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Lipid raft-associated β-adducin is required for PSGL-1-mediated neutrophil rolling on P-selectin

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ABSTRACT

Lipid rafts, a liquid-ordered plasma membrane microdomain, are related to cell-surface receptor function. PSGL-1, a major surface receptor protein for leukocyte, also acts as a signaling receptor in leukocyte rolling. To investigate the role of lipid raft in PSGL-1 signaling in human neutrophils, we quantitatively analyzed lipid raft proteome of human promyelocytic leukemia cell line HL-60 cells and identified a lipid raft-associated protein β-adducin. PSGL-1 ligation induced dissociation of the raft-associated protein β-adducin from lipid rafts and actin, as well as phosphorylation of β-adducin, indicating a transient uncoupling of lipid rafts from the actin cytoskeleton. Knockdown of β-adducin greatly attenuated HL-60 cells rolling on P-selectin. We also showed that Src kinase is crucial for PSGL-1 ligation-induced β-adducin phosphorylation and relocation. Taken together, these results show that β-adducin is a pivotal lipid raft-associated protein in PSGL-1-mediated neutrophil rolling on P-selectin. *J. Leukoc. Biol.* 97: 297–306; 2015.

Introduction

The recruitment of leukocytes to a site of inflammation is a highly complicated and strictly regulated process, including tethering, rolling, adhesion, diapedesis, transmigration, and chemotaxis of leukocytes [1–3]. PSGL-1 is constitutively expressed on microvilli of almost all kinds of leukocytes and functions as a common ligand for the 3 selectins [4–6]. At the early stage of inflammation, PSGL-1 interacts with P-selectin on activated platelets and endothelia, leading to the capture of free-flowing leukocytes. PSGL-1 also cooperates with CD44 and E-selectin ligand-1 to support leukocyte rolling on E-selectin expressed on endothelia [4, 7]. Extensive studies of mice lacking PSGL-1 have demonstrated that the interactions of P-, E-, and L-selectin with PSGL-1 are essential for leukocyte migration to the site of inflammation [8, 9].

Upon ligation with physiological ligands or antibody, PSGL-1 recruits many proteins to its cytoplasmic tail to trigger a series of intracellular signal events. The earliest signaling event is activation of the Src family kinase , which activate the ITAM2-containing adaptors [7, 10, 11]. During leukocyte rolling on P-selectin, PSGL-1 ligation induces the recruitment of the Syk into lipid rafts [12]. Activated Syk propagates serial activation of downstream mediators Bruton's tyrosine kinase, phospholipase Cγ2, and p38 MAPK [13]. Previous studies have demonstrated that c-Abl and p85 are activated by PSGL-1 ligation [14, 15]. Upon ligands binding, PSGL-1 starts to move laterally and finally forms clusters on the plasma membrane. This dynamic distribution of PSGL-1 is crucial for the cells to resist shear stress and stabilize the PSGL-1-cyto signaling complex. The lateral movement of membrane receptors is widely present, and this movement is closely related to a specialized subdomains of the plasma membrane known as "lipid rafts" [16, 17].

Lipid rafts are detergent-resistant membrane domains enriched in cholesterol and sphingolipid [18, 19]. These lipid rafts have been studied extensively as cellular signaling platforms in which protein–protein or protein–lipid interactions occur [20, 21]. Cell activation often causes lipid rafts to coalesce into larger entities. The coalescent rafts are more stable for concentrating signaling molecules and for ensuring efficient and sustained signal transduction [22]. Several cell-surface receptor proteins have been reported to execute their functions through association with lipid rafts [23–25], which have also been reported to

Abbreviations: C-adducin ΔP = depletion of prolines motif in C-addcuin, CTxB = cholera toxin subunit B, GM1 = ganglioside, HL-60 cell = human promyelocytic leukemia cell, LC-MS/MS = liquid chromatography-tandem mass spectrometry, MβCD = methyl-β-cyclodextrin, MNE = 25 mM 4-morpholineethanesulfonic acid, pH 6.5, 150 mM NaCl, 5 mM EDTA, MS = mass spectrometry, NP-40 = Nonidet P-40, Pic = piceatannol, PKC/A = protein kinase C/A, PP2 = phosphorylated peptide 2, PSGL-1 = P-selectin glycoprotein ligand-1, PY20 = phosphotyrosine antibody 20, rhP-Fc = recombinant human P-selectin Fc chimera, SH2/3 = Src homology 2/3, shRNA = small hairpin RNA, Syk = spleen tyrosine kinase

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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concentrate some proteins, such as Src and Syk, and to regulate PSGL-1-mediated leukocyte activation [7, 12]. Our previous work demonstrated that lipid raft disruption markedly inhibited the PSGL-1-induced β2 integrin activation [26]. Despite the significant advancements of lipid rafts as signaling organization platform, the dynamics of lipid raft-associated proteins in response to PSGL-1 ligation and the underlying mechanisms of these lipid raft-associated proteins in regulating PSGL-1-mediated leukocyte rolling events remain unclear.

In the present study, we showed that disruption of lipid rafts severely impaired the rolling of neutrophils on P-selectin. With the use of LC-MS/MS, we identified that β-adducin, a lipid raft-associated protein, is implicated in adhesion and rolling of neutrophils. Our data revealed that dissociation of β-adducin from lipid rafts is a critical trigger for releasing PSGL-1 resident lipid rafts from the underlying cortical actin cytoskeleton and for allowing the activation-induced lipid raft and PSGL-1 clustering to proceed.

MATERIALS AND METHODS

Reagents and antibodies

MβCD, cholesterol, filipin, PP2 (a specific inhibitor to Src kinase), genistein (inhibitor of several tyrosine kinases), Pic (a specific inhibitor to Syk kinase), PY20 (antiphosphotyrosine mAb), FITC-conjugated phalloidin, and non-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG were from Sigma-Aldrich (St. Louis, MO, USA). Antiphosphoserine antibody (Ab9332) was purchased from Abcam (Cambridge, United Kingdom). KPL-1 (the anti-PSGL-1 mAb, SC-13535), normal mouse IgG1 (SC-3877), and antibodies to flotillin-2 (SC-28320), β-adducin (SC-376063), Src (SC-32789), and Syk (SC-1240) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa-Fluor-488-conjugated CTxB was obtained from Molecular Probes (Eugene, OR, USA). P-Fc (rhP-Fc) was from R&D Systems (Minneapolis, MN, USA).

Neutrophil isolation and cell culture

Whole blood from healthy adult volunteers was drawn into heparinized syringes (10 U/ml). Neutrophils were isolated by Dextran 500 sedimentation, followed by Ficoll-Hypaque gradients, as described previously [27]. More than 95% of the isolated cells were polymorphonuclear leukocytes, and the viability was determined to be >98% by trypan blue exclusion. Neutrophils are short lived and should be used within 2–4 h of collection. HL-60 cells were purchased from the cell bank of the type culture collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in IMDM containing 10% FBS.

Cell ligation

HL-60 cells were resuspended in PBS and incubated with mouse IgG or KPL-1 at a concentration of 10 μg/ml at 4°C for 20 min and then ligated with a 20 μg/ml anti-mouse IgG (Fab-specific) $F(ab')_2$ fragment antibody at 37°C for the indicated time. For inhibition experiments, cells were preincubated with MβCD (5 mM), PP2 (10 μM), genistein (10 μM), and Pic (10 μM), respectively, or an equal volume of DMSO before ligation.

Immunofluorescence microscopy

The cells were ligated as described below, and then fixed with 4% paraformaldehyde at room temperature for 10 min. Then, the cells were treated with 3% BSA for 30 min and stained with anti-β-adducin antibody and tetramethylrhodamine isothiocyanate-conjugated anti-mouse IgG. The labeled cells were observed under a confocal fluorescence microscope (FluoView FV1000; Olympus Optical, Tokyo, Japan). To stain GM1, the cells were treated with 2 μg/ml CTxB-488 at 4°C for 30 min, followed by fixation with 4% paraformaldehyde for 10 min. For inhibition experiments, HL-60 cells were preincubated with 10 μM PP2 or with an equal volume of DMSO at 37°C for 30 min before ligation with KPL-1.

Detergent-resistant cell lysate fractions

Lipid raft purification was performed by discontinuous sucrose gradient centrifugation at 4°C. In brief, cells ($0.5–2 \times 10^8$) were lysed in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Brij 58, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 50 mM NaF, 10 mM sodium pyrophosphate, and 1 mM Na_3VO_4) for 30 min on ice, followed by centrifugation at 200 g for 10 min. The supernatant was collected and homogenized with 30 strokes in a loose-fitting Dounce homogenizer and gently mixed for 10 min on ice with an equal volume of 80% (wt/vol) sucrose in MNE buffer. Two layers of 30% (2 ml) and 5% (1 ml) sucrose in MNE buffer were added successively. The discontinuous gradient was spun for 18 h at 200,000 g at 4°C in Beckman MLS50 rotors (Beckman Coulter, Brea, CA, USA). Fractions collected from the gradient top were used immediately or kept frozen at −20°C until use.

Immunoprecipitation and Western blot assay

Cells were ligated with KPL-1 at 37°C for the indicated time. After ligation, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM each NaF, Na_3VO_4, and β-glycerophosphate, and 10 μg/ml aprotinin and leupeptin). After 30 min on ice, lysates were centrifuged at 12,000 *g* for 30 min. The supernatant was incubated with the indicated antibodies at 4°C overnight and then incubated for another 3 h with 30 μl protein A/G-Sepharose beads. After washing 3 times with lysis buffer, beads were subjected to SDS-PAGE and Western blot assay. For inhibitory experiments, cells were preincubated with MβCD, PP2, Pic, or an equal volume of DMSO at 37°C for 30 min before PSGL-1 ligation.

LC-MS/MS analysis

For LC-MS/MS, lipid raft samples were purified from unligated or KPL-1-ligated HL-60 cells. The raft pellet was diluted with an equal volume of 2× Laemmli buffer and separated by SDS-PAGE, followed by silver staining. The gel bands were sliced into 2 pieces and subjected to in-gel tryptic digestion. The extracted peptides from each gel piece were analyzed by LC-MS/MS, as described previously [28]. Protein identification results were extracted from SEQUEST (http://fields.scripps.edu/sequest/). The subcellular location, molecular function, and molecular weight of the identified proteins were analyzed by DAVID Bioinformatic Resources (http://david.abcc.ncifcrf.gov/home.jsp).

Membrane fractionation

The membrane fraction of HL-60 cells was processed as described previously [29] with a slight modification. HL-60 cells (1×10^7), with or without PSGL-1 ligation, were collected and resuspended in 1 ml ice-cold hypotonic buffer (42 mM KCl, 10 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 20 μg/ml aprotinin/leupeptin). After 20 min, the cells were homogenized by repeated passage through a 22 gauge needle (30 times). The lysate was centrifuged at 200 *g* for 10 min, and the supernatant (total fractions) was centrifuged at 13,000 *g* for 60 min at 4°C. The supernatant (cytosol fractions) was collected, and the pellets were lysed by adding 100 μl lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM PMSF, and 20 μg/ml aprotinin/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. The cytosol and membrane fractions were diluted with an equal volume of 2× Laemmli buffer and separated by SDS-PAGE.

Plasmid cloning

With the use of the HL-60 cDNA library as a template, N-adducin, C-adducin, C-adducin ΔP, and SH2 and SH3 domains were amplified by PCR by use of the indicated PCR primers (**Table 1**). The PCR product was digested with *Bam*HI and *Eco*RI and ligated into the same sites in pGEX-4T-2 to allow expression of

TABLE 1. Primers for indicated plasmids

Plasmids	Sequence
GST-N-adducin	5′CGGGATCCATGAGCGAAGAGACGGTC3′
	5′CGGAATTCTCACACGGAGCCCACCTCAT3′
GST-C-adducin	5′CGGGATCCCAGTGGGCCGGGAGCA3′
	5′CGGAATTCTCAGGACTCCACTTTCTC3′
GST-C-adducin (ΔP)	5′CGGGATCCCAGTGGGCCGGGAGCA3′
	5′CGGAATTCTCAGGACATGGGGCCGCT3′
GST-C-adducin (Y489F)	5′AATTTGTGCCTCTCTTTACTGACCCC3′
	5′AAGAGAGGCACAAATTGGTTTGGGT3′
GST-C-adducin (Y568F)	5′CGGGATCCCAGTGGGCCGGGAGCA3′
	5′CGGAATTCTCAGGACATGGGGCCGCT3′
Src-SH3	5′CGGGATCCACCTTTGTGGCCCTCTAT3′
	5′CGGAATTCTCAGGAGGGCGCCACGTA3′
Src-SH2	5′CGGGATCCAGGCTGAGGAGTGGTAT3′
	5′CGGAATTCTCACACGGTGGTGAGGC3′

these proteins in *Escherichia coli* cells. Mutations of 2 putative phosphorylation sites in β-adducin (Y489F and Y568F) were introduced by use of the pGEX-4T-2 β-adducin C-terminal domain plasmid with a 1-step overlap extension PCR method by use of the Easy Mutagenesis System (Transgen Biotec, Beijing, China). For shRNA preparation, annealed ds-shRNA oligonucleotides (sh1, 5′GCAGTCTTCCCGACATCTT3′; sh2, 5′GCAAGATCAGCAGTGTCTA3′) were cloned into the *Hpa*I and *Xho*I cloning sites of the lentiviral pLL3.7 vector.

GST precipitation assay

GST-fusion protein expression was induced with isopropyl-β-D-thiogalactoside. Cells were harvested in lysis buffer (20 mM Hepes, pH 7.5, 120 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mg/ml lysozyme, 1 mM PMSF, and 10 μg/ml each aprotinin and leupeptin) and homogenized by sonication. After centrifugation, GST-fusion proteins in supernatant were purified by glutathione-Sepharose 4B beads, according to the manufacturer's instructions. For GST pull-down assay, HL-60 cells were stimulated and lysed as mentioned above. Cell lysates were incubated with 10 μl glutathione-Sepharose 4B beads that had been preloaded for 4 h at 4°C with equal amounts of GST or GST-fusion proteins. After 2 h incubation, the beads were washed 3 times with 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA buffer, and bound proteins were eluted by boiling the beads in reducing SDS sample-loading buffer. After electrophoresis, proteins were transferred to polyvinylidene fluoride membrane for immunoblot analysis with the appropriate antibodies.

In vitro kinase assay

Src kinase was immunoprecipitated from the lysate supernatants of HL-60 cells, with or without KPL-1 ligation, by use of an anti-Src antibody. The immunoprecipitates were washed extensively with the kinase buffer (25 mM Tris-HCl, pH 7.5, 2 mM DTT, 5 mM β-glycerophosphate, 1 mM Na_3VO_4, and 10 mM $MgCl_2$). After preincubation at 37°C for 5 min, 40 μl reactants were initiated by the addition of 3 μg GST-fusion protein (GST, GST-C-adducin, GST-C-adducin Y489F and Y568F) and 5 μM ATP. Reactions were terminated by addition of 40 μl 2× Laemmli sample buffer, and the samples were subjected to SDS-PAGE and immunoblot.

Generation of lentiviruses and transfection

β-Adducin shRNAs were transfected into 293T cells, together with packaged mix (sPAX2 and pMD2; at a ratio of 4:3:1), to generate the respective lentiviruses. Viral stocks were prepared and were used to infect HL-60 cells, according to the manufacturer's introduction.

Cell adhesion assay under static conditions

P-Fc and control human IgG were diluted in PBS, added to 96-well tissue-culture plates (100 μl containing 5 μg/well) at 4°C overnight, and then blocked with BSA at 37°C for 2 h. Neutrophils or HL-60 cells were incubated with 1 μM calcein-acetoxymethyl ester at 37°C for 30 min. Thereafter, the cells were added to the triplicate wells (100 μl IMDM containing 5×10^4 cells), incubated on ice for 20 min, and then warmed rapidly at 37°C for 10 min. The percentage of adherent cells, before and after washing, was calculated by dividing the latter value by the former.

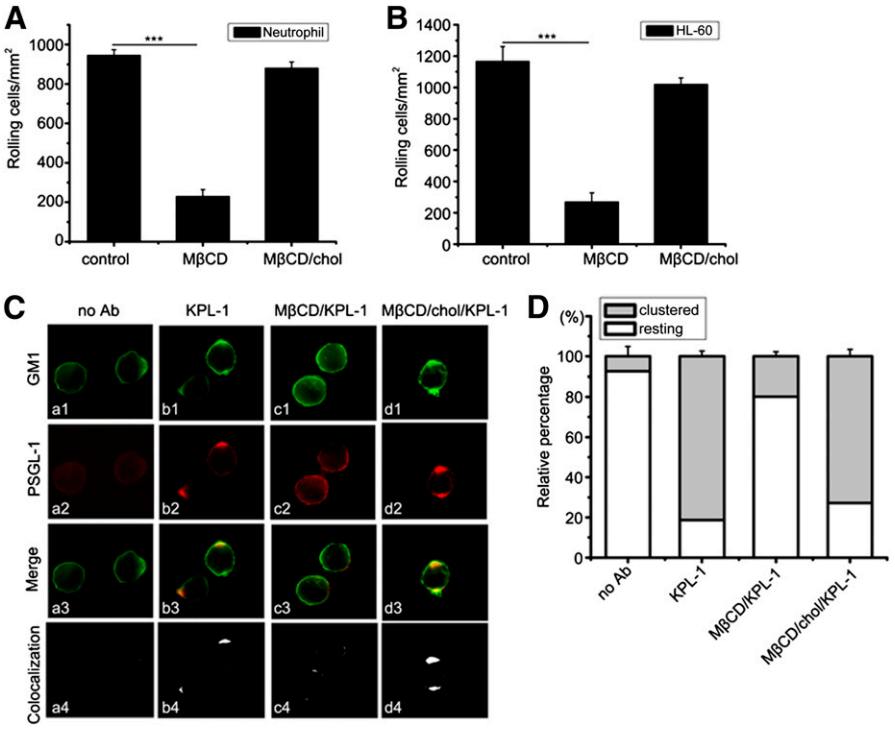

Figure 1. Lipid raft integrity is required for PSGL-1-mediated neutrophil rolling on P-selectin. (A and B) Untreated, 5 mM MβCD-treated, and 1 mM cholesterol (chol)-replenished neutrophils or HL-60 cells were perfused over the immobilized P-Fc, and their rolling on rP-selectin-coated surfaces at 1 dynes/cm^2 over a 5 min timespan was determined, as described in Materials and Methods. Bars represent mean ± SD of 3 independent experiments. ***$P < 0.001$. (C) Cells were pretreated with MβCD (5 mM) or replenished with cholesterol after MβCD treatment, then ligated with KPL-1 for indicated times, and stained with GM1 (green) and PSGL-1 (red) antibody. After washing, the cells were observed under a fluorescence microscope, and typical cells shown were chosen. Their colocalization in merged images was determined and indicated by white dots (bottom). (D) Cells were counted in 10 fields (20×), and the percentages were calculated by dividing the number of resting or clustered cells by the total cells. Data were obtained from >25 cells in each field.

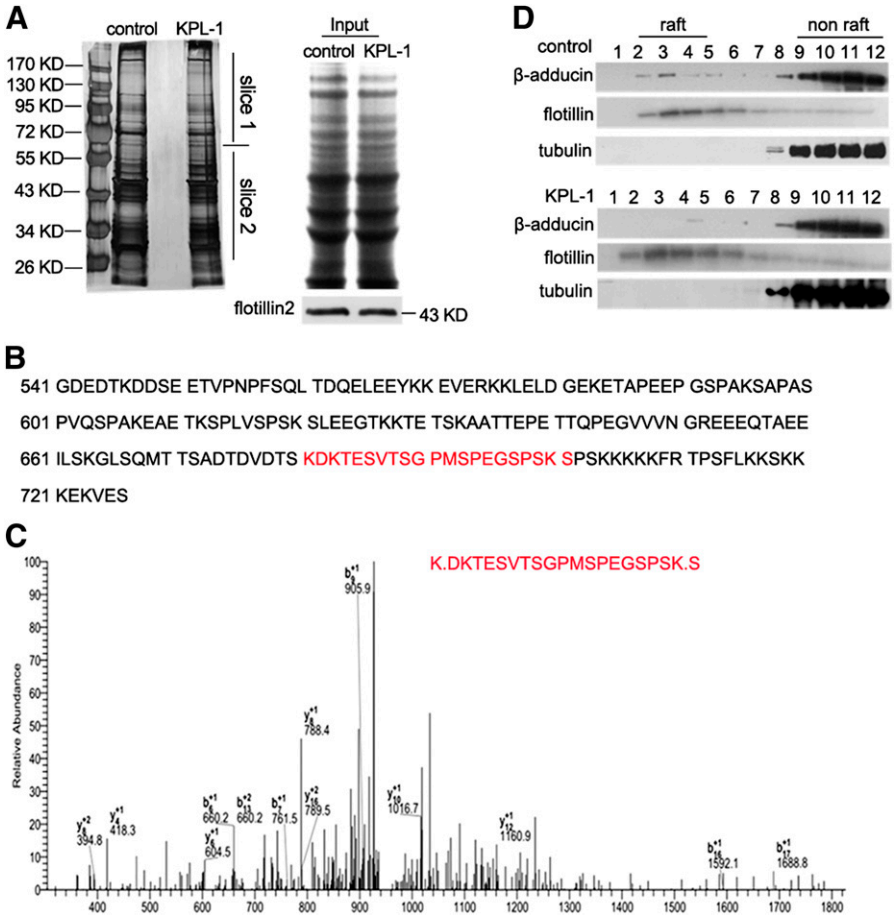

Figure 2. Identification of β-adducin as a lipid raft-associated protein in PSGL-1-ligated HL-60 cells. (A) Detergent-insoluble proteins were resolved on 10% SDS-PAGE and stained by silver staining. Gel bands were cut into 2 pieces and digested with trypsin. The resulting peptides were analyzed by LC-MS/MS (left). The equivalent amount of lysates from control and KPL-1-ligated cells was stained with Coomassie blue and immunoblotted with antiflotillin 2 antibody as input (right). (B) The positions of the peptides (red) within the β-adducin protein sequence is shown, which led to the identification of the β-adducin protein. (C) The special peptide spectra of β-adducin were derived from LC-MS/MS. (D) HL-60 cells, with or without KPL-1 ligation, were lysed and ultracentrifugated, and Western blot experiments were performed.

Cell adhesion assays under flow conditions

HL-60 cells rolling on P-selectin were measured in vitro by use of a parallel-plate flow chamber. Polystyrene Petri dishes were coated with P-Fc overnight and blocked at room temperature with 2% BSA in PBS for 30 min before use. HL-60 cells and shRNA-transfected HL-60 cells were washed, resuspended with IMDM, and perfused over the monolayer at constant shear stress (1 dyn/cm^2). The interaction between HL-60 cells and P-selectin was visualized and recorded by an inverted microscope (Olympus Optical), equipped with a camera (Panasonic, Yokohama, Japan) connected to a VCR and a computer monitor. Video images were evaluated afterwards, and the total number of adherent cells in 10 random fields of view (0.127 mm^2) was calculated.

Statistical analyses

Data were analyzed by 1-way ANOVA. The acceptable level of significance was $P < 0.05$. Data shown represent mean ± SD.

RESULTS

Lipid rafts regulate human neutrophil rolling on P-selectin and PSGL-1 distribution on the cell surface

In our previous work, we demonstrated that lipid rafts play a critical role in PSGL-1 ligation-induced β2 integrin activation in neutrophils. To investigate the role of lipid rafts in human neutrophil rolling on P-selectin, we treated neutrophil with 5 mM MβCD, which can effectively disrupt the integrity of lipid rafts via depleting cholesterol [30], and then the cells were perfused into flow chambers coated with P-selectin rP-Fc protein at a shear stress of 1.0 dyn/cm^2 to mimic the process of neutrophil rolling on inflammation-activated endothelia. The rolling events in cells treated with MβCD were decreased by 76% compared with the control samples. This inhibition was reversed by cholesterol replenishment (**Fig. 1A**). Furthermore, we used HL-60, a broadly used cell line in the study of neutrophil signaling. Preincubation of HL-60 cells with MβCD exhibited similar inhibitory effects on the rolling events, as observed in the experiment, by use of neutrophils, whereas cholesterol addition primarily rescued the inhibitory effect of MβCD on the rolling events (Fig. 1B). We also tested the effect of lipid raft on neutrophil rolling events by use of filipin (a cholesterol-sequestering drug). The results demonstrated that the effects of filipin (200 μg/ml) on cell rolling events are similar to those effects of cholesterol depletion by MβCD (Supplemental Fig. 1). The combined results indicate that lipid raft integrity is required for PSGL-1-mediated neutrophil rolling on P-selectin.

Antibody-mediated cross-linking of PSGL-1 has been used as a model system in studies regarding PSGL-1 function in vitro. Early leukocyte activation responses during their migration into tissues include a cell-shape change from spherical to polarized. To explore further the relationship between lipid rafts and PSGL-1, we labeled GM1 (a lipid raft marker) and PSGL-1. In resting HL-60 cell, both GM1 and PSGL-1 exhibited even distribution at the cell margins in control cells (Fig. 1C, a1–a4). After PSGL-1 ligation with KPL-1 for 5 min, GM1 aggregated (Fig. 1C, b1), and

TABLE 2. List of potential lipid raft-associated proteins by LC-MS/MS analysis

Identified protein IDs	Protein name	Subcellular location	Molecular function (part)
00064201	FERM and PDZ domain-containing 3 (C)	Cytoplasm	Regulate actin cytoskeletal organization
00001675	C-Type lectin domain family 5 (C)	Cell membrane	Immune response, signal anchor
00019904	β-Adducin[a] (C)	Cytoplasm, cytoskeleton, cell membrane	Membrane cytoskeleton-associated protein
00165955	MAPK15 (C)	Cytoplasm, cytoskeleton, nucleus	Cell survival/apoptosis
00332510	TRIO and F-actin-binding protein (C)	Cell membrane	Regulate actin cytoskeletal organization
00023502	CD64 (S)	Cell membrane	Innate immunity
00306604	Integrin β2 (S)	Cell membrane, cytoplasm, cytosol	Cell adhesion, cell migration
00297626	Syntaxin-binding protein 3 (S)	Cell membrane	Cell adhesion
00028108	Regulator of G-protein signaling 19 (S)	Cell membrane	Signal transduction inhibitor
00154755	Down syndrome cell adhesion molecule-like 1 (S)	Cell membrane	Cell adhesion
00290928	Guanine nucleotide-binding protein (G protein), α 13 (S)	Cell membrane	Guanine nucleotide-binding proteins
00303280	MAGUK family (S)	Cell membrane	Tumor suppression, receptor cluster

FERM, 4.1 protein, ezrin, radixin, moesin; PDZ, postsynaptic density protein, Drosophila disc large tumor suppressor, zonula occludens-1; C, resting; S, PSGL-1 ligation; MAGUK, membrane-associated guanylate kinase. [a]Target protein.

PSGL-1 clustered on almost all of the cells and formed a cap-like structure (Fig. 1C, b2). PSGL-1 colocalized with GM1 in most cells (Fig. 1C, b3 and b4). In contrast, 5 mM MβCD treatment dramatically inhibited the cap-like structure formation (Fig. 1C, c1–c4). However, with the replenishment of cholesterol, the colocalization of GM1 and PSGL-1 was recovered effectively (Fig. 1C, d1–d4). The percentages of the cells illustrated in Fig. 1C were calculated and shown in Fig. 1D, indicating that the majority of cells was presented in our result. All of these results suggest that lipid raft integrity is a prerequisite for PSGL-1 redistribution.

Identification of β-adducin as a lipid raft-associated protein in PSGL-1-ligated HL-60 cells

To screen lipid raft-associated proteins that may contribute to PSGL-1 function, we purified lipid raft and performed comparative MS (LC-MS/MS). HL-60 cells, with or without KPL-1 ligation, were lysed, and the lysates were centrifugated by use of discontinuous sucrose gradients. The lipid raft fractions were separated by SDS-PAGE and visualized by silver staining. Each gel lane was cut into 2 pieces and subjected to trypsin digestion and LC-MS/MS protein identification (**Fig. 2A**). The identical proteins in control and KPL-1-ligated cells and the proteins in the organelle membrane were filtered out during the analysis. To understand the function and subcellular location of the identified putative lipid raft-associated proteins, we used DAVID bioinformatics resources to perform bioinformatic analysis. The results revealed that the locations of 12 proteins were altered in response to PSGL-1 ligation, These proteins are involved in innate immunity, cell adhesion, cell migration, actin skeleton rearrangement, and so on (**Table 2**). It has been suggested that lipid raft aggregation may be controlled by cytoskeletal remodeling [31]. Among these identified proteins, β-adducin, a membrane skeleton protein, was the only one that could bind to the membrane and actin. The specific peptides of β-adducin and its spectra are shown in Fig. 2B and C.

To confirm the proteomic identification of β-adducin in response to PSGL-1 ligation in HL-60 cells, the β-adducin protein level in sucrose density gradient fractions from HL-60 cells was determined by Western blotting. As shown in Fig. 2D, the fractions 2–5 were considered as lipid raft fractions because of the high level of the lipid raft marker flotillin-2. Tubulin, a protein known to reside in nonlipid raft fractions, was present in fractions 8–12. The immunoblotting results with antibodies to β-adducin indicated that the amount of β-adducin in the lipid raft fraction purified from KPL-1-ligated HL-60 cells was reduced compared with the unligated cells (Fig. 2D). These data indicate that β-adducin dissociates from the lipid raft in response to PSGL-1 ligation; therefore, we finally selected β-adducin for further investigation.

β-Adducin is crucial for PSGL-1-mediated HL-60 cell rolling on P-selectin

During PSGL-1 signal transduction, signal complexes are commonly formed in its cytoplasmic domain [32]. To investigate whether β-adducin is in the same complex with PSGL-1, we performed an immunoprecipitation assay by use of the anti-PSGL-1 antibody. As shown in **Fig. 3A**, endogenous β-adducin coprecipitated with PSGL-1, and this association between PSGL-1 and β-adducin was reduced after PSGL-1 ligation. To determine whether β-adducin plays essential roles in cell rolling, we knocked down endogenous β-adducin by use of specific shRNA (Fig. 3B) and examined the rolling event of HL-60 cells by use of the flow chamber assay. We found that compared with control cells, the rolling events of β-adducin knockdown cells were reduced significantly (Fig. 3C). We also detected the P-selectin-binding activity by a static cell adhesion experiment and found that the adhesion events of β-adducin knockdown cells were reduced by ~25% (Fig. 3D).

It is generally accepted that the clustering of adhesion molecules is responsible for its function in adhesion and migration of leukocytes. Previous studies have suggested that β-adducin is involved in membrane and actin-related processes

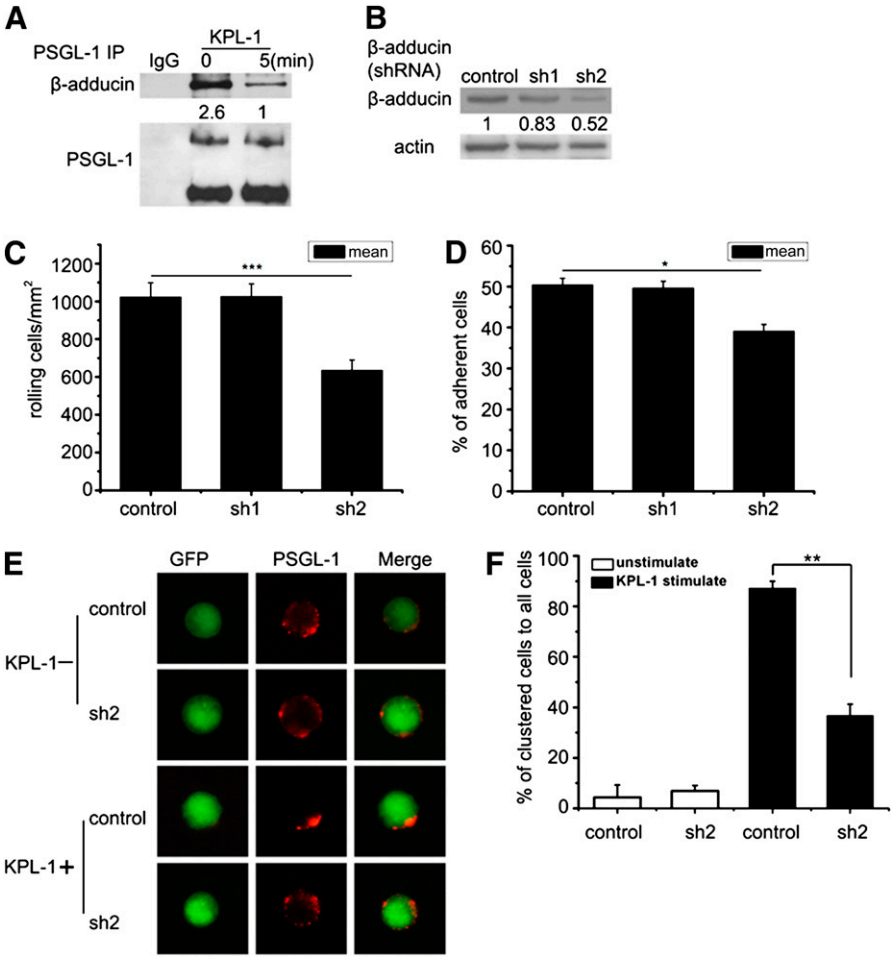

Figure 3. β-Adducin is crucial for PSGL-1-induced HL-60 cells rolling on P-selectin. (A) HL-60 cells were unligated or ligated with KPL-1, followed by PSGL-1 immunoprecipitation, and β-adducin and PSGL-1 were detected. (B) β-Adducin knockdown efficiency was detected by Western blotting. (C) Wild-type or β-adducin knockdown HL-60 cells rolling on the rP-selectin-coated surfaces at 1 dyne/cm^2 over a 5 min timespan was determined, as described in Materials and Methods. ***$P <$ 0.001. (D) Wild-type or β-adducin knockdown HL-60 cells adhered to 96-well plates coated with P-Fc. The percentage of sustained adhesion cells was calculated by dividing the fluorescence of plates after washing by the fluorescence measured before washing, multiplied by 100. Each experiment was repeated 3 times. *$P < 0.05$ compared with positive control. (E) shRNA to β-adducin was transfected into HL-60 cells, and the cells were unligated or ligated with KPL-1 and then stained for PSGL-1. (F) The ratio of clustered HL-60 cells treated as in E was calculated. Data are representative of 3 independent experiments. Bars represent mean ± SD of 3 independent experiments. **$P < 0.01$.

[33, 34]. This possibility prompted us to detect the relationship between the β-adducin and PSGL-1 cluster formation. As shown in Fig. 3E, PSGL-1 was clustered and formed a cap-like structure in PSGL-1-ligated HL-60 cells, whereas the extent of PSGL-1 clustering was weakened significantly in the β-adducin knockdown cells. The percentages of cells with cap-like structure to total cells were calculated in Fig. 3F. These data indicate that β-adducin is indispensable for PSGL-1-mediated neutrophil rolling and adhesion by regulating PSGL-1 clustering.

PSGL-1 ligation induces β-adducin phosphorylation and dissociation from actin cytoskeleton

With the consideration that serine residue and tyrosine residue are in the C-terminal actin-binding region of β-adducin, the phosphorylation status of β-adducin was examined after PSGL-1 ligation by KPL-1. We found that β-adducin was tyrosine phosphorylated in a time-dependent manner. The tyrosine-phosphorylation peak appeared at 3–5 min and remained detectable until 15 min (**Fig. 4A**, top). The serine-phosphorylation peak was detected at a later time-point. From our previous results, we hypothesized that β-adducin tyrosine phosphorylation is involved in PSGL-1-mediated early signaling events. To confirm that tyrosine kinases are responsible for β-adducin phosphorylation, we pretreated the HL-60 cells with genistein, which is an inhibitor of several tyrosine kinases, before PSGL-1 ligation. As shown in Fig. 4B, β-adducin phosphorylation was detected in KPL-1-ligated cells, and the phosphorylation level was inhibited after genistein treatment.

Adducin is a membrane skeleton protein that binds to the membrane and the actin cytoskeleton [33, 35]. The interaction of β-adducin with lipid rafts constitutively exists; however, the presence of β-adducin in lipid rafts was reduced greatly upon PSGL-1 ligation (Fig. 2D). This finding urged us to investigate the relation between β-adducin and actin cytoskeleton. The confocal images revealed that β-adducin and F-actin were distributed at the plasma membrane in unligated HL-60 cells (Fig. 4C, a1–a4). Upon PSGL-1 ligation for 5 min, F-actin was polarized to 1 pole of the HL-60 cells, whereas β-adducin was clearly underneath the membrane (Fig. 4C, b1–b4). However, the F-actin polarization was not detected in cells treated with MβCD, and β-adducin remained distributed around the plasma membranes (Fig. 4C, c1–c4). To validate the interaction between β-adducin and F-actin, we performed an immunoprecipitation assay by use of the anti-β-adducin antibody. Actin was present in β-adducin immunoprecipitation complexes in resting HL-60 cells. However, this association was obviously reduced in the PSGL-1 ligated cells (Fig. 4D). We also found that the dissociation of actin from β-adducin was strongly inhibited by genistein treatment. These data demonstrate that β-adducin phosphorylation induced by PSGL-1 ligation is crucial for its location.

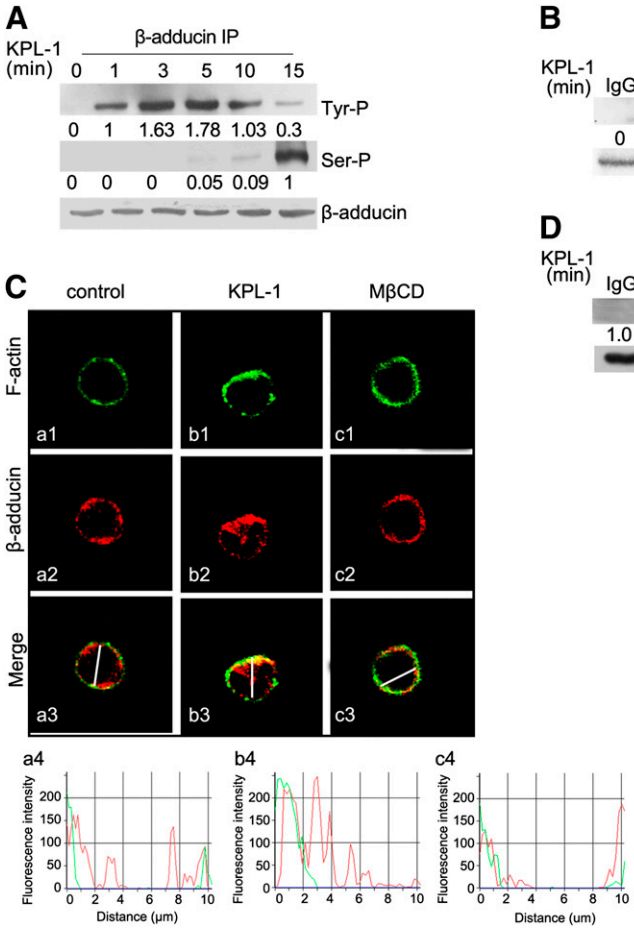

Figure 4. PSGL-1 ligation induces β-adducin phosphorylation and relocation. (A) HL-60 cells were ligated with KPL-1 for the indicated time and lysed. The lysates were subjected to coimmunoprecipitation by use of β-adducin antibody. Samples were subjected to SDS-PAGE and assessed by Western blotting by use of antibodies to tyrosine phosphorylation (Tyr-P) and serine phosphorylation (Ser-P). Then, the blots were stripped and reprobed with anti-β-adducin antibodies to demonstrate equal loading. (B) HL-60 cells were preincubated, with or without genistein (10 μM), before ligation and then immunoprecipitated (IP) with β-adducin antibody, followed by immunoblot analysis with anti-PY20. (C) HL-60 cells were treated, with or without MβCD, and then ligated with KPL-1 for 5 min. The treated cells were stained with phalloidin (green) and β-adducin (red) antibody. The fluorescence intensity of line scans in the merged images was analyzed by Image-Pro Plus software. (D) HL-60 cells were preincubated, with or without genistein (10 μM), before ligation, and then the cells were lysed. The lysates were subjected to coimmunoprecipitation by use of anti-β-adducin antibody, followed by immunoblot analysis with antiactin antibody. Data are representative of 3 independent experiments.

Src kinase is responsible for β-adducin phosphorylation

According to previous studies [34, 36] and the KinasePhos web server (http://kinasephos.mbc.nctu.edu.tw) predictions, we speculated that Src kinase and Syk kinase might be the candidates for phosphorylating the corresponding tyrosine residues in β-adducin. Next, we tested whether PSGL-1 ligation could trigger the activation of these kinases. Western blot analysis showed that Src and Syk kinases were activated in response to PSGL-1 ligation (**Fig. 5A**). We found that cells stimulated with P-selectin also induce Src and Syk activation (Supplemental Fig. 2). We used the specific inhibitors of these kinases to detect further the effect of the kinases on β-adducin phosphorylation through immunoprecipitation experiments. The results showed that PP2 (a specific inhibitor of Src kinase) treatment greatly reduced β-adducin phosphorylation, but Pic (a specific inhibitor of Syk kinase) treatment did not (Fig. 5B). Likewise, the level of β-adducin tyrosine phosphorylation increased upon P-selectin engagement, and PP2 treatment dramatically reduced β-adducin tyrosine phosphorylation (Fig. 5C). Next, we used the specific inhibitors to examine the effect of these kinases on β-adducin relocation. As shown in Fig. 5D, β-adducin was dissociated from the membrane in PSGL-1-ligated cells, and this distribution was greatly impaired in the cells pretreated with PP2. We examined whether Src kinase and β-adducin were involved in the same signaling event in response to PSGL-1 ligation. Results showed that P-selectin engagement leads to β-adducin relocation, and this relocation was greatly impaired in the cells pretreated with PP2 (Fig. 5E). As shown in Fig. 5F, endogenous β-adducin coprecipitated with Src kinase, and this association was significantly reduced by PSGL-1 ligation for 5 min. However, after 10 min of PSGL-1 ligation, β-adducin reassociated with the Src kinase. These data suggest that Src kinase is crucial for the regulation of β-adducin phosphorylation and relocation.

Src kinase interacts with the C-terminal domain of β-adducin

To determine further whether the interaction between Src kinase and β-adducin is direct and which domain or motif of β-adducin is responsible for the efficient interaction, we constructed GST-fusion proteins by use of β-adducin mutants, including N-adducin, (1–345 aa), C-adducin (346–726 aa), and C-adducin ΔP (346 –680 aa). As shown in **Fig. 6A**, Src kinase coprecipitated with GST-C-adducin but not GST-N-adducin, whereas the binding affinity of the C-adducin ΔP (prolines deletion) was markedly reduced compared with the C-adducin domain, particularly in the case of PSGL-1 ligation (Fig. 6B). To confirm the association of Src kinase and β-adducin, we performed a GST pulldown assay by use of the Src-SH2 and Src-SH3 domains. We found that β-adducin interacted with GST-Src-SH3 (Fig. 6C). To determine the mechanism for Src kinase activates β-adducin, we constructed the point mutants in the C-domain of β-adducin, GST-C-adducin Y489F and Y568F, and conducted an in vitro kinase assay by use of GST-fusion proteins as

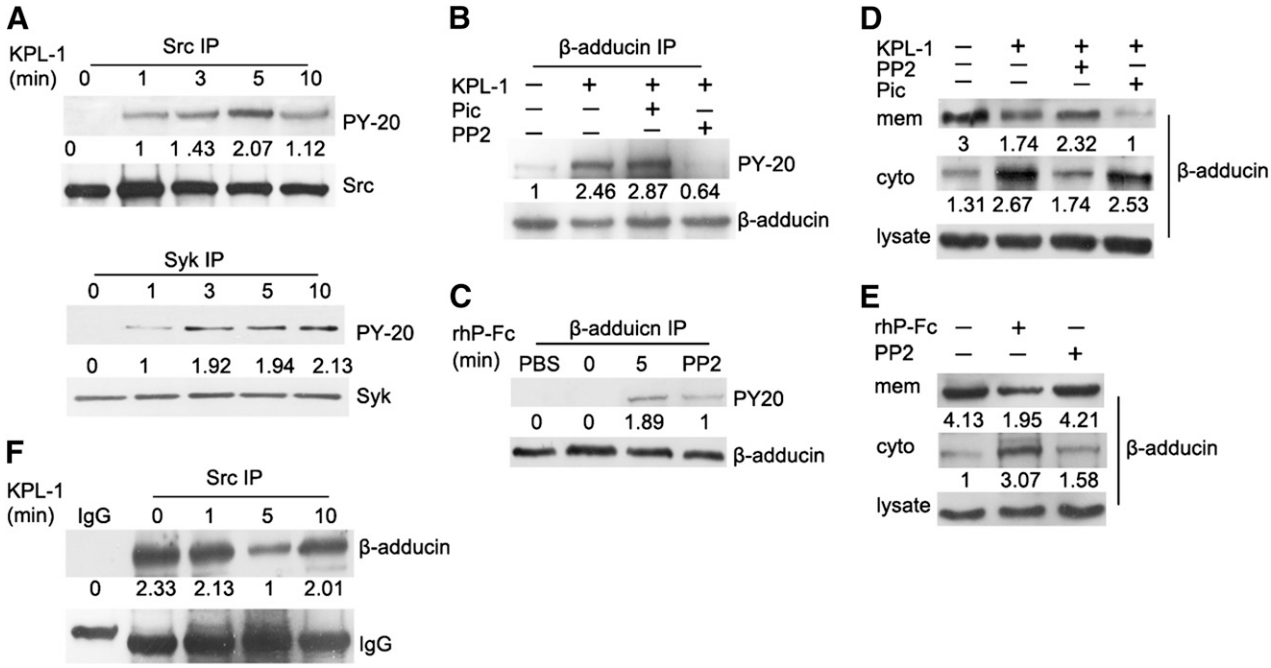

Figure 5. *β*-Adducin is a major effector of Src kinase in HL-60 cells. (A) HL-60 cells were ligated with KPL-1 for the indicated time and then immunoprecipitated with indicated antibodies. PY20 was used to detect the tyrosine phosphorylation of the immunoprecipitation complex. Then, the blots were stripped and reprobed with anti-Src and anti-Syk antibodies to demonstrate equal loading. (B) HL-60 cells were ligated with KPL-1 or not, followed by *β*-adducin immunoprecipitation. The tyrosine-phosphorylation of *β*-adducin was detected with PY20. For inhibition experiments, HL-60 cells were preincubated with PP2 (10 μM), Pic (10 μM), or DMSO before ligation. (C) HL-60 cells were stimulated with rhP-Fc or not, followed by *β*-adducin immunoprecipitation. For inhibition experiments, HL-60 cells were preincubated with PP2 (10 μM) before ligation. (D) HL-60 cells were unligated or ligated with KPL-1 for 5 min. Cytosol (cyto; middle) and membrane (mem; top) cell fractions were prepared, resolved by SDS-PAGE, and expression of *β*-adducin in each fraction was analyzed by immunoblotting with *β*-adducin antibody. The same protein amounts from each fraction were loaded in each lane. For inhibition experiments, HL-60 cells were preincubated with PP2 (10 μM), Pic (10 μM), or DMSO before ligation. (E) HL-60 cells were stimulated with rhP-Fc for 5 min and subcelluar fraction was prepared as described in D. For inhibition experiments, HL-60 cells were preincubated with PP2 (10 μM). (F) HL-60 cells were unligated or ligated with KPL-1 for the indicated time. Thereafter, the cells were lysed, and the supernatants were incubated with anti-Src antibody for immunoprecipitation as indicated. Immunoprecipitates were resolved by SDS-PAGE and then immunoblotted with anti-*β*-adducin antibody. IgG was used as a negative control. Data are representative of 3 independent experiments.

a substrate. As shown in Fig. 6D, the Y489F mutant was slightly less phosphorylated, whereas the level of Y568F mutant phosphorylation was reduced dramatically. The above results indicate that *β*-adducin interacts directly with Src kinase in vitro and that the *β*-adducin C-terminal domain and Src kinase SH3 play a pivotal role in their association. Additionally, Src kinase is responsible for the phosphorylation of Y568 at the C-terminal of *β*-adducin.

DISCUSSION

It is generally accepted that PSGL-1 mediates leukocyte rolling on selectin, and the lipid raft is implicated in the rolling of leukocytes [7, 12]. In our present study, we also demonstrated that lipid raft integrity is required for PSGL-1-mediated neutrophil rolling on P-selectin (Fig. 1). However, the mechanism of lipid rafts regulating leukocyte rolling remains to be elucidated further. Considerable evidence indicates that lipid rafts can concentrate or exclude some proteins to facilitate protein–protein interactions [37, 38]; however, the knowledge regarding proteins that associated with lipid rafts remains limited. In this study, we compared the difference in lipid raft proteins between control and PSGL-1-ligated HL-60 cells and identified *β*-adducin as a lipid raft-associated protein (Fig. 2) by use of LC-MS/MS and bioinformatic analyses, respectively. Ogawa and Rasband [39] have demonstrated that *β*-adducin can be enriched and identified from lipid raft fractions of myelinated nerve fibers by use of proteomic tools, such as MS. Here, we found first that *β*-adducin enriched in the lipid raft fractions of HL-60 cells, and the protein level in lipid raft was reduced in response to PSGL-1 ligation. The interaction between *β*-adducin and PSGL-1 was confirmed by immunoprecipitation and Western blotting (Fig. 3A). Our results clearly demonstrate that the knockdown of lipid raft-associated *β*-adducin markedly attenuates the rolling and adhesion of HL-60 cells on P-selectin (Fig. 3C and D), suggesting a key role of lipid raft-associated *β*-adducin in PSGL-1-mediated cell rolling on P-selectin.

Previous studies have demonstrated that adducin functions in capping the fast-growing ends of actin filaments [40, 41], and our previous study demonstrated that PSGL-1 ligation can induce actin cytoskeleton reorganization [42]. In the present study, we found that *β*-adducin dissociated from actin cytoskeleton in response to PSGL-1 ligation (Fig. 4C and D). Some studies have suggested that the cytoskeleton plays a crucial role in regulating lipid raft dynamic change [31, 43]. Here, we showed that *β*-adducin dissociated from the lipid raft in PSGL-1-ligated cells (Fig. 2D). These observations suggest that *β*-adducin dissociates from the lipid raft, and actin cytoskeleton is a prerequisite for lipid raft and PSGL-1 motility by releasing the

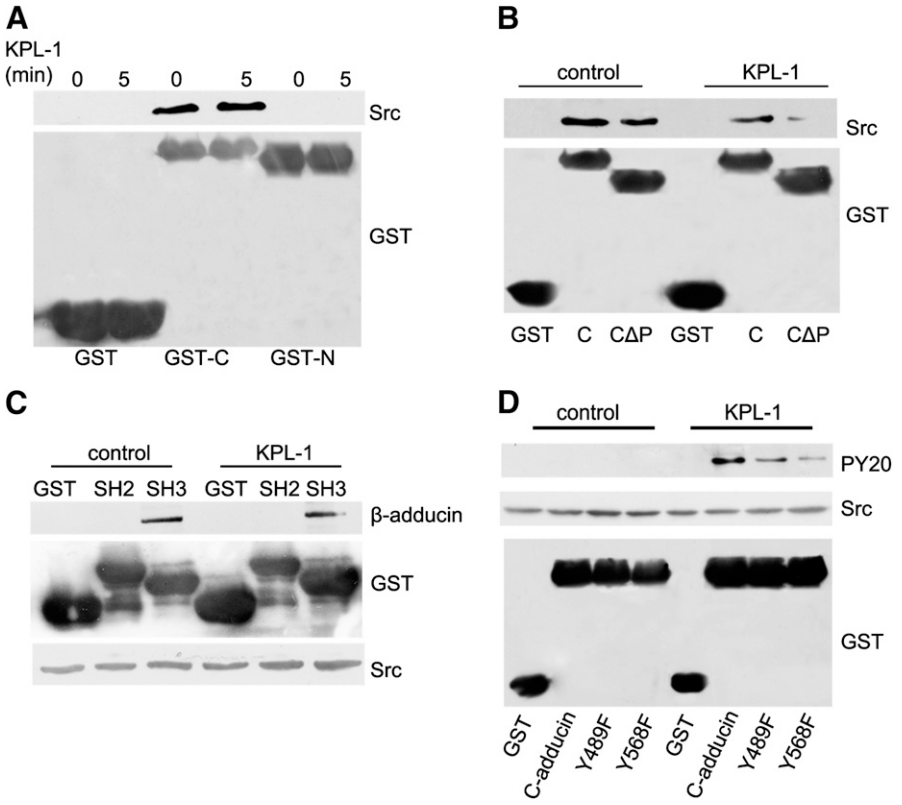

Figure 6. Src kinase constitutively interacts with β-adducin and regulates its phosphorylation. (A and B) Unligated or KPL-1-ligated HL-60 cells were lysed, and the lysates were incubated with GST, GST-N-adducin, GST-C-adducin, or GST-C (ΔP)-adducin (CΔP). Bound proteins were separated by SDS-PAGE and immunoblotted with anti-Src antibody. (C) Unligated or KPL-1-ligated HL-60 cells were lysed, respectively, and the lysates were incubated with GST, GST-Src-SH2, and GST-Src-SH3. Bound proteins were separated by SDS-PAGE and immunoblotted with antiadducin antibody. (D) Unligated or KPL-1-ligated HL-60 cells were lysed, respectively. β-Adducin phosphorylation was determined by in vitro kinase assay by use of GST-fusion proteins of β-adducin mutants (C-terminal domain, Y489F, and Y568F) as a substrate. Phosphorylation of GST-fusion proteins was tested by immunoblotting with antibody for phosphotyrosine (PY20). The level of GST-fusion proteins of β-adducin and Src was detected with the anti-GST antibody and anti-Src antibody to demonstrate equal loading.

membrane from the underneath actin cytoskeleton. PSGL-1 ligation in HL-60 cells induced a rapid phosphorylation of β-adducin (Fig. 4A), a modification known to regulate its linker function [34, 44]. The association of β-adducin phosphorylation and location was confirmed by coimmunoprecipitation experiments. The relocation of β-adducin was greatly inhibited by tyrosine kinase inhibitor treatment (Fig. 4D). Overall, our data suggest that β-adducin phosphorylation is crucial for its relocalization. We also found that the dissociation of β-adducin from lipid rafts and cytoskeleton was transient. β-Adducin reassociated with the membrane and actin at later time-points in response to PSGL-1 ligation, coinciding with the time when the PSGL-1 caps at 1 pole of the cell and localizes together with the coalesced lipid rafts (Supplemental Fig. 3). Thus, at these later times, β-adducin may link the cap structure with the underlying cortical cytoskeleton and stabilizes the cap structure. However, the temporal and spatial details regarding dynamic changes in β-adducin and the underlying mechanisms require further investigation.

Previous reports have demonstrated that adducin family proteins are phosphorylated at serine or threonine residues by PKC, PKA, and Rho-kinase [35, 45–48]. PKC phosphorylates adducin at Ser726, resulting in its dissociation from the actin filaments. In contrast, β-adducin is phosphorylated by Fyn at Y489, resulting in its translocation to the cell periphery [36]. In the present study, we demonstrated that PSGL-1 ligation induces Src kinase activation, and Src kinase is crucial for β-adducin phosphorylation and localization (Fig. 5B–D). With use of an in vitro protein-binding assay, we also found a direct interaction between Src kinase SH3 and β-adducin C-terminal domains, and the Y568 in the β-adducin C-terminal is crucial for its phosphorylation (Fig. 6). From these observations, we suggest that β-adducin tyrosine phosphorylation at Y568 is also required for its dissociation with actin or membrane.

In conclusion, we have provided evidence for the involvement of β-adducin in mediating PSGL-1 ligation-dependent distribution of PSGL-1 and lipid rafts by releasing PSGL-1 and lipid rafts from the underlying cortical actin cytoskeleton. Our data suggest that PSGL-1 resident lipid rafts are anchored by underlying cortical actin cytoskeleton under a resting condition. Upon PSGL-1 ligation, lipid raft-associated β-adducin becomes phosphorylated and then dissociates from the membrane and cortical actin cytoskeleton, resulting in PSGL-1 resident lipid rafts temporarily dissociated from the restrain of the actin cytoskeleton. Finally, PSGL-1 and lipid rafts form a cap structure, where the lipid raft may reassociate with the actin cytoskeleton to stabilize the structure. These data reveal a novel mechanism by which lipid rafts regulate leukocyte recruitment and underscore the potential of raft-targeting agents as effective anti-inflammation drugs.

AUTHORSHIP

T.X., X.W., and X.Z. designed the study, performed experiments, analyzed data, and wrote the manuscript. W.L. and C.Y. performed experiments. X.B. and Y.J. contributed to critical reading of the manuscript and guidance during the study.

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DISCLOSURES

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