Syk with Fak following thrombin stimulation. Since both PTKs are tyrosine phosphorylated following platelet activation, the molecular basis of this association may be the phosphotyrosine-SH2 interaction. In fibroblasts, Fak is associated with, and phosphorylated by, Src thus regulating the function of the downstream signaling molecules [20, 33]. However, Syk is not expressed in fibroblasts, and indeed we failed to detect the association of endogenous Fak with ectopically expressed wild type Syk in COS cells (data not shown). In hematopoietic lineages, the expression of some additional molecules may be required for this association. In addition, Syk activation occurs at an early phase of platelet activation, whereas that of Fak occurs at a later phase [5]. At present it remains unclear whether Syk can phosphorylate Fak directly. Further studies will be required to understand the cross talk between Syk/ZAP-70 PTKs and Fak.

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LEGENDS FOR FIGURES

Fig. 1. Thrombin induced tyrosine phosphorylation and relocation of Syk to the cytoskeletal fraction in human platelets.

1.5×10^9 human platelets were stimulated by thrombin for 300 s. A. Lysates of Triton-soluble and cytoskeletal fractions from non-activated (–) or activated platelets (+) by 0.1 U/ml of thrombin for 300 s were separated by adding CSK buffer followed by centrifugation as described in "EXPERIMENTAL PROCEDURES". The equal amounts of proteins from platelet subfractions or whole cell lysates were analyzed by immunoblot analysis with anti-Syk mAb or control mouse IgG. B. Equal amounts of Triton-soluble (lane 1) or cytoskeletal fractions (lanes 2, 3) from thrombin-activated platelets were immunoprecipitated with either anti-Syk mAb or control mouse IgG. Immunoprecipitates were analyzed by immunoblot analysis with anti-phosphotyrosine (anti-pTyr) mAb or anti-Syk mAb. The closed and open arrowheads indicate the position of Syk and Syk with mobility shift, respectively. 4.9 mg and 2.1 mg of proteins were recovered from the Triton-soluble and the cytoskeletal fractions from activated platelets, respectively. The position of the molecular mass marker are shown on the left (kDa).

Fig. 2. Association of Syk with the actin filament network.

A. 1.5×10^9 human platelets were stimulated by thrombin for the indicated times without or with pretreatment using 20 μM cytochalasin D for 15 min at 37°C Equal amounts of protein from the actin filament network were analyzed by immunoblot analysis with a polyclonal anti-Syk antibody or normal rabbit serum. B. The graph shows the results of densitometric analysis for the relative amount of Syk associated with the actin filament network at the indicated times without (○) or with (●) pretreatment using cytochalasin D. The data is representative of two experiments. The position of the molecular mass marker is shown on the left (kDa).

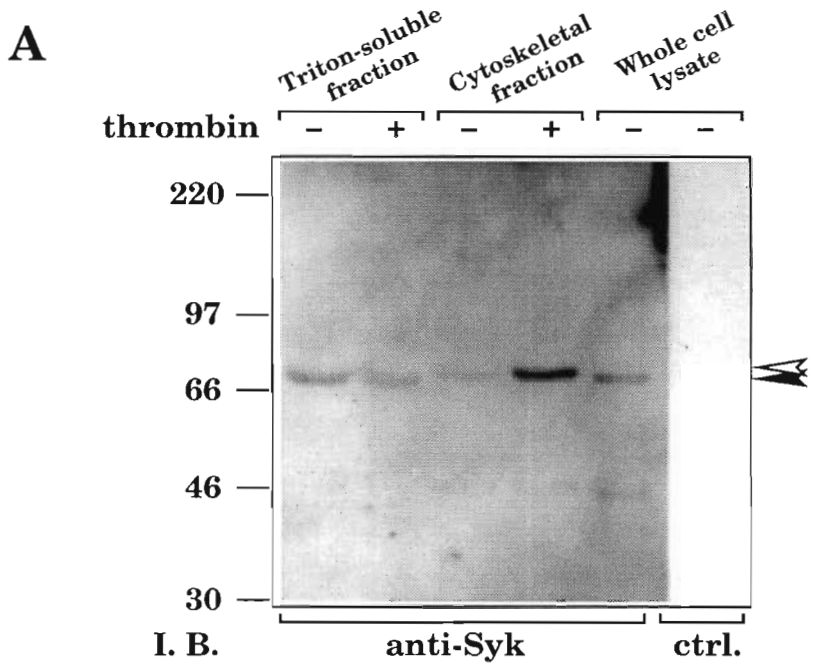
Fig. 3. The association of Syk with Fak in human platelets.

1.5×10^9 of human platelets were activated by 0.1 U/ml of thrombin for the indicated times (A), 300 s (B), or 90 s (C). A. Cell lysates from non-activated or activated platelets were immunoprecipitated with anti-Fak serum (BC3) or normal rabbit serum (ctrl.), following immunoblot analysis with anti-Syk mAb and anti-Fak serum (hFak1), respectively. B. Triton-soluble and cytoskeletal fractions from activated platelets were immunoprecipitated with anti-Fak serum (BC3), followed by immunoblot analysis with anti-Syk mAb. C. Lysates from non-activated (–) or activated platelets (+) by thrombin without (–) or with (+) pretreated platelets with 20 μM cytochalasin D (C.D.) or 200 μg/ml of Arg-Gly-Asp-

Ser (RGDS) peptide for 15 min at 37°C prior to thrombin stimulation were immunoprecipitated with anti-Fak serum (BC3). Immunoprecipitates were analyzed by immunoblot with anti-phosphotyrosine (anti-pTyr) mAb, anti-Syk mAb, and anti-Fak serum (hFak1). The closed arrowheads indicate the position of Syk and the open arrowheads indicate that of Fak. The data for the anti-Syk immunoblot from C were from the same polyvinylidene difluoride membrane filters which were reprobed. The position of the molecular mass marker are shown on the left (kDa).

Fig. 4. Interaction of wild type and various Syk mutants with the actin filament network in COS cells.

A. A schematic diagram of the porcine Syk mutants. [Arg395]Syk has a point mutation within the ATP binding site (Lys395 to Arg395) created by the polymerase chain reaction. Des-(350-628)-Syk is the kinase domain deletion mutant of porcine Syk, and des-(10-272)-Syk is the tandem SH2 domain-deleted mutant of porcine Syk. Anti-porcine Syk antibody recognized the linker region of porcine Syk. B. COS cells were transfected with control vector (pEF) (lane 1), pEF-porcine wild type Syk (lane 2), pEF-[Arg395]Syk (lane 3), pEF-des-(350-628)-Syk (lane 4), or pEF-des-(10-272)-Syk (lane 5), respectively. Equal amounts of protein from whole cell lysates or the actin filament network of COS cells were analyzed by immunoblotting using an anti-porcine Syk antibody. The arrowheads indicate the position of Syk or a series of Syk mutants. DNA construction and transfection of COS cells were performed as described in "EXPERIMENTAL PROCEDURES". The position of the molecular mass marker is shown on the left (kDa).


A
Triton-soluble fraction
Cytoskeletal fraction
Whole cell lysate
thrombin
–
+
–
+
–
–
220
97
66
46
30
I. B.
anti-Syk
ctrl.

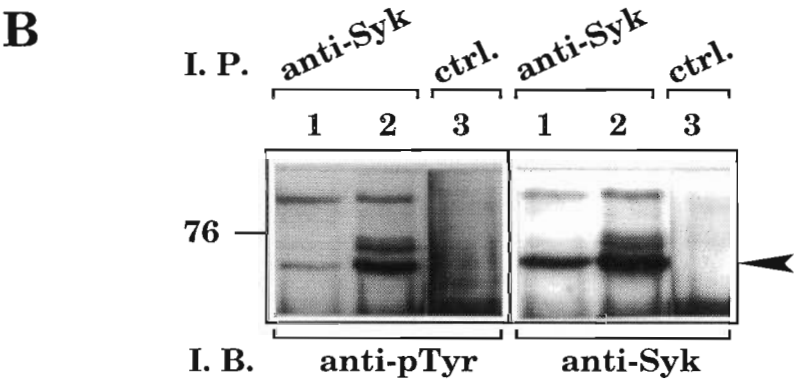
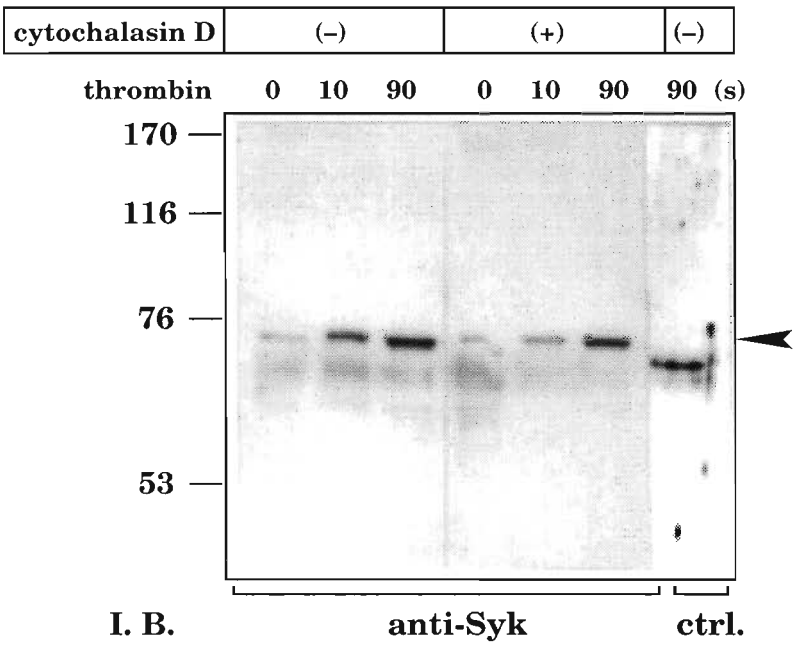

B
I. P.
anti-Syk
ctrl.
anti-Syk
ctrl.
1
2
3
1
2
3
76
I. B.
anti-pTyr
anti-Syk

Fig. 1

A


cytochalasin D
(–)
(+)
(–)
thrombin
0 10 90 0 10 90 90 (s)
170
116
76
53
I. B.
anti-Syk
ctrl.

B

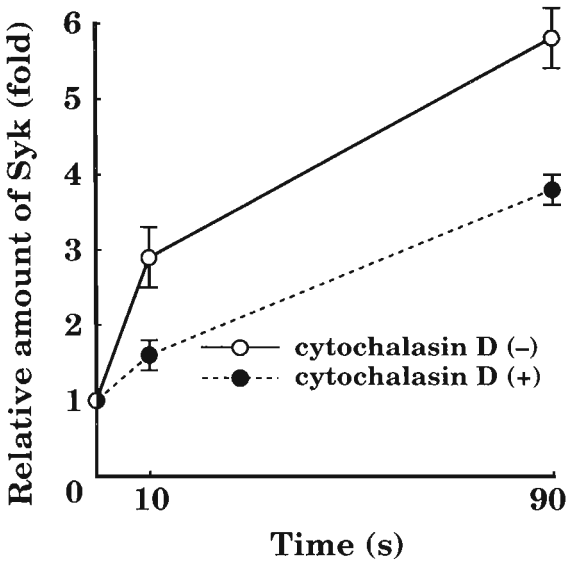
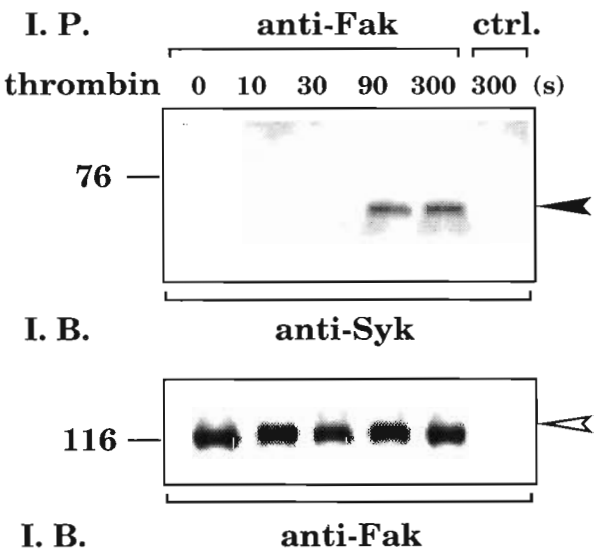

Relative amount of Syk (fold)
6
5
4
3
2
1
0
10
90
Time (s)
cytochalasin D (–)
cytochalasin D (+)

Fig. 2

A


I. P.
anti-Fak
ctrl.
thrombin
0
10
30
90
300
300
(s)
76
I. B.
anti-Syk
116
I. B.
anti-Fak

B

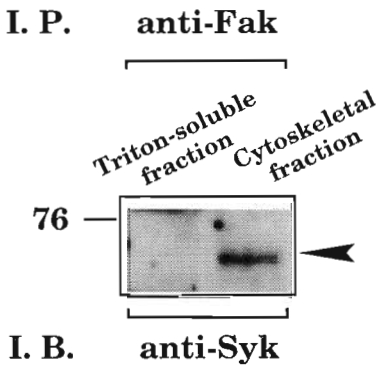

I. P.
anti-Fak
Triton-soluble fraction
Cytoskeletal fraction
76
I. B.
anti-Syk

Fig. 3 A, B

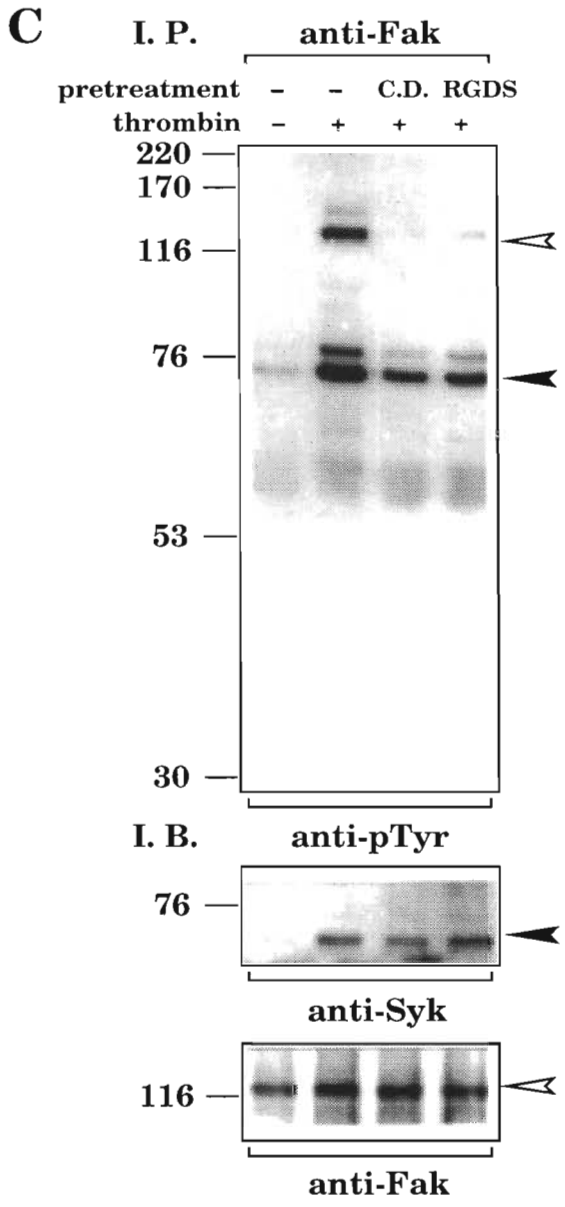

C
I. P.
anti-Fak
pretreatment - - C.D. RGDS
thrombin - + + +
220
170
116
76
53
30
I. B.
anti-pTyr
76
anti-Syk
116
anti-Fak

Fig. 3 C

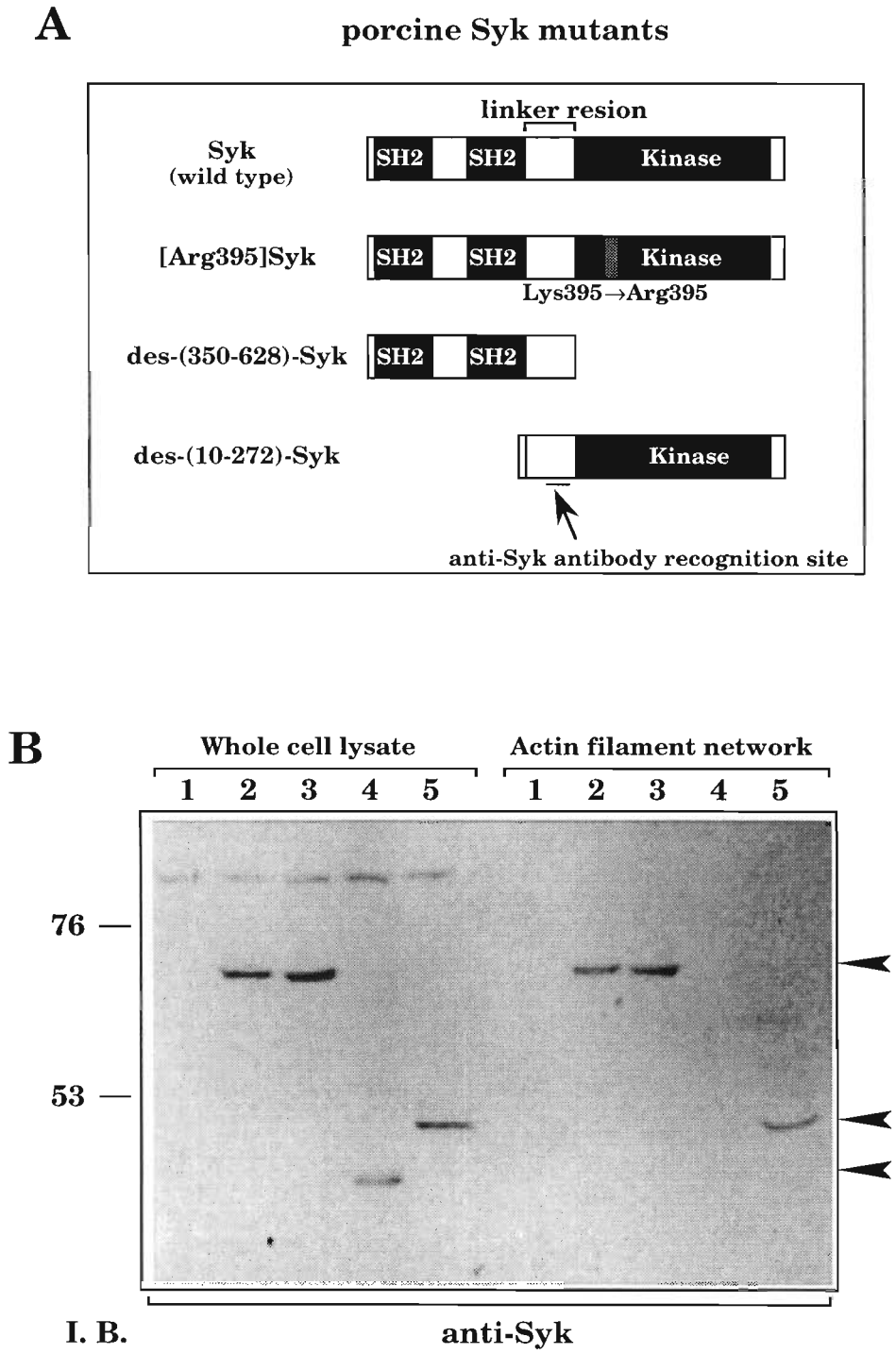

A
porcine Syk mutants
linker resion
Syk
(wild type)
SH2
SH2
Kinase
[Arg395]Syk
Lys395→Arg395
des-(350-628)-Syk
des-(10-272)-Syk
anti-Syk antibody recognition site
B
Whole cell lysate
Actin filament network
1 2 3 4 5
1 2 3 4 5
76
53
I. B.
anti-Syk

Fig. 4