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# ITAM-Based Interaction of ERM Proteins with Syk Mediates Signaling by the Leukocyte Adhesion Receptor PSGL-1

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## Summary

**P-selectin glycoprotein ligand 1 (PSGL-1) is a leukocyte adhesion molecule involved in cell tether and rolling on activated endothelium. Our work shows that PSGL-1 associates with Syk. This association is mediated by the actin-linking proteins moesin and ezrin, which directly interact with Syk in an ITAM-dependent manner. PSGL-1 engagement induces tyrosine phosphorylation of Syk and SRE-dependent transcriptional activity. Treatment of cells with the Syk inhibitor piceatannol and overexpression of either a Syk dead kinase mutant or an ITAM-mutated moesin abrogated PSGL-1-induced transcriptional activation. These data unveil a new functional role for the ERMs (ezrin/radixin/moesin) as adaptor molecules in the interactions of adhesion receptors and intracellular tyrosine kinases and show that PSGL-1 is a signaling molecule in leukocytes.**

## Introduction

Recruitment of leukocytes to inflammatory foci is a multistep process initiated by their interaction with the selectins expressed on activated endothelial cells, which mediate the rolling of leukocytes on endothelium (Kansas, 1996; McEver et al., 1995; Vestweber and Blanks, 1999). Selectins possess unique biophysical and molecular properties that account for their ability to mediate the transient tethering and rolling of leukocytes (Alon et al., 1996, 1997). The selectin family includes three membrane glycoproteins that display different patterns of expression and function. L-selectin is constitutively expressed by the majority of circulating leukocytes and is implicated in homing and leukocyte recruitment; P-selectin, expressed by activated platelets and activated endothelium, is rapidly translocated to the membrane from intracellular stores and promotes the tethering and rapid rolling of leukocytes, and has a predominant role in their recruitment (Robinson et al., 1999); finally, E-selectin is expressed de novo by activated endothelium and is involved in the slow rolling of leukocytes that precedes their firm attachment to endothelium. P- and E-selectins are also essential for the homing of hematopoietic progenitor cells to the bone marrow (Frenette et al., 1998).

PSGL-1, the best characterized selectin ligand, is expressed on almost all leukocytes (McEver and Cummings, 1997). Although PSGL-1 was initially identified as a ligand for P-selectin, it also interacts with L-selectin and E-selectin (Guyer et al., 1996; Sako et al., 1993). The relevant role of PSGL-1 in vivo was proved by the severe reduction in P-selectin-mediated cell rolling and early neutrophil recruitment in PSGL-1-deficient mice (Yang et al., 1999). Moreover, the in vivo role of PSGL-1 as an E- and P-selectin ligand has been reported (Hirata et al., 2000; Norman et al., 2000; Xia et al., 2002); however, there is no evidence that PSGL-1 is a ligand for L-selectin in vivo.

Besides its interaction with selectins, PSGL-1 also seems to have a role as a signal-transducing molecule. In this regard, it has been reported that the cytoplasmic tail of PSGL-1 interacts with ezrin and moesin (Alonso-Lebrero et al., 2000). Proteins of the ezrin/radixin/moesin (ERM) family function as membrane-actin cytoskeleton linkers (Mangeat et al., 1999) and play a key role in the formation of protrusive plasma membrane structures such as filopodia, microspikes, or microvilli (Bretscher et al., 2000; Vaheri et al., 1997; Yonemura and Tsukita, 1999). PSGL-1 is also a potent negative regulator of human hematopoietic progenitor cells (Levesque et al., 1999). Furthermore, the engagement of PSGL-1 induces tyrosine phosphorylation and activation of MAP kinases in human neutrophils (Hidari et al., 1997), as well as cytokine release by neutrophils, monocytes, and T cells (Celi et al., 1994; Damle et al., 1992; Weyrich et al., 1995, 1996) and activation of β2 integrin binding to ICAM-1 (Simon et al., 2000). However, the molecular mechanism which links PSGL-1 engagement and the subsequent intracellular signaling cascades remain unknown. In this paper, we report that PSGL-1 associates with Syk through its interaction with moesin and that the engagement of PSGL-1 promotes tyrosine phosphorylation of Syk and transcriptional activation of SRE element and c-*fos* promoter and gene expression. These data underline the importance of ERM proteins in connecting leukocyte adhesion receptors and tyrosine kinases.

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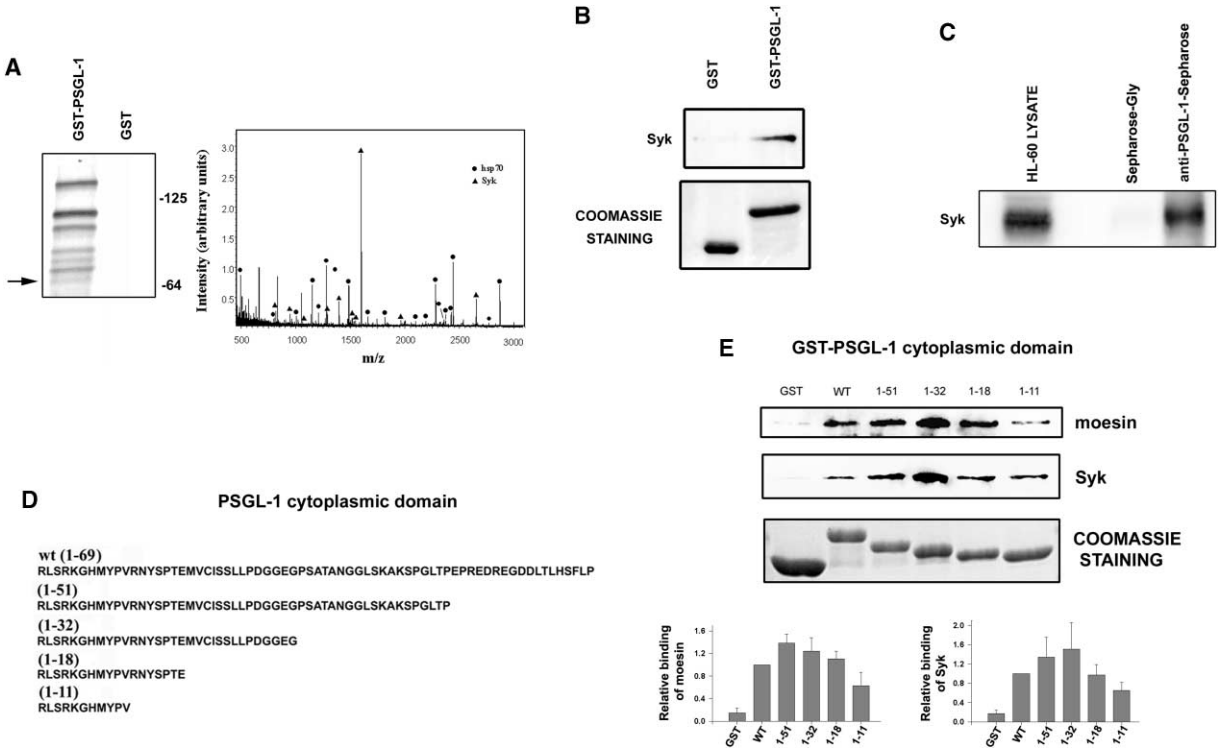



Figure 1. Association of PSGL-1 and Syk

(A) GST or the cytoplasmic tail of PSGL-1 fused to GST (GST-PSGL-1) was bound to glutathione-Sepharose beads and incubated with $^{35}$S-Met-labeled proteins from HL-60 cell lysates. The proteins pulled down were analyzed by SDS/PAGE (left). A protein band of 70 kDa (arrow) was digested with trypsin and analyzed by mass spectrometry (right). The peptides corresponding to the human tyrosine kinase Syk are labeled with a black triangle.

(B and C) Western blot analyses with specific anti-Syk mAb (4D10.1) of proteins pulled down from HL-60 cell lysates with the cytoplasmic tail of PSGL-1 fused to GST (GST-PSGL-1) bound to glutathione-Sepharose beads (B) or with PL2 specific anti-PSGL-1 mAb (C). Sepharose-glycine was used as control in (C).

(D) Amino acid sequences of the wild-type (wt) and several deletions of the cytoplasmic tail of PSGL-1.

(E) GST or the GST-fusion proteins containing the wt or the deletion mutants of the cytoplasmic tail of PSGL-1 were bound to glutathione-Sepharose beads and incubated with HL-60 cell extracts. The proteins pulled down were analyzed by Western blot with anti-moesin- or anti-Syk-specific antibodies. The relative binding capabilities of moesin and Syk to GST-cytoplasmic deletions of PSGL-1 were estimated by densitometric analysis comparing the amount of moesin and Syk bound to equal amounts of each fusion protein with the amount eluted from GST-PSGL-1 wild-type, as described previously by Yonemura et al., (1998). Values correspond to the arithmetic mean ± SE of three independent experiments.

## Results

### Syk Associates with the Cytoplasmic Domain of PSGL-1

To search for intracellular proteins that associate with PSGL-1, we incubated $^{35}$S-Met-labeled HL-60 cell lysates with a chimeric protein containing the cytoplasmic tail of PSGL-1 fused to GST. Several proteins specifically bound to the cytoplasmic domain of PSGL-1 (Figure 1A). Two of them, with apparent $M_r$ of 78 and 85 kDa, corresponded to moesin and ezrin (Alonso-Lebrero et al., 2000). A new protein band of 70 kDa was subsequently processed by in situ digestion with trypsin and analyzed by mass spectrometry. A database search yielded a clear match with Syk (Figure 1A). The association of Syk to PSGL-1 was verified by Western blot (Figure 1B). Furthermore, this interaction was confirmed by coimmunoprecipitation of Syk with PSGL-1 in HL-60 cells (Figure 1C).

To determine the minimal cytoplasmic sequence of PSGL-1 required for Syk association, protein deletion mutants of the PSGL-1 cytoplasmic domain were fused to GST (Figure 1D) and incubated with HL-60 cell lysates. Proteins pulled down with the different GST-PSGL-1 constructs were resolved by SDS-PAGE and analyzed by Western blot using anti-Syk and anti-moesin antibodies. As shown in Figure 1E, a juxtamembrane region of 18 amino acids of the PSGL-1 cytoplasmic tail was accounting for the association with Syk, which was coincident with the PSGL-1 binding region to moesin (Serrador et al., 2002a).

### Colocalization of SYK and PSGL-1 in Lymphoid Cells

To assess the subcellular localization of PSGL-1 and Syk, immunofluorescence experiments were carried out in different cells. The polarized mouse myeloma cell line NS1 was cotransfected with a cDNA construct containing human PSGL-1 fused to GFP and an expression plasmid containing the human Syk full-length cDNA. As shown in Figure 2A, PSGL-1 was concentrated in the

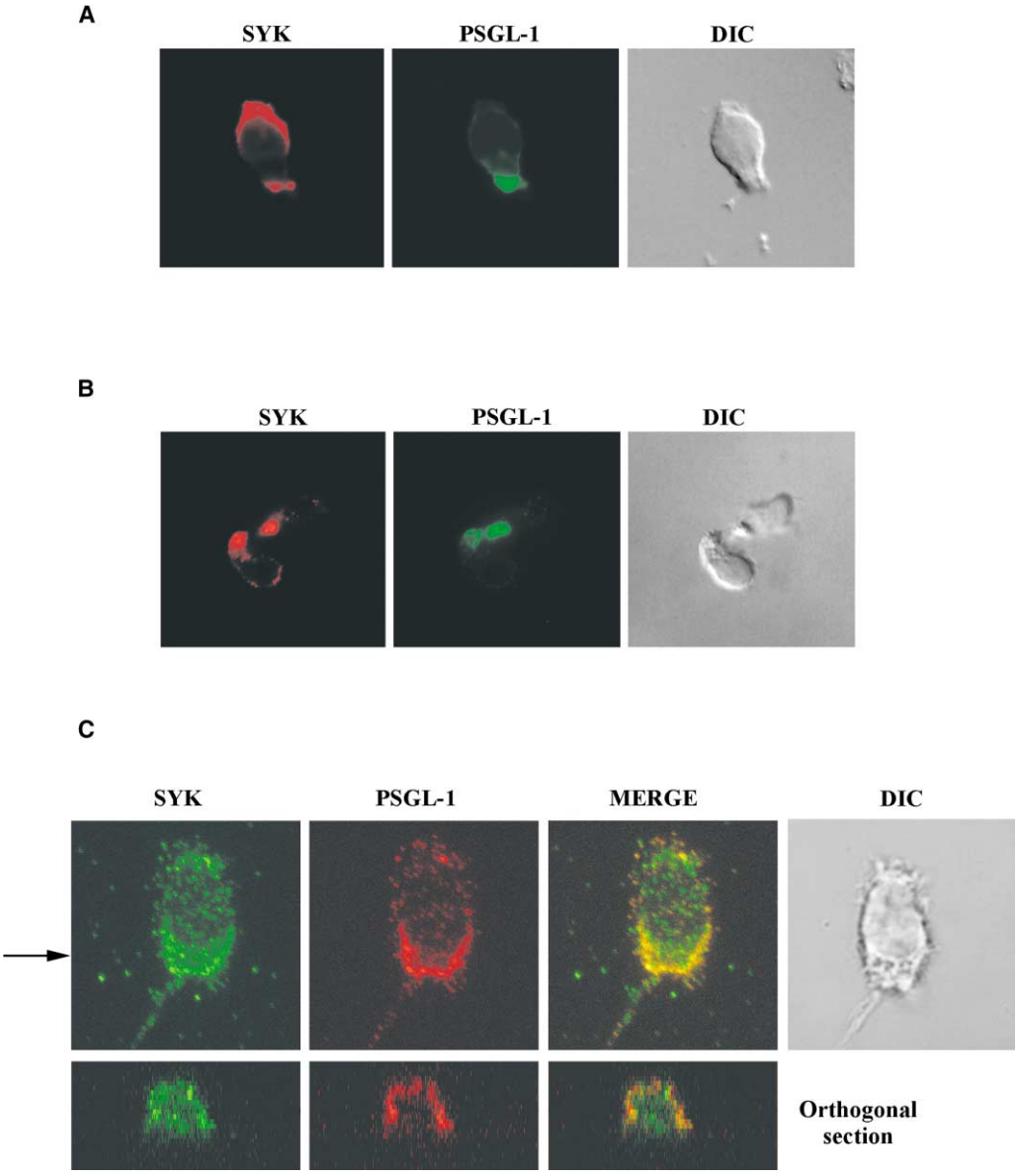



Figure 2. Colocalization of PSGL-1 and Syk in Lymphoid Cells

(A) NS1 cells were transiently cotransfected with PSGL-1-GFP and Syk cDNAs, grown on glass coverslips for 24 hr, fixed, and stained for Syk (red) with a polyclonal anti-Syk antibody. The subcellular distribution of PSGL-1 and Syk was analyzed by fluorescence microscopy. The corresponding differential interference contrast (DIC) image of the cell is shown.

(B) Human T lymphoblasts were double stained for PSGL-1 (green) and Syk (red), and their subcellular distribution was analyzed by fluorescence microscopy. The DIC image of cells is shown.

(C) Human T lymphoblasts were double stained for PSGL-1 and Syk and analyzed by confocal microscopy. In order to show the whole signal, a projection of the complete stack of confocal sections is depicted. Lower panels show an orthogonal section of the site of the uropod indicated by the arrow. The position of the cell is indicated in the differential interference contrast.

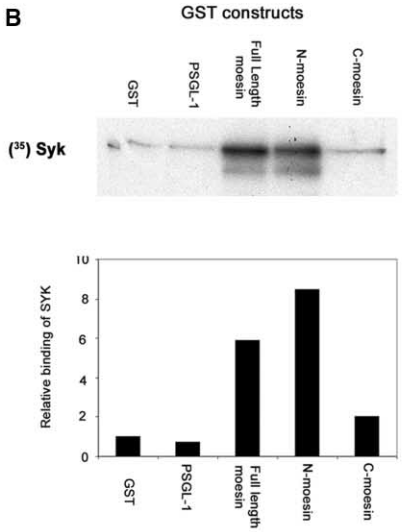
cell uropod, while Syk was located at both the uropod and cell leading edge. The distribution of endogenous PSGL-1 and Syk was also analyzed in T lymphocytes. In these cells, PSGL-1 was concentrated at the cellular uropod, and Syk was located at both cell poles (Figure 2B). Confocal analysis of the endogenous distribution of both molecules in T cells confirmed their colocalization at the trailing edge of polarized migrating cells (Figure 2C).

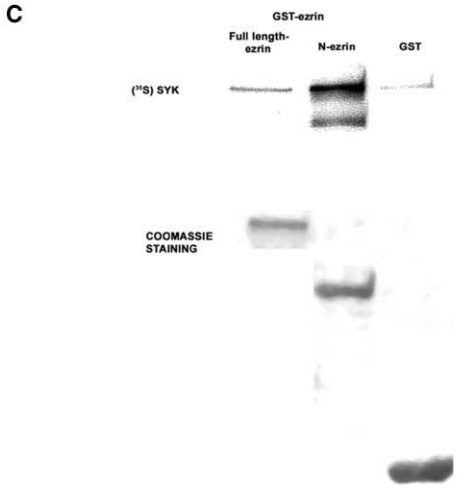
### Moesin Acts as an Adaptor Protein in PSGL-1-Syk Interaction

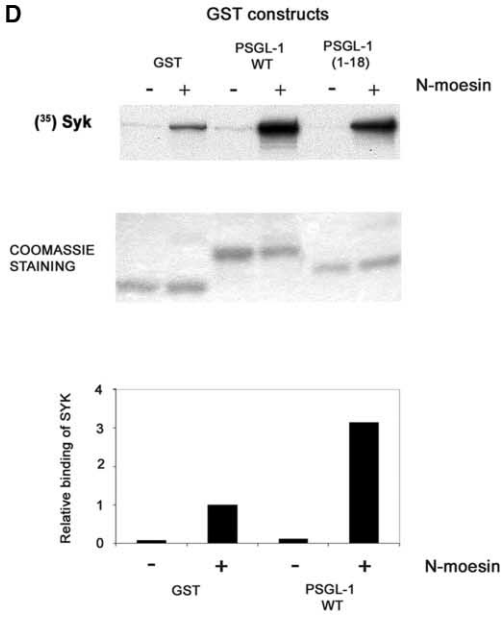
To investigate whether the association of PSGL-1 with Syk took place through a direct interaction, $^{35}$S-Met-Syk was incubated with the cytoplasmic domain of PSGL-1 fused to GST. We did not find any direct interaction between both proteins (Figure 3B). Since ERM proteins contain an ITAM-like motif in their amino-terminal region (Figure 3A, and Rozsnyay et al., 1996), it was of interest

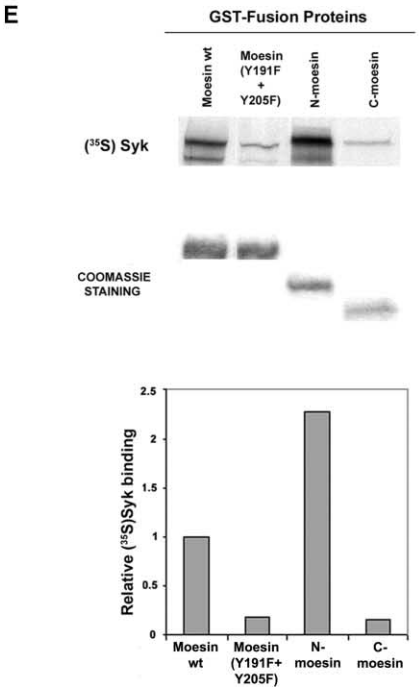
**A** **ITAM-LIKE MOTIF IN THE ERM PROTEIN FAMILY**

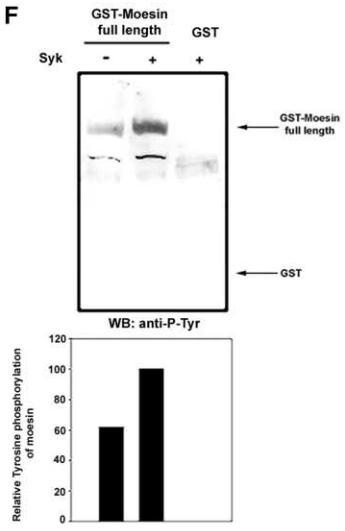
| | |
|---|---|
| Consensus ITAM motif | D/E(X)$_7$D/EXXYXXL/I(X)$_{10}$YXXL/I |
| ERM FAMILY | |
| Ezrin | E(X)$_6$DXXXXEYXXI(X)$_{10}$YXXI |
| Radixin | E(X)$_6$EDXXXEYXXI(X)$_{10}$YXXI |
| Merlin | E(X)$_6$DEXEXEYXXI(X)$_{10}$YXXI |
| Moesin | E(X)$_6$EDXXXEYXXI(X)$_{10}$YXXI |
| Moesin (Y191F + Y205F) | E(X)$_6$EDXXXEFXXI(X)$_{10}$FXXI |



to examine whether Syk and moesin could directly interact. Figure 3B shows that Syk bound to the full-length as well as to the amino-terminal region of moesin (N-moesin) but failed to interact with the carboxy-terminal region of the protein (C-moesin), suggesting that this interaction could take place through the ITAM-like motif of moesin. In addition, Syk also bound to ezrin (full-length and amino-terminal region), indicating that Syk interacts with other members of the ERM family (Figure 3C).

Then, we tested whether moesin could act as an adaptor molecule in Syk/PSGL-1 interaction. Protein binding assays with GST-PSGL-1 fusion protein bound to glutathion-Sepharose beads, $^{35}$S-Met-labeled Syk, and N-moesin demonstrated that PSGL-1 only interacted with Syk in the presence of N-moesin, indicating that moesin is able to link PSGL-1 and Syk (Figure 3D). To address the possible role of the ITAM-like motif of moesin in its interaction with Syk, the two tyrosines of this motif were mutated to phenylalanine (Y191F+Y205F) (Figure 3A). Protein binding assays showed that the Y191F+Y205F moesin mutant had completely lost the ability to associate with Syk (Figure 3E), demonstrating the key role of the moesin ITAM-like motif in the Syk-moesin interaction.

Moesin has been already reported to be tyrosine phosphorylated (Meyer et al., 1998). We next assessed whether the reticulocyte lysate had the ability to phosphorylate bacterially produced moesin in tyrosine. As shown in Figure 3F, moesin was tyrosine phosphorylated when the reticulocyte lysate was added, and the level of phosphorylation increased when Syk was previously translated by the reticulocyte lysate.

### PSGL-1 Engagement Induces Tyrosine Phosphorylation of Syk

The possible involvement of Syk in the signaling triggered through PSGL-1 was assessed. First, Syk tyrosine phosphorylation was analyzed after PSGL-1 engagement as an indicator of Syk activation. We found that the tyrosine phosphorylation level of Syk was clearly increased upon anti-PSGL-1 engagement, both in lymphocytes and U937 cells (Figures 4A and 4B). In contrast, no significant tyrosine phosphorylation of Syk was induced by engagement of HLA-A, B, or CD7, which were used as controls (Figure 4A). As shown in Figure 4B, piceatannol, which at low concentrations has been shown to selectively inhibit the activation of Syk as compared to the Src family kinases (Oliver et al., 1994), prevented the induction of Syk tyrosine phosphorylation by PSGL-1 engagement with either of the two specific anti-PSGL-1 mAbs used. To study whether Syk was also activated by interaction of PSGL-1 with P-selectin, U937 cells were incubated, under rotating conditions, at 37°C for 10 min on a monolayer of CHO cells stably expressing P-selectin. As shown in Figure 4C, the level of tyrosine phosphorylation of Syk was significantly increased upon the interaction of U937 cells with P-selectin-CHO cells, compared with untransfected CHO cells. Likewise, a similar induction of Syk phosphorylation was observed upon interaction of U937 cells with CHO cells stably transfected with E-selectin (Figure 4C).

### PSGL-1 Engagement Induces SRE-Dependent Transcriptional Activation

To assess whether the engagement of PSGL-1 was able to induce gene transcriptional activation, U937 cells were transfected with different reporter plasmids where luciferase expression is driven by multimers of the SRE or NFκB consensus sequences. Remarkably, the SRE-dependent transcriptional activity was clearly induced in cells treated with an anti-PSGL-1 mAb antibody (Figure 5A). In contrast, no significant induction was observed with control antibodies (Figure 5A). In addition, as shown in Figure 5B, the SRE transcriptional activity was significantly higher in U937 cells interacting with either P- or E-selectin, compared to a control protein, indicating that the interaction of PSGL-1 with P- or E-selectin was also able to induce gene transcriptional activation. Experiments in parallel evidenced that NFκB

Figure 3. Interaction of Syk with Moesin and Ezrin

(A) Consensus sequence for the ITAM motif and amino acid sequences found in the amino-terminal domains of the different proteins of the ERM family (the ITAM motif is underlined in each sequence). The amino acid sequence for the moesin mutant in which tyrosines 191 and 205 have been substituted by phenylalanine is also shown.

(B) GST and GST-fusion proteins containing the wt cytoplamic tail of PSGL-1, the full-length sequence of moesin, the amino-terminal domain of moesin (N-moesin), or the carboxy-terminal domain of moesin (C-moesin) were bound to Sepharose beads and incubated with $^{35}$S-Met-labeled Syk obtained by a transcription and translation-coupled system. After incubation, beads were centrifuged, washed, and boiled in sample buffer, and eluted proteins were analyzed by SDS-PAGE and autoradiography. A representative experiment is shown. Relative binding of Syk to GST-fusion proteins was estimated by densitometric analysis.

(C) GST or GST-fusion proteins containing the full-length sequence of ezrin or the amino-terminal domain of ezrin (N-ezrin) were bound to Sepharose beads and incubated with $^{35}$S-Met-labeled in vitro-translated Syk. The autoradiography and Coomassie blue staining of GST and GST-fusion proteins used are shown.

(D) GST and GST-PSGL-1 fusion proteins containing the wt cytoplasmic tail or the deletion mutant containing the 18-juxtamembrane amino acids of PSGL-1 were bound to Sepharose beads and incubated with $^{35}$S-Met-labeled in vitro-translated Syk, in the presence or absence of N-moesin. The proteins eluted from the beads were analyzed by SDS/PAGE. The autoradiography, Coomassie blue staining of the fusion proteins used, and the relative binding of Syk to the fusion proteins are shown.

(E) GST or GST-fusion proteins containing the wild-type full-length sequence of moesin, the ITAM-mutated moesin (Y195F+Y205F), the amino-terminal domain of moesin (N-moesin), or the carboxy-terminal domain of moesin (C-moesin) were bound to Sepharose beads and incubated with $^{35}$S-Met-labeled in vitro-translated Syk. The autoradiography, the Coomassie blue staining of GST and GST-fusion proteins used, and the relative binding of Syk to GST-fusion proteins, estimated by densitometry, are shown.

(F) GST and GST-moesin full-length fusion protein bound to Sepharose beads were incubated for 3 hr at 4°C with reticulocyte lysates control or containing Syk previously translated. Then, beads were washed and analyzed by Western blot with anti-phospho-tyrosine (anti-P-Tyr) mAb. The relative level of phosphorylation of the GST-moesin fusion proteins and GST has been calculated by densitometric analysis.

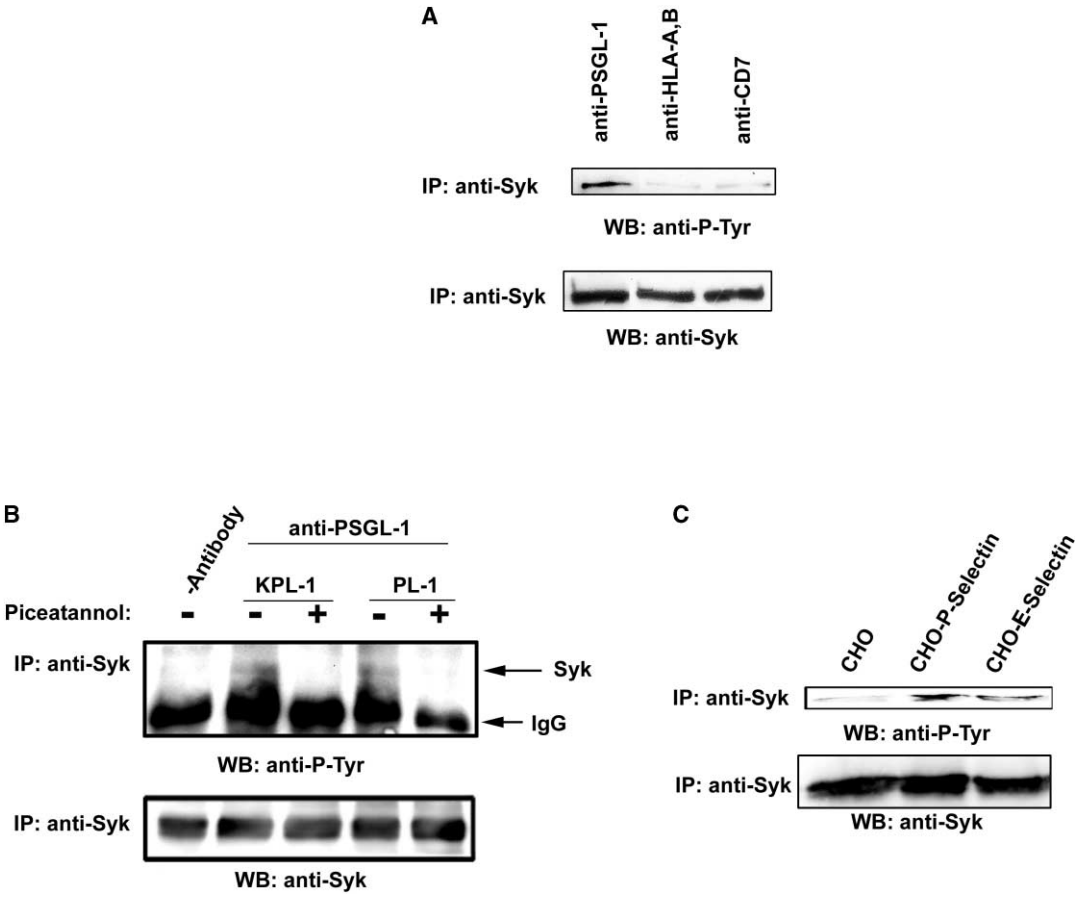



Figure 4. PSGL-1 Engagement Induces Tyrosine Phosphorylation of Syk

(A) T lymphoblasts were incubated at 37°C for 10 min with 10 μg/ml of anti-PSGL-1 (PL1), anti-HLA-A, B (W6/32), or anti-CD7 (3A1) mAbs. After treatment, cells were lysed and Syk was immunoprecipitated and analyzed by Western blot with anti-P-Tyr or anti-Syk mAbs.

(B) U937 cells were incubated at 37°C for 30 min in the presence or absence of 25 μM piceatannol and then for 10 min with anti-PSGL-1 (PL1 or KPL1) mAb. Syk was immunoprecipitated from lysates of treated cells and analyzed by Western blot with anti-P-Tyr or anti-Syk antibodies.

(C) U937 cells were incubated, under rotating conditions, at 37°C for 10 min on monolayers of control CHO cells or CHO cells stably transfected with P- or E-selectin. Nonadherent cells were harvested, and Syk was immunoprecipitated from the cell lysates and analyzed by Western blot with anti-P-Tyr or anti-Syk antibodies.

was barely activated, in agreement with previous results (Figure 5A and Weyrich et al., 1995). Since c-*fos* is a gene whose expression is highly dependent on SRE (Treisman, 1995), we tested whether PSGL-1 engagement resulted in the activation of the c-*fos* promoter. As expected, treatment of cells with an anti-PSGL-1 mAb antibody but not with a control antibody resulted in the transcriptional activation of both the c-*fos* promoter and the endogenous c-*fos* gene (Figures 5C and 5D, respectively).

### Transcriptional Activation Induced by PSGL-1 Is Mediated through the Moesin-Syk Complex

To ascertain whether Syk was directly implicated in the transcriptional activation induced through PSGL-1, U937 cells were pretreated with piceatannol before PSGL-1 engagement. A low concentration of this inhibitor was able to abolish the PSGL-1-mediated transcriptional activation of SRE and the endogenous c-*fos* gene expression (Figures 6A and 5D, respectively). Likewise, overexpression of a kinase dead mutant of Syk (Y518F+Y519F) was also able to prevent the SRE transcriptional activation by PSGL-1, while overexpression of wt Syk increased both the basal and the PSGL-1-induced activation of SRE (Figure 6B). These results demonstrated that Syk is involved in the PSGL-1-mediated signals that regulate induction of gene transcription.

Finally, we decided to investigate the relevance of the moesin-Syk interaction in the signaling triggered by PSGL-1. Overexpression in U937 cells of the moesin mutant Y191F+Y205F, which is unable to bind Syk, resulted in the inhibition of PSGL-1-induced SRE activation (Figure 6C). Moreover, overexpression of the wild-type moesin clearly increased both the basal and the PSGL-1-induced SRE transactivation. These results indicated that moesin, by coupling PSGL-1 and Syk, acts as a key adaptor molecule in the signaling pathway triggered by PSGL-1.

## Discussion

There is evidence for a role of PSGL-1 as a signal transmitting receptor in different cell types including neutro-

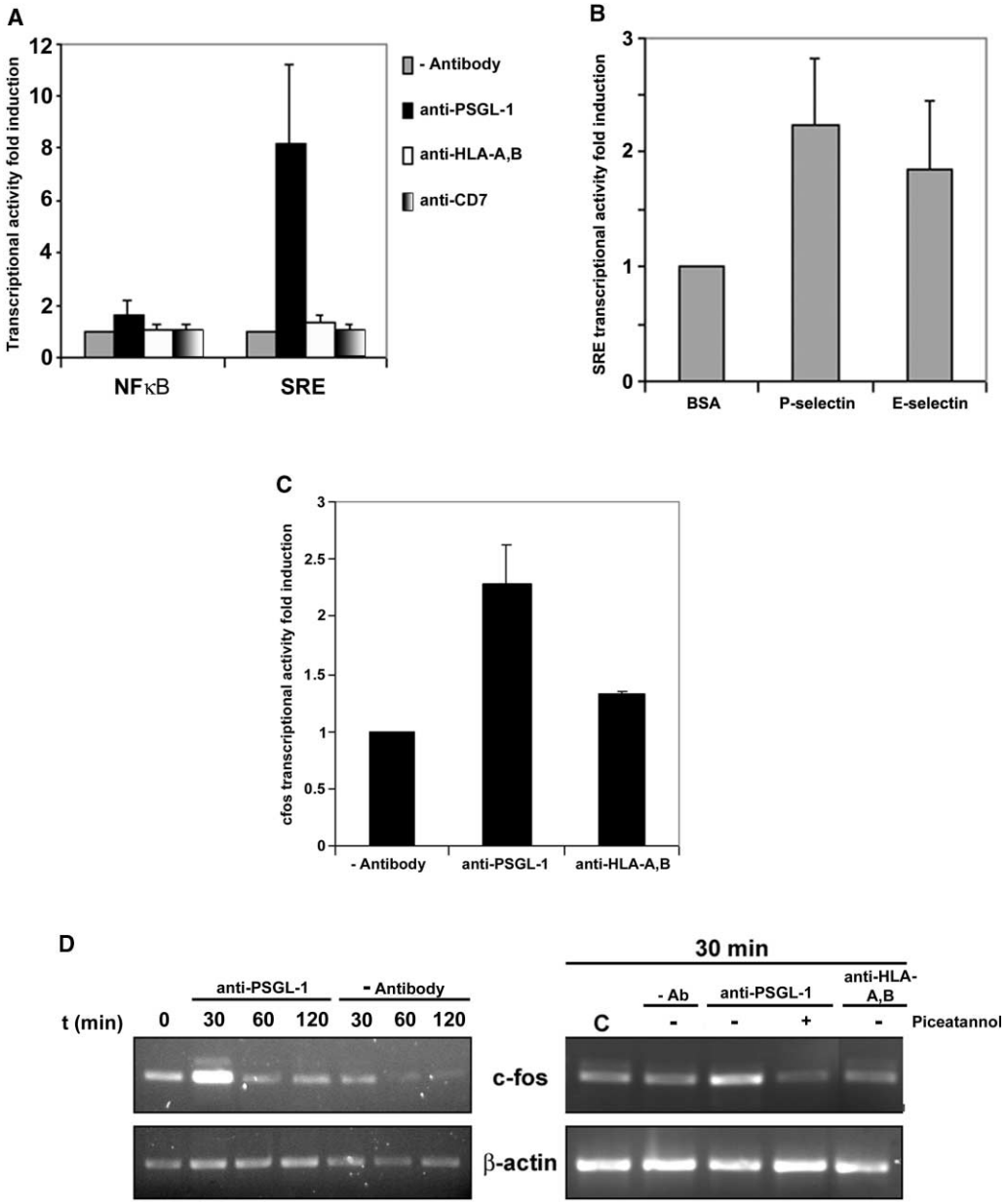



Figure 5. PSGL-1 Engagement Induces SRE-Dependent Transcriptional Activation

(A) U937 cells were transfected with luciferase reporter plasmids driven by NFκB or SRE multimers. Transfected cells were incubated at 37°C for 6 hr without antibody or with 10 μg/ml of anti-PSGL-1 (PL1), anti-HLA-A, B (W6/32), or anti-CD7 (3A1) mAbs. The data shown (fold induction of luciferase activity over the basal of the nontreated cells) represent mean ± SD of three independent experiments. All the Firefly luciferase values were corrected for the Renilla luciferase values obtained in each case.

(B) U937 cells were transfected with the SRE-luciferase reporter construct and incubated, at 37°C for 6 hr, on plates coated with P-selectin, E-selectin, or BSA. Then, cells were lysed and analyzed for luciferase activity. The data shown (fold induction of luciferase activity over the basal of the nontreated cells) represent mean ± SD of three independent experiments. All the Firefly luciferase values were corrected for the Renilla luciferase values obtained in each case.

(C) U937 cells ($2 \times 10^5$) transfected with the c-*fos*-luciferase reporter construct were incubated for 6 hr without antibody or with 10 μg/ml of anti-PSGL-1 (PL1) or anti-HLA-A, B (W6/32) mAbs. Then, cells were lysed and analyzed for luciferase activity. The data shown (fold induction of luciferase activity over the basal of the nontreated cells) represent mean ± SD of three independent experiments. All the Firefly luciferase values were corrected for the Renilla luciferase values obtained in each case.

(D) U937 cells ($10^7$ cells/ml) were incubated at 37°C for the indicated times with 10 μg/ml of anti-PSGL-1 (KPL1) or without antibody and then lysed and analyzed by RT/PCR for the expression of endogenous c-*fos* and β-actin genes (left panel). In the right panel, U937 cells ($10^7$ cells/ml), pretreated or not for 30 min at 37°C with 25 μM piceatannol, were incubated at 37°C for 30 min with 10 μg/ml of anti-PSGL-1 (KPL1) or anti-HLA-A,B (W6/32) antibodies, or without antibody. Then cells were lysed and analyzed by RT/PCR for c-*fos* and β-actin expression. The basal expression of c-*fos* and β-actin genes in untreated cells is also shown in the right panel (C).

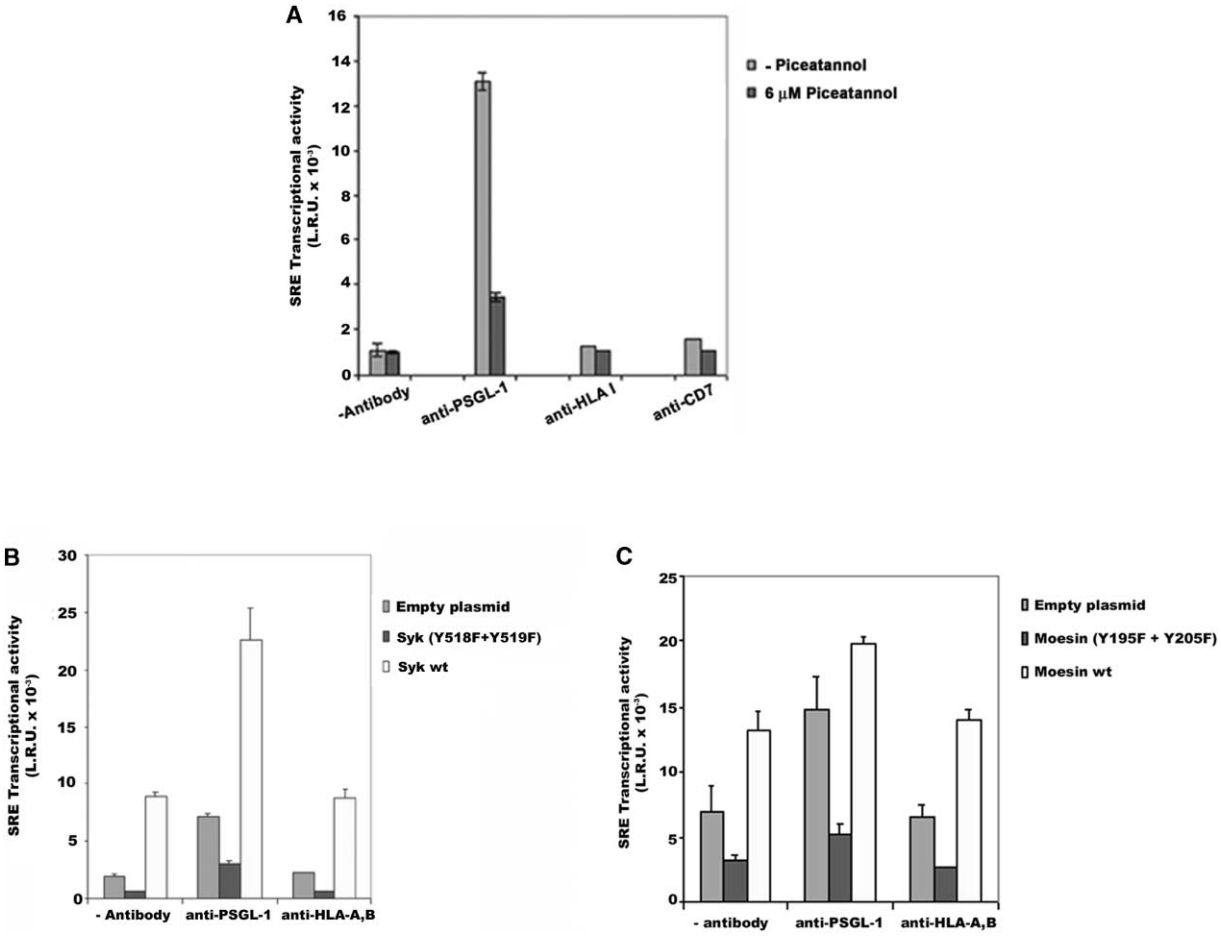



Figure 6. Transcriptional Activation Promoted by PSGL-1 Is Mediated by Syk and Moesin

(A) U937 cells were transfected with SRE-luciferase reporter construct. After 30 min of incubation at 37°C in the presence or absence of 6 μM piceatannol, $2 \times 10^5$ cells were incubated for 6 hr without antibody or with 10 μg/ml of anti-PSGL-1 (PL1), anti-HLA-A, B (W6/32), or anti-CD7 (3A1) mAbs. Then, cells were lysed and analyzed for luciferase activity. A representative experiment out of three independent ones is shown. Bars represent mean ± SD of triplicate samples. All the Firefly luciferase values were corrected for the Renilla luciferase values obtained in each case.

(B) U937 cells were transfected with empty pME18S plasmid or with the same vector expressing wt Syk or a kinase dead mutant Syk (Y518F+Y519F), in the presence of SRE-luciferase reporter construct. Transfected cells ($2 \times 10^5$) were then incubated for 6 hr without antibody or with 10 μg/ml of anti-PSGL-1 (PL1) or anti-HLA-A, B (W6/32) mAbs. After treatment, cells were lysed and analyzed for luciferase activity. A representative experiment out of three is shown. Bars represent mean ± SD of triplicate samples. All the Firefly luciferase values were corrected for the Renilla luciferase values obtained in each case.

(C) U937 cells were transfected with empty pCR3 plasmid or with the same vector expressing wt moesin or the moesin mutant (Y191F+Y205F), in the presence of SRE-luciferase reporter construct. Transfected cells ($2 \times 10^5$) were then incubated for 6 hr without antibody or with 10 μg/ml of anti-PSGL-1 (PL1) or anti-HLA-A, B (W6/32) mAbs. After treatment, cells were lysed and analyzed for luciferase activity. A representative experiment out of three independent ones is shown. Bars represent mean ± SD of triplicate samples. All the Firefly luciferase values were corrected for the Renilla luciferase values obtained in each case.

phils (Hidari et al., 1997), monocytes (Weyrich et al., 1995, 1996), T lymphocytes (Damle et al., 1992), and human hematopoietic stem cells (Levesque et al., 1999). However, the mechanism by which PSGL-1 triggers such signaling has not been elucidated so far. In this report we provide evidence for the association of PSGL-1 with Syk and demonstrate that this tyrosine kinase is activated upon engagement of PSGL-1 both in lymphocytes and U937 cells. In addition, signals elicited through PSGL-1 are able to induce expression of reporter plasmids driven by SRE, a finding consistent with the reported activation of Erk by PSGL-1 (Hidari et al., 1997). Accordingly, we have also found that PSGL-1 is able to transactivate the transcriptional activity of the c-*fos* promoter activity, as well as the endogenous c-*fos* gene, which is highly dependent on SRE (Treisman, 1995).

The transcriptional activation triggered by PSGL-1 requires the participation of Syk, as evidenced by its blockade with piceatannol as well as by overexpression of a dead kinase mutant of Syk. In addition, overexpression of the wild-type Syk was able to activate both the basal and the PSGL-1-dependent transcriptional activation. Thus, our data strongly suggest that the activation of SRE through PSGL-1 requires the activation of the protein tyrosine kinase Syk, which we have found to be

associated with PSGL-1. The weak activation of NFκB upon PSGL-1 engagement is in agreement with a previous work which reported that a costimulus was needed for the NFκB activation induced through PSGL-1 (Weyrich et al., 1995). The induction of expression of early-immediate activation genes upon PSGL-1 engagement, such as c-*fos*, is a relevant finding for the endothelial-leukocyte interaction that occurs during the extravasation process of leukocytes toward inflammatory foci. Thus, during the rolling of leukocytes on activated endothelium, the interaction of PSGL-1 with selectins triggers different signaling pathways that contribute to leukocyte activation. This phenomenon would also occur when leukocytes have been recruited to sites of inflammation, by the interaction of PSGL-1 with the selectins expressed on the surface of other leukocytes and platelets (Alon et al., 1996; del Pozo et al., 1997; Patel et al., 1995; Rainger et al., 1998). As in the case of PSGL-1, another mucin leukocyte receptor, CD43, interacts with moesin and ezrin (Serrador et al., 1998; Yonemura et al., 1998). It has been reported that CD43 triggers a signaling pathway mediated by Syk and that it induces the activation of AP-1 and the expression of CD69 (Santana et al., 2000; Tada et al., 1999). On the other hand, it has been recently reported that Syk acts as a tumor suppressor in human breast cancer cells (Coopman et al., 2000). This new functional role of Syk could be related to the function of PSGL-1 as suppressor of hematopoiesis (Levesque et al., 1999).

Our data indicate that PSGL-1 associates with Syk, but that this association is not direct and requires the presence of moesin, which acts as an adaptor protein. Hence, we found that Syk directly binds to moesin and ezrin through the ITAM-like motif of the amino-terminal domain of ERM proteins (Rozsnyay et al., 1996) and that PSGL-1 associates with Syk through these proteins. The interaction of PSGL-1 with moesin and ezrin has been previously reported (Alonso-Lebrero et al., 2000). Our data show that the indirect association of Syk to PSGL-1 is mediated through the juxtamembrane region of PSGL-1, a domain that is necessary and sufficient for moesin binding and that is responsible for its targeting to the uropod of the cell (Serrador et al., 2002a). On the other hand, we have also found that the mutation of the tyrosine residues of moesin ITAM abolishes both the Syk-moesin binding and the gene transactivation triggered by PSGL-1. Moesin can be phosphorylated in tyrosine, as shown in this work under our assay conditions and by others (Meyer et al., 1998). Therefore, it is feasible that in our binding assay conditions the moesin ITAM is phosphorylated, allowing a phosphotyrosine-dependent mechanism of Syk interaction. Nevertheless, the possibility cannot be ruled out that, as in the case of Syk-β3 integrin interaction (Gao et al., 1997; Woodside et al., 2001), a phosphotyrosine-independent binding of Syk to moesin could also occur. Altogether, these data indicate that ERM proteins act as adaptor molecules, allowing the coupling of PSGL-1 with Syk and thus the subsequent transmission of the PSGL-1-dependent signaling cascade.

Several other adhesion receptors, such as ICAM-3, ICAM-2, ICAM-1, L-Selectin, CD43, or CD44, have been found to interact with proteins of the ERM family (Heiska et al., 1998; Ivetic et al., 2002; Serrador et al., 1997; Yonemura et al., 1998). These molecules colocalize and interact with ERM proteins at the trailing edge of migratory lymphocytes (Serrador et al., 1997, 1998). Moreover, the functional role of the interaction of moesin with ICAM-3 in the membrane targeting of this receptor at the cell trailing edge during leukocyte migration has been recently reported (Serrador et al., 2002b) . On the other hand, a recent work revealed that ICAM-3 has an important role in the antigen scanning by T cells on APCs. However, ICAM-3 is excluded from the center of the immunological synapse (IS) and is localized in the actin-rich cellular protrusions (Montoya et al., 2002). PSGL-1 and CD43 are also excluded from the region of the immunological synapse, a phenomenon that, at least in the case of CD43, is mediated by ERM proteins (Allenspach et al., 2001; Delon et al., 2001; Montoya et al., 2002). Nevertheless, ezrin appears to have a critical role in the formation of the IS, as deduced from the inhibitory effect of the overexpression of a dominant-negative form of ezrin (N-ezrin) on the localization of the TCR at the central supramolecular antigen complex (SMAC) (Roumier et al., 2001). In this regard, our finding that ERM proteins bind Syk in an ITAM-dependent manner suggests that ezrin could act as an scaffold protein recruiting Syk, ZAP-70, and probably other intermediate and effector molecules. Accordingly, our preliminary results suggest that ZAP-70 is also able to associate to the ITAM motif of the ERMs (A.U., unpublished data). The possible role of ERMs as scaffold proteins for molecules of the TCR signaling complex in the IS would explain the inhibition of NFAT activation and IL-2 production observed by overexpression of N-ezrin (Roumier et al., 2001).

Altogether, our results allow us to postulate that ERM proteins, in addition to their function as linkers of membrane receptors with cytoskeleton (Mangeat et al., 1999; Yonemura and Tsukita, 1999), play an important role as adaptor proteins in intracellular signaling cascades. It is very likely that this novel function of ERM proteins has a critical role in the outside-in signaling of cell adhesion receptors lacking kinase activity or ITAM motifs in their cytoplasmic tails.

## Experimental Procedures

### Cell Culture and Transfection

The human myeloid cell lines HL-60 and U937 were grown in RPMI 1640 medium supplemented with 10% of fetal bovine serum at 37°C in a humidified atmosphere with 5% $CO_2$. The mouse myeloma NS1 cell line was grown in complete medium with 50 μM β-mercaptoethanol (Sigma Chemical Co., St Louis, MO). Human peripheral blood mononuclear cells (PBLs) were isolated from buffy coats over a Lymphoprep gradient (Nycomed Pharma AS, Oslo, Norway), and T lymphoblasts were obtained by stimulation for 48 hr with 5 μg/ml of phytohemaglutinin (PHA) and then treated with human rIL-2 (R&D Systems, Minneapolis, MN) for 8 to 15 days. Chinese hamster ovary (CHO) cells expressing a stably transfected human E- or P-selectin cDNA and the parental cell line (CHO-DUKX) were grown as described previously (Montoya et al., 1999).

NS1 cells were transiently transfected and processed for immunofluorescence as described (Serrador et al., 2002b). U937 cells were transfected by electroporation as described (Rubio et al., 1995) and then stimulated for 6 hr before being lysed for luciferase activity determination.

### Proteins and Antibodies

Human P-selectin and E-selectin were a gift of Dr. F.W. Luscinskas (Brigham and Women's Hospital, Boston, MA) and Dr. McEver (Medi-

cal Research Institute, Oklahoma, OK). Recombinant human fibronectin was purchased from Sigma. The anti-PSGL-1 PL1 and PL2 and KPL1 mAbs were kindly provided by Dr. R. McEver, and Dr. G. Kansas (Northwestern Medical School, Chicago, IL), respectively. The anti-Syk 4D10.1 and anti-phospho-tyrosine 4G10 mAb were purchased from Upstate Biotechnology (Lake Placid, NY), and the polyclonal anti-Syk (N-19) from Santa Cruz (Santa Cruz, CA). The anti-HLA-A,B W6-32 mAb has been previously described (Serrador et al., 1998) and monoclonal anti-CD7 3A1 was a gift of Dr. M. López-Botet (Pompeu Fabra University, Barcelona, Spain). The Syk inhibitor piceatannol was purchased from Calbiochem (La Jolla, CA).

### Protein Digestion, Extraction of Peptides, and Mass Spectrometry Analysis

SDS-PAGE gels were stained with colloidal Coomassie blue, and the band of interest excised and subjected to in situ digestion with trypsin as described (Shevchenko et al., 1996). A small aliquot (0.5 μl) of the extract was analyzed by MALDI-TOF mass spectrometry by using a Reflex III instrument (Bruker, Bremen, Germany) operating in reflection mode. Aliquots of 0.5 μl of the fractions to be analyzed were applied onto target and dried out. Then, 0.5 μl of saturated α-ciano-4-hydroxycinnamic acid matrix in water:acetonitrile (1:1) containing 0.1% TFA was added and dried out. Calibration was made internally by using characteristic trypsin autolysis products and matrix peaks. The proteins corresponding to the peptide mass fingerprints obtained by MALDI-TOF were identified by using MASCOT, MS-FIT, and Protein Prospector software packages. Positive matches were confirmed by checking that the candidate protein ranked first place in different computer analyses.

### Recombinant DNA Constructs

Moesin, N-moesin, ezrin, and N-ezrin constructs were provided by Dr. H. Furthmayr (Department of Pathology, Stanford University, Stanford, CA) and have been previously described (Serrador et al., 2002b). Point mutations in the ITAM-like motif of moesin were generated using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The GST-PSGL-1 constructs containing the full-length or different deletions of the cytoplasmic region of PSGL-1 and the pCDNA3 plasmids carrying the full-length cDNA of PSGL-1 or the cDNAs coding for PSGL-1 proteins with different deletions in their cytoplasmic tails, were generated by PCR and described elsewhere (Alonso-Lebrero et al., 2000; Serrador et al., 2002a) . The luciferase reporter plasmid carrying the multimers of SRE has already been described (Fluhmann et al., 1998). The luciferase reporter plasmid carrying the −711 to +45 segment of the c-*fos* promoter was obtained from Dr. J.M. Redondo (Centro de Biología Molecular, Madrid, Spain).

### Immunoprecipitation, Western Blot, In Vitro Translation, Binding Assay, and Pull-Down of Proteins from Cell Extracts with GST-Fusion Proteins

Cells were lysed and processed for immunoprecipitation and Western blot as described (Serrador et al., 1997). The plasmid containing the complete sequence of Syk cDNA under the Sp6 promoter was transcribed, translated, and isotope labeled by using a TNT-coupled rabbit reticulocyte system (Promega, Madison, WI) in the presence of Sp6 RNA polymerase and $^{35}$S-Met, according to the manufacturer's instructions. The isotope-labeled Syk protein obtained was then incubated with the GST-fusion proteins as described (Serrador et al., 2002b). In some cases, $^{35}$S-Met-labeled Syk was incubated with the GST-fusion protein in the presence of 3 μg of purified recombinant N-moesin. Proteins bound to the glutathione-Sepharose beads were resolved in SDS/PAGE under reducing conditions and then analyzed by fluorography and autoradiography. The pull-down of proteins from HL-60 cells extracts was carried out as previously described (Alonso-Lebrero et al., 2000)

### Fluorescence and Confocal Microscopy

Transiently transfected NS1 cells were allowed to adhere to glass coverslips by incubating overnight at 37°C. In the case of T lymphoblasts, coverslips were precoated with fibronectin (20 μg/ml), and cells were left to adhere for 20 min at 37°C. Then, cells were fixed and processed for immunofluorescence as described before (Yanez-Mo et al., 1998). For Syk staining, cells were first fixed and then permeabilized with 0.1% Triton X-100 for 30 s. Alexa 488-labeled goat anti-mouse antibody (Molecular Probes, Leiden, The Netherlands) or Rhodamine-Red X-labeled mouse anti-rabbit antibody (Jackson Immuno Research Laboratories, West Grove, PA), were used as secondary antibodies. For double immunostaining, after the staining with the anti-PSGL-1 mAb antibody, cells were fixed again, permeabilized, and treated for Syk staining. Laser scanning confocal microscopy was performed with a LEICA TCS-SP unit equipped with Argon and Helio/Neon laser beams and attached to a Leica DMIRBE inverted epifluorescence microscope using a ×100 oil immersion objective.

### Engagement of PSGL-1 with Antibody or Selectins

PBLs ($2 \times 10^7$ cells/ml) or U937 cells ($10^7$ cells/ml) were suspended in Hanks balanced saline solution HBSS containing 0.1% BSA and incubated for 10 min at 37°C with a saturating concentration of either PL1 or KPL1 anti-PSGL-1 mAbs or with different control mAbs (W632 anti-HLA-A, B or 3A1 anti-CD7). In some experiments, cells were incubated at 37°C for 30 min with Piceatannol at the indicated concentrations before adding the antibody.

For P- or E-selectin interaction assays, U937 cells ($4 \times 10^6$ cells/ml) suspended in Hanks balanced salt solution containing 0.1% BSA were incubated under rotating conditions (65 rpm) for 10 min at 37°C on monolayers of either control CHO cells or CHO cells stably transfected with P- or E-selectin, in 35 mm dishes.

### Luciferase Reporter Assay

For luciferase assays, $2 \times 10^5$ U937 transfected cells were stimulated with the indicated antibodies for 6 hr and then lysed in 25 μl of an appropriate reporter lysis buffer to measure both the Firefly and the Renilla luciferase (Promega). Luciferase activity was assayed in triplicate by luminometry using the Promega luciferase assay system. Luciferase activity was expressed either as fold increase relative to the basal activity seen in unstimulated cells or as total L.R.U. obtained. For assays carried out on P- or E-selectin, U937 transfected cells were incubated on plates coated with 10 μg/ml of either P- or E-selectin or 1% of BSA for 6 hr at 37°C before being lysed for luciferase activity. Control of transfection was determined by cotransfection of the plasmid containing the Renilla luciferase gene.

### Reverse Transcriptase and Polymerase Chain Reaction Assays

The RNA of U937-treated cells was isolated with the Ultraspec RNA isolation system (Biotech Laboratories, Inc., Houston, TX), and the AMV-RT (Promega) was used for reverse transcriptase (RT) reaction, following the manufacturer's recommendations. An aliquot of 2 μl of the RT reaction was analyzed by polymerase chain reaction (PCR) for both c-*fos* and β-actin expression using the Taq polymerase from Promega and the following pairs of primers: c-*fos* forward: 5′ AAGGAGAATCCGAAGGGAAAGGAATAAGATGGCT 3′; c-*fos* reverse: 5′ AGACGAAGGAAGACGTGTAAGCAGTGCAGCT 3′; β-actin forward: 5′ TGACGGGGTCACCCACACTGTGCCCATCTA 3′; β-actin reverse: 5′ CTAGAAGCATTTGCGGTGGACGATGGAGGG 3′.

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