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PI3K Is Involved in L-Selectin- and PSGL-1-Mediated Neutrophil Rolling on E-Selectin Via F-Actin Redistribution and Assembly

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ABSTRACT

L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) are adhesion molecules that play critical roles in neutrophil rolling during inflammation and lymphocyte homing. On the other hand they also function as signaling receptors to induce cytoskeleton changes. The present study is to investigate the signaling kinases responsible for the F-actin changes mediated by L-selectin and PSGL-1 during neutrophil rolling on E-selectin. Western blot analysis demonstrated that PI3K activation, peaking within 5 min, was induced by ligation of L-selectin and PSGL-1 with E-selectin, and that Vav1 (the pivotal downstream effector of PI3K signaling pathway involved in cytoskeleton regulation) was recruited to the membrane and tyrosine-phosphorylated, depending on PI3K. Furthermore, the F-actin redistribution and assembly mediated by ligation with E-selectin were blocked by LY294002, a PI3K specific inhibitor. Additional experiments showed that PI3K activity was involved in neutrophil rolling on E-selectin. However, Syk/Zap70, the well-known upstream kinase of PI3K, was not involved in this event. These data suggest that PI3K is required for the F-actin-based cytoskeleton changes during neutrophil rolling on E-selectin, which may consequently regulate the rolling event. J. Cell. Biochem. 110: 910–919, 2010.

KEY WORDS: PI3K; L-SELECTIN; PSGL-1; CYTOSKELETON; NEUTROPHIL ROLLING; SIGNAL TRANSDUCTION

Leukocyte transendothelial migration (TEM) is a tightly regulated process which is essential for both lymphocyte homing and neutrophil invasion at sites of inflammation. It is well known that the major steps in this process include: (a) rolling of leukocytes along the endothelial wall of postcapillary venules, (b) spreading and firm adherence of leukocytes to the endothelial cells, and (c) migration of leukocytes through the endothelial clefts to the underlying tissue [Butcher, 1991; Springer, 1994]. The rolling step is mediated by selectins and their counter receptors. Following exposure to inflammatory cytokines, such as tumor necrosis factor and interleukin-1, endothelial cells are induced to express E- and P-selectin [Zimmerman et al., 1992]. Several surface glycoproteins on neutrophils present oligosaccharide moieties that serve as counter receptors for E- and P-selectin [Blanks and Vestweber, 1999]. L-selectin and PSGL-1 are such glycoproteins, which are constitutively expressed on microvilli of almost all leukocytes and play critical roles in leukocyte rolling by recognizing E- and/or P-selectin [Simon et al., 2000; Green et al., 2004].

Upon ligation with physiological ligands or antibodies, L-selectin and PSGL-1 also function as signaling receptors that lead to an array of responses, including up-regulation of β_2 integrin-mediated adhesion to ICAM-1 [Levinovitz et al., 1993; Simon et al., 1995, 1999], oxidative burst, and secretion of cytokines such as TNFα and IL-8 [Laudanna et al., 1994; Hidari et al., 1997]. Besides, it has been reported that attachment of PSGL-1 to actin cytoskeleton is essential for leukocyte rolling on P-selectin, and destruction of cytoskeleton or elimination of the cytoplasmatic tail of PSGL-1 is associated with an increase in rolling velocities on P-selectin, a reduced number of adherent cells, and a reduced capability to stabilize rolling [Snapp et al., 2002]. Another work has shown that L-selectin's lateral mobility (clustering) in cell membrane is regulated by its interaction with actin which in turn fortifies leukocyte rolling [Mattila et al., 2005].

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L-selectin and PSGL-1 have also been demonstrated to activate a kind of cytoplasmic tyrosine kinase c-Abl, leading to F-actin redistribution and assembly [Ba et al., 2005; Chen et al., 2006]. All of these findings implicate the importance of L-selectin- and/or PSGL-1-dependent signaling in neutrophil rolling. However, up to now there is no direct link between neutrophil rolling and the cytoskeleton changes mediated by L-selectin and PSGL-1.

PI3K is a kind of lipid kinase in the cytosol. There are three classes in mammalian cells, among which class I PI3K has been well studied. It comprises a catalytic subunit and a regulatory adapter subunit [Otsu et al., 1991]. The catalytic subunit can be sub-divided into p110α, β, γ, and δ, and the regulatory subunit into p85α, p85β, and p55γ. The prototypical model for PI3K activation is that p110 is recruited to the activated epidermal growth factor receptor (EGFR, a kind of receptor tyrosine kinase) by p85 [Gillham et al., 1999; Yu et al., 2002]. After activation, PI3K phosphorylates its main substrate PtdIns(4,5)P2 into PtdIns(3,4,5)P3 which acts as a second messenger by binding the pleckstrin homology (PH) domain of diverse classes of signal transduction molecules, among which Vav1 acts as a pivotal regulator of cytoskeleton [Hornstein et al., 2004]. Accumulating evidences have indicated that PI3K plays a crucial part in cytoskeletal remodeling and cell migration [Cantrell, 2001; Brachmann et al., 2005]. In growth factor receptor signaling, PI3K has been shown to induce actin changes in the form of Rac-mediated lamellipodia and focal complexes, and Rho-mediated stress fibers and focal adhesions [Karin et al., 1996]. Upon fMLP stimulation, PI3Kδ is translocated to the leading edge of neutrophils and controls lamellipodia formation [Schymeinsky et al., 2007; Ferguson et al., 2007]. All these data suggest that PI3K might function as a critical kinase to regulate F-actin changes during neutrophil rolling. In the present work, we examined the role of PI3K in L-selectin- and PSGL-1-mediated neutrophil rolling on E-slectin. We found that upon ligation of L-selectin and PSGL-1 on neutrophils with E-selectin, PI3K was activated, which was consistent with the tyrosine phosphorylation and recruitment of Vav1 to the membrane. The activated PI3K could further regulate F-actin redistribution and assembly, and consequently regulate L-selectin- and PSGL-1-mediated neutrophil rolling on E-selectin. Thus, our work has demonstrated that PI3K is required for the regulation of neutrophil rolling on E-selectin via F-actin redistribution and assembly.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Dextran T-500 was purchased from Phamacia (Uppsala, Sweden). W6/32 (the anti-human HLA-ABC monoclonal antibody, mouse IgG) was purchased from eBioscence, Inc. DREG56 (anti-L-selectin monoclonal antibody, mouse IgG1, sc-18851), KPL1 (the anti-PSGL-1 monoclonal antibody, mouse IgG1, sc-13535), and antibodies to Vav1, Syk, Zap70 and p85 were purchased from Santa Cruz Biotechnology, Inc. rhE-selectin/Fc (Recombinant chimeric human E-selectin) is from R&D. PY20 (anti-phosphotyrosine monoclonal antibody), non-conjugated F-$(ab')_2$ fragment of goat anti-mouse IgG, FITC-conjugated Phalloidin, LY294002 [inhibitor to PI3K, which abolishes PI3K activity (IC50 = 0.43 μg/ml; 1.40 μM) but does not inhibit other lipid and protein kinases such as PI4 kinase, PKC, MAP kinase or c-Src], Piceatannol (inhibitor to Syk/Zap70), Latrunculin B, as well as other chemicals were from Sigma-Aldrich.

ISOLATION OF NEUTROPHILS

Whole blood from healthy adult volunteers was drawn into heparinized syringes (10 U/ml). Neutrophils were isolated by dextran T-500 sedimentation followed by Ficoll-hypaque gradients as previously described [Boyum, 1968] and resuspended at 4.0×10^7 cells/ml in HBSS buffer, pH 7.4, without Ca^{2+}. Before analysis, $CaCl_2$ was added to the buffer at a final concentration of 1.5 mM. Using the method presented here, neutrophils were found to remain unactivated for ~4 h after separation. More than 95% of the isolated cells were polymorphonuclear leukocytes, and the viability was determined to be >98% by trypan blue exclusion.

CELL STIMULATION

Neutrophils were resuspended in HBSS with 1.5 mM $CaCl_2$, incubated with W6/32, DREG56, KPL1 or rhE-selectin-Fc at a final concentration of 10 μg/ml at 4°C for 20 min, followed by another 20 min at 4°C with F(ab′)2 of goat anti-mouse IgG at a concentration of 20 μg/ml, and then ligated at 37°C for 10 min unless indicated otherwise. For inhibitory experiments, cells were preincubated with Latrunculin B (50 μM unless indicated, 10 min), LY294002 (50 μM unless indicated, 30 min), Piceatannol (5, 10, or 20 μM, 30 min), or equal volume of DMSO as described previously [Urzainqui et al., 2002; Wang et al., 2002, 2007; Srinivasan et al. 2003; Paruchuri et al. 2005; Abbal et al., 2006] before addition of antibodies or rhE-selectin.

After pretreatment of neutrophils with Latrunculin B at indicated concentrations, viability of the cells was measured by a LIVE/DEAD flow cytometric viability/cytotoxicity kit (Molecular Probes, Eugene, OR) following the manufacturer's suggestion.

FLOW CHAMBER ASSAY

Recombinant E-selectin was dissolved in TBS. Polystrene Petri dishes were coated with 100 μl of 2.5 μg/ml rhE-selectin-Fc overnight at 4°C, blocked at room temperature with 2% human serum albumin in TBS for 30 min.

Neutrophil rolling on E-selectin was measured in vitro using a parallel-plate flow chamber (Glyco Tech, Rockville, MD). Neutrophils were washed and resuspended in HBSS with 1.5 mM $CaCl_2$ before analysis. In some experiments, cells were pretreated with DREG56, KPL1, LY294002 or Latrunculin B at indicated concentrations. Cells were perfused over the rhE-selectin-Fc monolayer via a syringe pump at a shear stress of 1.2 dyn/cm^2. The interaction between neutrophils and E-selectin was visualized and recorded by an inverted microscope (Olympus Optical, Tokyo, Japan) equipped with a camera (Panasonic, Yokohama, Japan) connected to a VCR and a television monitor. After 1 min perfusion with PBS/Ca^{2+}/Mg^{2+}/BSA flow buffer, cells entered the chamber and started to interact with E-selectin. Perfusion experiments were performed for 3 min.

Video images were evaluated afterwards, and the total number of rolling cells in 10 random fields of view (0.127 mm^2) of perfusion

period for 1 min (2–3 min) was counted by digital imagine processing. Experiments were performed at least three times.

STATISTICAL ANALYSIS

Data are expressed as means ± SD. The statistical significance of differences between means was determined by one-way ANOVA.

IMMUNOFLUORESCENCE MICROSCOPY

To detect the distribution of F-actin after ligation of L-selectin and PSGL-1 with primary and secondary antibodies or with rhE-selectin-Fc and secondary antibodies, neutrophils were fixed with 1% paraformaldehyde for 15 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS (containing 5 mM EDTA and 2% FBS) for 10 min. After washing with PBS, cells were stained with 3.3×10^{-7} M FITC-conjugated Phalloidin at room temperature for 20 min, washed and investigated under a fluorescence microscope. For inhibitor-blocking experiments, the inhibitor (LY294002) was incubated with cells at 4°C for 30 min before ligation.

FLOW CYTOMETRY

The quantification of F-actin in neutrophils was performed by flow cytometry. Briefly, the resting or stimulated neutrophils were fixed, permeabilized, stained with 3.3×10^{-7} M FITC-conjugated Phalloidin at room temperature for 20 min, and washed with PBST. Cells were examined on a FACScan flow cytometer, and the results were expressed as relative fluorescence index (RFI) by dividing the fluorescence value of the experimental groups by that of the control group.

IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Neutrophils were isolated and stimulated with antibodies or rhE-selectin-Fc as described above. The stimulations were stopped by transferring the cells to an ice bath followed by a transient spin at 1,300 rpm. The pellets were suspended in cold lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM each of NaF, Na_3VO_4, and glycerolphosphate, and 20 μg/ml aprotinin/leupeptin] on ice for 30 min and centrifuged at 13,000*g* for 30 min at 4°C, and the supernatants were pre-cleared with protein A sepharose beads (50% slurry) and incubated with p85 antibody at 4°C with gentle rotation for 3 h. Then, 50 μl of protein A was added and rotated for another 1 h. The immunoprecipitates were washed six times with lysis buffer and resolved by SDS–PAGE. Proteins were transferred to the nitrocellulose membranes using chilled transfer buffer (25 mM Tris, 192 mM glycerin, and 20% methanol) at 100 V for 1 h. After protein transfer, nitrocellulose membranes were washed with TBST (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) for more than three times and immediately incubated with 3% BSA and then incubated with primary antibody and HRP-conjugated secondary antibody at 37°C for 1 h. Chemiluminescent detection was performed by using ECL plus Western-blotting reagents.

SUBCELLULAR FRACTIONATION

The membrane of neutrophils was processed as previously described [Martin et al., 2000] with a slight modification. Briefly, cells were suspended in ice-cold hypotonic buffer (42 mM KCl, 10 mM HEPES [pH 7.4], 5 mM $MgCl_2$, 10 μg/ml each aprotinin and leupeptin) and incubated on ice for 15 min. The lysates were centrifuged at 200*g* for 10 min, and the supernatant was centrifuged at 13,000*g* for 60 min at 4°C. The supernatant (cytosol) was collected and the pellets were resuspended in lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, and 10 μg/ml each aprotinin and leupeptin), vortexed for 5 min at 4°C, and centrifuged again at 13,000*g* for 60 min at 4°C. The supernatant representing the membrane fractions was saved. Both the cytosol and membrane fractions were diluted with equal volume of 2 × Laemmli buffer and separated by SDS–PAGE.

RESULTS

NEUTROPHIL ROLLING ON E-SELECTIN IS MEDIATED BY L-SELECTIN AND PSGL-1

We first tested the roles of L-selectin and PSGL-1 in neutrophil rolling on E-selectin, since the sLe^x (sialyl Lewisx) on L-selectin and PSGL-1 can be recognized by E-selectin [Green et al., 2004]. After blocking L-selectin or PSGL-1 with DREG56 or KPL1, rolling events were decreased by 48% and 60%, respectively, compared with the control sample. Preincubation of neutrophils with DREG56 plus KPL1 inhibited the rolling events to 25%, and pretreatment with EGTA almost totally blocked the rolling events (Fig. 1). This result indicates that neutrophil rolling on E-selectin is mainly mediated by L-selectin and PSGL-1.

LIGATION OF L-SELECTIN AND PSGL-1 WITH E-SELECTIN TRIGGERS F-ACTIN REDISTRIBUTION AND ASSEMBLY

Our previous work has shown that L-selectin or PSGL-1 antibody ligation induced F-actin-based cytoskeleton redistribution and assembly in neutrophils [Ba et al., 2005; Chen et al., 2006]. Here we ligated both of the above-mentioned adhesion molecules and analyzed the distribution and assembly of F-actin. As shown in Figure 2A, in resting neutrophils (no antibody was used for ligation). F-actin was evenly distributed. Ligation of neutrophils with DREG56

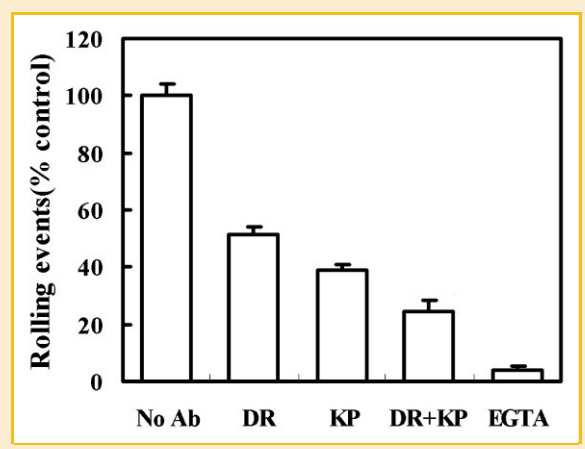

Fig. 1. L-selectin and PSGL-1 mediate neutrophil rolling on E-selectin. Neutrophils were incubated on ice for 30 min in the absence (No Ab) or presence of DREG56 (DR), KPL1 (KP) or EGTA, and perfused over the immobilized rhE-selectin-Fc (rhE-Fc). Rolling events (% control) were calculated as percentage of rolling events of the experimental groups to that of the control groups (No Ab)

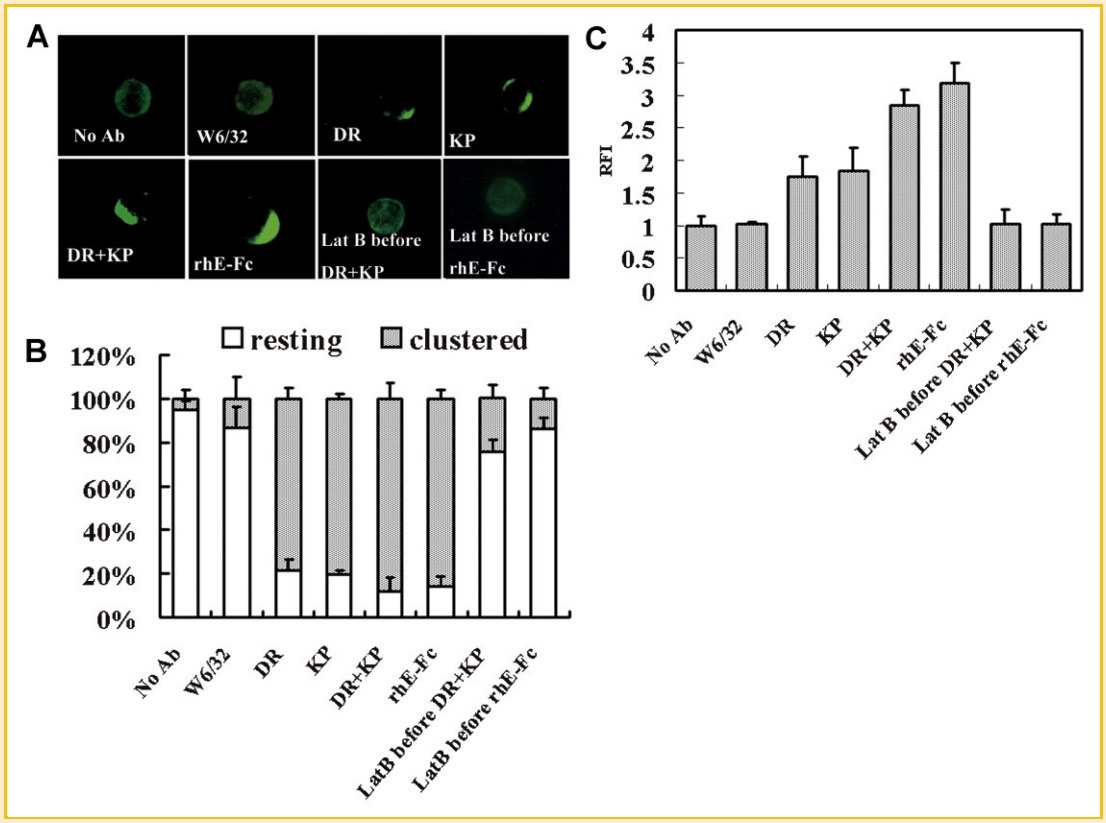

Fig. 2. F-actin redistributes and assembles in response to L-selectin and PSGL-1 ligation with E-selectin. A: Neutrophils were ligated with indicated antibodies or rh-E-selectin-Fc as described in materials and methods. F-actin was visualized by staining with FITC-conjugated phalloidin. For the inhibitory experiments, neutrophils were preincubated with Latrunculin B (Lat B). B: Neutrophils were counted in 5–10 fields (20×) and the percentages were calculated by dividing the number of resting or clustered cells by the total number. Data were obtained from >25 cells for each field. C: Neutrophils were pretreated as described in (B), and the amount of F-actin was detected by flow cytometry. The results were expressed as relative fluorescence index (RFI) which divides the fluorescence value of the experimental groups by that of the control group (No Ab). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

or KPL1 induced a redistribution of F-actin into clusters (these cells were clustered cells), while ligation with rhE-selectin-Fc or with DREG56 plus KPL-1 induced more obvious F-actin clusters. When neutrophils were pretreated with Latrunculin B, the redistribution and clustering induced by L-selectin and PSGL-1 ligation were inhibited. The ratio of the number of resting or clustered cells to the total cell number was shown in Figure 2B, indicating that the majority of cells had been shown in Figure 2A.

Besides, we analyzed the quantitative level of F-actin-based cytoskeleton changes. As shown in Figure 2C, when neutrophils were directly fixed without antibody ligation, the fluorescent value was chosen as the basic control. W6/32 ligation only induced a negligible increase. Ligation with DREG56 or KPL1 enhanced the RFI to 1.75 and 1.83, respectively; while ligation with both antibodies increased the RFI to 2.84 and with E-selectin increased it to 3.19. Pretreatment of neutrophils with Lautrunculin B inhibited the ligation-induced F-actin assembly almost to the basal level.

NEUTROPHIL ROLLING ON E-SELECTIN IS DEPENDENT ON THE F-ACTIN REDISTRIBUTION AND ASSEMBLY IN RESPONSE TO L-SELECTIN AND PSGL-1 LIGATION

Since cytoskeleton is vital for cell movement, and Latrunculin B inhibited the F-actin redistribution and assembly in response to E-selectin ligation (Fig. 2), we used Latrunculin B here to test the involvement of F-actin changes (redistribution and assembly) in neutrophil rolling on E-selectin. As shown in Figure 3A (unblocked), neutrophil rolling on E-selectin decreased in a dose dependent manner with Latrunculin B concentrations, indicating that F-actin redistribution and assembly were crucial for the rolling event. After incubation of neutrophils with DREG56 plus KPL1, the rolling events did not decrease with increasing concentrations of Latrunculin B (Fig. 3A, blocked), suggesting that the de novo F-actin changes induced by L-selectin and PSGL-1 ligation could further support neutrophil rolling on E-selectin. Additional assay revealed that live neutrophils varied between 92% and 96% (Fig. 3B), indicating the viability was not altered by Latrunculin B.

PI3K IS ACTIVATED BY L-SELECTIN AND PSGL-1 LIGATION

Redistribution and assembly of F-actin is regulated by many kinases, such as c-Abl, PKCθ, Vav1 and Rac [Dustin and Cooper, 2000; Villalba et al., 2000, 2001; Ba et al., 2005; Chen et al., 2006]. Tyrosine phosphorylation and recruitment to the membrane are critical to activate kinases. Therefore, we investigated which kinases play a critical role in the F-actin changes in response to neutrophil rolling on E-selectin by detecting the state of tyrosine phosphorylation of proteins in the membrane fractions. After ligation of

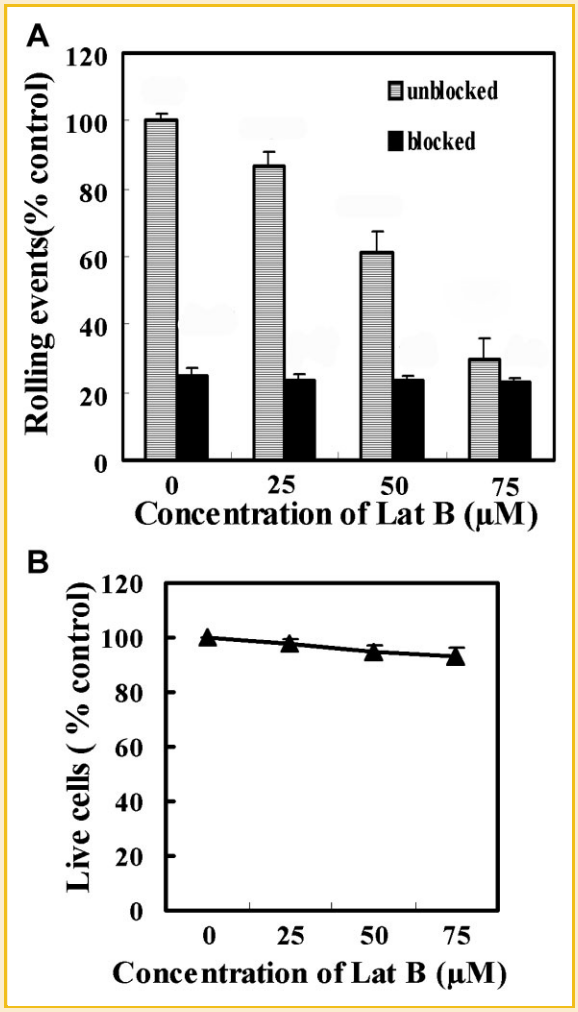

Fig. 3. Neutrophil rolling on E-selectin is dependent on F-actin redistribution and assembly. A: Some neutrophils were pretreated with Latrunculin B at indicated concentrations before perfusion (unblocked). Others were incubated with DREG56 and KPL-1 on ice for 30 min and then pretreated with Latrunculin B before perfusion (blocked). B: Neutrophils were treated as described in (A) (unblocked) and cell viability was analyzed to ensure that the viability was not altered by Latrunculin B. Rolling events (% control) were calculated as percentage of rolling events of the experimental groups to that of the control groups (0 μM Lat B).

neutrophils with rh-E-selectin-Fc for specified time between 1 and 10 min, we found in the membrane fractions that a kind of ~85 kDa protein was tyrosine-phosphorylated in a time-dependent way, and peak phosphorylation appeared at 5 min and remained detectable until 10 min (Fig. 4A, top left). According to its molecular weight, we speculated it might be p85, the regulatory subunit of PI3K. Consequently, we performed another Western blot analysis with the antibody to p85 after stripping the same membrane and thereby the phosphorylated protein was confirmed to be p85 (Fig. 4A, top right). Similarly, the third Western blot analysis was performed as a control, using a kind of serine-phosphorylation antibody, indicating that the tyrosine phosphorylation of p85 was more relative to our experiment than serine phosphorylation (Fig. 4A, bottom left). Next, we investigated the source of p85 which appeared in the membrane fractions and found that p85 was recruited to the membrane, at least in part, from the cytosol (Fig. 4B). Taking into account the fact that tyrosine phosphorylation of p85 is crucial for PI3K activation [Cantrell, 2001], this result suggests that PI3K is activated after L-selectin and PSGL-1 ligation.

Vav1 is a kind of well-known cytoskeleton regulator [Hornstein et al., 2004]. One of its activation ways is to bind PIP3 (the PI3K product) and to be brought to the membrane and activated by tyrosine phosphorylation [Cantrell, 2001]. Based on this, we further ascertained PI3K activation by analyzing the membrane recruitment of Vav1. Western blot analysis revealed that Vav1 was recruited to the membrane from the cotosol after neutrophil ligation with DREG56, KPL1 or rh-E-selectin-Fc (Fig. 5A). When neutrophils were pretreated with LY294002, a specific inhibitor to PI3K, the recruitment of Vav1 to the membrane was inhibited to the basal level (Fig. 5B), and the phosphorylation of Vav1 was no longer detectable at the corresponding sites, in spite of ligation (Fig. 5B). This result demonstrates that PI3K can be activated by L-selectin and PSGL-1 ligation, and this is a prerequisite for PI3K to act as a regulator of F-actin changes during neutrophil rolling on E-selectin.

PI3K ACTIVITY IS REQUIRED FOR NEUTROPHIL CYTOSKELETON REDISTRIBUTION AND ASSEMBLY

As we have shown earlier, PI3K was activated after ligation of L-selectin and PSGL-1 on neutrophils, and F-actin changes were crucial for neutrophil rolling on E-selectin. Together with the accumulating reports that PI3K is a key kinase in signal transduction, especially in actin reorganization [Karin et al., 1996; Cantrell, 2001; Carricaburu et al., 2003; Brachmann et al., 2005; Schymeinsky et al., 2007; Ferguson et al., 2007], these data inspired us to find out whether PI3K is involved in the neutrophil cytoskeleton changes induced by L-selectin and PSGL-1 ligation. As shown in Figure 6A, ligation of neutrophils with antibodies or rhE-selectin-Fc induced F-actin redistribution. Preincubation of neutrophils with 50 μM LY294002, an appropriate concentration sufficient to block PI3K activity, inhibited the F-actin redistribution to a similar state with that of the resting cells. The percentages of the cells illustrated in Figure 6A were calculated and shown in Figure 6B, indicating the majority of cells had been shown in our result. Apart from the inhibitory effect of LY294002 on the F-actin spatial changes mediated by L-selectin and PSGL-1, the use of 50 μM LY294002 also brought the F-actin assembly down to the basal level (Fig. 6C). This result indicates that PI3K activity is required for the F-actin redistribution and assembly induced by L-selectin and PSGL-1 ligation.

PI3K ACTIVITY IS REQUIRED FOR L-SELECTIN- AND PSGL-1-MEDIATED NEUTROPHIL ROLLING ON E-SELECTIN

In order to analyze the relationship between PI3K and neutrophil rolling, we preincubated neutrophils with LY294002 at different concentrations from 5 to 75 μM, as was pointed out in Figure 7A. We found that 5 μM LY294002 inhibited neutrophil rolling events on E-selectin to 67%, compared with equal volume of DMSO. When the concentration was increased to 50 μM, the decrease was about 44%, while further increase in the concentration of LY294002 to 75 μM did not enhance any inhibitory effect, indicating that LY294002

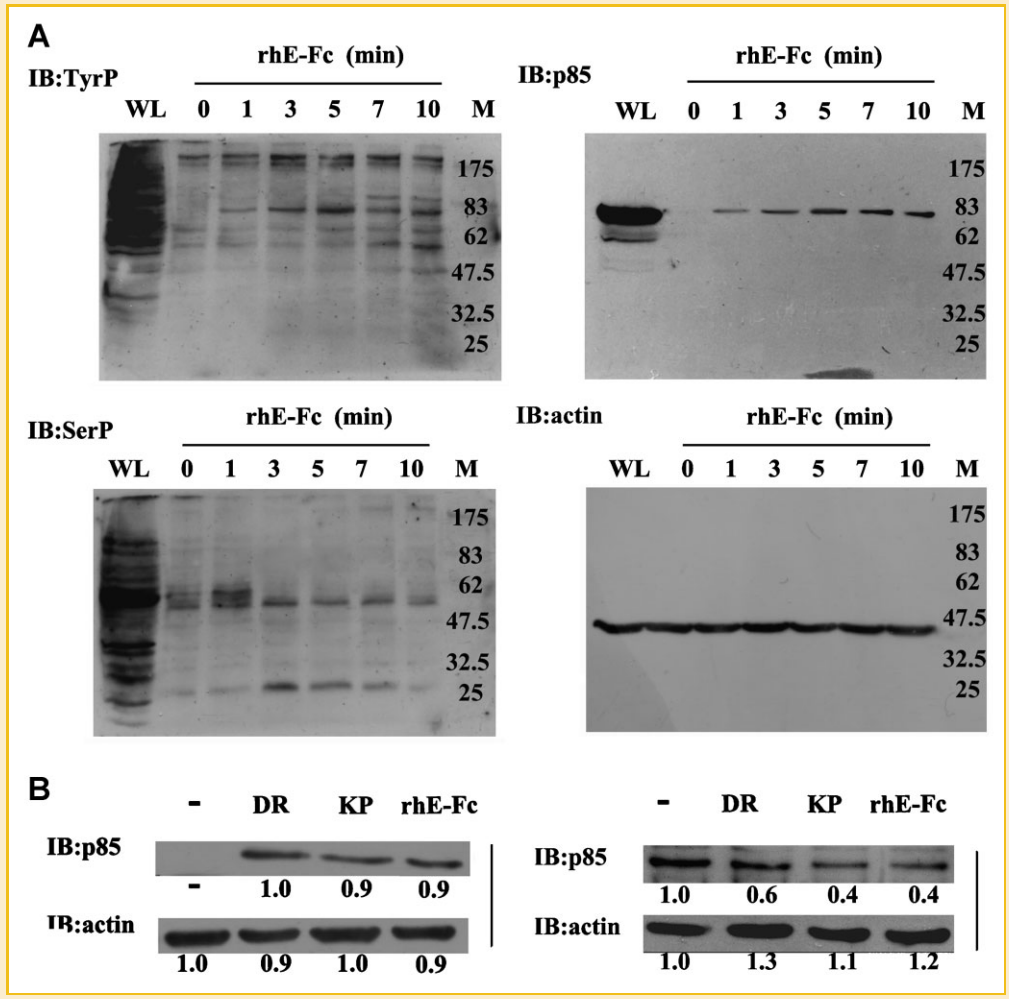

Fig. 4. PI3K is tyrosine-phosphorylated and recruited to the membrane in response to L-selectin and PSGL-1 ligation. A: Neutrophils were stimulated with rhE-Fc for indicated times and the membrane fractions were collected and detected sequentially with antibodies to tyrosine phosphorylation (TyrP), p85 and serine phosphorylation (SerP). The same aliquots of each membrane fraction were loaded and actin was detected as a loading control. M: pre-stain marker; WL: whole lysis of neutrophils. B: Neutrophils were stimulated with rhE-Fc for 7 min. The membrane (left) and the cytosol (right) were collected. p85 was detected by Western blotting, with actin as a loading control. Western blotting results were analyzed by Image-Pro Plus 6.0.

inhibited neutrophil rolling on E-selectin in a dose dependent manner, and at the concentration of 50 μM, LY294002 reached the maximum inhibitory effect. So we can draw a conclusion that PI3K activity is required for neutrophil rolling on E-selectin.

Previous observations have shown that PI3K is downstream of Syk/Zap70 in BCR signaling [Beitz et al., 1999; Moon et al., 2005] and that Syk and Zap70 are important in leukocyte rolling and signaling [Abbal et al., 2006; Chen et al., 2007]. Whether Syk/Zap70 functions in PI3K-required neutrophil rolling on E-selectin remains unknown. Additional flow chamber assays were performed to examine the effect of piceatannol, a widely used specific inhibitor to Syk/Zap 70, on neutrophil rolling. As shown in Figure 7B, the variation of piceatannol concentrations within the widely-used scale (5–20 μM) only had negligible effects on the rolling events, indicating that Syk/Zap70 was not involved in this event, which was consistent with the previous study [Abbal et al., 2006]. Since Syk and Zap70 have been found to interact with p85 [Moon et al., 2005], we next detected whether Syk/Zap70 and p85 were in the same immunocomplex. Notably, Syk, rather than Zap70 bound p85 in a constitutive way in the immunoprecipitation experiments with antibody to p85 (Fig. 7C), implying that Syk/Zap70 might not function as the upstream kinase of PI3K in neutrophil rolling on E-selectin.

DISCUSSION

Here we have characterized a previously unknown signaling pathway downstream of L-selectin and PSGL-1, which is required for the L-selectin- and PSGL-1-mediated and F-actin-based neutrophil rolling on E-selectin. This signaling pathway depends on p85 tyrosine phosphorylation and subsequent recruitment of Vav1 to the cell membrane. By documenting the involvement of PI3K in the F-actin changes and the neutrophil rolling on E-selectin mediated by L-selectin and PSGL-1, we hereby provide a direct

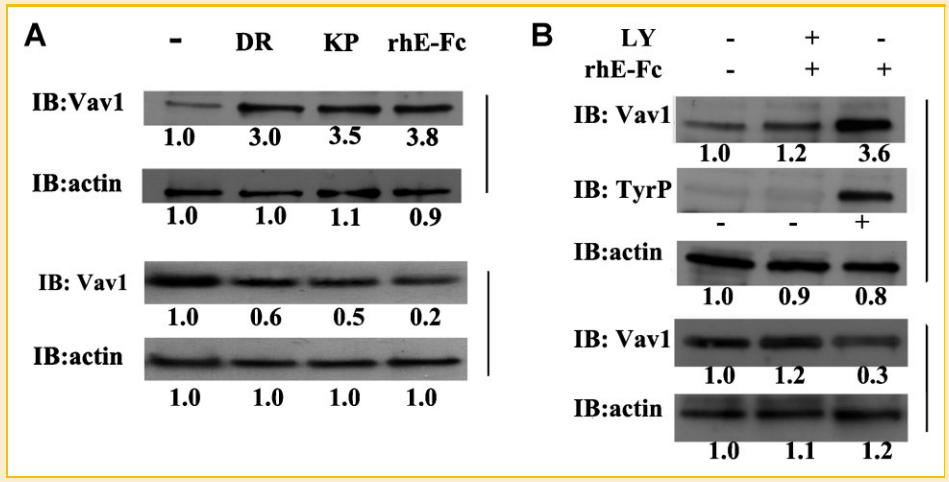

Fig. 5. Vav1 is tyrosine-phosphorylated and recruited to the membrane dependent on PI3K. A: Neutrophils were stimulated with rhE-Fc for 7 min. The membrane (top) and the cytosol (bottom) were collected. Vav1 and its tyrosine phosphorylation were detected by Western blotting. B: Neutrophils were pretreated with or without LY294002 (LY) before ligation with rhE-selectin-Fc, or left neither inhibited nor ligated, and then the membrane (top) and the cytosol (bottom) were analyzed with antibodies to Vav1 and tyrosine phosphorylation.

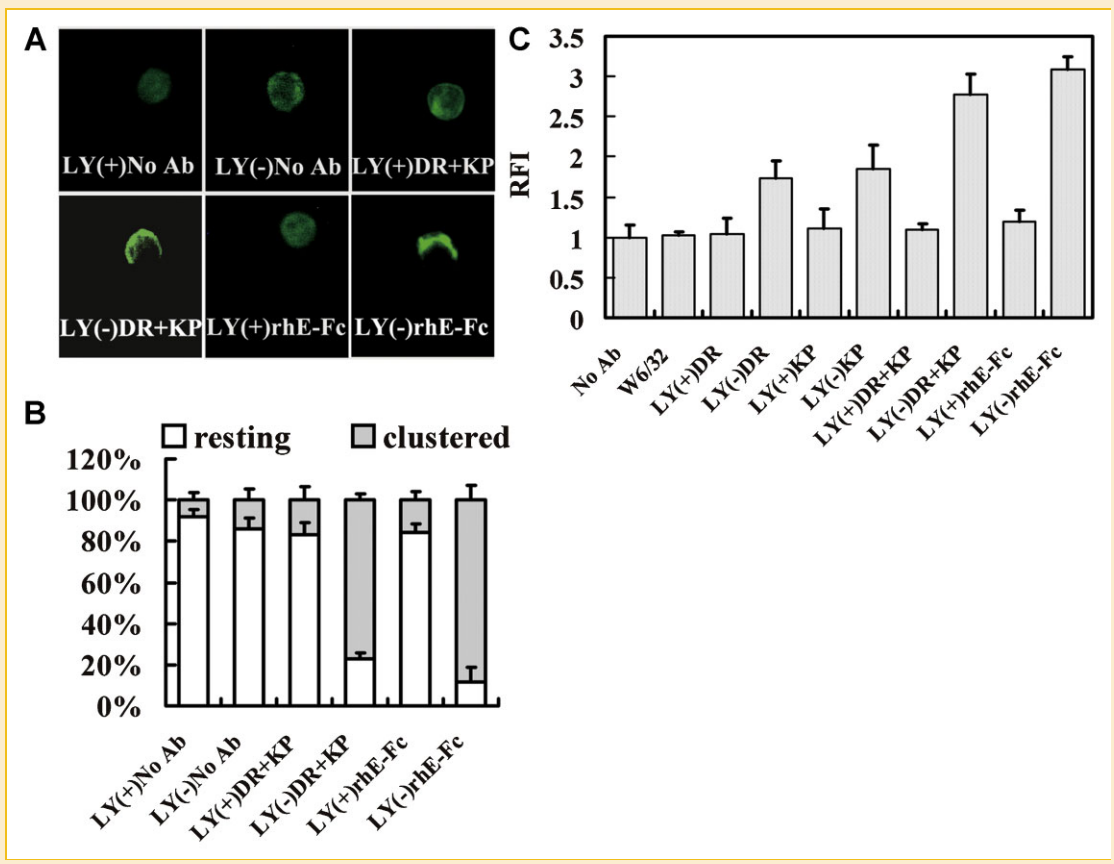

Fig. 6. PI3K activity is required for neutrophil F-actin redistribution and assembly. A: Neutrophils were preincubated with 50 μM LY294002 or DMSO [LY(+) or (−)] and then ligated with or without DREG56, KPL1 or rhE-selectin-Fc. F-actin was visualized by staining with FITC-conjugated phalloidin. B: The number of resting or clustered cells was counted and the percentages were calculated as described in Figure 2B. C: The amount of F-actin in neutrophils treated as indicated in Figure 6A was detected by flow cytometry, and the values were expressed as RFI. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

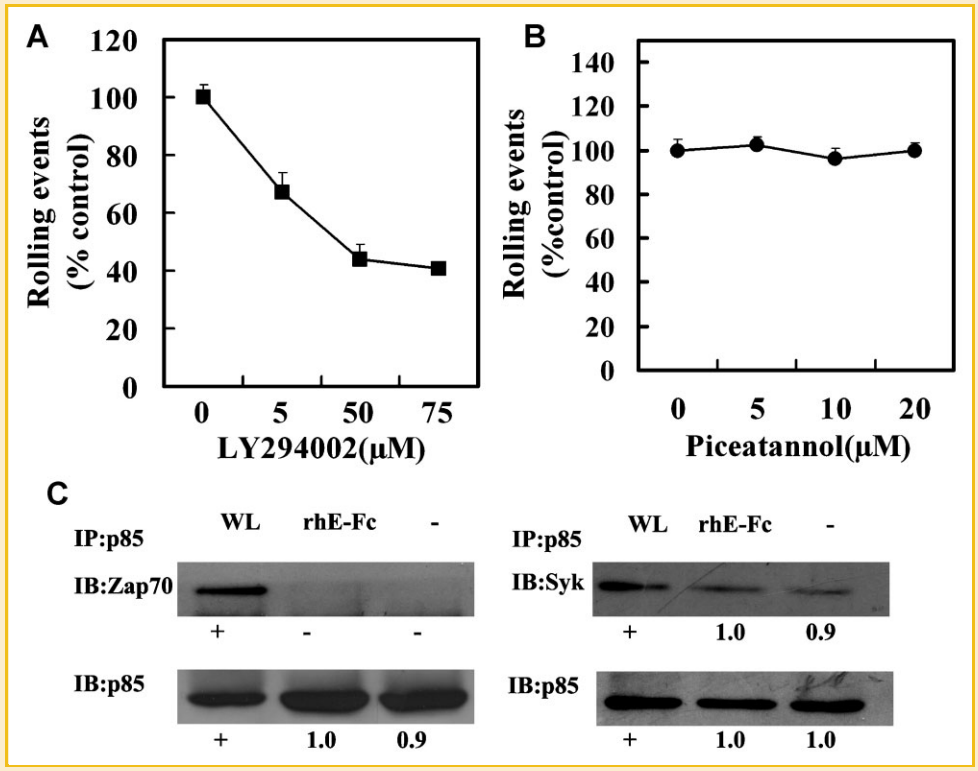

Fig. 7. PI3K activity is required for neutrophil rolling on E-selectin. A: Neutrophils were preincubated with equal volume of DMSO, or 5, 50, 75 μM of LY294002 respectively on ice for 30 min, washed and perfused in the flow chamber assay. Results were calculated as described in Figure 1. B: Neutrophils were preincubated with equal volume of DMSO, or 5, 10, 20 μM of Piceatannol, respectively, and the rolling events were counted and valued after flow chamber assay. C: Neutrophils were stimulated with rhE-Fc for 5 min, and immunoprecipitation experiments with antibody to p85 were performed as described in materials and methods. The results of Western blotting probing Syk/Zap70 were analyzed by Image-Pro Plus 6.0.

evidence for function of adhesion molecules not only as cell adhesion receptors but also as signal transducers.

L-selectin and PSGL-1 are appropriately positioned. Both are constitutively expressed on membrane microvilli and contain multiple N- and O-linked sLe^x [Moore et al., 1994; Bruehl et al., 1996; Somers et al., 2000; Green et al., 2004]. During inflammation, both E-selectin and P-selectin are expressed on activated endothelium. However, E-selectin is unique because its affinity to monovalent sLe^x is 10-fold greater than P-selectin [Somers et al., 2000]. These reports encouraged us to focus on neutrophil rolling on E-selectin in our experiments. Pretreatment of neutrophils with antibody to either L-selectin or PSGL-1 exhibited a great decrease of cell rolling on E-selectin. Notably, although dual blockage of both L-selectin and PSGL-1 exhibited a greater inhibitory effect than single blockage with either DREG56 or KPL1, rolling events were not completely inhibited. Whereas EGTA chelation almost completely inhibited the rolling events (Fig. 1). This suggests that some other undefined ESL-1-like proteins might be expressed on neutrophils.

During leukocyte TEM, active remodeling of actin cytoskeleton goes with cell spreading, firm adherence and subsequent migration through the endothelium clefts [Butcher, 1991; Springer, 1994], implying the prominent functions of cytoskeleton in leukocyte migration. The mechanisms to regulate cytoskeleton have been largely studied, most of which focus on the cytoskeleton-related proteins such as cofilin, L-plastin, and ERM protein. However, the initial signaling kinases crucial for cytoskeleton regulation are still not well-known. Our previous papers have reported that c-Abl kinase is regulated by L-selectin or PSGL-1 ligation in neutrophils and involved in F-actin changes [Ba et al., 2005; Chen et al., 2006]. Here, we demonstrate that another signaling pathway, involving PI3K, is required for F-actin-based cytoskeleton redistribution and assembly (Fig. 6). However, we could not detect p85 in the c-Abl immunoprecipitation complex after ligation of neutrophils with L-selectin and PSGL-1 antibodies, nor could we detect c-Abl in the p85 immunoprecipitation complex (data not shown). This might be explained that c-Abl is not required for the PI3K activity [von Willebrand et al., 1998], or c-Abl and PI3K play roles parallelly. Furthermore, tyrosine phosphorylation of many cytoplasmic kinases has been found pivotal for initiation and amplification of extracellular signals transduced by membrane receptors [Houtman et al., 2005; Xu et al., 2008]. We detected p85 phosphorylation and recruitment to the membrane after L-selectin and PSGL-1 ligation with E-selectin. In this way, PI3K activation was analyzed, which was further confirmed by analyzing the recruitment of P13K downstream Vav1 to the cell membrane (Figs. 4 and 5).

Given that PI3K activity is important for neutrophil F-actin changes (Fig. 6), together with the fact that F-actin assembly and redistribution are essential for neutrophil rolling on E-selectin (Fig. 3), we hypothesized that PI3K might function in neutrophil rolling on E-selectin. This hypothesis was confirmed by the result

shown in Figure 7A, which suggests that PI3K may serve as a critical cytoplasmic kinase to transduce signals from L-selectin and PSGL-1 to F-actin-based cytoskeleton and to regulate neutrophil rolling.

When trying to investigate the upstream kinases of PI3K in neutrophil rolling on E-selectin, we found that Syk and p85 existed in the same immunocomplex. However, their co-existance was not dependent on E-selectin stimulation (Fig. 7C). This result implies that the Syk-p85 complex is not required for the rolling event or other kinases may be involved to activate PI3K. In addition, another kind of protein (~60 kDa, Fig. 4A, IB: TyrP) in the membrane fractions was also intensely phosphorylated on tyrosine in response to E-selectin stimulation. However, its tyrosine phosphorylation appeared later than that of p85, so we speculate that the protein may not be an upstream kinase of PI3K. It's interesting that the protein whose molecular weight is higher than 25 kDa (Fig. 4A, IB: SerP) was phosphorylated on serine before p85 tyrosine phosphorylation. Although we could not clearly know the significance of the protein's serine phosphorylation, it's possible for it to act as a PI3K-upstream kinase.

Taken together, our present data further support the signaling role of L-selectin and PSGL-1 and we ascertain a novel function of PI3K as a regulator of F-actin changes during neutrophil rolling on E-selectin.

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