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A Novel Glycosylphosphatidyl Inositol-Anchored Protein on Human Leukocytes: A Possible Role for Regulation of Neutrophil Adherence and Migration

Kichiya Suzuki,* Tadashi Watanabe,* Shin-ichi Sakurai,* Kazuhisa Ohtake,* Taroh Kinoshita,* Akemi Araki,* Teizo Fujita,* Hiroshi Takei,* Yuji Takeda,* Yukiko Sato,* Takao Yamashita,* Yoshihiko Araki,* and Fujiro Sendo2 *

We report here a novel glycosylphosphatidyl-inositol (GPI)-anchored glycoprotein on human leukocytes. Treatment of neutrophils with a mAb (3H9) to this molecule sequentially up-regulates and down-regulates β2 integrin-dependent adhesion of these cells as well as their transendothelial migration in vitro. In addition, this mAb simultaneously modulates the avidity of β2 integrin for its ligand, IC3b, with kinetics similar to those observed in 3H9 modulation of neutrophil adherence. This mAb also induces β2 integrin-dependent cytoskeletal remodeling. This novel GPI-anchored protein (GPI-80) is highly homologous with Vanin-1, a recently reported GPI-anchored protein that is expressed on perivascular thymic stromal cells and is involved in thymus homing in mice. The finding that both GPI-80 and Vanin-1 are 40% homologous with human biotinidase suggests the existence of a biotinidase superfAMILY of molecules that may be involved in the regulation of leukocyte trafficking. The Journal of Immunology, 1999, 162: 4277–4284.

Extravasation of leukocytes is an essential preliminary event in various inflammatory and immune responses. This process begins with the adherence of leukocytes to the endothelium through interactions of several kinds of cell adhesion molecules (CAM) (1), which is then followed by cycles of adhesion to and de-adhesion from the endothelium while the leukocytes move through the endothelium. In this past decade, we have broadened our understanding of the early phase of leukocyte extravasation, consisting of the rolling of leukocytes on the endothelium, mediated mainly by selectin molecules and their ligands, followed by firm adhesion via integrins that are partially induced by activation of leukocytes through chemokines present on the endothelium (2). As a result, our knowledge has been greatly advanced in terms of what has been learned about molecular interactions among various CAMs (3, 4). However, little is known about the mechanisms involved in the late phase of extravasation, diapedesis, which consists of rapid cyclic leukocyte adhesion to and de-adhesion from the endothelium concurrent with their locomotion through the endothelium. The main questions pertaining to this late stage could be summarized as follows. First, how do leukocytes begin their crawl along the endothelium toward the extravascular tissues (5, 6)? Second, what are the mechanisms involved in the combined processes of cyclic adhesion and de-adhesion and cytoskeletal remodeling, and what roles do they play in cell locomotion (7)? Finally, it is generally known that a large portion of leukocytes, neutrophils especially, can detach themselves from the endothelium and begin to recirculate in the blood. However, the molecular mechanisms by which leukocytes commit themselves to extravasation or to detachment and subsequent recirculation are largely unknown.

As a means of studying these problems, we established a mAb, tentatively designated 3H9, that modulates neutrophil adherence and induces its motility. The 3H9-reactive molecule (3H9RM) is mainly expressed on neutrophils and monocytes that are the most motile and can easily extravasate (8). Activation of β2 integrin seems to be a prerequisite for modulation of neutrophil function by this mAb, because an increase in intracellular free calcium ([Ca2+]i) evoked by 3H9 treatment was observed only when β2 integrin had been preactivated through cross-linking of CD18 by a relevant mAb (9). The finding that 3H9 not only inhibits neutrophil adherence but also induces motility led us to speculate that this mAb may not block the reaction between CAM(s) and their ligand(s), but recognize a molecule that may regulate neutrophil adherence and migration.

In the present report, we describe the molecular cloning of 3H9RM and discuss the biological significance of this molecule in the regulation of adherence and migration of neutrophils.

Materials and Methods
Reagents and Abs
RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS (BioWhittaker, Walkersville, MD) was used as the incubation medium. Reagents and Abs were purchased from the companies in parentheses. These were fibrinogen and FMLP (Sigma, St. Louis, MO); phosphatidylinositol phospholipase C (PIPLC), digoxigenin

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(DIG)-11-dUTP, alkaline phosphatase-conjugated sheep anti-DIG Fab, and diosside 2-chloro-5-(4-methoxy)pyrrol-1,2-dioxetane-3,2’-(5’-chloro) tricyclo[3.3.1.1 3,7 ]decan-4yl-1-phenyl phosphate (CDP-Star) (Boehringer Mannheim, Indianapolis, IN); dextran 200,000 and Triton X-100 (Wako, Osaka, Japan); human bone marrow cDNA library (cat. no. HL5005SB), Marathon cDNA amplification kit, and human 12-lane multiple tissue Northern (MTN) Blot (cat. no. 7780) (Clontech Laboratories, Palo Alto, CA); mouse mAbs to human CD18 (MHH23, DAKO, Glostrup, Denmark); and TS1/18.1.2.11 (American Type Culture Collection, Manassas, VA). 3H mAb was obtained by immunizing BALB/c mice with phorbol myristate acetate-treated human peripheral blood neutrophils (8). TCY-3 is an IgG1 mouse mAb used as a control for 3H (8). Fab and Fab′2 was obtained by methods shown by Hagiwara et al. (10) and Lamoyi and Nisonoff (11), respectively.

Neutrophil adherence assay
Details of this assay have been described elsewhere (12). Briefly, Falcon 3072 plates (Becton Dickinson Labware, Franklin Lakes, NJ, were coated with 50 μg/ml FCS or fbrinogen before being washed with RPMI 1640 medium. A total of 50 μl of neutrophil suspensions (5 × 10 6 cells/ml) in RPMI 1640, 50 μl of a solution of stimulants, and 100 μl of FCS or fibronogen were added to FCS or fibronogen-coated plates and incubated for varying periods of time at 37°C. The percentage of adherence inhibition was calculated using the following formula: [percent inhibition] = ([(OD570 nm in the presence of TCY-3) – (OD570 nm in the presence of 3H)]/OD570 nm in the presence of TCY-3) × 100.

Neutrophil transendothelial migration
We followed the method described by Yong and Lien (13). HUVEC monolayers of cell culture inserts (Nunc, Becton Dickinson, Tokyo, Japan) were precultured for 4 h with human rIL-1β (10 U/ml) and washed three times with warm PBS before being transferred to wells containing fresh medium (30% FCS, RPMI 1640) for migration experiments. A total of 300 μl of 10°C-labeled neutrophil suspension (1.5 × 10 6 cells) containing 10 μg/ml of 3H mAb or a control mAb, TCY-3, was placed in the upper chamber of the cell culture inserts and the culture plate was incubated for varying periods of time at 37°C in a humidified 5% CO 2 atmosphere. The culture inserts were removed from the chamber, washed, dried and dried, and the percentage of migration inhibition was calculated using the following formula: [percent inhibition] = [(cpm in the presence of TCY-3 – cpm in the presence of 3H)/cpm in the presence of TCY-3] × 100.

Eic3b rosette assay
The details of this assay have been described elsewhere (14). Briefly, to obtain C3b, C3 purified from fresh human serum was incubated with factors B and D. Sensitized sheep erythrocytes were incubated sequentially with Clg1, C4h1, and oxidized C2h2a to prepare EAC14ox2y. These complement-coated sheep erythrocytes were then incubated with C3 and used as EC3b. Purified neutrophils (4 × 10 6 ) were mixed with EC3b in RPMI 1640 containing 1 mg/ml BSA. The tubes were rotated for 30 min at 37°C, and the number of rosette-forming cells was counted under microscopy. Neutrophils bearing more than two RBC were considered rosetting cells. The percentage of rosetting was calculated using the following formula: [percent rosetting] = (percent of rosetting-forming cells in the presence of TCY-3 and FMLP – percent of rosetting-forming cells in the presence of 3H and FMLP)/percent of rosetting-forming cells in the presence of TCY-3 and FMLP) × 100.

F actin distribution in cells as seen under confocal microscopy
Human neutrophils (1 × 10 6 /ml) in an incubation buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl 2, 10 mM glucose, 20 mM HEPES, pH 7.4) were treated with 10 μg/ml of 3H or control TCY-3 for 15 min on ice. In another experimental group, 50 μg/ml anti-CD18 mAb (TS1/18.1.2.11) was added to 3H. The reaction mixtures were then incubated on fibronectin-coated coverslips for 0.15, or 30 min at 37°C. The reason why we used fibronectin is that this matrix protein was better than fibrinogen to obtain sharp figures in a confocal microscopy, and neutrophil adherence to fibronectin is also β2 integrin-dependent (15). Following incubation, coverslips were treated for 3 min on ice with University of Wisconsin solution (107 M CaCl 2, 30 mM sucrose, 5 mM MgSO 4, pH 7.4) containing 0.1% saponin and were then fixed with 4% paraformaldehyde for another 30 min on ice. Samples were stained for 120 min on ice with immunostaining buffer (Dulbecco’s PBS supplemented with 10% heat-inactivated FCS and 2 mg/ml BSA) containing 0.3% rhodamin-phalloidin. The specimens were examined with a confocal laser microscope (TCS, Lica, Hiderberg, Germany).

Screening of a cDNA library, DNA, sequencing, and sequence analysis
Using a method described by Mierendorf et al. (16), screening of a cDNA library constructed from human bone marrow in the phage vector Agt11 (Clontech) was performed using 3H mAb as a specific probe. Positive phage DNA was purified using LambdaSoloa (Promega, Madison, WI) after filtration and chromatography of the manufacturer’s instructions. After sequencing, the phage DNAs were digested with EcoRI purified using low-gelling-temperature agarose gel electrophoresis, then subcloned into pHBluescript II SK(-) phagemid vector (Stratagene, La Jolla, CA). Deletion mutants were prepared from both strands by unidirectional digestion with exonuclease III and mung bean nuclease. All clones were sequenced by the dideoxynucleotide termination method (17) using a DNA sequencer (Model 373A, Applied Biosystems, Foster City, CA). To obtain a full-length cDNA, 5’ and 3’ rapid amplification of cDNA ends (RACE) (18) were conducted with a Marathon cDNA amplification kit (Clontech) as follows. Using 1 μg of poly(A) + RNA from human peripheral blood cells, cDNA was synthesized with avian myeloblastosis virus reverse transcriptase and the oligo(dt) primer (5’TTCCTGAATTCACGGCCGCTT30NNN3). The cDNA was blunted and ligated to the adaptor oligonucleotide (5’-TGAATTCGACTGACCTAGGCTGGCTCGAGGCGCTGCCCGAGGT3’). To obtain the 5’-RACE fragment, the cDNA was amplified by PCR with anchor primer 1 (5’-CAGCTCCTAATACGACTACTAGGCTC3’) and the antisense primer corresponding to nucleotides 367–389 of 3H9RM (glycosylphosphatidylinositol) (GIPI-80) from Fig. 3 downward, see below) cDNA. A nested PCR was then performed with anchor primer 2 (5’-GCTCTAGATCATGTTGGCTCAGACCGGC3’) and an antisense primer corresponding to nucleotides 459–480 of 3H9RM cDNA. To obtain the 3’-RACE fragment, cDNA was amplified by PCR with anchor primer 1 and a sense primer corresponding to nucleotides 148–168 of 3H9RM cDNA. Both the 5’- and 3’-RACE fragments were fused at the overlapping regions by PCR, then amplified with anchor primer 1 and the oligodt primer to obtain the full length of 3H9RM cDNA. The nucleotide sequence and the secondary structure of the deduced amino acid sequence were then compared with those previously reported for other proteins by computer-aided sequence analysis using the GENETYX-MAC program (Software Development, Tokyo, Japan).

Northern blot analysis
Preparation of the DIG-labeled cDNA fragment synthesized by PCR was used as a probe for the detection of mRNA as described previously (19). In brief, 3H9RM (GIPI-80) cDNA (see below) was amplified with primer pairs (a sense primer corresponding to nucleotides 148–168 of 3H9RM (GIPI-80) cDNA and an antisense primer corresponding to nucleotides 367–389 of the 3H9RM (GIPI-80) cDNA in the presence of DIG-11-dUTP. As the positive control, a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (20) probe was used. Human 12-lane MTN Blot (Clontech) was hybridized with the DIG-labeled cDNA fragment at 68°C for 6 h as described previously (19). A positive signal(s) was visualized by CDP-Star according to the method described by Engler-Blum et al. (21).

Amino acid sequencing of purified 3H9RM (GIPI-80)
Purified neutrophils were lysed in ice-cold 20 mM phosphate buffer, pH 7.0, containing 60 mM N-octyl-β-D-glucoside, 1 mM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotinin. Insoluble fractions were clarified at 13,000 × g for 60 min, and the supernatants were used as a source of crude neutrophil AGs for purification of 3H9RM by affinity chromatography. The supernatants were loaded onto a 3H9-coupled Affi-Gel 10 column (Bio-Rad, Hercules, CA). After washing, bound 3H9RM was eluted with elution buffer (0.1 M acetic acid). After dialyzing against 20 mM phosphate buffer, pH 7.0, 3H9RM-positive fractions were boiled in SDS sample buffer with 2 ME, subjected to electrophoresis through 10% SDS-PAGE, and subsequently blotted onto a polyvinylidene difluoride membrane. The proteins were stained with Coomasie Brilliant Blue R250 and the amino acid sequence were determined using the HPG 100SA protein sequencing system (Hewlett Packard, Meriden, CT).
_percentages of migrating cells at 30, 60, 90, and 120 min of incubation in the presence of TCY-3 were 5.1 ±

_mAb (TS1/18.1.2.11) (10_m6 concentrations were 1.16 ±

_in the presence of 10_m6 the presence of FMLP alone at 5, 15, 30, and 60 min of incubation were 26.2 ±

6_M FMLP for 15, 60, or 120 min in the presence of 1.0, 5.0, and 10.0 mg/ml were 0.78 ± 0.04, 0.70 ± 0.04, 0.82 ± 0.04, 0.76 ± 0.02, 0.75 ± 0.02, 0.78 ± 0.03, and 0.78 ± 0.04, respectively, and those after 60 min incubation at these concentrations were 1.16 ± 0.01, 1.23 ± 0.04, 1.38 ± 0.06, 1.19 ± 0.00, 1.22 ± 0.04, 1.34 ± 0.09, and 1.22 ± 0.03, respectively. C, Transendothelial migration. 51Cr-labeled neutrophils were incubated in cell culture inserts in the presence of 3H9 or TCY-3 as described in Materials and Methods. Percentages of migrating cells at 30, 60, 90, and 120 min of incubation in the presence of TCY-3 were 5.1 ± 1.4, 18.8 ± 3.0, 27.2 ± 5.0, and 37.4 ± 5.5, respectively (n = 5). *, p < 0.05 between the 3H9 group and TCY-3 group.

_B, 3H9 effects on neutrophil adherence at different concentrations. Human neutrophils in plates precoated with FCS in RPMI 1640 were incubated with 102 M FMLP plus 3H9 (10 μg/ml) or FMLP plus anti-human CD18 mAb (TS1/18.1.2.11) (10 μg/ml). The reaction mixtures were incubated for varying periods of time at 37°C. Percentages of cells forming rosettes in the presence of FMLP alone at 5, 15, 30, and 60 min of incubation were 26.2 ± 3.1, 31.8 ± 2.3, 40.0 ± 6.1, and 38.6 ± 4.2, respectively (n = 3). *, p < 0.05.

Results

3H9 augments the early phase but inhibits the late phase of β2 integrin-dependent neutrophil adherence, transendothelial migration, and iC3b binding avidity.

We examined the effects of 3H9 on FMLP-stimulated neutrophil adherence to plastic plates precoated with FCS or fibrinogen. These adherences were β2 integrin-dependent, because at any time of incubation the adherence was completely inhibited by anti-CD18 mAb. In contrast, 3H9 enhanced the early phase (15 min) of these β2 integrin-dependent neutrophil adhesions, but inhibited them at the late phase (60 and 120 min). On the other hand, a mAb to HLA did not affect this adherence (Fig. 1A). To observe the concentration dependency of the effects of 3H9, neutrophils were incubated for 15 or 60 min in the presence of FMLP and varying concentrations of 3H9. After 15 min incubation, 3H9 enhanced the adherence from the point of 100 ng/ml. On the other hand, after 60 min incubation 3H9 inhibited the adherence from the point of 50 ng/ml (Fig. 1B). Then, we examined whether the whole IgG molecule was required for the observed phenomena and whether cross-linking of 3H9 affected the effects of 3H9. When the addition of 5 μg/ml 3H9 whole IgG molecule inhibited 41.0 ± 6.4% of neutrophil adhesion in the presence of 10−6 M FMLP at 60 min incubation, the addition of the same concentrations of its Fab, F(ab′)2 and whole IgG in the presence of 5 mg/ml anti-mouse Ig showed 31.8 ± 6.3, 35.8 ± 5.4, and 32.9 ± 4.6% inhibition, respectively. The result that Fab and F(ab′)2 of 3H9 showed neutrophil adherence inhibition comparable to whole 3H9 IgG, and the addition of anti-mouse Ig did not augment the effect of 3H9 suggests that the Fc portion of this mAb or cross-linking of 3H9RM by 3H9 is not required for the effects of 3H9.

FIGURE 1. Modulation by 3H9 of β2 integrin-dependent neutrophil adherence, transendothelial migration, and iC3b binding avidity. A, Neutrophil adherence. Human neutrophils were in plates precoated with FCS or fibrinogen in RPMI 1640 and incubated with 10−6 M FMLP for 15, 60, or 120 min in the presence of 10 μg/ml of 3H9 (•), anti-CD18 mAb (TS1/18.1.2.11) (○), a mAb to monomorphic HLA-A,B,C (MB40.5, American Type Culture Collection) (□), and TCY-3. OD values of FCS-coated group at 570 nm in the presence of the control mAb, TCY-3, were 0.88 ± 0.04, 0.55 ± 0.08, or 0.34 ± 0.02 at 15, 60, or 120 min, respectively (n = 3). OD values of the fibrinogen-coated group in the presence of TCY-3 were 1.23 ± 0.06, 0.99 ± 0.05, or 0.50 ± 0.06 at 15, 60, or 120 min of incubation, respectively (n = 3). Percentage of adherence inhibition was calculated as follows: [percent inhibition of adherence = (OD values in the presence of the TCY-3 – OD values in the presence of 3H9, anti-CD18 mAb or anti-HLA mAb)/OD values in the presence of TCY-3] × 100%. *, p < 0.05. B, 3H9 effects on neutrophil adherence at different concentrations. Human neutrophils in plates precoated with FCS in RPMI 1640 were incubated with 10−6 M FMLP for 15 (●) or 60 (○) min in the presence of various concentrations of 3H9 (n = 3). OD values at 570 nm in the presence of the control mAb, TCY-3, after 15 min incubation at concentrations of 0.005, 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 mg/ml were 0.78 ± 0.04, 0.70 ± 0.04, 0.82 ± 0.04, 0.76 ± 0.02, 0.75 ± 0.02, 0.78 ± 0.03, and 0.78 ± 0.04, respectively, and those after 60 min incubation at these concentrations were 1.16 ± 0.01, 1.23 ± 0.04, 1.38 ± 0.06, 1.19 ± 0.00, 1.22 ± 0.04, 1.34 ± 0.09, and 1.22 ± 0.03, respectively. C, Transendothelial migration. 51Cr-labeled neutrophils were incubated in cell culture inserts in the presence of 3H9 or TCY-3 as described in Materials and Methods. Percentages of migrating cells at 30, 60, 90, and 120 min of incubation in the presence of TCY-3 were 5.1 ± 1.4, 18.8 ± 3.0, 27.2 ± 5.0, and 37.4 ± 5.5, respectively (n = 5). *, p < 0.05 between the 3H9 group and TCY-3 group. D, iC3b binding. Modulation of the iC3b binding activity of human neutrophils by 3H9. Human neutrophils were mixed with EiC3b in the presence of 1 × 10−6 M FMLP plus 3H9 (10 μg/ml) (●) and FMLP plus anti-human CD18 mAb (TS1/18.1.2.11) (10 μg/ml) (○). The reaction mixtures were incubated for varying periods of time at 37°C. Percentages of cells forming rosettes in the presence of FMLP alone at 5, 15, 30, and 60 min of incubation were 26.2 ± 3.1, 31.8 ± 2.3, 40.0 ± 6.1, and 38.6 ± 4.2, respectively (n = 3). *, p < 0.05.
We then examined the effect of 3H9 on neutrophil transendothelial migration in vitro. Transmigration of neutrophils seeded on HUVEC, which had been stimulated with IL-1 for 4 h, was significantly enhanced by 3H9 at 30 min incubation in the presence of FMLP in the lower chambers of the apparatus used for the leukocyte transendothelial migration assay. On the other hand, with longer incubation periods, 3H9 inhibited migration (Fig. 1C).

To explore the mechanisms involved in the above phenomena, we used the iC3b rosette assay to examine the effect of 3H9 on the binding avidity of FMLP-stimulated neutrophils to iC3b, a ligand to Mac-1 and p150, 95. The number of iC3b neutrophils forming rosettes in the presence of FMLP was reduced by the addition of anti-CD18 mAb. In contrast, when 3H9 was added to FMLP, it enhanced iC3b binding activity of neutrophils at 5 and 15 min but inhibited it at 30 and 60 min (Fig. 1D). The effects of 3H9 on iC3b neutrophil binding were similar to those seen with β2 integrin-dependent neutrophil adhesion in that 3H9 enhanced binding in the early phase, but inhibited it in the late phase. 3H9 modulation of the iC3b binding avidity of neutrophils did not occur via a change in the level of expression of β2 integrin on these cells, because treatment with 3H9 did not affect the expression of CD18 on neutrophil surfaces (data not shown).

3H9 induces a change in F actin distribution in neutrophils

Our previous results showed that 3H9 induced neutrophil motility, although the total F actin content of these cells did not change with 3H9 treatment, unlike treatment with FMLP (9). To assess the effect of 3H9 on the cytoskeleton, we examined F actin localization in neutrophils treated with this mAb alone, because if treatment were conducted in the presence of FMLP, it would surely make the results more difficult to interpret inasmuch as FMLP itself induces changes in F actin localization. Many protrusions were observed in the F actin of cells incubated with this Ab for 30 min at 37°C, but only small changes were detected in cells treated with a control Ab, TCY-3, at this point in time. The change in F actin distribution induced by 3H9 was β2 integrin-dependent because it was inhibited by anti-CD18 mAb (Fig. 2). This result suggests that 3H9RM is involved in β2 integrin-dependent cytoskeletal remodeling.

3H9RM is a novel GPI-anchored glycoprotein that is homologous to Vanin-1 expressed on mouse perivascular tissues and involved in prethymic cell homing to the thymus

An attempt was then made to isolate the cDNA, which encodes 3H9RM using a human bone marrow cDNA expression library with 3H9 mAb as the probe. A clone isolated using molecular cloning techniques, encodes a 522 amino acid sequence (Fig. 3). Distribution of the 3H9RM message was examined by Northern blot analysis as shown in Fig. 4. A single signal was observed mainly at the position of ∼2.0 kb in mRNA obtained from human peripheral blood leukocytes. This value is similar to the total number of nucleotides (2034 bp) in the 3H9RM cDNA. Spleen, lung, placenta, colon, and liver, which contain relatively large amounts of blood, also showed a very faint signal. Hydrophobicity plot analysis revealed a strong hydrophobicity at the N-terminal portion of the deduced polypeptide (data not shown). These first 23 amino acids form a hydrophobic core, typical of the signal sequence of an integral membrane protein. Using the sliding window/matrix scoring method and the −1, −3 rule for predicting signal cleavage sites (22, 23), the amino acid positions between Gln-23 and Asp-24 were suggested as cleavage sites for the signal peptidase. The N-terminal amino acid sequence of the affinity-purified 3H9RM is shown in Table I. These results allowed us to conclude that the cDNA sequence consists of a full-length mature 3H9RM coding region (499 amino acids) with a leader peptide of 23 amino acids. Furthermore, the m.w. deduced from this clone is 58,000, which is identical with that obtained by Western blotting after treatment of 3H9RM with N-glycanase (data not shown). Hydrophyt analysis revealed two signal sequences at the N- and C-terminals of the molecule, suggesting that 3H9RM is a GPI-anchored protein. We examined neutrophil expression of 3H9RM after treatment with PIPLC and found it to have been almost completely abolished (Fig. 5). We also examined the expression of this molecule on neutrophils of patients with paroxysmal nocturnal hemoglobinuria (PNH), which is a typical GPI-anchored protein-deficient disease. In two of these patients, ∼50% of neutrophils lacked 3H9 expression, a finding quite similar to that obtained for CD59, a GPI-anchored protein (Fig. 6). Based on the above mentioned results, this novel GPI-anchored protein is now designated as GPI-80 for 3H9RM. An amino acid homology search revealed that GPI-80 is 39% homologous to human bisiodin (24) (Fig. 7). Furthermore, GPI-80 has 59% amino acid sequence homology with Vanin-1, a recently reported GPI-anchored perivascular molecule involved in thymus homing in mice (25) (Fig. 7), which also showed ∼40% molecular homology with biotinidase. The position of cysteine in these proteins is completely conserved.

Discussion

We have described here a novel GPI-anchored protein tentatively designated GPI-80 that may regulates β2 integrin-mediated cell adhesion, in vitro transendothelial migration, and motility of neutrophils.

The molecular mechanisms involved in integrin-mediated cell adhesion have been previously discussed from various standpoints (26, 27). Based on the finding that mutations that eliminate the highly conserved cytoplasmic domain of the α subunit of αIIbβ2 integrin increase ligand-binding affinity, inside-out signaling has been proposed as essential for the regulation of integrin functions (28, 29), and a cytoplasmic regulator of LFA-1 integrin, cytohesin-1, has been reported as a likely candidate for this inside-out
signaling (30). On the other hand, intermolecular reactions outside the plasma membrane between the ectodomain of \( \beta_2 \) integrin and GPI-anchored proteins such as urokinase-type plasminogen activator (uPA) receptor (uPAR) (31, 32), Fc\( \gamma \)RIIIB (CD16b) (33–35), and CD14 (36) have recently been recognized as important in the regulation of \( \beta_2 \) integrin function. Furthermore, the \( \beta_2 \) integrin-mediated intermolecular reaction is considered to be a prerequisite for signal transduction through these GPI-anchored proteins into the cytoplasm (37, 38). In addition, it has been proven that uPAR is also involved in the regulation of \( \beta_1 \) integrin-dependent cell adhesion and migration (39).

The present finding that GPI-80 is a GPI-anchored protein that may be involved in the regulation of \( \beta_2 \) integrin-mediated adherence of neutrophils and our previous results showing that preactivation of \( \beta_2 \) integrin is required for a \([Ca^{2+}]_i\) increase by cross-linking with 3H9 (9) are consistent with the fact that the \([Ca^{2+}]_i\) increase induced by uPAR stimulation with uPA is only observable when \( \beta_2 \) integrin is also present on cell surfaces (31). Our preliminary results from light (Sakurai et al., unpublished observations) and electron microscopy (Sato et al., unpublished observations) showing that GPI-80 and \( \beta_2 \) integrin are colocalized on neutrophil surfaces may support the assumption that the effect of

**FIGURE 3.** Nucleotide sequence of 3H9RM (GPI-80) cDNA and derived amino acid sequence. The consensus translational start site of Kozak sequence at nucleotides 2–10 is double-underlined. The stop codon at nucleotides 1570–1572 is shown by an asterisk. The vertical arrow between Thr-22 and Gln-23 indicates the putative site of signal peptide cleavage. The potential N-glycosylation sites (Asn residue, just before X aa-Ser/Thr) are shown by closed circles. The possible C-terminal sequence of GPI-anchored cleavage site (\( \tau \), \( \tau \), \( \tau \)) are highlighted in a black box (between Ser-491 and Ala-499), and a predicted transmembrane domain is underlined (between Leu-503 and Met-519).
3H9 is proximal to the functions of β2 integrin. However, because we have no definitive evidence yet showing physical association of 3H9RM with β2 integrins as shown for other integrin-associated GPI-anchored proteins, we do not know the mode of reaction of 3H9RM with integrins at this time.

Although little is known of the precise mechanisms involved in the sequential up- and down-regulation of β2 integrin-dependent neutrophil adherence and transendothelial migration induced by 3H9 treatment, these may be at least partially due to the modulation of β2 integrin avidity of the neutrophil for its ligand because the kinetics of cell adhesion, transendothelial migration, and iC3b rosette formation were similar in the sense that all these activities were enhanced at an early stage of 3H9 exposure and were inhibited at a late stage. Furthermore, these opposite effects of 3H9 may eliminate the possibility that it is the kind of mAb that activates β2-dependent cell adhesion (40). Inasmuch as the ligands for GPI-anchored proteins that have relationships with integrin functions, uPAR, CD16b, and CD14; uPA, IgG, and LPS/LPS binding protein complex, respectively, are in plasma or sera, the GPI-80 ligand may also be in plasma or sera. Activity of the mAb, 3H9 may mimic the function of this unknown GPI-80 ligand to stimulate neutrophils. Regarding the possible mechanisms of GPI-80-mediated regulation of β2 integrin-dependent cell adhesion and locomotion, although speculative, molecular interactions between GPI-80 and its hypothetical ligand(s) may modulate β2 integrin-mediated functions of neutrophils, similar to the interactions between uPA and uPAR in myeloid cells (31, 32). Very recently, it has been reported that leukocyte recruitment via β2 integrin in vivo is impaired in uPAR-deficient mice, suggesting that integrin associated GPI-anchored proteins are important for regulation of leukocyte recruitment (41). We would like to examine whether GPI-80 has some roles in vivo for the regulation of leukocyte extravasation through the establishment of GPI-80-overproducing or -deficient animals.

The homology between GPI-80 and Vanin-1 and homology of these two molecules with biotinidase (Fig. 7) presents us with a puzzling but interesting problem. We are unable at this point to speculate on the involvement of biotinidase-like-functions in GPI-80 regulation of integrin-mediated cell adhesion and motility, as already reported in the case of Vanin-1, which has no biotinidase activity (25). However, there is ~60% molecular homology between GPI-80 and Vanin-1, and these two molecules share a

Table I. N-terminal amino acid sequence analysis of 3H9RM (GPI-80)

<table>
<thead>
<tr>
<th>Partial Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-Gln-Tyr-</td>
</tr>
<tr>
<td>Phe-Ile-Ala-Ala-Val-Tyr-Glu-</td>
</tr>
<tr>
<td>His-Ala-Val-Leu-Pro-</td>
</tr>
</tbody>
</table>

Alignment of partial amino acid data obtained from the deduced amino acid sequence of GPI-80 cDNA (A) and the N-terminal sequence of affinity-purified 3H9RM (B). Amino acid residues identical to the deduced sequence of GPI-80 cDNA are boxed.
similar function in the sense that both may be involved in the regulation of leukocyte trafficking. This suggests the existence of a family of molecules that regulates leukocyte extravasation through as yet unknown mechanisms. We are now searching for other potential members of this family using genetic engineering techniques.

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References


