Type IV Collagen-Binding Proteins of Neutrophils: Possible Involvement of L-Selectin in the Neutrophil Binding to Type IV Collagen

By Kazuhsa Iwabuchi, Isao Nagaoka, Akimasa Someya, and Tatsuhsa Yamashita

To isolate type IV collagen-binding proteins, 125I-labeled human-neutrophil extracts were chromatographed on a type IV collagen-Sepharose column. The affinity chromatography-separated fraction contained the four reactive proteins with apparent molecular masses of 28, 49, 67, and 95 kD on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis indicated that the 95-kD proteins contained both L-selectin and nonspecific cross-reacting antigen 90 (NCA90), and that the 67-kD protein was the 67-kD elastin/laminin-binding protein (67BP). The data obtained with the type IV collagen-affinity chromatography and the immunoaffinity chromatographies using anti-L-selectin and anti-NCA90 monoclonal antibodies (MoAbs) have shown that L-selectin is closely associated with 67BP and the 49-kD protein, and that NCA90 is associated with 67BP, the 28-kD and 49-kD proteins. Among these binding proteins, sialic acid residues were contained in 67BP, L-selectin, and NCA90, but not in the 28-kD and 49-kD proteins. Sialidase treatment completely abolished both the binding affinity of the type IV collagen-binding proteins to type IV collagen and the neutrophil adherence to type IV collagen-coated plastic. Thus, the sialic acid residues of 67BP, L-selectin, and NCA90 seem to be important for the binding of neutrophils to type IV collagen. Furthermore, L-selectin IgG chimeric protein directly bound to type IV collagen-Sepharose column, and anti-L-selectin MoAb DREG56 inhibited the neutrophil adherence to type IV collagen-coated plastic by 51%. These observations suggest that L-selectin likely plays a role in the neutrophil binding to type IV collagen, although neutrophils have several kinds of adhesion molecules for type IV collagen such as L-selectin, NCA90, 67BP, and the 28-kD and 49-kD proteins.

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DURING AN ACUTE inflammatory response, neutrophils bind to endothelial cells and migrate through basement membrane toward the focus of inflammation. Type IV collagen is the predominant component of the basement membrane, and serves as a scaffold for the adherence and migration of neutrophils. It is thought that neutrophils have adhesion molecules for type IV collagen, and some molecules have been reported. The anti-CD18 monoclonal antibody (MoAb) has inhibited the binding of phorbol myristate acetate-stimulated human neutrophils to type IV collagen, suggesting that CD18 is involved in the binding of activated neutrophils. The 67-kD protein has been isolated from the guanidine-HCl extract of human neutrophils; this protein has been proved to be identical to the 67-kD Elastin/Laminin binding protein (67BP) of the elastin receptor of elastin-producing cells. However, guanidine-HCl is known to be a strong protein denaturing and solubilizing agent, and may disturb the functions of the adhesion molecules.

In this report, to evaluate the adhesion molecules for type IV collagen, we isolated and characterized the type IV collagen-binding proteins from human neutrophils using affinity chromatography in the physiologic buffer containing mild nonionic detergent NP-40. The results obtained suggest that in addition to 67BP, neutrophils have L-selectin (CD62L), nonspecific cross-reacting antigen 90 (NCA90, CD66c) as type IV collagen-binding proteins, and that sialic acid residues of these molecules are important for the binding.

MATERIALS AND METHODS

Materials. Type IV collagen (type VI, from human placenta), phenylmethylsulfonyl fluoride (PMSF), chymostatin, and bovine serum albumin (BSA, Fraction V), were obtained from Sigma Chemical Co (St Louis, MO); Sialidase (Arthrobacter ureafaciens), lactoperoxidase, and glucose oxidase from Boehringer Mannheim (Mannheim, Germany); and Sialic acid (Sigma Chemical Co (St Louis, MO). Anti-CD18 MoAb L130 (IgGl) was kindly provided by Dr R.P. Mechan (Pulmonary Research, the Jewish Hospital at Washington University, St Louis, MO). Anti-L-selectin (CD62L) MoAb SK11 (available as Leu 8, IgG2a), anti-CD18 MoAb L130 (IgGl), anti-CD44 MoAb L178 (IgGl), anti-ICAM-1 (CD54) MoAb LB-2 (IgG2b), and anti-Sialyl-LeX CSLEX1 (IgM) were purchased from Becton Dickinson (San Jose, CA); anti-CD11a MoAb SPV-L7 (IgGl), anti-CD11b MoAb Bearl (IgGl), anti-CD11c MoAb FK24 (IgGl), anti-CD18 MoAb CLB-LFA-1/I (IgGl) from Seikagaku-kogyo Co Ltd (Tokyo, Japan); anti-CD44 MoAb J-173 (IgGl), anti-L-selectin MoAb DREG56 (IgGl), and anti-NCA95 (CD66b) MoAb 80H3 (IgGl) from Immotech S.A. (Marseille, France); anti-NCA90 (CD66c) MoAbs CLBgran10 (IgGl) and 49.30 from Sanbio (Uden, The Netherlands); anti-L-selectin MoAb TQ12 (IgGl) from Coulter Immunology (Hialeah, FL). In control experiments, mouse IgG and human IgG from normal serum (Chemicon International Inc, Temecula, CA) were used.

Isolation of neutrophils. Antiaggregated peripheral blood with 0.1 vol of 4% (wt/vol) sodium citrate was obtained from normal volunteers. Neutrophils were isolated by Polymorphprep (Nycomed Pharma AS, Oslo, Norway) centrifugation according to the method of the manufacturer, and suspended at more than 95% of the cells were neutrophils. The isolated neutrophils were immediately used for surface labeling and adherence assay without activation.

Cell surface labeling and preparation of cell extract. Neutrophils were labeled with 125I by the lactoperoxidase/glucose oxidase method as described previously. Neutrophils were pretreated with 1 mmol/L diisopropylfluorophosphate for 15 minutes on ice and then

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365

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labeled. Labeled cells (5 × 10^7 cells) were lysed with 3 mL of the lysis buffer, 50 mmol/L sodium phosphate buffer, pH 7.5, containing 0.5% NP-40, 2 mmol/L MgCl₂, 1 mmol/L CaCl₂, 2 mmol/L PMSF, and 20 μg/mL chymostatin) for 30 minutes at 4°C. The insoluble materials were removed by centrifugation at 1,500g for 15 minutes at 4°C, and the resultant supernatant was further centrifuged at 15,000g for 30 minutes to obtain the detergent-solubilized materials.

**Isolations of type IV collagen-binding proteins.** Using the affinity chromatography, type IV collagen-binding proteins were isolated from the neutrophil extracts as described previously. All purification procedures were performed at 4°C. Type IV collagen-Sepharose column was prepared by the coupling of type IV collagen (2 mg/mL) to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden) as described by the manufacturer, and treated with 10 mg/mL BSA to block the nonspecific binding. To remove nonspecific binding proteins, the detergent-solubilized materials were first applied to a Sepharose 4B column (1.5 × 5.7 cm, 10 mL) equilibrated with buffer B (50 mmol/L sodium phosphate buffer, pH 7.5, containing 100 mmol/L NaCl, 0.25% NP-40, 2 mmol/L MgCl₂, 1 mmol/L CaCl₂, 2 mmol/L PMSF, and 20 μg/mL chymostatin). The flow-through fraction of the Sepharose 4B column was pumped onto the type IV collagen-Sepharose column (0.5 × 6.4 cm, 5 mL) equilibrated with buffer B, and cycled over the column overnight. Then the column was sequentially washed with about 10 column volumes of buffer B, 2 mL of 10 mg/mL BSA in buffer B, and buffer B. The binding proteins were eluted from the affinity column with a linear 0- to 2-mol/L NaCl gradient in buffer B. In some experiments, the binding proteins were eluted from the affinity column with 10 mmol/L EGTA in buffer B without CaCl₂, before eluting with the NaCl gradient.

**Immunofinity chromatography.** The binding proteins obtained from the type IV collagen-Sepharose chromatography were characterized with immunofinity chromatography techniques. To prepare immunofinity columns, anti- L-selectin MoAb SK11 and anti-NCA90 MoAb CLBgran10 were coupled to Affi-Gel Hβ (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer’s instructions. Anti-KLH MoAbs X39 (IgG2a) and X40 (IgG1) (Becton Dickinson) were used as class much control MoAbs, and also coupled to Affi-Gel Hβ. The NaCl eluate from the type IV collagen-Sepharose chromatography was applied to NAP-10 columns (Pharmacia LKB Biotechnology) equilibrated with buffer B containing 10 mg/mL BSA. The desalted fractions were applied to class-match control MoAbs and, also coupled to Affi-Gel Hβ. The NaCl eluate from the type IV collagen-Sepharose chromatography was applied to NAP-10 columns (Pharmacia LKB Biotechnology) equilibrated with buffer B containing 10 mg/mL BSA. The desalted fractions were collected, applied to class-match control MoAbs and, also coupled to Affi-Gel Hβ columns equilibrated with buffer B, and cycled over the columns overnight at 4°C. The flow-through fractions were collected, applied to SK11- or CLBgran10-coupled Affi-Gel Hβ columns, and then cycled over the columns overnight at 4°C. After sequential washing with buffer B, 10 mg/mL BSA in buffer B and buffer B containing 0.5 mol/L NaCl, the bound proteins were eluted from the columns with 4 mol/L NaSCN in buffer B.

**Polyacrylamide gel electrophoresis and blotting.** Affinity-isolated proteins were run on 5% to 15% linear gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under the reducing condition as described previously, and the proteins were electrophoretically onto PVDF membranes (Immobilon-P™, Millipore, Bedford, MA). Autoradiography was performed by exposing the electrophoretically transferred membranes to Kodak X AR film (Eastman Kodak, Rochester, NY) at -80°C with intensifying screens (Dupont Lightning Plus, Wilmington, DE). The isolated proteins were also evaluated with Western blotting. The membranes were incubated with murine MoAbs at 4°C overnight and then incubated with biotinylated goat-antimurine IgG+ M (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The reacted proteins were detected by ECL (Amersham International plc, Buckinghamshire, UK). To detect sialic acid residues of the binding proteins, the membranes were sequentially incubated with 2 μg/mL biotinylated *Sambucus sieboldiana* (SSA) lectin (Homen Corp, Tokyo, Japan) for 1 hour at room temperature, and peroxidase-labeled streptavidin (Zymed Laboratories, Inc, San Francisco, CA) for 15 minutes. After extensive washing, the sialic acid residues were detected by ECL.

**Binding of L-selectin chimeric protein to type IV collagen.** L-selectin-IgG chimeric protein (LEC-IgG) containing Ca²⁺-dependent (C-type) lectin-, epidermal growth factor- (EGF-), and duplicated complement binding-domains was kindly gifted by Dr L. A. Lasky (Genentech, Inc, San Francisco, CA). LEC-IgG (20 μg) in 1 mL buffer B containing 10 mg/mL BSA was applied to a type IV collagen- or fibronectin-Sepharose column (0.5 mL) equilibrated with buffer B, and cycled over the columns overnight at 4°C. After extensive washing with buffer B, bound materials were eluted with 10 mmol/L EGTA in buffer B without CaCl₂. The eluate was run on 5% to 15% SDS-PAGE under the nonreducing condition, and then transferred to Immobilon membrane. The membrane was incubated with peroxidase-conjugated goat IgG to human IgG Fc (Organon Teknika Co, Durham, NC), followed by ECL detection.

**Effect of sialidase-treatment on the binding to type IV collagen.** The NaCl eluate from the type IV collagen-Sepharose chromatography was desalted with NAP-10 columns, and incubated with or without 50 μU/mL *Arthrobacter ureafaciens* sialidase at 37°C for 15 hours. After incubation, reaction mixtures were added with the equal volumes of 20 mg/mL BSA containing 20 mmol/L CuSO₄ in buffer B, and chromatographed on type IV collagen-Sepharose columns. After extensive washing, the binding proteins were eluted from the columns with a linear 0- to 2-mol/L NaCl gradient in buffer B.

**Adherence assay.** Neutrophil adherence to type IV collagen-coated plastic wells were assayed by estimating adherent cells in terms of the cell-associated ⁵¹Cr according to the method of Gallin and Rosenthal with slight modification. Briefly, neutrophils (10⁵ cells) were labeled with 1 μCi ⁵¹Cr at 4°C for 1 hour with gentle shaking. After washing twice with buffer A without divalent caions, labeled neutrophils were resuspended in buffer A at 1 × 10⁶ cells/mL. Type IV collagen-coated wells were prepared using Corning 24-well plastic plates (Iwaki Glass Co, Tokyo, Japan) as described previously. Labeled neutrophils (2 × 10⁵ cells) were added to each well, and incubated in the presence of 20 μg/mL MoAbs, 200 mmol/L lactose, or 50 μU/mL sialidase at 37°C for 30 minutes with gentle shaking (30 rpm). After incubation, the wells were washed twice with buffer A, the attached cells were lysed in 200 μL of 1% Triton X 100. The lysates were counted using a γ-counter (model 1282 CompuGamma; Pharmacia LKB Biotechnology).

**RESULTS**

**Isolation and identification of type IV collagen-binding proteins.** Type IV collagen-binding proteins were isolated by applying ¹²⁵I-labeled neutrophil extracts on a type IV collagen-Sepharose affinity column. The bound proteins were eluted as a single radioactive peak with about 0.5 mol/L NaCl from the column (Fig 1A). SDS-PAGE analysis showed that the peak fraction contained the four radioactive proteins with the molecular masses of 28, 49, 67, and 95 kD under the reducing condition (Fig 1B). Immunoblot analysis showed that the 95-kD protein reacted with anti-L-selectin (CD62L) MoAb SK11. The 95-kD protein also reacted with anti-NCA90 MoAb CLBgran10. These observations indicate that the 95-kD type IV collagen-binding proteins contain both L-selectin and NCA90. In contrast, the 67-kD protein reacted with anti-67BP MoAb BC2α, indicating that the 67-kD protein is identical to 67BP. However, the 28-kD and 49-kD proteins did not react with anti-L-selectin, NCA90,
or 67-kD elastin/laminin binding protein MoAb. None of the isolated proteins reacted with the MoAbs against CD11a, CD11b, CD11c, CD15, CD18, CD44, CD54, and NCA-95 (CD66b) (data not shown).

Effect of sialidase treatment on the binding. It has been shown that sialic acid residues, especially sialyl Lewis^x structure (SLe^x), of L-selectin and NCA90 are important for the binding to their ligands. Among the isolated proteins, the 67- and 95-kD proteins reacted with SSA lectin, which specifically recognizes sugar chains containing sialic acid residues (Fig 2A). In contrast, the 28-kD and 49-kD proteins did not react with SSA lectin. By sialidase treatment, sialic acid residues of the 67- and 95-kD proteins were almost completely removed, although the mobilities of these binding proteins were not apparently changed on autoradiograms. Immunoblot analysis showed that the mobilities of the 67-kD protein (67BP) and 95-kD protein (L-selectin) were not affected by the sialidase treatment. However, the mobility of the 95-kD protein corresponding to NCA90 was slightly changed. Interestingly, the sialidase treatment completely abolished the binding activities of these proteins (Fig 2B). Therefore, the sialic acid residues of the 67- and 95-kD proteins seem to be critical for the binding to type IV collagen.

As seen in Fig 2A, the 150-kD band was stained with SSA-lectin. However the 150-kD protein was not detected by autoradiography (Figs 1 through 3). Therefore, the 150-kD protein might be an intracellular protein that contains sialic acids and binds to type IV collagen.

Characterization of the binding to type IV collagen. L-selectin contains the C-type lectin-like binding domain that is thought to mediate the Ca^{2+}-dependent binding to its ligands. Therefore, we determined the Ca^{2+}-dependency of the binding proteins. As shown in Fig 3A, 10 mmol/L EGTA eluted the binding proteins from the affinity column. SDS-PAGE analysis showed that the fraction contained the 49-, 67-, and 95-kD proteins, but not the 28-kD protein. The NaCl-gradient further eluted the 28-, 49-, 67-, and 95-kD proteins from the column. The EGTA-eluted 95-kD protein reacted with anti-L-selectin MoAb, but not with anti-NCA90 MoAb. On the other hand, the NaCl-eluted 95-kD protein reacted with anti-NCA90 MoAb, but not with anti-L-selectin MoAb. Anti-67BP MoAb reacted with both the EGTA- and NaCl-eluted 67-kD proteins.

Next, the proteins obtained from type IV collagen-affinity column (Fig 1A) were chromatographed on immunoaffinity columns, which were prepared using anti-L-selectin or anti-NCA90 MoAb. When type IV collagen-binding proteins were applied to the anti-L-selectin MoAb SK11-coupled Affi-Gel HZ column, the 49-, 67-, and 95-kD proteins were isolated (Fig 4). In case of using the anti-NCA90 MoAb CLBgran10 immunoaffinity column, the 28-, 49-, 67-, and 95-kD proteins were obtained. Immunoblot analysis showed that the anti-L-selectin MoAb-immunoaffinity column bound proteins contained the 67-kD (67BP), and 95-kD (L-selectin) proteins, whereas anti-NCA90 MoAb immunoaffinity column-bound proteins contained the 67-kD (67BP) and 95-kD (NCA90) proteins.

The above observations suggest that type IV collagen-binding proteins of neutrophils are separated into two...
Fig 2. Effect of sialidase-treatment on the binding (A) Western blot analysis of sialidase-treated type IV collagen-binding proteins. The adsorbed fraction obtained from the type IV collagen-Sepharose column (Fig 1A) was incubated with (+) or without (−) 50 mU/mL sialidase for 15 hours. After incubation, reaction mixtures were run on 5% to 15% SDS-PAGE under the reducing condition. The electrophoresed proteins were transferred to Immobilon membranes. After autoradiography, the membranes were probed with biotinylated-SSA lectin (Sialic acid), anti-L-selectin MoAb SK11 (L-selectin), anti-NCA90 MoAb CLBgran10 (NCA90), and anti-67BP MoAb BCZ60 (67BP). (B) Type IV collagen-affinity chromatography of the sialidase-treated binding proteins. The adsorbed fraction obtained from the type IV collagen-Sepharose column (Fig 1A) was incubated with (+) or without (−) 50 mU/mL sialidase for 15 hours. After incubation, the reaction mixtures were applied to a type IV collagen-Sepharose column, and the bound materials were eluted with the NaCl gradient. The eluates were run on 5% to 15% SDS-PAGE under the reducing condition, followed by autoradiography. df, dye front. Molecular masses of the binding proteins are shown at the left side of each panel.

Fig 3. Elution of the type IV collagen binding proteins with EGTA. (A) Elution profile of type IV collagen-Sepharose column. Surface-labeled neutrophils were extracted and subjected to a type IV collagen-Sepharose column, and the bound proteins were eluted with 10 mmol/L EGTA and then eluted with the linear NaCl gradient. (B) Immunoblot analysis. The EGTA-eluted (EGTA) and NaCl-eluted (NaCl) fractions (Fig 3A) were electrophoresed on 5% to 15% SDS-PAGE under the reducing condition. The electrophoresed proteins were transferred to Immobilon membrane, autoradiogramed, and then probed with MoAbs SK11 (L-selectin), CLBgran10 (NCA90), and BCZ60 (67BP). Molecular masses of the binding proteins are shown at the left side of the panel.
groups: the 49-kD, 67-kD (67BP), and 95-kD (L-selectin) proteins make a complex which binds to type IV collagen Ca\(^{2+}\)-dependently; another complex is consisted of the 28-kD, 49-kD, 67-kD (67BP), and 95-kD (NCA90) proteins, which binds to type IV collagen Ca\(^{2+}\)-independently.

**Binding of LEC-IgG to type IV collagen.** Our data indicate that L-selectin is a possible type IV collagen-binding protein of neutrophils. To determine whether L-selectin binds to type IV collagen, LEC-IgG containing C-type lectin like-, EGF-, and duplicated complement binding-domains of L-selectin\(^{22}\) was applied to a type IV collagen-Sepharose column. As shown in Fig 5, LEC-IgG bound to a type IV collagen-Sepharose column, but not to a fibronectin-Sepharose column, which was used to see the nonspecific binding. These results indicate that the LEC-IgG can directly bind to type IV collagen.

**Effect of MoAbs and sialidase on neutrophil adherence to type IV collagen.** To further elucidate the role of L-selectin on neutrophil binding to type IV collagen, we examined the effect of anti-L-selectin MoAb DREG56 on neutrophil adherence to type IV collagen. As shown in Fig 6, DREG56 inhibited the neutrophil adherence to type IV collagen-coated plastic by 51% ± 6%, whereas control murine IgG hardly affected the adherence. These results suggest that L-selectin is involved in the binding of neutrophils to type IV collagen.

The affinity chromatography experiments have indicated that the sialic acid residues of the 67- and 95-kD proteins are required for the binding to type IV collagen (Fig 2B). Sialidase treatment decreased the neutrophil adherence to type IV collagen-coated plastic by 89% ± 4% (mean ± SE). The inhibitory effect of sialidase was interfered with a sialidase inhibitor (10 mmol/L CuSO\(_4\)\(_{3-}\)).\(^{30}\) Anti-SLe\(^{\text{x}}\) MoAb CSLEX-1, which reacted with the 95-kD proteins (L-selectin and NCA90) but not with the 67-kD protein (data not shown), inhibited the neutrophil adherence to type IV collagen-coated plastic by 70% ± 6% (Fig 6). Thus, sialic acid residues of L-selectin, NCA90, and 67BP are likely to be important for the neutrophil binding to type IV collagen. 67BP has the galactose-binding lectin properties, and eluted from the type IV collagen-affinity column with galactose sugars such as lactose.\(^{6}\) In this study, lactose (200 mmol/L) inhibited the neutrophil adherence to type IV collagen-coated plastic by 26% ± 4%. However, neutrophil adherence to type IV collagen was not affected by up to 20 \(\mu\)g/mL anti-67BP MoAb BCG7 (data not shown), suggesting that BCG7 recognizes the domain of 67BP, which is not involved in the binding to type IV collagen.

**DISCUSSION**

The purpose of the present study was to identify the adhesion molecules of neutrophils for type IV collagen. We have isolated the 28-, 49-, 67-, and 95-kD proteins from human neutrophils using the type IV collagen-affinity chromatography. Immunoblot analyses have indicated that the 95-kD proteins contain L-selectin and NCA90, and that the 67-kD protein is identical to 67BP. L-selectin is a member of selectin family of cell adhesion molecules containing C-type lectin like-, EGF-, and complement binding-domains.\(^{12}\) NCA90 is a member of glycoproteins belonging to the carcinoembryonic antigen family within the Ig supergene family, and is specifically expressed on neutrophils.\(^{31}\) 67BP was originally isolated as a component of the elastin/laminin receptor of fibroblasts.\(^{32}\) Senior et al\(^{6}\) chromatographed the neutrophil material that was extracted using 0.5% octyl-\(\beta\)-glucoside and 3 mol/L guanidine HCl, on the type IV collagen-affinity
column in the presence of divalent cation-chelating reagent EDTA, and then isolated only 67BP. In contrast, we have extracted neutrophils with a mild nonionic detergent NP-40 in physiologic buffer. Then, the extract was chromatographed on the affinity column in the presence of divalent cations, because neutrophil adherence is divalent cation-dependent. Thus, the differences in the experimental conditions might lead to the different results.

Sialidase treatment completely abolished the binding affinity of type IV collagen-binding proteins (Fig 2), and inhibited the neutrophil adherence to type IV collagen-coated plastic (Fig 6). Sialic acid residues are contained in 67BP, L-selectin, NCA90, but not in the 28-kD and 49-kD proteins, suggesting that the 28- and 49-kD proteins are not likely to be important for the neutrophil binding to type IV collagen. Furthermore, anti-SLe^a MoAb CSLEX-1 inhibited the neutrophil adherence to type IV collagen-coated plastic. Thus, SLe^a of L-selectin and NCA90, and sialic acid residues of 67BP are likely to be important for the binding of neutrophils to type IV collagen.

The 28-kD and 49-kD proteins did not react with any kinds of MoAbs. In preliminary experiments, we have identified the N-terminal sequences of the 28-kD and 49-kD proteins as ARXTQPXLGYEAYG and TVYPNGTXPV, respectively, using the Direct-protein Microsequencing Techniques. The database searches indicated that the sequences of the 28-kD and 49-kD proteins did not exhibit homology to the proteins so far reported.

Type IV collagen is reported to contain fucose and sialic acid residues through N-linked oligosaccharides. Actually, we have observed that type IV collagen used in this report reacted with both SSA-lectin and anti-SLe^a MoAb CSLEX-1 (K.I., unpublished observation, March 1994). However, the sialidase- or CSLEX-1-treatment of type IV collagen-Sepharose resins did not affect the binding of L-selectin, NCA90, 67BP, and the 28-kD and 49-kD proteins (data not shown). Therefore, sialic acid residues of type IV collagen are not considered to be involved in the interactions of the binding proteins with type IV collagen.

Anti-L-selectin MoAb DREG56 inhibited the neutrophil adherence to type IV collagen-coated plastic by 51%. Anti-SLe^a MoAb CSLEX-1, which recognizes both L-selectin and NCA90, inhibited the neutrophil adherence by 70%. Furthermore, neutrophil adherence to type IV collagen-coated plastic was inhibited by 26% with lactose which eluted 67BP containing the galactose binding lectin property from the type IV collagen-affinity chromatography. These observations indicate that L-selectin, NCA90, and 67BP are all likely to be involved in the binding of neutrophils to type IV collagen, although L-selectin seems to substantially mediate the binding.

Lymphocyte L-selectin has been shown to be closely asso-
associated with other receptors such as TCR/CD3 complex. In this study, neutrophil L-selectin, 67BP, and the 49-kD protein were eluted from the type IV collagen-affinity column with Ca\(^{2+}\)-chelating reagent EGTA, and bound to anti-L-selectin MoAb immunoaffinity column (Figs 3 and 4). These results suggest that neutrophil L-selectin, together with 67BP and the 49-kD protein, likely makes a complex, and this complex Ca\(^{2+}\)-independently binds to type IV collagen. In contrast, NCA90, 67BP, and the 28-kD, and 49-kD proteins were not eluted with EGTA, but eluted with the NaCl gradient. The anti-NCA90 MoAb immunoaffinity column adsorbed NCA90, 67BP, and the 28-kD and 49-kD proteins. Thus, it seems that the 28-kD, 49-kD, 67-kD (67BP), and 95-kD (NCA90) proteins likely make a complex, and the complex binds to type IV collagen Ca\(^{2+}\)-independently.

67BP was present in both the L-selectin-containing complex and the NCA90-containing complex (Fig 3). It has been shown that the binding of 67BP to type IV collagen does not require divalent cations. However, the complex containing L-selectin and 67BP bound to type IV collagen Ca\(^{2+}\)-dependently. Furthermore, LEC-IgG bound to type IV collagen Ca\(^{2+}\)-dependently (Fig 5). Therefore, 67BP does not seem to be important for the binding of the L-selectin-containing complex to type IV collagen. It has been revealed that NCA90-mediated cell adherence is Ca\(^{2+}\)-independent using Chinese hamster ovary cells transfected with the NCA90 gene. Thus, both 67BP and NCA90 likely play a role in the binding of the NCA90-containing complex to type IV collagen.

Several investigators have shown that type IV collagen can modulate neutrophil functions. Low concentration of type IV collagen (<1 μg/mL) and its 7S fragment induce neutrophil migration, whereas high concentration of type IV collagen (>20 μg/mL) and its NC1 domain inhibit superoxide generation and degranulation of neutrophils. Type IV collagen binding activates intracellular killing of neutrophils. Therefore, type IV collagen seems to modulate the neutrophil functions via its receptor(s). The type IV collagen-binding proteins isolated in this study, such as L-selectin, NCA90, and 67BP, may function as the receptors.

It is well known that L-selectin is immediately shedding after neutrophil activation, whereas NCA90 is upregulated. Preliminarily, we have observed that type IV collagen-bound L-selectin was downregulated by FMLP (10^{-9} mol/L) stimulation of neutrophils, whereas NCA90 and 67BP were markedly upregulated (data not shown). Therefore, the adhesion molecules involved in the binding of neutrophils to type IV collagen are likely to be changed during the cell activation. Selectins are shown to be important in the initial neutrophil contact and rolling on endothelial cells. However, when a blood vessel wall is damaged and endothelial continuity is disrupted, circulating and nonactivated (L-selectin-expressing) neutrophils could recognize and bind to type IV collagen of subendothelial basement membranes. In contrast, activated neutrophils may recognize and bind to type IV collagen through NCA90 and 67BP. After the binding to type IV collagen, neutrophils would leave the circulation into particular inflammatory sites. Further studies on the regulation of the neutrophil-adhesion molecules for type IV collagen are helpful for the elucidation of the migration mechanisms of neutrophils.

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Type IV collagen-binding proteins of neutrophils: possible involvement of L-selectin in the neutrophil binding to type IV collagen

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