Binding of ADAM28 to P-selectin Glycoprotein Ligand-1 Enhances P-selectin-mediated Leukocyte Adhesion to Endothelial Cells*

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ADAMs (a disintegrin and metalloproteinases) are a recently discovered gene family of multifunctional proteins with the disintegrin-like and metalloproteinase domains. To analyze the biological functions of ADAM28, we screened binding molecules to secreted-type ADAM28 (ADAM28s) by the yeast two-hybrid system and identified P-selectin glycoprotein ligand-1 (PSGL-1). Binding between the disintegrin-like domain of ADAM28s and the extracellular portion of PSGL-1 was determined by yeast two-hybrid assays, binding assays of the domain-specific recombinant ADAM28s species using PSGL-1 stable transfectants and leukocyte cell lines expressing native PSGL-1 (HL-60 cells and Jurkat cells), and co-immunolocalization and co-immunoprecipitation of the molecules in these cells. Incubation of HL-60 cells with recombinant ADAM28s enhanced the binding to P-selectin-coated wells and P-selectin-expressing endothelial cells. In addition, intravenous injection of ADAM28s-treated HL-60 cells increased their accumulation in the pulmonary microcirculation and alveolar spaces in a mouse model of endotoxin-induced inflammation. These data suggest a novel function that ADAM28s promotes PSGL-1/P-selectin-mediated leukocyte rolling adhesion to endothelial cells and subsequent infiltration into tissue spaces through interaction with PSGL-1 on leukocytes under inflammatory conditions.

A disintegrin and metalloproteinases (ADAMs) are a recently discovered gene family of membrane-anchored and secreted proteins that have proteolytic and/or adhesive properties (1, 2). At present, more than 30 members have been identified in humans (3, 4). The precursor forms of ADAMs (pro-ADAMs) are composed of propeptide, metalloproteinase, disintegrin-like, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic domains (4, 5). Roles of ADAMs include cell surface processing of membrane proteins such as tumor necrosis factor-α by ADAM17 (6), heparin-binding epidermal growth factor by ADAM9 (7), ADAM12 (8), and ADAM17 (9) and amyloid precursor protein by ADAM10 (10) and ADAM17 (11). ADAMs are also reported to digest various proteins, including type IV collagen by ADAM10 (12) and insulin-like growth factor binding protein-3 (IGFBP-3) by ADAM12 (13) and ADAM28 (14). On the other hand, accumulated lines of evidence have shown that the disintegrin-like domain of many ADAM members interacts with integrins (3, 15), although a recent study on crystal structures suggested that the integrin-binding motif within the disintegrin-like domain is structurally inaccessible for protein binding (16). The secreted form of ADAM9 has recently been reported to lead to invasion by binding to α6β4 and α2β1 integrins (17). The cysteine-rich domain of ADAM12 is known to interact with syndecans on mesenchymal cells, leading to the β1 integrin-mediated cell spreading (18). The disintegrin-like and cysteine-rich domains of ADAM13 bind to laminin and fibronectin (19). However, information about the binding molecules of ADAMs and their functional modulation by these interactions is still limited.

ADAM28 is expressed by human peripheral blood lymphocytes in two alternative forms, i.e. a prototype membrane-anchored form (ADAM28m) and a secreted form (ADAM28s) (20). The disintegrin-like domain of ADAM28 is reported to interact with integrins α6β1, α4β7 and α9β1 on lymphocytes in an activation-dependent manner of the integrins (21, 22). The metalloproteinase domain of ADAM28 has the zinc-binding catalytic-site consensus sequence, and ADAM28 cleaves myelin basic protein (23), CD23 ectodomain (24), and IGFBP-3 (14). In addition, we have reported that ADAM28 is overexpressed in non-small cell lung carcinomas with correlations to carcinoma cell proliferation and lymph node metastasis (25), and we have recently shown that ADAM28 plays a role in breast carcinoma cell proliferation through enhanced bioavailability of insulin-like growth factor-1 by selective digestion of IGFBP-3 of the insulin-like growth factor-I-IGFBP-3 complex (26). Thus, these data suggest the possibility that ADAM28 is involved in various cellular and tissue reactions such as cell-cell and cell-matrix interactions, cell motility, shedding of cell surface proteins, and
cell proliferation. However, little is known about the biological functions of human ADAM28 and their molecular mechanisms under pathophysiological conditions.

In this study, we screened ADAM28s-interacting proteins by the yeast two-hybrid system and identified P-selectin glycoprotein ligand-1 (PSGL-1). Our data provide the evidence that the binding between the disintegrin-like domain of ADAM28s and the extracellular portion of PSGL-1 enhances the interaction promotes accumulation of PSGL-1-expressing cells in the lung microcirculation and alveolar spaces in a mouse model of endotoxin-induced inflammation. Based on our results, we propose a novel pathway by which ADAM28s is involved in the promotion of leukocyte rolling adhesion to blood vessel endothelial cells and the subsequent migration into tissue spaces under inflammatory conditions.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid System—MATCHMAKER Gal4 two-hybrid system 3 and the MATCHMAKER human lung cDNA library were purchased from Clontech. cDNA fragment encoding the disintegrin-like (Dis), cysteine-rich (CR), and secreted-alloproteinase domain of ADAM28 (25) or anti-ADAM28 mAb (297–2F3) specific to the met-alloproteinase domain of ADAM28 (25) was co-introduced into strain AH109 with the corresponding plasmid vector (Invitrogen), generating pFASTBac1-pro-ADAM28s, pCMVTag4a-Dis/CR/SS, and pCMVTag4a-PSGL-1 library with the corresponding primers (Table 1). The product was cloned into the pGBKT7 vector, generating pGBKT7-Dis, pGBKT7-CR, and pGBKT7-SS vectors (Fig. 1A). These plasmids were co-introduced into strain AH109 with pACT2 vector containing a cDNA fragment encoding full-length PSGL-1 (pACT2-PSGL-1) and assayed according to our previous methods (27).

**Establishment of Stable Transfectants Expressing PSGL-1**—cDNA encoding human full-length PSGL-1 was prepared by PCR from the cDNAs derived from HL-60 cells (a human myeloid cell line) using the corresponding primers (Table 1), and cloned into pCMVTag4a (Stratagene, La Jolla, CA), generating pCMVTag4a-PSGL-1. Stable transfectants expressing PSGL-1 or mock transfectants were prepared by transfection of pCMVTag4a-PSGL-1 or pCMVTag4a vector alone to COS7 cells as described previously (28), and 35 stable transfectants were established. The expression of PSGL-1 and ADAM28 in COS7 cells, mock transfectants, and PSGL-1 transfectants was examined by immunoblotting with anti-PSGL-1 monoclonal antibody (mAb) (KPL1 mAb; Santa Cruz, CA), anti-ADAM28 mAb (297–2F3) specific to the metalloprotease domain of ADAM28 (25) or anti-β-actin antibody (Ab) (A5441; Sigma), and by flow cytometry with KPL1 mAb or nonimmune mouse IgG (Santa Cruz Biotechnology) as described previously (28).

**Expression and Purification of Recombinant ADAM28s**—pCMVTag4a-pro-ADAM28s containing the full-length ADAM28s cDNA with its signal peptide was previously prepared (14). pCMVTag4a-Pro/Met having a cDNA fragment encoding the signal peptide, propeptide (Pro), and metalloprotease (Met) domains was prepared by PCR from pCMVTag4a-pro-ADAM28s using the corresponding primers (Table 1). cDNA fragments encoding the signal peptide and the Dis, CR, and SS domains were amplified by PCR using the pCMVTag4a-Pro/Met vector to fuse the signal peptide to the amino terminus of the Dis/CR/SS domains, generating pCMVTag4a-Dis/CR/SS. The FLAG-tagged cDNA fragments encoding ADAM28s species were subcloned into pFASTBac1 vector (Invitrogen), generating pFASTBac1-pro-ADAM28s, pFASTBac1-Pro/Met, and pFASTBac1-Dis/CR/SS. The vec-

### TABLE 1

| Vector | Oligonucleotide sequence |
|--------|--------------------------|
| pGBK7-Dis/CR/SS | Forward, 5’-GGAAATTCATGATTTTCCTTCGC-3’ |
| pGBK7-Dis | Reverse, 5’-GGAAATTCATGATTTTCCTTCGC-3’ |
| pGBK7-CR | Forward, 5’-GGAAATTCATGATTTTCCTTCGC-3’ |
| pGBK7-SS | Reverse, 5’-GGAAATTCATGATTTTCCTTCGC-3’ |
| pCMVTag4a-PSGL-1 | Forward, 5’-GGAAATTCATGATTTTCCTTCGC-3’ |
| pCMVTag4a-Pro/Met | Reverse, 5’-GGAAATTCATGATTTTCCTTCGC-3’ |
| pCMVTag4a-Dis/CR/SS Signal peptide | Forward, 5’-GGAAATTCATGATTTTCCTTCGC-3’ |
| Dis/CR/SS domains | Reverse, 5’-GGAAATTCATGATTTTCCTTCGC-3’ |

**Yeast Two-hybrid Assay**—cDNA fragments encoding the Dis, CR, or SS domain of ADAM28s were amplified by PCR using pGBK7-Dis/CR/SS plasmid with the corresponding primers (Table 1). The products were cloned into the pGBK7 vector, generating pGBK7-Dis, pGBK7-CR, and pGBK7-SS vectors.
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tors were infected to insect Sf9 cells, and the recombinant full-length pro-ADAM28s (rpro-ADAM28s) and its deletion mutants consisting of the Dis, CR, and SS domains (rDis/CR/SS) or the Pro and Met domains (rPro/Met) were purified by anti-FLAG M2-agarose affinity gels (Sigma) according to our previous methods (14).

**Binding Assay of rADAM28s Species to PSGL-1-expressing Cells**—Parental COS7 cells, mock transfectants, and stable transfectants expressing PSGL-1 were incubated with 125I-labeled rpro-ADAM28s, rDis/CR/SS, or rPro/Met (1 nM each), and the percentage of bound activity to added activity was calculated by counting the radioactivity using a γ-counter (29). A similar binding assay was performed with HL-60 cells and Jurkat cells (a T-lymphoma cell line). The competitive inhibition study was performed by incubation of stable transfectants, HL-60 cells, and Jurkat cells with a 10-fold excess amount of nonlabeled rADAM28s species prior to the binding assay. The binding assay was also carried out in the presence of 1 μM KB-R7785, an ADAM inhibitor (a gift from Dr. Koichiro Yoshino, Carnabiobience, Kobe, Japan) (8, 26). For the inhibition studies, these cells were incubated with anti-PSGL-1 mAb (KPL1 mAb or PL1 mAb; Santa Cruz Biotechnology), anti-PSGL-1 polyclonal Ab (H-300 Ab; Santa Cruz Biotechnology), or nonimmune IgG prior to the binding assays.

**Laser Scanning Confocal Microscopy**—Cell suspensions of HL-60 and Jurkat cells were incubated with rpro-ADAM28s in phosphate-buffered saline containing 2% fetal bovine serum (JRH Bioscience, Lenexa, KS) or buffer alone. After incubation with anti-FLAG M2 mAb (Sigma) or nonimmune mouse IgG, they were plated on glass slides, fixed with methanol/acetic acid/formaldehyde (27, 28), and incubated with goat anti-PSGL-1 polyclonal Ab (C-19 Ab; Santa Cruz Biotechnology) or nonimmune goat IgG (R & D System, Minneapolis, MN). Following incubation with fluorescein isothiocyanate (FITC)- or rhodamine-conjugated secondary Ab (Dako Corp., Glostrup, Denmark), all preparations were viewed under an Olympus laser scanning confocal microscope Fluoview FV300 (Olympus, Tokyo, Japan) (27).

**Immunoprecipitation of rpro-ADAM28s-PSGL-1 Complex**—HL-60 and Jurkat cells were incubated with 125I-labeled rpro-ADAM28s, and supernatants of the cell lysates were subjected to immunoprecipitation with KPL1 mAb, anti-FLAG M2 mAb, or nonimmune mouse IgG according to our previous methods (28). The immunoprecipitates were immunoblotted with KPL1 mAb as described above. 125I-Labeled rpro-ADAM28s on the same membranes was detected by an imaging plate and the BAS-2000 system (Fuji Photo Film Co., Tokyo, Japan).

**Adhesion Assay of HL-60 Cells to Immobilized P-selectin**—Prior to the adhesion assay, the glycosylation patterns of HL-60 and Jurkat cells were examined by flow cytometry using CSLEX-1 Ab (BD Biosciences) and NCC-ST-439 Ab (Nippon Kayaku Co., Tokyo, Japan), which recognize sialyl-Lewis X and both nonsulfated and 6-sulfatated sialyl-Lewis X on core 2-type O-glycans, respectively. Microtiter plates (NalgeNunc, Rochester, NY) were coated with human P-selectin (R & D Systems) or bovine serum albumin fraction V (BSA; Sigma) in phosphate-buffered saline containing 1 mM CaCl₂ and 1 mM MgCl₂. Suspensions of HL-60 cells, which were labeled with 2',7'-bis (2-carboxyethyl)-5 (6)-carboxyfluorescein tetrakis (acetoxyethyl) ester (BCECF-AM; Sigma) (30), were incubated with rpro-ADAM28s, rDis/CR/SS, or rPro/Met and allowed to adhere to the microtiter plates for 10 min at 4°C. The adherent cells were observed by an inverted microscope, and a percentage of adherent cells to added cells was calculated by quantitating the fluorescence intensity (Dainippon Sumitomo Pharma Co., Osaka, Japan). For inhibition studies, the cells were incubated with rpro-ADAM28s in the presence of H-300 Ab, KPL1 mAb, or nonimmune IgG prior to the adhesion assay. Similarly, the adhesion assay was performed with active rADAM28s (14). Shedding of PSGL-1 from HL-60 cells after treatment with rpro-ADAM28s or active rADAM28s was analyzed by flow cytometry using KPL1 mAb or nonimmune mouse IgG.

**Adhesion Assay of HL-60 Cells to Thrombin-stimulated Human Umbilical Vein Endothelial Cells (HUVECs)**—Primary HUVECs were prepared (31) and characterized by the expression of factor VIII-related antigens and CD31 by immunohistochemistry (29, 32). HUVECs in 24-well plates were stimulated without or with 1 unit/ml thrombin (Sigma) for 5 min and fixed with 1% paraformaldehyde (33). Suspensions of BCECF-AM-labeled HL-60 cells were reacted with rpro-ADAM28s, rDis/CR/SS, rPro/Met, active rADAM28s, or buffer alone, distributed over the plates of HUVECs, and incubated for 10 min at room temperature with shaking at 60 rpm with an orbital shaker (Taitec, Saitama, Japan) (33). Cells attached to HUVECs were observed by an inverted microscope, and the fluorescence intensity of the cell lysates was measured. Inhibition studies using anti-PSGL-1 Abs (H-300 Ab and KPL1 mAb) or nonimmune IgG were also performed.

**Adhesion and Transendothelial Migration Study of HL-60 Cells in a Mouse Model**—Systemic inflammation was induced by intraperitoneal injection of lipopolysaccharide (LPS; Sigma) or phosphate-buffered saline in adult C57BL/6 mice (9–10 weeks; SLC, Shizuoka, Japan) 18 h prior to the experiments (34, 35). BCECF-AM-labeled HL-60 cells in RPMI 1640 medium (Sigma) containing 25 mM HEPES were incubated with rpro-ADAM28s, rDis/CR/SS, rPro/Met, or buffer alone and then injected into mouse tail veins. After 12 h, the lungs were resected under deep anesthesia. Frozen sections were examined by a fluorescence microscope, and the number of HL-60 cells was determined by counting fluorescent cells in at least 10 distinct sections per animal. For the inhibition study, mice were treated with neutralizing anti-P-selectin mAb (RB40.34 mAb; BD Biosciences) 2 h before the cell injection (36). To localize the endothelial cells, frozen sections fixed with 4% paraformaldehyde were subjected to immunofluorescent staining using rat anti-CD31 mAb (MEC13.3; BD Biosciences) and rhodamine-conjugated secondary Ab (Immunotech). Bronchoalveolar lavage (BAL) fluids were prepared (37), and the fluorescence intensity of the cell lysates was measured by plate reader. Care of the animals was in accordance with the Guidelines for the Care and Use of Laboratory Animals of Keio University School of Medicine, and our experiments have been approved by the University Animal Welfare Committee.
showed definite growth on high stringency plates, whereas only negligible background growth was observed with those of pGBKT7-CR, pGBKT7-SS, or pGBKT7 vector alone (negative control) with pACT2-PSGL-1 (Fig. 1B). When the α-galactosidase activity of each transformants was measured, the transformants of pGBKT7-Dis/CR/SS or pGBKT7-Dis with pACT2-PSGL-1 showed higher activity as compared with other transformants (Fig. 1C). These data strongly suggest that the Dis domain of ADAM28s interacts with PSGL-1.

**Binding of Recombinant ADAM28s Species to PSGL-1-expressing Cells**—The expression levels of PSGL-1 in the 35 stable transfectants varied by immunoblotting and flow cytometric analyses. Thus, we selected two clones with low expression (clones 7 and 18) and two clones with high expression (clones 14 and 17) for further studies of binding assays (Fig. 2, A and B). To examine the binding activity of recombinant ADAM28s species to the transfectants, we purified rpro-ADAM28s, rPro/Met, and rDis/CR/SS, which showed bands of 65, 48, and 18 kDa under reducing conditions on SDS-PAGE, respectively (Fig. 2C), iodinated them, and then counted radioactivity bound to the clones. Because ADAM28 is reported to interact with integrin α4β1 and α4β7 in an activation-dependent manner (22), the binding assays were carried out in the absence of MnCl2, an activator of the integrins, to avoid their effects on the assays. As shown in Fig. 2, D–F, the binding activity of 125I-labeled rpro-ADAM28s and rDis/CR/SS to the transfectants was increased depending on the expression levels of PSGL-1 and significantly higher than that of mock transfectants or parental COS7 cells (p < 0.05 or p < 0.01), whereas the binding activity of rPro/Met to the clones was not different from that of mock transfectants or parental cells (data not shown for parental cells). The binding of rpro-ADAM28s and rDis/CR/SS to the high expressing clone was competitively inhibited and decreased to basal levels by addition of nonlabeled rpro-ADAM28s and rDis/CR/SS, respectively (Fig. 2, D and E). Similarly, the binding activity of rpro-ADAM28s and its deletion mutants was examined using cell lines expressing native PSGL-1 (HL-60 cells and Jurkat cells), both of which showed negligible expression of ADAM28 by RT-PCR or immunoblotting (data not shown). As shown in Fig. 2, G and H, 125I-labeled rpro-ADAM28s and rDis/CR/SS could bind to these cells, whereas 125I-labeled BSA had only a background binding activity. The specific binding of rpro-ADAM28s and rDis/CR/SS was confirmed by a competitive inhibition study using nonlabeled rpro-ADAM28s or rDis/CR/SS. When the assays using the stable transfectants or the cell lines were performed in the presence of an ADAM inhibitor, KB-R7785, which inhibits the catalytic activity of ADAM28s (26), no changes in the binding activity were observed by the treatment (data not shown). Thus, the metalloprotease activity was not required for binding, supporting the data that rDis/CR/SS lacking the metalloprotease domain can bind to the stable transfectants (Fig. 2E) and the PSGL-1-expressing cell lines (Fig. 2, G and H).

**Binding of ADAM28s to the Extracellular Decamer Repeats of PSGL-1**—We examined the region of PSGL-1 that interacts with ADAM28s by inhibition studies using three different anti-PSGL-1 Abs, which recognize the tyrosine sulfation consensus sequence motif of the receptor-binding domain (KPL1 mAb),

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**Statistical Analysis**—Results between the two independent groups were determined by the Student’s t test. Comparisons among more than three groups were determined by the Bonferroni/Dunn test. Statistical analyses were carried out using StatView statistical software (SAS Institute Inc. Cary, NC) on a personal computer. p values less than 0.05 were considered significant.

**RESULTS**

**Screening of Proteins That Interact with ADAM28s and Determination of Its Interacting Domain**—To seek binding proteins to ADAM28s, we screened 1.0 × 107 clones of human lung cDNA library by the yeast two-hybrid system using a cDNA fragment encoding the Dis, CR, and SS domains of ADAM28s as bait (Fig. 1A), and isolated 215 positive clones. Among the clones, five were identified as human PSGL-1; they encoded the decamer repeats, transmembrane and cytoplasmic domains of PSGL-1. Then we further performed yeast two-hybrid assays to determine the ADAM28s domain involved in the binding with PSGL-1. As shown in Fig. 1, A and B, plasmid DNAs encoding different domains of ADAM28s (pGBK7-Dis/CR/SS, pGBK7-Dis, pGBK7-CR, and pGBK7-SS) were co-introduced with that encoding full-length PSGL-1 (pACT2-PSGL-1) into S. cerevisiae strain AH109. The yeast transformants of pGBK7-Dis/CR/SS or pGBK7-Dis with pACT2-PSGL-1 and positive control transformants expressing p53 and SV40
the nontyrosine-sulfated sequence of the receptor-binding domain (PL1 mAb), and the decamer repeats, transmembrane and cytoplasmic domains of PSGL-1 (H-300 Ab) (Fig. 3A). The binding activity of ¹²⁵I-labeled rpro-ADAM28s or rDis/CR/SS to HL-60 and Jurkat cells was significantly inhibited by incubation of the cells with H-300 Ab in a dose-dependent manner, but the other two Abs (KPL1 mAb and PL1 mAb), nonimmune mouse IgG or nonimmune rabbit IgG, had no such effect (Fig. 3, B–D and data not shown for Jurkat cells). Similar inhibition was obtained by incubation of the PSGL-1 transfectants (clone 17) with H-300 Ab but not with KPL1 mAb, PL1 mAb, or nonimmune IgG (Fig. 3, E and F). These data strongly suggest that ADAM28 binds to the extracellular decamer repeats of PSGL-1.

Co-localization of rpro-ADAM28s and PSGL-1 on Cell Surfaces of HL-60 and Jurkat Cells—HL-60 and Jurkat cells were incubated with rpro-ADAM28s or buffer alone, and then observed by double immunofluorescent staining for ADAM28s and PSGL-1 under a confocal microscope. As shown in Fig. 4A, PSGL-1 was localized on the cell surfaces of HL-60 cells regardless of the treatment with rpro-ADAM28s. Importantly, rpro-ADAM28s was immunolocalized on the cell surfaces only after the cells were incubated with rpro-ADAM28s. A merged image of the double staining showed yellow staining on most cells (Fig. 4A). However, no such staining patterns were obtained when the cells were incubated with rPro/Met and then subjected to double immunostaining for rPro/Met and PSGL-1 (data not shown). In addition, the cells incubated with rpro-ADAM28s or rPro/Met exhibited no immunostaining with nonimmune IgG (data not shown). Similar results were obtained with Jurkat cells (data not shown). To further study the interaction between ADAM28s and PSGL-1, immunoprecipitation was carried out with anti-PSGL-1 mAb, anti-FLAG M2 mAb for rpro-ADAM28s, or nonimmune IgG (negative control) in HL-60 and Jurkat cells, which had been incubated with ¹²⁵I-labeled rpro-ADAM28s. As shown in Fig. 4B, rpro-ADAM28s of 65 kDa and PSGL-1 of ~120 kDa were co-immunoprecipitated from the cell lysates (data not shown for Jurkat cells). All these data indicate the co-localiza-
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FIGURE 3. Binding of ADAM28s to the extracellular decamer repeats of PSGL-1. A, schematic drawing of the domain structure of PSGL-1. Rb, receptor binding domain; Decamer repeats, 15–16 stretches of 10 amino acids each; TMD, transmembrane domain; Cyto, cytoplasmic domain. The epitopes of anti-PSGL-1 Abs (KPL1 mAb, PL1 mAb, and H-300 Ab) are indicated. B–D, inhibition of rpro-ADAM28s and rDis/CR/SS binding to HL-60 cells with H-300 anti-PSGL-1 Ab. HL-60 cells (B–D) were incubated with H-300 Ab, KPL1 mAb, PL1 mAb, nonimmune rabbit IgG (20 μg/ml each) for 30 min at 37 °C and then incubated with 125I-labeled rpro-ADAM28s (8) and rDis/CR/SS (D) for 30 min at 37 °C. Relative binding activity was calculated by radioactivity. C, various concentrations of H-300 anti-PSGL-1 Ab (3) or nonimmune rabbit IgG (C) were incubated with HL-60 cells, and relative binding activity was determined. Bars indicate mean ± S.D. (n = 5), **, p < 0.01. E and F, inhibition of rpro-ADAM28s and rDis/CR/SS binding to PSGL-1 stable transfectants with H-300 anti-PSGL-1 Ab. Transfectants with high expression of PSGL-1 (clone 17) were incubated with H-300 Ab, KPL1 mAb, PL1 mAb, nonimmune rabbit IgG or mouse IgG (20 μg/ml each), and relative binding ratios were calculated. Bars, mean ± S.D. (n = 5), **, p < 0.01.

FIGURE 4. Co-localization of rpro-ADAM28s and PSGL-1 on the cell surfaces of HL-60. A, co-immunofluorescence of rpro-ADAM28s and PSGL-1 by double immunofluorescent staining. HL-60 cells (1 × 10⁴ cells in 100 μl) were incubated with rpro-ADAM28s (100 μg/ml) or buffer alone for 30 min at 37 °C. After washing, the cells were incubated with anti-FLAG M2 mAb (10 μg/ml) for 30 min at 4 °C, followed by incubation with FITC- or rhodamine-conjugated secondary antibodies. Bars, 10 μm. B, co-immunoprecipitation of ADAM28s and PSGL-1 from HL-60 cells. After incubation of the cells (1 × 10⁶ cells) with [125I]-labeled rpro-ADAM28s (500 ng), cell lysates were subjected to immunoprecipitation (IP) with KPL1 anti-PSGL-1 mAb, anti-FLAG M2 mAb, or nonimmune mouse IgG (2 μg/ml each). PSGL-1 and ADAM28s were detected by immunoblotting (IB) with KPL1 anti-PSGL-1 mAb and autoradiography (AR), respectively.
inhibited to the original level with H-300 Ab and completely with KPL1 mAb. Because active rADAM28s has metalloproteinase activity, flow cytometric analysis was performed to examine the shedding of PSGL-1. As shown in Fig. 5E, the expression levels of PSGL-1 on the cell surfaces were not changed after treatment with either rpro-ADAM28s or active rADAM28s, suggesting that PSGL-1 is resistant to ADAM28s activity. Collectively, these data suggest that both rpro-ADAM28s and active rADAM28s enhance the PSGL-1/P-selectin-mediated cell binding through their interaction with the extracellular decamer repeats of PSGL-1.

Enhancement of PSGL-1/P-selectin-mediated Endothelial Cell Adhesion with ADAM28s—To determine whether ADAM28s affects the interaction of HL-60 cells with endothelial cells, we carried out a cell adhesion assay using HUVECs on constantly shaking with an orbital shaker (33). In accordance with the previous report that thrombin activates P-selectin expression on HUVECs (33), the adhesion rate of HL-60 cells to thrombin-stimulated HUVECs was significantly higher than that to nonstimulated HUVECs (p < 0.01) (Fig. 6A). When HL-60 cells were incubated with rpro-ADAM28s or rDiS/CR/SS prior to the adhesion assay, adhesion activity to thrombin-treated HUVECs was significantly enhanced compared with the original level (p < 0.01), although the activity was not changed with the cells incubated with rPro/Met (Fig. 6A). Similarly, the enhanced adhesion of HL-60 cells to HUVECs was observed by treatment with active rADAM28s (p < 0.01). Importantly, the enhanced adhesion with rpro-ADAM28s or active rADAM28s was abolished by treatment of the cells with H-300 anti-PSGL-1 Ab but not with nonimmune rabbit IgG (Fig. 6A). In addition, the basal and enhanced HL-60 cell adhesion rates to thrombin-stimulated HUVECs were inhibited to
original levels by incubation with KPL1 mAb against the receptor-binding domain of PSGL-1 (Fig. 6B), indicating that the adhesion is based on PSGL-1 binding to P-selectin. Thus, these data suggest that both rpro-ADAM28s and active rADAM28s promote the HL-60 cell adhesion to thrombin-stimulated HUVECs through ADAM28s/PSGL-1 interaction.

Effects of ADAM28s on Accumulation and Transendothelial Migration of HL-60 Cells in Mouse Lungs—Because ADAM28s increased PSGL-1/P-selectin-mediated cell adhesion to endothelial cells in vitro, we further examined in vivo the effects of ADAM28s on tethering and rolling and subsequent transendothelial migration of HL-60 cells using a mouse model of endotoxin-induced inflammation; BCECF-AM-labeled HL-60 cells were injected into mouse tail veins, and accumulated cells in the lungs were counted by fluorescence microscopy. As shown in Fig. 7, A and B, only a negligible level of HL-60 cells was present in the lungs of control nontreated mice (1.44 ± 0.52 cells/mm²), but the level was significantly increased in the lungs of LPS-treated mice (15.04 ± 5.50 cells/mm²) (p < 0.01). When HL-60 cells were incubated with rpro-ADAM28s, rDis/CR/SS, or rPro/Met prior to the assay, the accumulation in the lungs was significantly enhanced with rpro-ADAM28s (39.59 ± 1.95 cells/mm²) and rDis/CR/SS (38.19 ± 5.78 cells/mm²) as compared with that without the incubation (p < 0.01), but no significant change was observed with rPro/Met (18.47 ± 2.70 cells/mm²) (Fig. 7B). The promoting effect of the ADAM28s on the accumulation was not observed in control nontreated mice (data not shown). In addition, the enhanced accumulation with rpro-ADAM28s was prevented to a basal level by treatment of the mice with anti-P-selectin Ab (Fig. 7B). Immunohistochemistry of the lung endothelial cells with anti-CD31 antibody revealed that some HL-60 cells, which had been incubated with rpro-ADAM28s or rDis/CR/SS, are present in the alveolar spaces (Fig. 7A, panel 6, and data not shown for rDis/CR/SS-treated cells). To further study the effects of ADAM28s on HL-60 cell emigration into alveolar spaces, the cell fluorescence intensity in BAL fluids was measured. As shown in Fig. 7C, the intensity was significantly higher in the lungs from mice injected with rpro-ADAM28s-treated HL-60 cells as compared with that from control mice that received untreated cells (p < 0.05). Thus, all these data show that ADAM28s enhances tethering and migration of HL-60 cells in vivo through ADAM28s/PSGL-1 interaction.

DISCUSSION

In this study, we provided the first evidence that ADAM28s binds to PSGL-1 through interaction between the Dis domain of ADAM28s and the extracellular portion of PSGL-1, and that this interaction on cell surfaces enhances PSGL-1/P-selectin-mediated leukocyte adhesion to endothelial cells and subsequent transendothelial migration in vitro and in vivo. Based on these findings, we propose that ADAM28s is a factor that promotes leukocyte adhesion to endothelial cells and transendothelial migration into tissue spaces.

The interaction between ADAM28s and PSGL-1 discovered by the yeast two-hybrid system was further supported by the data of binding assays, immunoprecipitation, and co-immunolocalization of ADAM28s and PSGL-1. Because the ADAM28s species could bind to Jurkat cells expressing PSGL-1, which lacks the core 2-type O-glycans and sialyl-Lewis X, ADAM28s is considered to bind to PSGL-1 through a protein-protein interaction without requiring the sugar moiety of PSGL-1. The data of the yeast two-hybrid assay and binding assays using rpro-ADAM28s and its deletion mutants indicated that the Dis domain is involved in the binding with PSGL-1, although our study did not identify the motif in the Dis domain responsible for the binding. Most ADAMs such as ADAM9, -12, -15, -19, and -28 interact with several integrins, mainly αβ1 and/or α9β1 (15, 40), and this interaction is generally thought to occur through binding between integrins and the integrin-binding motif (RX_aDLPEF), called the disintegrin-loop, in the Dis domains of most ADAM species (41). However, residues
located outside of the disintegrin loop are also known to participate in integrin recognition of ADAM28 (42). In addition, a recent study on the crystal structure has demonstrated that the disintegrin-loop is packed against the CR domain and stabilized by a disulfide bridge within the Dis domain and suggested that the loop of membrane-anchored ADAM is inaccessible for other proteins (16). In the present study, we did not determine whether membrane-anchored ADAM28m can also interact with PSGL-1. However, because secreted-type ADAMs lack most of the CR domain as well as the whole EGF-like, transmembrane, and intracytoplasmic domains, it is possible to speculate that the Dis domain of ADAM28s is not rigidly packed, leaving the domain open to interact with PSGL-1. Thus, the interaction between the ADAM28 Dis domain and PSGL-1 may be selective to ADAM28s, although this possibility must be clarified by future studies. On the other hand, our inhibition study using anti-PSGL-1 Abs has shown that the extracellular decamer repeats of PSGL-1, a motif different from the selectin-binding domain, are essential at least in part to the binding with the ADAM28s Dis domain.

One of the most intriguing findings in this study is that the interaction of ADAM28s with PSGL-1 enhanced the binding of HL-60 cells to immobilized P-selectin and the adhesion to P-selectin-expressing HUVECs. Previous studies have shown that ADAM28 binds to α4β1, α4β7, and α9β1 integrins in an activation-dependent manner of the integrins (21, 22). However, the enhanced effect of ADAM28s on the PSGL-1/P-selectin-mediated function was evident in the absence of MnCl2, which is essential to the activation of integrins (21, 43). Instead, both basal and ADAM28s-promoted binding levels to P-selectin were abrogated with anti-PSGL-1 Abs. Thus, these data exclude the possibility that the ADAM28s-promoted binding is because of the direct interaction between ADAM28s and integrins or secondary to the stimulation of PSGL-1 action through the integrin-derived intracellular signals. Accordingly, our data strongly suggest that the binding of ADAM28s to P-selectin is itself enhances the PSGL-1/P-selectin interaction and promotes the cell adhesion to HUVECs.

The molecular mechanisms of the promoting effect of ADAM28s on the PSGL-1/P-selectin-mediated function...
remain unclear at present. However, because the flow cytometric analysis showed no changes of PSGL-1 protein expression on the cell surface after incubation with ADAM28s species, the enhanced effect is not from altered expression levels of PSGL-1 but may be due to subtle changes of PSGL-1. Several possibilities can be considered as follows. First, conformational changes of PSGL-1 may be induced after the ADAM28s interaction with the decamer repeats of PSGL-1, facilitating the cells to gain access to P-selectin. Second, binding of ADAM28s to PSGL-1 may cause redistribution of PSGL-1 to the tips of microvilli of leukocytes, which is essential to the interaction with P-selectin (44). Third, tyrosine sulfation and/or glycosylation of PSGL-1, both of which are important to the PSGL-1 function (45), might be promoted after interaction with ADAM28s. Finally, dimerization of PSGL-1, which is required for optimal recognition of P-selectin (46), may be enhanced after binding with ADAM28s.

The last possibility, however, seems unlikely, because our preliminary study has provided no changes in the dimerization by the rpro-ADAM28s treatment (data not shown). Other possibilities should be tested by further work.

Because rpro-ADAM28s, active rADAM28s, and rDis/CR/SS all promoted the binding between PSGL-1 and P-selectin, regardless of the presence or absence of ADAM inhibitor, it is evident that metalloproteinase activity of ADAM28s is not a prerequisite for the enhanced effect on the binding. Mocarhagin, a venom ADAM-like metalloproteinase, is known to cleave PSGL-1 to a soluble form (47), and forced expression of ADAM10 or aspartyl proteinase BACE1 increases shedding of PSGL-1 from the cell surfaces by cleaving different sites of the juxtamembrane region of PSGL-1 (48). Thus, this prompted us to examine whether ADAM28s has a sheddase activity for PSGL-1. However, we observed no definite shedding of PSGL-1 by flow cytometric analysis of HL-60 cells after incubation with active ADAM28s, suggesting that PSGL-1 is resistant to ADAM28s. Nevertheless, our study did not completely exclude the possibility that ADAM28 may be involved in the turnover of PSGL-1 after binding to PSGL-1 on the cell membrane, because only a short incubation time (45 min) was used in this study. In addition, because shedding of cell adhesion molecules is carried out by cleavage in cis and in trans (49), P-selectin shedding by ADAM28 should be studied in future studies.

Tethering and rolling of leukocytes to the vessel wall in the microcirculation initiate their transendothelial migration into the inflammatory tissues, and this step is mediated by the molecular interaction of PSGL-1 expressed on the leukocytes with P-selectin on the endothelial cells (50, 51). The PSGL-1 expression by leukocytes is primarily constitutive (52). On the other hand, P-selectin is constitutively expressed but emerges on the cell surface upon stimulation of endothelial cells with factors such as thrombin (33). Thus, accumulated lines of evidence suggest that in acute inflammation, the PSGL-1/P-selectin-mediated tethering and rolling of leukocytes is directed mainly by the endothelial cell surface expression of P-selectin (53). In this study, however, we have provided the in vitro and in vivo data that the leukocyte adhesion to endothelial cells is promoted through interaction between PSGL-1 and ADAM28s. In addition, our parallel immunohistological studies demonstrated that ADAM28 is co-localized with PSGL-1 on neutrophils and monocytes (macrophages) within the pulmonary vessels and alveolar spaces in the human lungs of acute bronchopneumonia, but not normal lung tissues.1 Thus, our data in this study suggest the possibility that ADAM28 plays a role in the leukocyte infiltration under the acute inflammatory conditions by modulating the initial step of the transendothelial migration of leukocytes.

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