Two Proteins Modulating Transendothelial Migration of Leukocytes Recognize Novel Carboxylated Glycans on Endothelial Cells[1]

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We recently showed that a class of novel carboxylated *N*-glycans was constitutively expressed on endothelial cells. Activated, but not resting, neutrophils expressed binding sites for the novel glycans. We also showed that a mAb against these novel glycans (mAbGB3.1) inhibited leukocyte extravasation in a murine model of peritoneal inflammation. To identify molecules that mediated these interactions, we isolated binding proteins from bovine lung by their differential affinity for carboxylated or neutralized glycans. Two leukocyte calcium-binding proteins that bound in a carboxylate-dependent manner were identified as S100A8 and annexin I. An intact N terminus of annexin I and heteromeric assembly of S100A8 with S100A9 (another member of the S100 family) appeared necessary for this interaction. A mAb to S100A9 blocked neutrophil binding to immobilized carboxylated glycans. Purified human S100A8/A9 complex and recombinant human annexin I showed carboxylate-dependent binding to immobilized bovine lung carboxylated glycans and recognized a subset of mannose-labeled endothelial glycoproteins immunoprecipitated by mAbGB3.1. Saturable binding of S100A8/A9 complex to endothelial cells was also blocked by mAbGB3.1. These results suggest that the carboxylated glycans play important roles in leukocyte trafficking by interacting with proteins known to modulate extravasation. ***The Journal of Immunology*****, 2001, 166: 4678–4688.**

Recruitment of leukocytes into sites of inflammation depends on a cascade of molecular events, much of which has been delineated in the last decade. Cloning and definition of the roles played by selectins, integrins, and the endothelial adhesion molecules have contributed to the vast literature on the existing paradigm of leukocyte emigration (reviewed in Ref. 1). However, little is known about molecules involved in transmigration across the endothelium, and subsequent processes in the migration of leukocytes. How the system down-regulates extravasation and what leads to the cascade of events being perpetuated in chronic inflammation are also less well established.

Our previous work on a library of bovine lung oligosaccharides led us to find a novel carboxylated glycan that was constitutively expressed on mammalian endothelial cells (2, 3). These glycans interacted with cell surface molecules on activated, but not resting, neutrophils (3). A mAb against this novel modification inhibited extravasation of neutrophils and monocytes in a murine model of peritoneal inflammation (3). This inhibition of cell trafficking correlated with increased sequestration but reduced transmigration of leukocytes adherent to the endothelium of the mesenteric venules (3). To identify molecules involved in the process, we searched for proteins capable of binding these sugar chains. We report here that two soluble proteins of predominant leukocyte origin, which are externalized on activation and known to modulate neutrophil and monocyte extravasation interact specifically with the novel carboxylated glycans of bovine lung.

Materials and Methods

Materials

The following materials were obtained from the sources indicated: platelet-activating factor (PAF)[4] and PMA, Sigma (St. Louis, MO); ionomycin, Calbiochem (La Jolla, CA); DEAE Sephadex A25 and Sephadex G25, Pharmacia (Uppsala, Sweden); Affigel-10, Bio-Rad (Hercules, CA); $Na^{125}I$, American Radiolabeled Chemicals (St. Louis, MO); Iodobeads iodination reagent, GelCode Blue, and bis-sulfosuccinimidyl suberate (BS^3), Pierce (Rockford, IL); Phenyl-650C Toyopearl Resin, TosoHaas (Montgomeryville, PA); mouse anti-bovine annexin I, Biogenesis (Sandown, NH); mouse anti-human S100A8 and S100A9, Bachem Bioscience (King of Prussia, PA); purified mouse IgG1 isotype standard, BD PharMingen (La Jolla, CA); annexin I cDNA, American Type Culture Collection (ATCC; Manassas, VA; ATCC number 65115); expression vector pET23b, Novagen (Madison, WI); restriction enzymes and T4 DNA ligase, Promega (Madison, WI); nucleotide primers, Genset (La Jolla, CA); Western blotting chemiluminescence detection reagent kit, Amersham Pharmacia Biotech (Piscataway, NJ); [2-^{3}H]mannose, American Radiolabeled Chemicals. mAb mAbGB3.1 directed against the novel carboxylated glycan was generated as described (3). Calf pulmonary artery endothelial cells (CPAE) were purchased from ATCC, and HUVECs were from Cell Applications (San Diego, CA).

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[4] Abbreviations used in this paper: PAF, platelet-activating factor; 0.1 M or 0.3 M *asialo*-COO^- or *asialo*-CONHMe glycopeptides, bovine lung glycopeptides charge fractionated on a DEAE column and desialylated, or desialylated and carboxylate-neutralized by methylamidation; NP-40, Nonidet P-40; BS^3, bis-sulfosuccinimidyl suberate; MALDI, matrix-assisted laser desorption ionization; CPAE, calf pulmonary artery endothelial cells.

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Generation and fractionation of bovine lung glycopeptides

Five grams of bovine lung acetone powder was extensively digested with proteinase K at 50°C for 24 h in 0.1 M Tris-HCl, pH 7.5. The mixture was heat-inactivated by boiling at 100°C for 5 min, was centrifuged, and the supernatant was dialyzed against 3 × 4 liters of water by using a molecular mass cut-off of 3500 Da. The glycopeptides then were loaded on a 75-ml column of DEAE Sephadex A25 equilibrated in 10 mM Tris-HCl, pH 8.0, washed with 10 column volumes of starting buffer, and eluted sequentially with 0.1 M, 0.3 M, and 1 M NaCl in starting buffer. These fractions are hereafter simply called "0.1 M, 0.3 M, or 1 M" glycopeptides. The eluates were individually dialyzed extensively against water by using a molecular mass cut-off of 1000 Da and tested for their ability to inhibit binding of mAbGB3.1 to immobilized bovine lung glycans. Briefly, 96-well plates were coated with 50 ng of BSA neoglycoproteins containing total bovine lung glycopeptides coupled to BSA by using glutaraldehyde (see preparation of neoglycoproteins for immunization and screening; Ref. 3) for 4 h at 37°C. Plates were washed and blocked with 3% BSA in PBS overnight at 4°C. Wells were incubated with purified mAbGB3.1 IgG at 10 ng/ml in PBS containing 1% BSA and 0.05% Tween 20, in the absence or presence of varying concentrations of the above glycopeptides, in a total assay volume of 100 μl for 2 h at room temperature. The plates then were washed and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG, followed by development with *p*-nitrophenyl phosphate substrate. Absorbance was measured at 405 nm in an ELISA plate reader.

Monosaccharide compositional analysis of the 0.1 M and 0.3 M glycopeptides obtained in a similar fractionation experiment were analyzed by using a high pH anion-exchange chromatography-pulse amperometric detection system as described earlier (2). Sialic acid content was determined using resorcinol assay.

Generation of an affinity column for the isolation of cognate proteins from bovine lung

From the above fractionation, 0.1 M and 0.3 M NaCl eluates were used individually to generate affinity columns. The glycopeptides were first desialylated by mild acid treatment (10 mM HCl, 30 min at 100°C) to remove sialic acids, split into two equal aliquots, and lyophilized. These are referred to in the text as "0.1 M or 0.3 M *asialo*-COO^- glycopeptides." The carboxylate groups on one aliquot were modified by two cycles of methylamidation as described earlier (2). This treatment neutralized ~80% of the carboxylate groups (not shown). These glycopeptides are referred to in the text as "0.1 M or 0.3 M *asialo*-CONHMe glycopeptides." The glycopeptides were coupled to BSA by using glutaraldehyde (3), and the BSA neoglycoproteins then were coupled to Affigel-10 following manufacturer's recommendations. The efficacy of methylamidation of carboxylates was determined by loss of reactivity with mAbGB3.1. Briefly, 96-well plates were coated with 250 ng of the individual BSA neoglycoproteins in PBS for 4 h at 37°C. Plates were washed and blocked with 3% BSA in PBS overnight at 4°C. Wells were incubated with purified mAbGB3.1 IgG at 10 ng/ml in PBS containing 1% BSA and 0.05% Tween 20, in a total assay volume of 100 μl for 2 h at room temperature. The plates then were developed as indicated above.

Affinity chromatography of bovine lung extracts

One hundred grams wet weight of bovine lung was homogenized with a BioHomogenizer (Biospec Products, Barletsville, OK) in PBS containing EDTA-free protease inhibitors, 10 mM DTT, 1 mM $CaCl_2$, and 1% Nonidet P-40 (NP-40). The suspension was centrifuged at 650 × *g* for 15 min and then at 10,000 × *g* for 30 min. The supernatants (~2.5 g of protein) first were precleared by passing over a 2-ml BSA-Affigel column, and equal volumes then were loaded on BSA-bovine lung glycopeptide affinity columns or the corresponding carboxylate-neutralized columns run in PBS (10 mM phosphate, 150 mM NaCl), pH 7.5. Unbound proteins were washed out with 10 column volumes of starting buffer, and bound proteins were eluted with 50 mM sodium citrate in 10 mM phosphate buffer (no change in pH or net ionic strength over loading/wash buffer). The eluates were then dialyzed, lyophilized, reconstituted in PBS, and analyzed on SDS-PAGE gels.

SDS-PAGE and Western blot analysis

Affinity-purified bovine lung or neutrophil lysate proteins were separated on 12% SDS-polyacrylamide gels under reducing conditions and visualized by silver staining or by using Gelcode Blue. For immunodetection, proteins were electroblotted onto nitrocellulose membranes. The membranes were blocked overnight with 3% BSA in PBS, washed with PBS containing 0.05% Tween 20, and incubated with mouse anti-bovine annexin I, mouse anti-human S100A8, or mouse anti-human S100A9 diluted in PBS containing 1% BSA and 0.05% Tween 20 for 1–2 h at room temperature. This was followed by incubation with alkaline phosphatase or peroxidase-conjugated goat anti-mouse IgG. Bound proteins were visualized by incubating with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, or by using chemiluminescence detection reagents.

N-terminal sequence analysis

Bovine lung proteins eluted from the glycopeptide affinity column were separated on 12% SDS polyacrylamide gels run in electrophoretic buffer containing 0.1 M thioglycolate, and transferred to polyvinylidene difluoride membranes. Transfer was conducted at 80 mV for 2 h and proteins stained with 0.1% Coomassie brilliant blue in 40% methanol. Individual bands were excised after destaining in 40% methanol and 7.5% acetic acid, and N-terminal Edman sequencing was performed on a Model 494 Procise Sequencer (Applied Biosystems, Foster City, CA).

Binding studies on human neutrophil lysates

Human neutrophils were isolated from fresh peripheral (EDTA-anticoagulated) blood from healthy volunteers by differential migration through mono-poly resolving medium (Ficoll-Hypaque d1.114) followed by hypotonic lysis of residual erythrocytes. The preparations were >98% granulocytes as confirmed by using Turk's staining (0.01% crystal violet in 3% acetic acid). Cells were lysed by sonication in PBS containing protease inhibitors, and debris was removed by centrifugation at 10,000 × *g* for 30 min. Specificity of binding was determined by using three different approaches.

Binding to BSA-glycopeptides immobilized on ELISA plates. Plates (96-well) were coated with 250 ng of BSA-coupled 0.3 M *asialo*-COO^- or *asialo*-CONHMe glycopeptides in PBS for 4 h at 37°C. Plates were washed and blocked with 3% BSA in PBS overnight at 4°C. The wells then were incubated with neutrophil lysate proteins in 10 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, and 1.3 mM $CaCl_2$ for 2 h at room temperature. This was followed by incubation with anti-bovine annexin I Ab or anti-human S100A8 or anti-S100A9 for 2 h at room temperature. Wells then were washed and incubated with alkaline phosphatase conjugated goat anti-mouse IgG, followed by development with p-nitrophenyl phosphate substrate.

Binding to the glycopeptide affinity columns. Lysate proteins (~1 mg) were individually precleared on a BSA-Affigel column, and equal volumes then were loaded on BSA-bovine lung glycopeptide affinity columns or the corresponding carboxylate-neutralized columns run in PBS (10 mM phosphate, 150 mM NaCl), pH 7.5. Unbound proteins were washed out with 10 column volumes of starting buffer, and bound proteins were eluted with sodium citrate in phosphate buffer, without change in ionic strength, as described above for bovine proteins. The unbound fractions and eluates were concentrated on YM10 membranes and equivalent proportions of each were analyzed after separation on 12% SDS gels by silver staining and western blotting by using mouse anti-bovine annexin I, mouse anti-human S100A8, or mouse anti-human S100A9 as described above for bovine proteins.

Depletion of annexin I and S100 proteins from human neutrophil lysates by incubation with bovine lung glycopeptides. Plates (96-well) were coated with BSA-coupled 0.3 M *asialo*-COO^- lung glycopeptides and blocked as above. Human neutrophil lysates were sequentially incubated in each of four wells for 30 min/well at 37°C in the above-mentioned buffer, in presence or absence of 200 μM 0.3 M *asialo*-COO^- glycopeptides or *asialo*-CONHMe glycopeptides. After incubation, the supernatants from the incubation wells were collected and protein depletion analyzed by immunoblotting by using the respective Abs. For detection of S100A8/A9 complexes, the proteins in the supernatants were stabilized by chemical cross-linking by using BS^3 according to Staros (4). The cross-linker was added from a fresh 2-mM stock solution to neutrophil lysates from the above depletion experiments to a final concentration of 200 μM, and the mixture was incubated at room temperature for 30 min. Reactions were quenched by the addition of 1 M Tris to a final concentration of 10 mM and samples stored at −80°C before assay. Cross-linked samples were incubated with 2-ME before separation on SDS-PAGE.

Neutrophil adhesion assays

Microtiter plates (96-well) were coated with 250 ng/well of BSA-conjugated 0.3 M *asialo*-COO^- glycopeptides for 4 h at 37°C. Control wells had BSA alone. Plates were washed and blocked with 3% BSA in PBS overnight. Neutrophils were isolated as described above. Cells were resuspended at 5×10^6 cells/ml in HBSS buffer containing 0.2% BSA and used within 30 min after isolation. A total of 0.5×10^6 neutrophils in the above buffer containing 1 μM PAF were added to each well and incubated for 30

min at 37°C in the presence and absence of varying amounts of modifiers, in a total volume of 200 μl. Unbound cells were removed by washing with HBSS. Bound cells were quantitated by assaying myeloperoxidase as described (3). Assays were performed in duplicate.

Flow cytometric assays

Neutrophils were isolated as above. Cells were resuspended at 3×10^6/ml in HBSS, incubated in the presence or absence of 1 μM PAF or 100 ng/ml PMA/1 μM ionomycin for 15 min at 37°C, and pelleted by centrifugation at $300 \times g$ for 10 min. Activated and unactivated neutrophils were incubated with mouse monoclonal anti-annexin I or anti-S100A8 (both IgG1 mouse Abs) or isotype IgG1 mouse control Ab in HBSS containing 1% BSA (each Ab at 1 μg/million cells), followed by FITC-conjugated anti-mouse IgG. They were analyzed by flow cytometry with a FACScan (Becton Dickinson, Mountain View, CA) equipped with CellQuest software, and gated by the side scatter and forward scatter filters.

Purification of human S100A8/A9 complex from peripheral human neutrophils

All steps were conducted at 4°C unless otherwise indicated. Ammonium sulfate precipitation was conducted as described (5). Briefly, 100 ml of peripheral blood was obtained from healthy volunteers and neutrophils was obtained by Ficoll-Hipaque centrifugation as indicated above. The cells were lysed in PBS containing 1 mM DTT, 1% NP-40, and protease inhibitors and subjected to ultracentrifugation at $160{,}000 \times g$ for 30 min. The protein in the supernatant was adjusted to 2 mg/ml with the above buffer, and the solution stirred on ice. Solid ammonium sulfate was slowly added to a final concentration of 75% (w/v) and stirred for 1 h. The solution the was centrifuged at $10{,}000 \times g$ for 30 min and the supernatant dialysed against $3\times$ 4 liters of the above buffer for 24 h. The preparation was concentrated on YM10 membranes and applied to a 10-ml column of Phenyl-650C resin equilibrated in 0.1% trifluoroacetic acid (6). The unbound proteins were washed off with 3 column volumes of starting buffer and the bound proteins eluted with a gradient of 0–50% acetonitrile in the starting buffer. S100A8/A9 proteins eluted at 35–50% acetonitrile and were >90% pure as determined by SDS gels. The pooled fractions were lyophilized and reconstituted in PBS containing protease inhibitors and stored at −20°C.

Expression and purification of recombinant human annexin I

Nucleotide sequences encoding human annexin I were amplified by PCR by using a cDNA clone containing the complete coding sequence as template and the oligonucleotides Ann1_up: 5′-GTCGATAACATATGGCAATGG TATCAGAATTCC-3′ and Ann1_down: 5′-AACGTACTTCATATGTTAG TTTCCTCCACAAAGAGCC-3′ as primers. This resulted in a 1067-bp fragment containing the entire annexin I cDNA flanked by *Nde*I restriction sites. The fragment was digested with *Nde*I and cloned into the expression vector pET23b by using standard procedures (7), resulting in the plasmid pWE151. Proper insertion and DNA sequences were confirmed by established DNA sequencing methods by using primers oVW144: 5′-GAT GCT GAT GAA CTT-3′, oVW145: 5′-AAG TTC ATC AGC ATC-3′, oVW146: 5′-CCA CAA CTT CGC-3′, and oVW147: 5′-GCG AAG TTG TGG-3′. This revealed a conflict from the published sequence (GenBank accession number NM_0007000) at position 288 ($T_{288}G$). The sequences of two other clones showed the same alteration. However, the T to a G does not alter the encoded amino acid and thus could be a polymorphism. The expression construct then was used to transform *Escherichia coli* strain BL21(DE3). Three hours after induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside, the cells, grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin, were harvested by centrifugation and resuspended in 25 mM Tris-HCl buffer, pH 8.0. Soluble fractions were obtained by freeze-thaw lysis in three to five cycles in the above buffer. The lysates were loaded on a DEAE Sephadex A25 column equilibrated with the above buffer, and the flow-through fractions were collected. The molecular mass of purified annexin I was determined by SDS gels and matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (Perseptive Biosystems, Cambridge, MA).

Radioiodination of proteins

Purified proteins were radioiodinated by using $Na^{125}I$ and Iodo-Beads Iodinating Reagent as per the manufacturer's protocols. Unbound label was removed on a Sephadex G25 column, followed by concentration of the void volume fractions by using Centricon10 concentrators.

ELISAs with purified proteins

Immunoassays with purified proteins in the absence or presence of varying concentrations of 0.3 M *asialo*-COO^- glycopeptides or 0.3 M *asialo*-CONHMe glycopeptides were done as described above for neutrophil lysates.

Precipitation of mannose-labeled glycoproteins

CPAE cells were labeled with ([2-^{3}H]mannose at 20 μCi/ml for 24 h in α-MEM containing 10% FBS. Cells were harvested by trypsinization, washed three times in PBS, and lysed in PBS containing protease inhibitors and 0.5% NP-40. Protein-rich lysates were prepared by ultrafiltration on YM10 membranes. Purified S100A8/A9 or annexin I proteins were immobilized on Affigel-10 and mixed with the labeled glycoproteins from CPAE cells in 10 mM HEPES buffer (described above for neutrophil lysate ELISAs) after preclearing the lysates with BSA-Affigel 10. For immunoprecipitations with mAbGB3.1, lysates were precleared with normal mouse IgG-protein G beads and then incubated with protein G coupled to mAbGB3.1 IgG in PBS. After overnight incubation at 4°C, the individual pellets were washed free of all unbound label until no more counts appeared in the washes, and the radioactivity associated with the pellets was measured. For cross-binding, label associated with each pellet first was eluted by using high salt (5 M $MgCl_2$, which eluted >70% bound label). The eluates were repeatedly desalted on YM10 membranes and resuspended in the respective buffers. Label eluted from mAbGB3.1 gels was mixed with immobilized S100A8/A9 or annexin I and vice versa. Again, after overnight incubation at 4°C, the individual pellets were washed free of all unbound label and counted.

Surface binding of iodinated S100A8/A9 to endothelial cells

HUVECs were grown in endothelial cell growth medium (Cell Applications) at 37°C in 5% CO_2. Cell staining and Western blotting were performed to confirm expression of mAbGB3.1 Ags. HUVECS at passages 3–4 were used in the binding assays. Cells were grown to confluence in 12-well plates and activated with 1 μM PAF for 4 h. Activated and unactivated cells were washed with cold HBSS and then incubated with 4–80 nM ^{125}I-S100A8/A9 in 1 ml of HBSS or 20 nM labeled complex in the absence and presence of modulators after activation at 4°C for 2 h. The reaction was stopped by rapid removal of unbound label, and the cells were washed three times with cold HBSS. They were solubilized in 1 M NaOH followed by addition of an equal volume of 1 M HCl, and the radioactivity associated with the lysates was measured with a gamma counter.

Results

We chose whole bovine lung as a source of the binding proteins because it would have considerable trapped blood and sequestered neutrophils; in addition, we reasoned that the lung tissue might contain potential physiological binding proteins derived from blood vessels, as lung tissue is enriched in endothelial cells.

Preparation and characterization of bovine lung glycopeptides for affinity chromatography

We had earlier shown that mAbGB3.1 specifically recognizes carboxylate groups (3). We made an affinity column enriched for the novel carboxylate on bovine lung glycopeptides. Proteinase K-digested glycopeptides from bovine lung acetone powder were fractionated on a DEAE Sephadex column into 0.1 M, 0.3 M, and 1 M NaCl fractions as indicated in *Materials and Methods*. Each anionic fraction in the above preparation inhibited binding of the Ab to bovine lung proteins, but the neutral glycopeptides did not (Fig. 1). Based on earlier work, the 1 M fractions probably include a complex array of glycosaminoglycan chains (2). For this reason, and because the most consistent results were obtained with 0.1 M and 0.3 M fractions, the latter were further processed. Also, for most inhibition assays described in the text, desialylated 0.3 M glycopeptides were used at 100–200 μM final concentration.

Monosaccharide compositional analysis of the 0.1 M and 0.3 M fractions from a typical preparation indicated the presence of sugars commonly found in *N*- and *O*-glycans (Table I), but with no evidence of any uronic acids typical of glycosaminoglycan chains. In separate experiments, to determine the proportion of the unknown carboxylate groups in the two fractions, oligosaccharides were released from the 0.1 M or 0.3 M glycopeptides by hydrazinolysis, desialylated by mild acid treatment, and end-labeled with

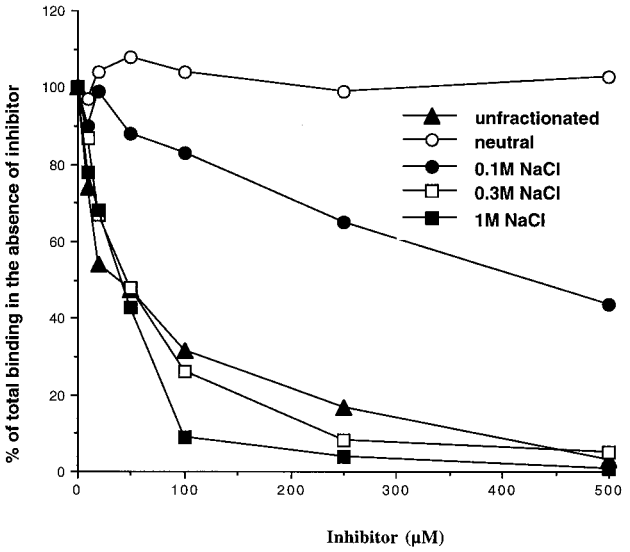

FIGURE 1. Inhibition of mAbGB3.1 binding to BSA-conjugated bovine lung glycopeptides by various DEAE fractions of bovine lung glycopeptides. BSA-conjugated total bovine lung glycopeptides were coated onto microtiter plates (50 ng/ml), and blocked with 3% BSA in PBS. Wells were incubated with mAbGB3.1 (10 ng IgG/ml), in the presence or absence of varying concentrations of the DEAE-fractionated glycopeptides. The concentration of inhibitor glycopeptides was determined by neutral sugar estimation (assuming 5 hexoses/glycopeptide). The different fractions are referred to by the concentration of NaCl used in elution. Binding in the absence of inhibitor was considered 100%. The 1 M fractions gave variable results with different preparations (data not shown). Each point is the mean of two determinations.

[^{3}H]sodium borohydride. Labeled oligosaccharides were subjected to methanolysis to remove sulfate esters and convert the carboxylates to methyl esters. The neutralized oligosaccharides then were subjected to base treatment to regenerate the carboxylate groups. Charge fractionation before and after the treatments showed that ~29% and 33% of the glycans in 0.1 M and 0.3 M fractions contained the novel carboxylate species respectively. This was further confirmed by QAE analysis of [^{3}H]acetic anhydride-labeled desialylated 0.1 M or 0.3 M glycopeptides before and after carboxylate neutralization by methylamidation by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) and methylamine (both results not shown).

Generation of an affinity column for the isolation of the binding proteins

Based on the above findings, and to eliminate any sialic acid-dependent binding, we chose to use desialylated carboxylate-enriched glycopeptides to make the affinity column. Henceforth, these are referred to in the text as "0.1 M or 0.3 M *asialo*-COO$^-$ glycopeptides." To serve as negative controls, carboxylate groups on aliquots of the glycopeptides were neutralized by methylamidation by EDC/NHS and methylamine (2). Henceforth, these are referred to in the text as "0.1 M or 0.3 M *asialo*-CONHMe glycopeptides." These glycopeptides then were individually conjugated to BSA by using glutaraldehyde. The reactivity of 0.1 M and 0.3 M BSA-*asialo*-COO$^-$ glycopeptides against mAbGB3.1 was substantially reduced (~75% and 95%, respectively) after carboxylate neutralization (not shown). These carboxylate-enriched or neutralized glycopeptide-BSA neoglycoproteins then were individually coupled to Affigel-10 to make the affinity columns.

Table I. *Monosaccharide compositional analysis of bovine lung glycopeptides*[a]

Monosaccharide	Nanomoles per Milligram Dry Weight	
	0.1 M glycopeptides	0.3 M glycopeptides
Sia	269	140
Fuc	32	13
$GalNH_2$	63	67
$GlcNH_2$	128	95
Gal	161	112
Man/Xyl	97	31

[a] Bovine lung glycopeptides obtained by charge separation were analyzed using a CarboPac PA1 column (Dionex). Sialic acid was determined by the resorcinol assay. 0.1 M and 0.3 M glycopeptides contained ~18% of carbohydrates and ~11% of carbohydrates by weight, respectively. The gradient used does not separate mannose and xylose.

A very small fraction of the proteins from whole bovine lung bind to the columns in a carboxylate-specific manner

A high concentration of immobilized negatively charged sugars could function simply as an ion exchanger rather than as an affinity matrix. To minimize such nonspecific ionic interactions, we conducted initial binding under physiological ionic strength and pH by using phosphate buffer in the presence of 150 mM NaCl. Because citrate is an inhibitor of mAbGB3.1 binding to bovine lung proteins (3), we then eluted the column with 50 mM citrate in phosphate buffer, (i.e., keeping the pH and ionic strengths unchanged). Under these conditions, <0.0001% of the loaded proteins bound to and eluted from the column. Of the five or more protein bands that were detected on silver stained gels, three proteins of masses 35, 37, and 30 kDa did not bind to columns on which carboxylate groups were neutralized (Fig. 2*A*). As seen in Fig. 2*B*, the three proteins completely rebound to the unmodified column after dialysis to remove citrate, and could be eluted with as little as 5 mM citrate. Bands of >48 kDa also were present in the unbound fractions, and their binding was not carboxylate-dependent (Fig. 2*A*).

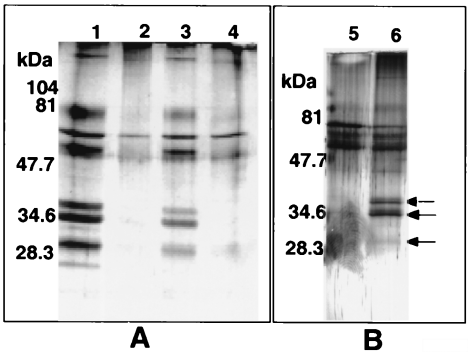

FIGURE 2. Proteins from bovine lung bind to lung glycopeptides in a carboxylate-specific manner. Bovine lung extracts were loaded on affinity columns and eluted with 50 mM citrate as described in *Materials and Methods*. Silver staining of proteins eluted from the bovine lung glycopeptide affinity column *A*. Eluates are from: *lane 1*, column containing immobilized 0.1 M *asialo*-COO$^-$ bovine lung glycopeptides; *lane 2*, corresponding control column containing immobilized 0.1 M *asialo*-CONHMe glycopeptides; *lane 3*, column containing immobilized 0.3 M *asialo*-COO$^-$ bovine lung glycopeptides; and *lane 4*, corresponding control column with 0.3 M *asialo*-CONHMe glycopeptides. *B*, Eluted proteins from *lanes 1* and *3* were pooled, dialyzed, and reloaded onto the column containing immobilized 0.1 M *asialo*-COO$^-$ lung glycopeptides; *lane 5*, unbound fractions; *lane 6*, proteins eluted by 5 mM citrate in starting buffer. Arrows indicate protein bands that bound and rebound in a carboxylate-dependent manner.

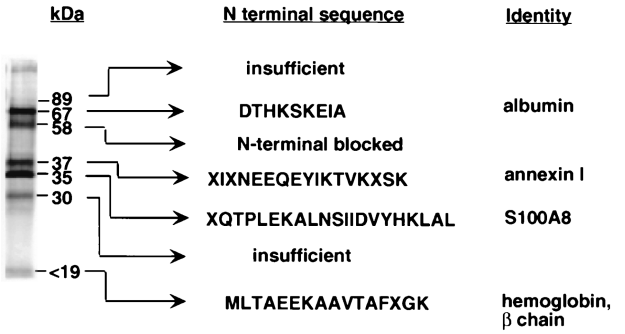

FIGURE 3. N-terminal sequence analysis of bovine lung proteins eluted from the carboxylate-enriched glycopeptide columns. Bovine lung proteins eluted from the glycopeptide affinity column were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Proteins were visualized using 0.1% Coomassie brilliant blue. Individual bands were excised and N-terminal Edman sequencing performed on a Model 494 Procise Sequencer.

N-terminal sequence analysis of the 37- and 35-kDa proteins show that they are calcium-binding proteins

Fig. 3 shows the N-terminal sequences of the bound proteins. BLAST searches for sequence similarity of the proteins in the GenBank data bases showed that the sequence of the 37-kDa band was >80% similar to a sequence beginning with the 13th residue of a leukocyte calcium binding protein, annexin I. Native annexin I is known to be blocked at the N terminus (8). The identity of annexin I was further confirmed in immunoblots by using a commercially available mouse mAb against bovine annexin I. A doublet was recognized on the blots, the native (37 kDa), and a "clipped"(~35.5 kDa) form, which is devoid of the first 12 amino acids, as deduced from the protein sequencing data (not shown).

The N-terminal 21 amino acids of the 35-kDa band shared >80% sequence identity with an EF hand motif calcium binding protein of the S100 family, namely migration inhibitory factor-related protein 8 (MRP8 or S100A8). Commercial mAbs against distinct but uncharacterized epitopes of human S100A8 did not recognize the bovine homologue (not shown). S100A8 may not be a very conserved protein; for example, human S100A8 protein shares only ~59% sequence homology with that of the mouse (9, 10).

S100A8 has a molecular mass of 10.8 kDa. However, the protein we isolated with the N-terminal sequence of S100A8 had a molecular mass of 35 kDa. It is known that the protein often exists and physiologically functions as a Ca^{2+}-dependent complex with S100A9 (or MRP14), another protein of the S100 family (11). The complexes have molecular masses of 24.5 kDa [S100A8 · S100A9 heterodimer], 35 kDa [$(S100A8)_2$·S100A9 heterotrimer], and 48.5 kDa [$(S100A8)_2$·$(S100A9)_2$ heterotetramer]. Though disulfide-linked complexes can be generated in vitro from purified S100A8 and S100A9 proteins, heteromeric complexes formed in vivo are known to be noncovalently linked and break down on SDS gels under both reducing and nonreducing conditions (11). They have been identified by electrospray ionization mass spectrometry (12) and the two-hybrid system (13). It remains to be seen how we were able to isolate the 35-kDa [$(S100A8)_2$·S100A9] trimer as a non-dissociable complex. We did not obtain a sequence for S100A9 on the 35-kDa band. This could be attributable to an N-terminal block, as is known with native human S100A9 (14). Table II compares the N-terminal sequences of a few mammalian S100A8 and annexin I proteins.

The 89- and 30-kDa protein bands were insufficient for sequencing. The 58-kDa protein was N-terminally blocked, and the 67-kDa protein was bovine albumin. The <19-kDa bands had sequences similar to the β-chain of hemoglobin and also variable overlapping sequences that could not be matched with any known protein(s).

Human homologues of the S100 proteins and annexin I also bind to the novel carboxylated bovine lung glycans

The two affinity-isolated bovine proteins showed specific binding to bovine lung glycans immobilized on microtiter plates, as detected by using radiolabeled bovine S100A8/A9, or an Ab against annexin I (data not shown).

Next, we sought to determine whether human homologues of S100A8/A9 complexes and annexin I showed similar binding by using three different approaches. First, we checked and confirmed specific binding of the proteins from human neutrophil lysates to carboxylated but not to neutralized glycans, by direct binding to immobilized glycans on ELISA (Fig. 4, *A* and *B*). Next, we analyzed binding of human neutrophil lysate proteins to the carboxylated and carboxylate-neutralized glycopeptide columns under the same conditions that were used for bovine proteins. Both S100A8/A9 and annexin I bound specifically to the carboxylated, but not the neutralized species (Fig. 4, *insets*). Bound proteins also consisted of few other minor bands that were detected on silver-stained gels (not shown). The identity of these were not determined and is beyond the scope of the present study. Approximately 95% of the total loaded proteins did not bind to the columns, and because S100A8, S100A9, and annexin I together account for ~35% of the total neutrophil cytosolic protein, the load probably exceeded the column capacity. Analysis was done on reducing gels to see whether any of the unusually nondissociable S100 complexes (as seen with the bovine S100A8/A9 heterotrimer) were present in the bound fractions. In fact, a small fraction of the bound

Table II. *N terminal sequences of mammalian S100A8 and annexin I*

		GenBank Accession No.	Sequence
S100A8	Rabbit	D17405	M P T D L E N S L N S I I F V Y H K C S L
	Mouse	X87966	M P S E L E K A L S N L I D V Y H N Y S N
	Human	M21005	M L T E L E K A L N S I I D V Y H K Y S L
	Bovine[a]		X Q T P L E K A L N S I I D V Y H K L A L
Annexin I	Rabbit	U24656	M A M V S E F L K Q A W F I D N E E Q D Y I N T V K T Y K
	Mouse	M69250	M A M V S E F L K Q A R F L E N Q E Q E Y V Q A V K S Y K
	Human	NM_000700	M A M V S E F L K Q A W F I E N E E Q E Y V Q T V K S S K
	Bovine	X56649	M A M V S E F L K Q A W F I E N E E Q E Y I K T V K G S K
	Bovine[a]		X I X N E E Q E Y I K T V K X S K

[a] Sequences obtained in the present study.

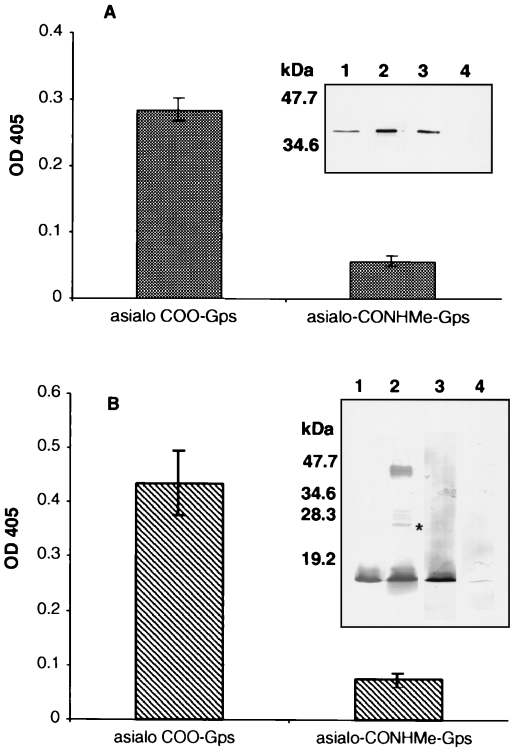

FIGURE 4. Human homologues of the S100 proteins and annexin I bind to the novel carboxylated bovine lung glycans. Neutrophil lysates were incubated with BSA-conjugated 0.3 M *asialo*-COO$^-$ glycopeptides immobilized on microtiter plates and the wells developed with anti-annexin I (*A*), anti-S100A8 (*B*), or anti-S100A9 (not shown) as described in *Materials and Methods*. Each point is the mean ± SD of three determinations. *Insets*, Neutrophil lysates were loaded onto carboxylated (*lanes 1* and *2*) or neutralized (*lanes 3* and *4*) glycopeptide affinity columns and eluted with citrate as described in *Materials and Methods*. Unbound and bound fractions were analyzed by Western blotting using anti-annexin I (*A*) or anti-S100A8 (*B*). *Lanes 1* and *3* represent unbound fractions and *lanes 2* and *4* bound fractions. The identity of the S100A8/A9 heterodimer (marked by asterisk) in the glycopeptide-bound fractions (*B*, *lane 2*) was confirmed by a separate immunoblotting with anti-S100A9 (not shown).

human S100 proteins consisted of a stable heterodimer, the identity of which was confirmed by a separate immunoblot with S100A9 (not shown). However, a majority of the proteins broke down to monomeric forms. Also, the identity of a set of ~42- to 45-kDa anti-S100A8 reactive bands that bound to the column could not be determined because they did not react with anti-S100A9, and their masses did not correspond to any known S100A8/A9 complexes.

Next, we conducted depletion assays in which lysates from activated human neutrophils first were incubated with immobilized *asialo*-COO$^-$ glycopeptides, and the residual proteins in the supernatants were analyzed by immunoblots. Because the S100 protein complexes from neutrophil lysates broke down into monomers on SDS-PAGE, the intensity of staining in immunoblots was beyond the linearity of reaction for any quantitative evaluation. Therefore, to determine whether physiologically relevant multimeric complexes showed specific binding, we stabilized the complexes by chemical cross-linking before SDS-PAGE analysis. Because crude lysates were used in the cross-linking, it is conceivable that nonspecific cross-linking can occur. Therefore, we have identified only the bands that were still strongly stained with increasing dilution of both anti-S100A8 and anti-S100A9. As seen in Fig. 5, S100A8 homodimer as well as S100A8/A9 heterodimer and heterotrimer were depleted from the incubation mixture after binding

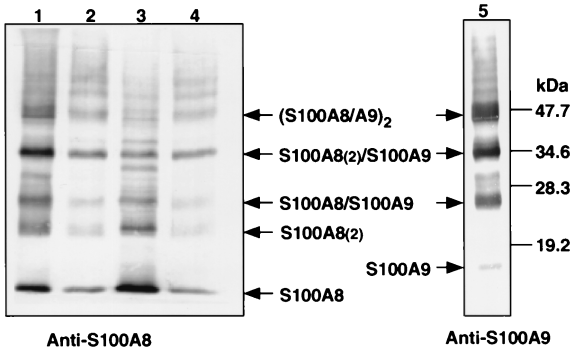

FIGURE 5. Human S100A8 binds to the carboxylated glycans as a heteromeric complex with S100A9. Freshly activated human neutrophil lysates were incubated with BSA-conjugated 0.3 M *asialo*-COO$^-$ bovine lung glycopeptides coated on microtiter plates as described in *Materials and Methods*. Supernatants after incubation were collected and analyzed for depletion of S100A8 by immunoblotting by using anti-S100A8 after stabilizing the protein complexes by cross-linking by using BS^3. *Lane 1*, At zero time; *lane 2*, after binding to the immobilized glycopeptides for 2 h; *lane 3*, after binding to the immobilized glycopeptides for 2 h in the presence of 200 μM soluble 0.3 M *asialo*-COO$^-$ glycopeptides; *lane 4*, after binding to the immobilized glycopeptides for 2 h in the presence of 200 μM 0.3 M *asialo*-CONHMe glycopeptides. The identity of each heteromeric immunoreactive band was further confirmed by a separate detection using anti-S100A9 (*lane 5*).

to the glycopeptides (*lane 2*). The binding of the heterodimer and homodimer, but not the heterotrimer, was blocked if the incubation was done in the presence of bovine lung 0.3 M *asialo*-COO$^-$ glycopeptides but not by *asialo*-CONHMe glycopeptides (*lanes 3* and *4*, respectively). A separate immunoblotting with anti-human S100A9 confirmed the identity of the heteromeric complexes (*lane 5*). Therefore, the differences in the binding and stability of the human and bovine heteromeric complexes could be a species difference. S100A8 monomer, arising from breakdown of any residual noncross linked complexes, also showed similar binding specificity. The monomer probably does not represent the component involved in the actual binding, because complex formation is considered an essential prerequisite for the biological functions of these S100 proteins (14).

Residual proteins from the above incubations also were separately analyzed by immunoblots by using anti-annexin I. As shown in Fig. 6, annexin I from the neurophil lysates appeared as the native (37 kDa) and the clipped (~35.5 kDa) doublet, which is devoid of the first 12 amino acids, based on our sequencing data. Whereas the intact protein was >90% depleted from the neutrophil

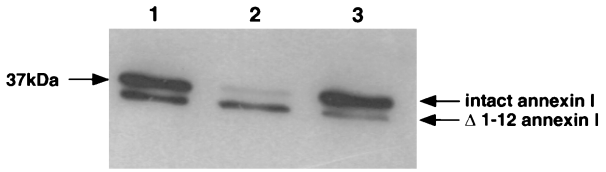

FIGURE 6. An intact N terminus may be necessary for human annexin I to bind to the carboxylated glycans. Freshly activated human neutrophil lysates were incubated with BSA-conjugated 0.3 M *asialo*-COO$^-$ bovine lung glycopeptides coated on microtiter plates as described in *Materials and Methods*. Supernatants after incubation were collected and analyzed by immunoblots with anti-annexin I and chemiluminescence detection. *Lane 1*, At zero time; *lane 2*, after binding to the immobilized glycopeptides for 2 h; *lane 3*, after binding to the immobilized glycopeptides for 2 h in the presence of 200 μM soluble 0.3 M *asialo*-COO$^-$ glycopeptides.

preparations after binding to the carboxylate-enriched glycopeptides, the truncated protein was not (*lane 2*), suggesting that the N-terminal 1–12 amino acids may be important for optimal binding under these conditions. Reactivity of the intact human protein was blocked in the presence of 0.3 M *asialo*-COO^- glycopeptides (*lane 3*), suggesting specificity for the novel carboxylate.

Effects of anti-annexin I, anti-S100A8, and anti-S100A9 on neutrophil binding to immobilized carboxylated glycans

We previously showed that when the novel carboxylated glycans are immobilized on plastic, they supported the binding of freshly activated human neutrophils (3). This binding was completely blocked by mAbGB3.1 and by soluble *asialo*-COO^- glycopeptides, but not by the *asialo*-CONHMe glycopeptides. To determine whether this interaction was in fact mediated by annexin I or the S100 proteins, here we tested the effects of anti-annexin I, anti-S100A8, and anti-S100A9 on the binding of neutrophils to BSA-conjugated 0.3 M *asialo*-COO^- glycopeptides. As shown in Fig. 7, although anti-annexin I and anti-S100A8 had little or no inhibitory effects, anti-S100A9 significantly reduced binding, as did mAbGB3.1, indicating that neutrophil binding to the novel glycans may be principally mediated by S100A9.

Annexin I and S100 proteins are expressed on the cell surface of neutrophils on activation

S100A8 and S100A9 are cytosolic proteins, but membrane-associated forms have been identified in monocytes on differentiation or activation (15, 16). It is conceivable that neutrophil binding to immobilized glycans is attributable to translocation of S100A8/A9 complexes to the surface on activation. To test this possibility, we determined surface expression of annexin I and S100A8/A9 before and after activation of neutrophils by flow cytometric analysis. S100A8 and annexin I are not expressed on resting neutrophils but are detected on the cell surface within 15 min of activation with PMA/ionomycin (Fig. 8) or PAF (data not shown). This also explains our earlier findings that neutrophils acquire surface binding sites for the glycans on short-term activation (3).

For further characterization of binding, and for functional studies, and to verify whether the interactions are mediated by the proteins themselves or in association with other molecules, we purified human S100A8/S100A9 and annexin I.

Purified human S100A8/A9 complex and annexin I show specific binding to the novel glycans

S100A8/A9 was purified from fresh human neutrophil cytosolic fractions because they contain abundant amounts of the proteins

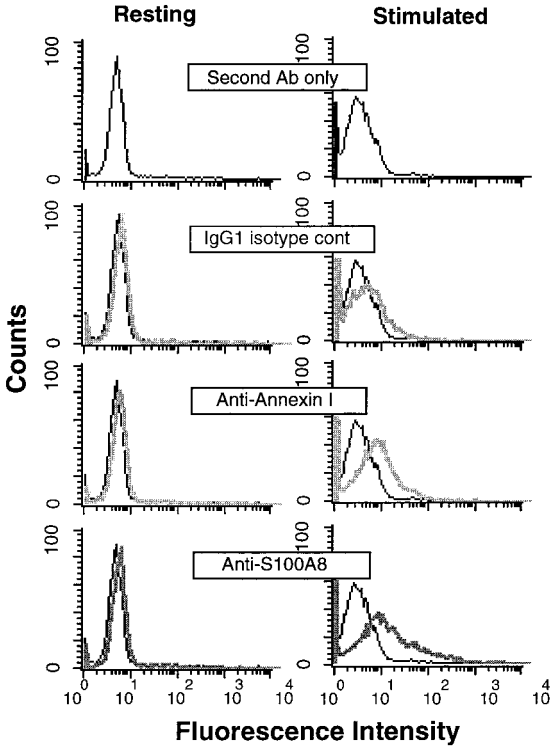

FIGURE 8. Annexin I and S100A8 are expressed on the cell surface of neutrophils on activation. Freshly isolated human neutrophils were activated as described in *Materials and Methods*. Stimulated or resting neutrophils were incubated with anti-annexin I, anti-S100A8, or an isotype-matched control Ab followed by FITC-conjugated anti-mouse IgG. Cells stained with secondary Ab only were used as negative controls and provide the background staining in the plot overlays.

(30–45% of total cytosolic proteins; see Ref. 17). We exploited the high solubility of S100 proteins in ammonium sulfate (5) to obtain a preliminary enrichment of ~80% before further purification of the proteins (to ~95% purity) on a hydrophobic column (6). The proteins stayed as heteromeric complexes through purification, were even retained on YM10 and YM30 ultrafiltration membranes, and could not be separated into individual proteins without denaturation. The molecular masses of the purified proteins as determined by SDS gels were 11 kDa and 14 kDa for S100A8 and S100A9, respectively (not shown). Purified S100A8/A9 complex showed the same specificity of binding as the proteins from the

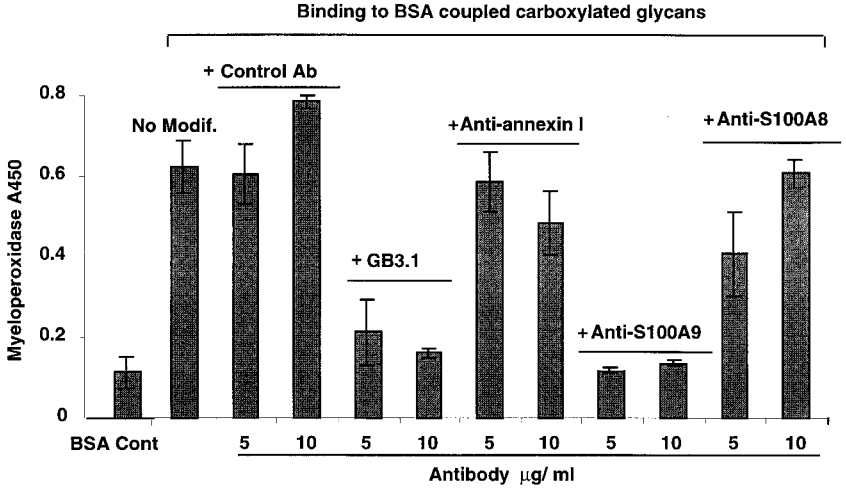

FIGURE 7. Neutrophil adhesion to immobilized carboxylated glycans is blocked by mAbGB3.1 and anti-S100A9. Neutrophil adhesion to immobilized carboxylated glycans was performed as described in *Materials and Methods*. Each point is the mean ± SD of two to four determinations.

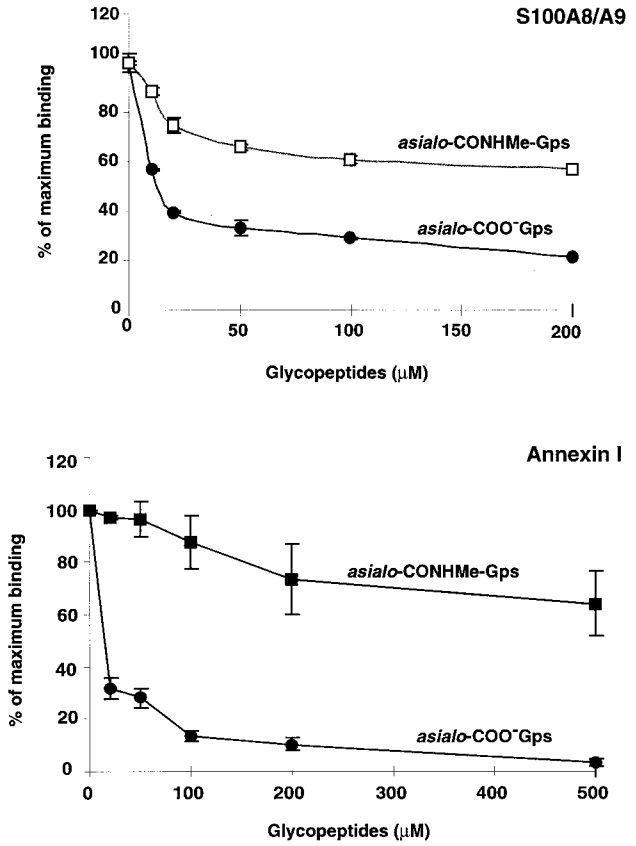

FIGURE 9. Purified human S100A8/9 and annexin I show specific binding to immobilized bovine lung glycans. Purified S100A8/A9 complex (*top*) or annexin I (*bottom*) were incubated with BSA-conjugated 0.3 M *asialo*-COO^- glycopeptides coated onto microtiter plates and the wells developed as described in *Materials and Methods*. Each point is the mean ± SD of two determinations.

initial human neutrophil cytosol (Fig. 9, *top*). Binding to BSA-coupled *asialo*-COO^- bovine lung glycopeptides immobilized on microtiter plates was inhibited in the presence of 0.3 M *asialo*-COO^- glycopeptides but much less effectively by the *asialo*-CONHMe glycopeptides.

Annexin I purified from various mammalian tissues contains a significant proportion of protein proteolytically cleaved at the amino terminus (18). To obtain sufficient quantities of full-length annexin I for functional studies, we cloned the entire coding sequence of human annexin I into the expression vector pET23b as described under *Materials and Methods*. The expression construct was transformed into the *E. coli* strain BL21(DE3). Freeze-thaw lysates from the transformed *E. coli* contained full-length annexin I with apparent molecular mass of 38 kDa as determined by SDS gels and MALDI mass spectrometry (not shown). Because the N-terminal regions of annexins are extremely sensitive to proteolytic cleavage, rapid purifications with fewer steps are generally recommended for all annexins. In our preparation, >95% enrichment from freeze-thaw lysates was possible by using a DEAE column to which the protein did not bind. Purified recombinant annexin I bound to BSA-coupled 0.3 M *asialo*-COO^- bovine lung glycopeptides that were immobilized on microtiter plates (Fig. 9, *bottom*). Again, the specificity of this binding was demonstrated by a dose-dependent inhibition in presence of *asialo*-COO^- glycopeptides, but not by *asialo*-CONHMe glycopeptides.

Purified S100A8/A9 complex and annexin I bind to a subset of endothelial glycoproteins recognized by mAbGB3.1

We previously showed that a major fraction of the mAbGB3.1-reactive carboxylated epitopes are carried on *N*-linked glycans and that mAbGB3.1 recognized several proteins on endothelial cells (3). In keeping with this, mAbGB3.1 consistently immunoprecipitated ~13–15% of [2-^{3}H]mannose-labeled glycoproteins from CPAE cells (Table III). To determine whether the same glycoproteins were also recognized by the S100A8/A9 complex or annexin I, cross-binding assays were conducted as described in *Materials and Methods*. Essentially, purified proteins were immobilized on Affigel-10 and mixed with [2-^{3}H]mannose-labeled glycoproteins from CPAE cells (precleared with BSA-Affigel-10). A separate immunoprecipitation of labeled glycoproteins was simultaneously conducted by using mAbGB3.1 after preclearing the lysates with normal mouse IgG-protein G Sepharose. As shown in Table III, S100A8/A9 complex precipitated 9% of mannose-labeled glycoproteins from CPAE cells as compared with 14% of label immunoprecipitated by the Ab. When the precipitated proteins were eluted either from the S100A8/A9 complex or mAbGB3.1 and cross-bound, S100A8/A9 complex precipitated about one-third of the label eluted from mAbGB3.1, whereas mAbGB3.1 immunoprecipitated almost two-thirds of the label eluted from the S100A8/A9 complex. These experiments showed that the S100A8/A9 complex bound to a subset of endothelial glycoproteins recognized by mAbGB3.1. In comparison, immobilized annexin I precipitated only ~2–3% of mannose-labeled glycoproteins from endothelial cells (not shown). This may indicate differences in binding affinities or may suggest that more of the annexin I-reactive epitopes could be present on *O*-glycans.

Table III. *S100A8/A9 complex recognizes a subset of [2-^{3}H]mannose-labeled CPAE glycoproteins immunoprecipitated by mAbGB3.1*[a]

Sample Processing		[2-^{3}H]Mannose-Labeled CPAE Glycoproteins (cpm)		% Bound Radioactivity (mean ± SD)
Primary binding to	Rebinding to	Total added	Bound to gel	
mAbGB3.1	–	22,949	2,674	
		22,565	3,132	13.5 ± 1.8
		11,160	1,768	
		11,206	1,420	
S100A8/A9	–	20,386	1,698	8.7 ± 0.5
		10,286	928	
mAbGB3.1	S100A8/A9	4,951	1,569	31.5 ± 0.2
		1,961	615	
S100A8/A9	mAbGB3.1	1,074	693	62.5 ± 2.8
		504	305	

[a] Sequential binding of mannose-labeled CPAE glycoproteins to S100A8/A9 or mAbGB3.1 was done as described in *Materials and Methods.*

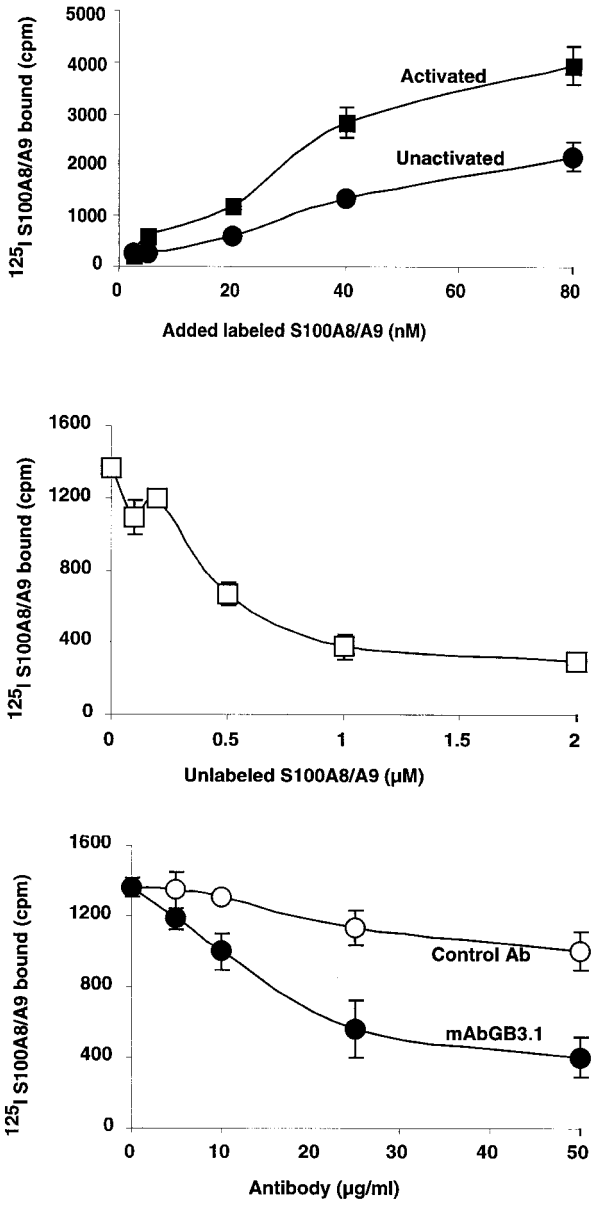

FIGURE 10. Cell surface binding of ^{125}I-S100A8/A9 to endothelial cells. HUVECs were grown to confluence in 12-well plates and incubated with 4–80 nM ^{125}I-S100A8/A9 in 1 ml of HBSS at 4°C for 2 h before and after activation with PAF (*top*) or 20 nM labeled complex in the absence (*middle*) and presence (*bottom*) of modulators after activation. The reaction was stopped by rapid removal of unbound label, and the cells were washed three times with cold HBSS. Cells were solubilized and the radioactivity associated with the lysates was measured with a gamma counter. Each point is the mean ± SD of two determinations.

Cell surface binding of ^{125}I-S100A8/A9 to activated endothelial cells is competed by mAbGB3.1

We found dose-dependent and saturable binding of ^{125}I-S100A8/A9 on PAF-activated HUVECs. Binding to unactivated cells was 2-fold less (Fig. 10, *top*). Binding to activated cells was specific because it was competed by increasing amounts of unlabeled ligand, with half-maximal competition of specific binding at ~500 nM (Fig. 10, *middle*), and also by mAbGB3.1, the blocking being as effective as competition with unlabeled ligand, whereas a control Ab had minimal effect (Fig. 10, *bottom*). Binding was also inhibited by anti-S100A8 and anti-S100A9 (not shown).

Efforts to identify annexin I binding sites on endothelial cells by using radiolabeled annexin I were hampered by degradation of annexin I on iodination, a problem also observed by other laboratories (19). Biotinylation did not destroy the protein but abolished binding of both annexin I and S100A8/A9 to the glycans (not shown), suggesting involvement of critical lysines in the interactions.

Discussion

We previously showed that novel carboxylated glycans are constitutively expressed on mammalian endothelial cells (2, 3) and bind to activated neutrophils. We also showed that a mAb against this novel modification inhibited neutrophil and monocyte efflux across murine mesenteric venular endothelium (3). In this report, we identify the leukocyte proteins that mediate these effects. We provide several lines of evidence to show that three inflammation-related calcium-binding proteins, annexin I and S100A8 and S100A9, bind to the novel glycans.

Annexin I present in the bovine lung extract could have originated from the lung tissue, the blood cells, or both. The lung also contains annexins II and V (20), but repeated isolation of annexin I alone on the columns showed that its binding was specific. The specificity of binding may be imparted by the biologically active N terminus (~40 aa in length), which is unique for each member of the annexin family (21). The N-terminal alanine of intact annexin I is acetylated and resistant to Edman degradation (8); the presence of the Δ1–12 annexin I enabled identification of the sequence, though the intact form was also isolated (as found in immunoblots, not shown). In contrast, the S100A8/A9 complex present is exclusively in leukocytes (17, 22) and probably originated from the trapped blood or sequestered neutrophils in bovine lung. Subsequently, we found that human homologues of annexin I and S100A8 from neutrophil lysates also bound to the glycopeptide affinity columns and immobilized glycans in a carboxylate-dependent manner (Fig. 4).

The two migration inhibitory factor-related proteins S100A8 and S100A9 belong to the large family of S100 proteins, the members of which have the EF hand calcium-binding structures (for a recent review, see Ref. 14). These heteromeric complexes are found in sera of patients with cystic fibrosis, chronic bronchitis, and rheumatoid arthritis, suggesting extracellular roles (23–25). Well-defined functions of S100A8 and S100A9 either as individual proteins or as complexes has been elusive. Existing evidence suggests that they may be involved in macrophage differentiation and inflammation. When monocytes extravasate through the endothelium and become resident macrophages, these Ags are lost (26). However, at sites of chronic inflammation in patients with rheumatoid arthritis, sarcoidosis, and tuberculosis, tissue macrophages continue to express S100A8/A9 (26, 27). These proteins have also been found coating vascular endothelium adjacent to marginating neutrophils and monocytes, suggesting that secretion of S100A8/A9 on to vessel walls is involved in the migration of leukocytes into tissues (28). Kerkhoff et al. (29) report that only monocytes expressing the S100A8/A9 complex preferentially migrate through the microvascular endothelium. How the S100 proteins mediate these interactions with the endothelium is not clearly understood. Recent studies show that S100A8/A9 binds to unsaturated fatty acids with high affinity and may be involved in arachidonic acid metabolism (30–32). Cell surface S100A8/A9 binding sites have also been detected on human leukemia cell lines (6). S100A9 also is shown to modulate adhesion of neutrophils to fibrinogen via β_2 integrin Mac1, by recognizing a distinct but uncharacterized pertussis toxin-sensitive G protein-coupled receptor on neutrophils (33).

In this study, we provide several lines of evidence that S100A8/A9 could function by directly interacting with novel carboxylated glycans on endothelial cells. First, S100A8/A9 heteromeric complexes of S100A8/A9 are depleted from neutrophil lysates after binding to the glycans, and this binding is carboxylate-dependent (Figs. 4*B* and 5). Adhesion of neutrophils to the immobilized glycans is effectively inhibited by both anti-S100A9 and mAbGB3.1 (Fig. 7). Purified S100A8/A9 complex I binds to the novel glycans in a carboxylate-dependent manner (Fig. 9). S100A8/A9 complex cross-reacts with mannose-labeled glycoproteins immunoprecipitated by mAbGB3.1 (Table III). Finally, specific binding of S100A8/A9 to endothelial cell surface is blocked by mAbGB3.1 (Fig. 10). Recombinant S100A8 alone also does not show specific binding to the glycans (data not shown), suggesting that heteromeric assembly of the S100 proteins was necessary for this interaction. The binding also appears exclusive to S100A8/A9 because binding of two other S100 proteins S100A1 and S100B remained constant between carboxylated and neutralized glycans under these conditions (data not shown).

Annexin I, also called lipocortin I, is a member of a family of 13 distinct calcium-binding proteins characterized by a common C-terminal core structure consisting of four or eight highly conserved repeating motifs, which bind calcium and phospholipids, and a variable N terminus (21). Annexin I is distributed in different tissues, but is abundant in cells of the hemopoietic lineage, particularly granulocytes and monocytes, where it makes up as much as 2–4% of total cytosolic protein (34).

A growing body of evidence from studies on inflammation in animal models and humans suggests that annexin I potently inhibits neutrophil extravasation (reviewed in Ref. 35). Annexin I also is strongly induced by glucocorticoids and is considered to mediate some of the well-known antiinflammatory effects of these hormones (36). The N terminus of annexin I has been shown to play an important modulatory role in the biological effects of the protein. For example, it has been shown by several studies that the N-terminal peptide Ac 2–26 mimics the potent antiinflammatory effects of intact annexin I (35). The amino-terminal domain regulates interactions with membranes (37), and is directly involved in binding to S100 proteins (38, 39). Most notably, the N-terminal peptide Ac2–12, which in our study appears essential for binding to the novel glycans (Fig. 6), has been shown to be as effective in detaching adherent neutrophils from murine postcapillary venules as the intact protein (40). An endogenous protease catalyzes cleavage at tryptophan 12 of intact annexin I in a Ca^{2+}-dependent manner (41), and proteolysis is believed to regulate the action of externalized annexin I (36). The mechanisms by which annexin I and its peptide mimetics affect neutrophil extravasation have remained elusive so far. Annexin I binding sites on neutrophils and monocytes have been identified, and the protein has recently been shown to be a ligand for the formyl peptide receptor (42, 43). Annexin I also has been reported to regulate the mitogen-activated protein kinase/extracellular signal-regulated kinase signaling pathway (44).

Here we provide evidence that annexin I could function by interacting with novel carboxylated glycans. Annexin I from human neutrophil lysates shows carboxylate-dependent binding to the glycans (Fig. 4*A*). Depletion experiments suggest that the N-terminal 1–12 amino acids of annexin I may be important for optimal binding (Fig. 6). Recombinant full-length human annexin I also binds to the novel glycans in a carboxylate-dependent manner (Fig. 9). However, the precise presentation or the structure of the glycans recognized by annexin I and S100A8/A9, and the physiological processes mediated in vivo by the two proteins could differ. For example, annexin I precipitates only ~2–3% of mannose-labeled glycoproteins from endothelial cells compared with 9% by S100A8/A9 (not shown), suggesting that some recognition epitopes preferred by annexin I could be on *O*-glycans. Also, unlike S100 proteins, annexin I does not modulate neutrophil adhesion to endothelial monolayers but still impedes neutrophil emigration (Ref. 45; G.S. and H.H.F., unpublished results). It recently has been shown that annexin I inhibits monocyte adhesion to microvascular endothelial cells, this again involving its N-terminal domain (46). This may explain why we find increased adhesion of leukocytes to endothelial cells in the presence of mAbGB3.1 after induction of peritonitis in mice (3).

We previously had shown neutrophils acquired carboxylate-dependent binding sites for the novel glycans after short-term activation (3). The novel glycans also bind to secretions from activated neutrophils (not shown). Annexin I and S100 proteins are cytosolic and lack classical leader peptide sequences and transmembrane domains. We observe that S100A8/A9 and annexin I are expressed on the surface of neutrophils immediately after activation (Fig. 8). Earlier studies have found that S100A8 and S100A9 translocate from the cytosol to the membrane and intermediate filaments in a calcium-dependent manner (14–16). Neutrophil activation with opsonized zymosan also correlates with translocation of S100A8 to plasma membrane (47). Secretion of the S100 proteins is believed to be tubulin-, energy-, and protein kinase C-dependent (48). Cell surface expression of annexin I after neutrophil adhesion to endothelial monolayers also has been demonstrated (34, 44, 45). These findings conform with the characteristics of annexin I and S100A8 and S100A9 as a class of proteins that includes growth factors and the S-type lectins or galectins, which are secreted by a Golgi-independent pathway (49, 50).

Our findings that the novel carboxylated endothelial glycans bind to soluble leukocyte proteins that are secreted on activation and that are known to mediate inflammatory responses suggest the existence of a novel pathway in acute inflammation. Efforts are now underway in our laboratories to isolate and characterize endothelial glycoproteins that carry the novel carboxylated glycans, to identify binding partners involved in mediating the functions of S100A8/A9 and annexin I, and to decipher the signaling mechanisms by which these proteins bring about their inflammatory responses in vivo.

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