Engagement of P-selectin Glycoprotein Ligand-1 Enhances Tyrosine Phosphorylation and Activates Mitogen-activated Protein Kinases in Human Neutrophils*

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Kazuya I.-P. Jwa Hidari‡, Andrew S. Weyrich§, Guy A. Zimmerman§, and Rodger P. McEver¶

From the J.W. K. Warren Medical Research Institute and the Departments of Medicine and Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, and the Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104 and the Nora Eccles Harrison Cardiovascular Research and Training Institute and the Department of Internal Medicine, University of Utah Health Sciences Center, Salt Lake City, Utah 84122

During inflammation, P-selectin on activated platelets and endothelial cells initiates adhesion of leukocytes through interactions with P-selectin glycoprotein ligand-1 (PSGL-1). We investigated whether ligation of PSGL-1 also transmits signals into leukocytes. Neutrophils incubated with anti-PSGL-1 monoclonal antibodies, but not with Fab fragments of these antibodies, rapidly increased tyrosine phosphorylation of proteins with relative molecular masses of 105–120, 70–84, and 42–44 kDa. PSGL-1-dependent adhesion of neutrophils to P-selectin increased tyrosine phosphorylation of similarly sized proteins. Cytochalasin B did not prevent the tyrosine phosphorylation induced by ligation of PSGL-1, suggesting that an intact cytoskeleton is not required for signaling. Engagement of PSGL-1 activated the GTPase Ras through a mechanism that did not require tyrosine phosphorylation of PSGL-1 or association of the Shc–Grb2–Sos1 complex with PSGL-1. Engagement of PSGL-1 activated the 42–44-kDa extracellular signal-regulated kinase family of mitogen-activated protein (MAP) kinases through a pathway that required activation of the MAP kinase kinase. Ligation of PSGL-1 also stimulated secretion of interleukin-8. The tyrosine kinase inhibitor, genistein, blocked tyrosine phosphorylation and secretion of interleukin-8, whereas the MAP kinase kinase inhibitor PD98059 partially inhibited secretion of interleukin-8. Tyrosine phosphorylation stimulated through PSGL-1 on selectin-tethered leukocytes may propagate a signaling cascade that is integrated with signals generated by other mediators.

During inflammation, interactions of selectins with their cell-surface carbohydrate ligands initiate the rolling of leukocytes on the vessel wall (reviewed in Refs. 1 and 2). P-selectin, expressed on activated platelets and endothelial cells, and E-selectin, expressed on activated endothelial cells, bind to ligands on myeloid cells and subsets of lymphocytes. L-selectin, expressed on leukocytes, binds to ligands on other leukocytes and on endothelium. Thus, selectins promote leukocyte-leukocyte, leukocyte-platelet, and leukocyte-endothelial cell interactions under shear forces.

The selectins recognize a variety of sialylated and fucosylated glycans, and P- and L-selectin also bind sulfated glycans such as heparin and sulfatides (3). However, the selectins bind with higher affinity to only a few glycoproteins on leukocytes or endothelial cells. One of the best characterized is P-selectin glycoprotein ligand-1 (PSGL-1) (reviewed in Ref. 4). PSGL-1 is a dimeric mucin expressed on the microvilli of leukocytes that interacts with all three selectins (5–9). P-selectin binds to an N-terminal region of PSGL-1 that must be modified with at least one core-2, sialylated and fucosylated O-glycan and at least one tyrosine sulfate (10–15). L-selectin also binds to the N-terminal domain of PSGL-1 (16–19). PSGL-1 mediates adhesion of leukocytes to P-selectin under both static and shear conditions (20–22). Interactions of PSGL-1 with L-selectin contribute to shear-dependent neutrophil aggregation (16) and leukocyte-assisted recruitment of other leukocytes to selectin surfaces (17).

Selectin-mediated adhesion facilitates juxtacrine signaling of leukocytes by positioning the cells near locally generated lipid autacoids and chemotactants (23). These mediators activate leukocyte integrins, which strengthen adhesion and direct transendothelial migration. Cell-cell contacts through selectins may themselves transduce signals that are integrated with those produced by lipids and chemotactants. The combined signals may produce leukocyte responses that are not observed with either individual stimulus. For example, adhesion of neutrophils to P-selectin enhances β2-integrin activation and potentiates intracellular calcium transients in response to low concentrations of an agonist (23–25). Monocytes that attach to P-selectin mobilize the transcription factor NF-κB and secrete cytokines in response to platelet-activating factor or the chemokine RANTES; both responses are prevented when adhesion is blocked by Fab fragments of mAbs to PSGL-1 or to P-selectin (26, 27).

PSGL-1-dependent signaling pathways in leukocytes are not well understood. Phosphorylation of intracellular proteins is an important proximal regulator of cellular responses to many extracellular stimuli (28). Here we demonstrate that engagement of PSGL-1 on neutrophils rapidly enhances tyrosine phosphorylation of several proteins; it also activates the GTPase

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† To whom correspondence should be addressed: W. K. Warren Medical Research Institute, University of Oklahoma Health Sciences Center, 825 N.E. 13th St., Oklahoma City, OK 73104. Tel.: 405-271-6480; Fax: 405-271-3137; E-mail: rodger-mcever@ouhsc.edu.

‡ The abbreviations used are: PSGL-1, P-selectin glycoprotein ligand-1; Me2SO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; ICAM-1, intercellular adhesion molecule-1; IL-8, interleukin-8; MAP kinase, mitogen-activated protein kinase; mAb, monoclonal antibody; MBP, myelin basic protein; MEK, MAP kinase kinase; PAGE, polyacrylamide gel electrophoresis.
Ras and the MAP kinases, ERK-1 and/or ERK-2. These data suggest a mechanism for signaling through a specific mucin ligand for selectins on leukocytes.

MATERIALS AND METHODS

Proteins and Antibodies—P-selectin was purified from human platelets as described (29). The anti-PSGL-1 mAbs PL1 and PL2 and the anti-P-selectin mAbs G1 and S12 (both Fab\(^\gamma\) fragments) were prepared as described (20, 30). Fab fragments of PL1 were prepared using the Immunopure Fab Preparation kit (Pierce). The anti-L-selectin mAb DREG200 (51) was kindly provided by Dr. Takashi Kei Kishimoto (Boehringer Ingelheim Pharmaceuticals). The control mAb MOPC21 and the anti-CD11a mAb HI111 were purchased from Sigma and Pharmingen (San Diego, CA), respectively. The anti-ICAM-1 mAb 18E3D was a gift from Drs. Joel Hayflick and Pat Hoffman (ICOS Corp.). All these mAbs were of the mouse IgG1 subclass and, unless otherwise described, were prepared as purified, intact IgM. Murine anti-

pp125\(F\alpha R\) mAb 2A7 was a gift from Drs. Michael Schaller and Thomas Parsons (University of Virginia School of Medicine). Rabbit polyclonal anti-phosphorysphine serum, horseradish peroxidase-conjugated recombinant anti-phosphotyrosine antibody, murine anti-SoS1 mAb (clone 35), and rabbit polyclonal anti-Shc IgG (product number S14630) were obtained from Transduction Laboratories (Lexington, KY). A control lysate of J2 (37) was kindly provided by Dr. Western blotting was also obtained from Transduction Laboratories. Horseradish peroxidase-conjugated murine anti-ERK1,2 mAb (clone ERK-7D8) was from Zymed Laboratories. Rabbit polyclonal anti-ERK-1 IgG (product number sc-93), anti-ERK-2 IgG (sc-154), anti-Grb2 IgG (product number sc-255), and rat anti-H-Ras mAb (clone 259) and murine anti-Shc mAb (clone PG-787) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Shc IgG (product number sc-63) was from Santa Cruz Biotechnology. Anti-Grb2 IgG (product number sc-154) was from Santa Cruz Biotechnology. Anti-Shc IgG (product number sc-63) was from Santa Cruz Biotechnology. Anti-Grb2 IgG (product number sc-154) was from Santa Cruz Biotechnology.

Cell lysates were resolved by SDS-PAGE under reducing conditions and transferred to an Immobilon-P membrane (Millipore) using a transblot apparatus (Millipore). The membranes were probed with specific antibodies, and bound antibodies were detected with a chemiluminescence technique (20).

Immunocomplex Kinase Assay—An immunocomplex kinase assay was performed as described (35), with slight modifications. Briefly, the treated cells were lysed in a buffer consisting of 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM Na\(\_2\)VO\(_3\), 1 mM \(\beta\)-glycerophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml each of aprotinin and leupeptin, and 20 mM Tris, pH 7.5, and boiled for 10 min. In some experiments, lysates were also prepared from mononuclear cells isolated from the same donor (33). Cell lysates were resolved by SDS-PAGE under reducing conditions (34), and the proteins were transferred to an Immobilon-P membrane (Millipore) using a transblot apparatus (Millipore). The membranes were probed with specific antibodies, and bound antibodies were detected with a chemiluminescence technique (20).

Fluorescently labeled mAbs or P-selectin were incubated with the cells for 20 min at 37 °C before they were incubated with PL1 IgG in the presence of 20 \(\mu\)Ci of \(\gamma\)-\(\text{[32P]ATP}\) and 0.5 mg/ml kinase substrate, myelin basic protein (MBP), at 30 °C for 20 min. The reaction was terminated by boiling with SDS sample buffer for 5 min. The samples were resolved by SDS-PAGE under reducing conditions. The gel was then stained for phosphorylated proteins or subjected to autoradiography.

\(\text{\[^{32}P\] Labeling of Neutrophils and Measurement of Ras Activation—Neutrophils were metabolically labeled for 3 h at 37 °C with 2 Ci/ml (NEN Life Science Products, 8.8 Ci/\(\mu\)mole) in prewarmed phosphate-free RPMI 1640 medium supplemented with 2% dialyzed, phosphate-free fetal calf serum. The ratio of \(\text{[^{32}P]}\) labeled GTP-GDP bound to Ras was determined as described (36, 37), with minor modifications. Briefly, cells were incubated with 40 \(\mu\)g/ml PL2 or HI111 for various intervals and then lysed in 25 mM Hepes, pH 7.5, 120 mM NaCl, 20 mM MgCl\(_2\), 10 mM NaF, 10 mM Na\(\_2\)VO\(_3\), 1 mM Na\(\_3\)VO\(_4\), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 50 \(\mu\)g/ml aprotinin, and 50 \(\mu\)g/ml leupeptin. The lysates were cleared by centrifugation, and the supernatants were incubated at 4 °C for 2 h with anti-H-Ras mAb-conjugated agarose (sc-33AC, Santa Cruz). The immune complexes were washed five times with lysis buffer and then incubated in 20 mM EDTA for 20 min at 65 °C. After centrifugation, the eluted guanine nucleotides in the supernatant were resolved in a polyethyleneimine cellulose plate coated with fluorescein isothiocyanate (J. T. Baker Inc.) in a running buffer of 0.75 mM KH\(_2\)PO\(_4\), pH 3.5. The labeled nucleotides were visualized by autoradiography and quantified on a PhosphorImager (Molecular Dynamics). Unlabeled GDP and GTP standards analyzed in parallel were visualized with fluorescent light.

Immunoprecipitation—To determine whether PSGL-1 or She was tyrosine-phosphorylated, neutrophils were rapidly lysed in 25 mM Hepes, pH 7.5, 120 mM NaCl, 10 mM Na\(\_2\)VO\(_3\), 50 \(\mu\)g/ml aprotinin, 50 \(\mu\)g/ml leupeptin, and 1% SDS, boiled for 5 min, and then briefly sonicated. The lysate was cleared by centrifugation, and the supernatant was incubated with antibody agarose for 2 h at 4 °C. After centrifugation, the eluted guanine nucleotides in the supernatant were resolved in a polyethyleneimine cellulose plate coated with fluorescein isothiocyanate (J. T. Baker Inc.) in a running buffer of 0.75 mM KH\(_2\)PO\(_4\), pH 3.5. The labeled nucleotides were visualized by autoradiography and quantified on a PhosphorImager (Molecular Dynamics).

Unlabeled GDP and GTP standards analyzed in parallel were visualized with fluorescent light.

Immunoblotting—After treatment, neutrophils were rapidly sedimented, resuspended in boiling lysis buffer (2% SDS, 5 mM EDTA, 5 mM Na\(\_2\)VO\(_3\), 1 mM \(\beta\)-glycerophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml each of aprotinin and leupeptin, and 20 mM Tris, pH 7.5), and boiled for 10 min. In some experiments, lysates were also prepared from mononuclear cells isolated from the same donor (33). Cell lysates were resolved by SDS-PAGE under reducing conditions (34), and the proteins were transferred to an Immobilon-P membrane (Millipore) using a transblot apparatus (Millipore). The membranes were probed with specific antibodies, and bound antibodies were detected with a chemiluminescence technique (20).

Measurement of IL-8 Secretion from Neutrophils—Neutrophils were suspended in a concentration of 5.5 \(\times\) 10\(^5\) cells/ml with 0.5% FCS containing polyvinylin B (10 \(\mu\)g/ml). The cells were preincubated for 10 min at 37 °C with genistein (100 \(\mu\)M) or an equivalent volume of Me\(\_2\)SO. In other experiments, the cells were preincubated for 45 min at 37 °C with PD98059 (10 \(\mu\)M) or with an equivalent volume of Me\(\_2\)SO. The neutrophils were then incubated for 8 h at 37 °C with buffer only, with 40 \(\mu\)g/ml anti-PSGL-1 mAb PL1, or with 40 \(\mu\)g/ml anti-ICAM-1 mAb 18E3D. The cell suspensions were then centrifuged at 15,000 \(\times\) g for 10 min, and the
cell-free supernatants were collected for measurement of IL-8 secretion by enzyme-linked immunosorbent assay (27). More than 95% of the neutrophils excluded trypan blue after the 8-h incubation, confirming that they remained viable under the conditions of the experiment.

**RESULTS**

**Binding of mAbs or P-selectin to PSGL-1 on Neutrophils Increases Tyrosine Phosphorylation of Several Proteins**—To determine whether ligation of PSGL-1 could affect tyrosine phosphorylation in neutrophils, we incubated intact cells with the anti-PSGL-1 mAb PL1, which blocks binding of PSGL-1 to P-selectin (20). The treated cells were rapidly lysed in boiling SDS buffer, and the lysates were subjected to immunoblotting with anti-phosphotyrosine antibodies. PL1 increased tyrosine phosphorylation of several proteins with relative molecular masses of 105–120, 70–84, and 42–44 kDa (Fig. 1). Because of variable background, the increased tyrosine phosphorylation of the 42–44-kDa proteins was occasionally not observed (compare Fig. 1A with Fig. 1B). Phosphorylation increased within 1–2 min, reached a maximum within 10 min, and persisted for at least 30 min (Fig. 1A and data not shown). Tyrosine phosphorylation was not enhanced when neutrophils were incubated with MOPC21, an isotype-matched nonbinding mAb, or with H111, an isotype-matched anti-CD11a mAb that binds to neutrophils (Fig. 1B). PL2, a nonblocking mAb that binds to a different epitope on PSGL-1 (20, 38), caused the same pattern of tyrosine phosphorylation as that observed with PL1 (data not shown).

Fab fragments of PL1 did not increase tyrosine phosphorylation, but addition of Fab fragments of goat anti-mouse IgG to neutrophils preincubated with PL1 Fab induced tyrosine phosphorylation like that observed with intact PL1 IgG. Saturation of FcRγII and FcRγIII receptors with specific Fab fragments did not prevent PL1 IgG-induced tyrosine phosphorylation (data not shown). These data suggest that cross-linking of PSGL-1 is required to signal tyrosine phosphorylation and that signaling does not require co-ligation of Fcy receptors. PL1 increased tyrosine phosphorylation of proteins with mobilities that were similar to those phosphorylated upon binding of the anti-L-selectin mAb DREG200 (Fig. 1B and Ref. (39)). PL1 also increased tyrosine phosphorylation in neutrophils pretreated with cytochalasin B, suggesting that an intact cytoskeleton is not required for this response (Fig. 1B).

The experiments in Fig. 1 demonstrate that engagement of PSGL-1 with specific mAbs triggers tyrosine protein phosphorylation in neutrophils. To determine whether interaction of PSGL-1 with P-selectin itself increases tyrosine phosphorylation, we incubated neutrophils on immobilized P-selectin or on control surfaces. Adhesion of neutrophils to P-selectin induced a profile of tyrosine-phosphorylated proteins that was similar to that observed after treatment with PL1 (Fig. 2A). This response was not observed when cells attached to poly-D-lysine, a nonspecific adhesive substrate (Fig. 2B). Tyrosine phosphorylation required cell adhesion to P-selectin, because both adhesion and tyrosine phosphorylation were prevented by pretreatment of the dish with G1, a blocking mAb to P-selectin, but not with S12, a nonblocking mAb to P-selectin. Furthermore, monovalent Fab fragments of PL1 prevented both adhesion and tyrosine phosphorylation (Fig. 2B). These data demonstrate that binding of P-selectin to PSGL-1 increases tyrosine phosphorylation in neutrophils. The results support the physiological relevance of the tyrosine phosphorylation observed after engagement of PSGL-1 with mAbs (Fig. 1), and they confirm that the response does not require co-engagement of Fcγ receptors.

**Engagement of PSGL-1 Activates the MAP Kinases, ERK-1 and/or ERK-2**—The 42- and 44-kDa phosphorylated proteins have electrophoretic mobilities that are similar to those of MAP kinases (35, 40). We therefore examined whether MAP kinases were activated by occupancy of PSGL-1. Neutrophils incubated with PL1 for various times were lysed and subjected to immunoprecipitation with control rabbit IgG or with rabbit antibodies to the MAP kinases, ERK-1 and ERK-2. The kinase activities of the immunoprecipitates were examined using MBP as substrate. PL1 increased the activity of ERK-1 and/or ERK-2 in a time-dependent manner (Fig. 3). The effect was specific for the ERK proteins, because no increase in kinase activity was observed in the control immunoprecipitates. The kinetics of ERK activation were similar to the kinetics of tyrosine phosphorylation of the 42- and 44-kDa proteins. These data demonstrate that signaling through PSGL-1 increases ERK activity.

Sequential activation of the GTPase Ras and the kinases Raf and MEK is the best characterized pathway for activation of MAP kinases (41). To determine whether engagement of PSGL-1 activated ERK-1 and/or ERK-2 through MEK, we preincubated neutrophils in the presence or absence of the MEK inhibitor PD98059 and then incubated the cells with PL2. The activity of ERK-1,2 was measured in immunoprecipitates from cell lysates. PD98059 completely prevented the induction of ERK activity through PSGL-1 (Fig. 4). This result demonstrates that MAP kinase activation occurs downstream of MEK.
and GDP in lysates from 32P-labeled cells that were incubated in neutrophils, we quantified the levels of Ras-associated GTP activity was measured as in Fig. 3. Similar results were obtained in two incubated with PL2 for 10 min at 37 °C. The cells were lysed, and ERK in neutrophils pretreated with the MEK inhibitor PD98059. 107 cells) were preincubated in the presence or absence of PD98059 (10 μM) for 45 min at room temperature. The cells were then incubated with PL2 for 10 min at 37 °C. The cells were lysed, and ERK activity was measured as in Fig. 3. Similar results were obtained in two other experiments.

To determine whether engagement of PSGL-1 activated Ras in neutrophils, we quantified the levels of Ras-associated GTP and GDP in lysates from 32P-labeled cells that were incubated with the anti-PSGL-1 mAb PL2 or the control anti-CD11a mAb H111. PL2, but not H111, rapidly increased the percentage of GTP associated with Ras, with peak levels observed after 1–2 min and a return to basal levels by 10 min (Fig. 5). These experiments indicated that engagement of PSGL-1 activated Ras but do not demonstrate whether Ras activation is required for activation of MEK or ERK.

Cross-linking of growth factor receptors (41) and some integrins (42) induces tyrosine phosphorylation of their cytoplasmic domains. This provides binding sites for the SH2-containing adaptor protein Shc, which recruits the Grb2/Sos1 complex to the plasma membrane in a tyrosine phosphorylation-dependent manner. The nucleotide exchange protein Sos1 then activates Ras (41). We investigated whether engagement of PSGL-1 might activate Ras in this manner. 32P-Labeled neutrophils were incubated with PL2 for various intervals and then lysed and analyzed by SDS-PAGE and autoradiography. The labeled cells remained responsive, because engagement of PSGL-1 activated Ras in the labeled cells (see Fig. 5) and induced tyrosine phosphorylation in mock-labeled cells (data not shown). Many cellular proteins were phosphorylated in the labeled cells (Fig. 6A), but PSGL-1 immunoprecipitated from the cell lysates was not detectably phosphorylated, and no other phosphorylated protein coprecipitated with PSGL-1 (Fig. 6B). We next immunoprecipitated PSGL-1 or Shc from lysates of PL2-treated neutrophils, and we examined the immunoprecipitates by immunoblotting with anti-phosphotyrosine antibodies. Neither protein was detectably phosphorylated on tyrosine (Fig. 7, A and B). Furthermore, when PSGL-1 immunoprecipitates were immunoblotted with antibodies to Grb2 or Sos1, no detectable association of either protein with PSGL-1 was observed (Fig. 7, C and D). Indeed, little or no Sos1 was detected in neutrophils, although the anti-Sos1 antibody readily identified Sos1 in a lysate of PC12 cells (Fig. 7D). These results suggest that engagement of PSGL-1 does not induce phosphorylation of its cytoplasmic tail, activation of Shc, or association of Grb2/Sos1 with PSGL-1 in neutrophils.

Engagement of PSGL-1 Elicits Tyrosine Phosphorylation-dependent IL-8 Secretion from Neutrophils—To determine whether PSGL-1-dependent tyrosine phosphorylation is required to generate effector responses in neutrophils, we treated cells with the anti-PSGL-1 mAb PL1 or, as a control, the isotype-matched anti-ICAM-1 mAb 18E3D, in the presence or absence of the tyrosine kinase inhibitor, genistein. After an 8-h incubation, we measured the cell-free supernatants for release of the chemokine, IL-8. PL1, but not 18E3D, significantly increased IL-8 secretion by the neutrophils (Fig. 8A). The increased IL-8 secretion from neutrophils after engagement of PSGL-1 was not due to endotoxin contamination of PL1 because 1) boiled PL1 did not increase IL-8 secretion, 2) a Limus assay failed to detect endotoxin in the PL1 preparation, and 3) the endotoxin inhibitor, polymyxin B, was included in the cell suspensions. The tyrosine kinase inhibitor, genistein, abrogated the PL1 induction of IL-8 secretion (Fig. 8A). Parallel experiments confirmed that genistein blocked PL1-induced protein tyrosine phosphorylation (Fig. 8B). These data demonstrate that engagement of PSGL-1 stimulates secretion of IL-8 through a pathway that probably requires tyrosine phosphorylation.

We also examined whether the MEK inhibitor, PD98059, affected PSGL-1-induced secretion of IL-8. Neutrophils were pretreated with MeSO diluent or with PD98059 and then incubated for 8 h in the presence or absence of PL1. In pooled data from three independent experiments, IL-8 secretion (ng/
ml, mean ± S.E.) was as follows: Me2SO, 0.09 ± 0.04; PD98059, 0.11 ± 0.01; Me2SO plus PL1, 0.61 ± 0.18; PD98059 plus PL1, 0.40 ± 0.15. PD98059 partially inhibited the PL1-induced secretion of IL-8. Although the inhibition did not reach statistical significance in the pooled data, PD98059 partially inhibited secretion in each independent experiment. These data suggest that engagement of PSGL-1 stimulates secretion of IL-8 through both MAP kinase-dependent and -independent pathways.

**DISCUSSION**

Interactions of PSGL-1 with P-selectin mediate the initial tethering of leukocytes to activated platelets or endothelial cells at sites of infection or tissue injury (20, 22, 27). These tethers lead to juxtacrine signaling of leukocytes by lipid autacoids and chemokines that are expressed by activated endothelial cells or platelets (23, 43). Here we demonstrate that ligation of PSGL-1 on human neutrophils with mAbs or P-selectin increased protein tyrosine phosphorylation, activated the ERK family of MAP kinases, and induced secretion of IL-8. The tyrosine kinase inhibitor, genistein, blocked IL-8 secretion, suggesting that PSGL-1-mediated tyrosine phosphorylation initiates biologically important responses in neutrophils. Engagement of PSGL-1 with P-selectin propagates tyrosine phosphorylation of PSGL-1 and Shc, and association of PSGL-1 with Grb2 or Sos1 in neutrophils. Neutrophils (1.5 × 10⁶) were incubated with PL2 for the indicated times at 37 °C and then lysed. A, an aliquot of each lysate was subjected to SDS-PAGE and autoradiography to demonstrate incorporation of [32P] into many proteins. B, PSGL-1 was immunoprecipitated from each cell lysate. The immunoprecipitates were resolved by SDS-PAGE and then analyzed by autoradiography or immunoblotting with PL2. The autoradiograms were exposed at −80 °C for 5 h in A and for 62 h in B.

**FIG. 7.** Engagement of PSGL-1 does not induce detectable tyrosine phosphorylation of PSGL-1 in neutrophils. [32P]-Labeled neutrophils (1 × 10⁶) were incubated with PL2 for the indicated times at 37 °C and then lysed. A control lysate from PC12 cells was subjected to immunoblotting to demonstrate the presence of Grb2 in the cells. D, the total lysate from 2 × 10⁶ neutrophils was analyzed by immunoblotting. A control lysate from PC12 cells was used to demonstrate that the anti-Sos1 antibody was active. The exposure times for the chemiluminescent signals in the immunoblots were as follows: A, 16 h for anti-Tyr(P) and 2 min for PL2; B, 1.5 h for anti-Tyr(P) and 30 min for anti-Shc; C, 1 h for anti-Grb2 and PL2 in the immunoprecipitation (IP) experiment, and 3.5 min for anti-Grb2 blotting of the total lysate; D, 3.5 h for anti-Sos1 and 12 min for PL2 in the IP experiment, 1.5 h for PL2 blotting of total lysate, and 1.5 h for anti-Sos1 blotting of the total lysate and the PC12 lysate.

To elicit other effector responses, PSGL-1-dependent signals may require integration with signals generated by other mediators. Tyrosine phosphorylation increased when neutrophils were incubated with anti-PSGL-1 mAbs that were presented as bi- or monovalent Fab fragments. This suggests that tyrosine phosphorylation requires clustering of PSGL-1, which itself is a dimer (5). Adhesion of neutrophils to immobilized P-selectin rapidly induced the same profile of tyrosine-phosphorylated proteins, supporting the physiologic relevance of the response. Ligation of PSGL-1 also activated the MAP kinases ERK-1 and/or ERK-2. MAP kinases must be tyrosine-phosphorylated to be activated (41); therefore, ERK-1 and ERK-2 represent a portion of the 44- and 42-kDa proteins that are tyrosine-phosphorylated upon ligation of PSGL-1.

The pathway for PSGL-1-induced activation of the ERKs requires further characterization. ERK activation appears to be downstream of MEK, because the MEK inhibitor PD98059 blocked the ability of anti-PSGL-1 mAbs to activate the MAP kinases in neutrophils. Engagement of PSGL-1 activated the GTPase Ras. The mechanism for Ras activation is obscure, because ligation of PSGL-1 did not cause detectable phosphorylation of PSGL-1, activation of Shc, or association of PSGL-1 with the Grb2-Sos1 complex. Indeed, the cytoplasmic domain of PSGL-1 lacks canonical sequences for binding of the SH2 or SH3 domains found in many signaling proteins. GTP-bound Ras might bind to and activate Raf, leading to activation of MEK and ERKs. However, engagement of some integrins may bypass Ras to activate MEK and MAP kinases (44). It is conceivable that cross-linking of PSGL-1 initiates a similar Ras-independent pathway to signal MEK and MAP kinases in neutrophils.

The identities of the other tyrosine-phosphorylated proteins in neutrophils are unknown. Recently it was reported that adhesion of human T cells to P-selectin induces tyrosine phosphorylation of the focal adhesion kinase, pp125FAK, but the role of PSGL-1 in this response was not examined (45). Although pp125FAK was reportedly found in neutrophils (46), we have
The mechanisms by which engagement of PSGL-1 or L-selectin initiates tyrosine phosphorylation in neutrophils are unknown. Both molecules are concentrated in the microvillous tips of leukocytes (20, 52). PSGL-1 or L-selectin may interact with the cytoskeleton under some conditions (53–55). But cytochalasins do not block induction of tyrosine phosphorylation through either protein, suggesting that an intact cytoskeleton is not required for this response (Ref. 39 and this study). Cytochalasins, in contrast, prevent the protein tyrosine phosphorylation and activation of MAP kinases that occur after cross-linking of integrins (35, 40, 56–59). Engagement of L-selectin on cultured T cell lines induces rapid tyrosine phosphorylation of L-selectin, binding of L-selectin to Grb2 and Sos, and the activation of Ras and MAP kinases; these responses require the presence of the T cell Src kinase p56lck (60). It is not known whether engagement of L-selectin on neutrophils induces similar responses. Cross-linking of L-selectin activates β2 integrins in naive, but not memory, peripheral lymphocytes (61). This finding indicates that engagement of L-selectin or PSGL-1 need not use identical signaling mechanisms in different classes of leukocytes. We did not detect tyrosine phosphorylation of PSGL-1 or binding of PSGL-1 to Grb2 or Sos-1 in neutrophils that were incubated with anti-PSGL-1 mAbs.

We found that engagement of PSGL-1 was sufficient to induce secretion of IL-8 from neutrophils. In most situations, however, interactions of PSGL-1 with P-selectin do not directly activate leukocyte effector responses, but they do enhance the abilities of other mediators to elicit such responses. Thus, adhesion of neutrophils to P-selectin does not directly induce intracellular Ca2+ fluxes, superoxide generation, granule secretion, or functional activation of β2 integrins. However, adhesion augments these responses upon exposure to platelet-activating factor, a potent lipid mediator that is co-expressed with P-selectin on activated endothelial cells (23, 24). Activated platelets may use P-selectin plus other mediators to cooperatively induce superoxide generation and integrin up-regulation in adherent neutrophils (62–66). Under shear stress, neutrophils rolling on P-selectin rapidly modify the velocity of β2 integrin-dependent migration in response to fMet-Leu-Phe or platelet-activating factor (67). Adhesion of monocytes to P-selectin does not elicit cytokine synthesis and secretion, but it potentiates the ability of other mediators to elicit such responses. Thus, adhesion of neutrophils to P-selectin does not directly induce integrin-dependent leukocyte aggregation and leukocyte adhesion to the vessel wall.
The mechanisms that integrate signals transduced by adhesion molecules and other receptors in leukocytes are largely unknown. MAP kinases may be important participants because they are activated by different classes of conventional signal-transducing receptors (70) as well as by certain adhesion molecules (35, 39, 40). The kinetics of activation of ERK-1,2 upon engagement of PSGL-1 are sufficiently rapid to mediate signaling responses in leukocytes (23, 24, 67). Activation of other kinases and phosphorylation of their targets may also be important for signal integration. The use of cooperative signals implies that activation through a single pathway need not be robust.

Interactions of PSGL-1 with P-selectin (and possibly E- and L-selectin) are sufficient to induce tyrosine phosphorylation and activate MAP kinases in human neutrophils. This suggests that tyrosine phosphorylation represents a critical proximal step in PSGL-1-mediated signaling. Determining how phosphorylation is regulated may help explain how signaling through PSGL-1 is integrated with other pathways that activate leukocytes at sites of inflammation or thrombosis (43).

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