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Identification of Histone H2B as a Regulated Plasminogen Receptor[†]

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ABSTRACT: Tethering of plasminogen to cell surfaces controls plasmin formation and, thereby, influences pericellular proteolysis and cell migration. Modulation of cellular plasminogen binding sites provides a mechanism for regulation of these events. In this study, two distinct models, phorbol ester-stimulated adhesion of U937 monocytoid cells and culturing of peripheral blood neutrophils, treatments which modulate plasminogen binding sites, have been examined to determine the molecular basis for the upregulation of plasminogen receptors. Membranes were isolated from cell populations, with and without upregulated plasminogen binding capacities, and analyzed by [¹²⁵I]plasminogen ligand blotting of gel transfers. Approximately 15 different [¹²⁵I]plasminogen-binding proteins were discerned in the membrane fractions, and only relatively minor differences in the intensities of individual bands were noted in the different cell populations. The notable exception was the presence of a 17 kDa band, which was selectively and markedly enhanced in the membranes from cells with enhanced plasminogen binding capacities. The 17 kDa protein was isolated from both cell types, and amino acid sequencing of peptide fragments identified the same protein, histone H2B. Increased expression of histone H2B was observed on stimulated U937 cells and cultured neutrophils by confocal microscopy with an antibody raised to the carboxy-terminal octopeptide sequence of histone H2B. This antibody or its Fab fragments substantially decreased the level of binding of plasminogen to these cultured neutrophils and stimulated U937 cells that exhibited elevated levels of binding but not to nonstimulated cells. Thus, histone H2B represents a regulated plasminogen receptor, which contributes significantly to the plasminogen binding capacity of cells.

Most cell types, including human leukocytes, bind plasminogen in a specific and saturable manner (1). The bound plasminogen can be converted to plasmin, which mediates pericellular proteolysis that facilitates cell migration and matrix remodeling and, thereby, influences leukocyte recruitment during an inflammatory response (2–4). The plasminogen binding capacity of neutrophils, monocytes, and lymphocytes is high, at 10⁴–10⁶ molecules bound per cell, and that of certain transformed monocytoid and lymphoblastoid cell lines can exceed 10⁷ molecules per cell. With this high capacity, it is not surprising that several plasminogen receptors have been identified on leukocytes (4). α -Enolase was the first of these to be identified as a plasminogen binding protein on the surface of U937 monocytoid cells (5) and now has been implicated as a plasminogen receptor on many cell types (6–8). The annexin II heterotetramer serves as a plasminogen binding protein on leukemic promyelocytes and certain leukocytic cell lines (9) as well as endothelial cells (10). Although the contributions of the individual subunits of the annexin II heterotetramer to plasminogen binding remain unresolved (11, 12), both in vitro and in vivo data support the functional role of this complex in pericellular proteolysis (13, 14). TIP49a (15) and

integrin α M β 2 (16) also function as plasminogen binding proteins on leukocytic cells. In addition, many other cell types, ranging from human to bacterial origin and from normal to transformed cells, express plasminogen binding proteins [e.g., endothelial cells (10, 17, 18), fibroblasts (19), human glomerular epithelial cells (20), neuroblastoma cells (21), breast and colon cancer cells (22–24), and streptococci (25, 26)]. Most of the identified plasminogen binding proteins have or are cleaved to have a carboxy-terminal lysine, which can interact with the kringles of plasminogen. The role of the carboxy-terminal lysyl residues in mediating plasminogen binding has been demonstrated by the observations that (i) carboxy-terminal lysyl analogues, such as ϵ -aminocaproic acid (EACA),¹ inhibit binding of plasminogen to cells and (ii) type B carboxypeptidases, which remove carboxy-terminal lysyl (or arginyl) residues, reduce the level of binding of plasminogen to cells (27). Even though not all of the identified plasminogen receptors contain a C-terminal lysine, EACA can still block the interactions, suggesting that

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¹ Abbreviations: EACA, ϵ -aminocaproic acid; PMA, phorbol 12-myristate 13-acetate; HBSS, Hanks' balanced salt solution; DPBS, Dulbecco's phosphate-buffered saline; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; BSA, bovine serum albumin; LDH, lactic acid dehydrogenase; CpB, carboxypeptidase B; PVDF, polyvinylidene difluoride; SBTI, soybean trypsin inhibitor; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; HNE/CMK, human neutrophil elastase inhibitor methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethyl ketone; CG/CMK, cathepsin G inhibitor benzyloxycarbonyl-Gly-Lue-Phe-chloromethyl ketone; SEM, standard error of the mean.

an internal basic amino acid assumes an orientation that mimics a C-terminal lysine (21).

Once bound to cell surfaces, plasminogen is more readily activated to plasmin than free plasminogen (28–30), and the active enzyme is partially protected from inactivation by α_2 -antiplasmin and α_2 -macroglobulin (28, 31). Moreover, the intrinsic enzymatic activity of cell-bound plasmin is augmented (32). Because plasmin has a broad substrate specificity, which includes a variety of extracellular matrix proteins, other enzymes, such as metalloproteinases, and several growth factors, its activity must be tightly regulated (27, 33). Modulation of their plasminogen binding capacity provides cells with a control mechanism for regulating plasmin generation at cell surfaces. Indeed, several circumstances under which leukocytic cells modulate their plasminogen binding capacity have been reported (reviewed in ref 27). Upon stimulation of U937 monocytoid cells with phorbol myristate acetate (PMA), adherent and nonadherent cell populations can be separated; the plasminogen binding capacity decreases slightly in adherent cells and increases dramatically, as much as 17-fold, in the nonadherent cells (34). Stimulation of these cells with interferon- γ or vitamin D₃ also results in a substantial increase in their level of binding of plasminogen (35, 36). Similarly, when monocytoid cells (THP-1 or U937 cells) are provided with an adhesive substrate, including fibronectin, vitronectin, or laminin, an adherent and a nonadherent cell population can be separated, and the plasminogen binding capacity can increase by 3-fold in the nonadherent cells and can decrease in the adherent cells (37). A particularly striking modulation (>30-fold) in plasminogen binding occurs in the culture of human neutrophils and monocytes (38).

In the examples cited above, the modulation of plasminogen binding capacity is marked, but the mechanisms underlying these changes are unclear. In this study, we have examined changes in plasminogen binding proteins on the surfaces of leukocytes using PMA stimulation of U937 monocytoid cells and culture of human peripheral blood neutrophils as model conditions for modulation of plasminogen binding. Our results lead to the identification of a new regulated plasminogen receptor on these cell types that contributes significantly to the changes in the plasminogen binding capacity of the cells.

EXPERIMENTAL PROCEDURES

Proteins. Glu-plasminogen was isolated from fresh plasma using lysine–Sepharose affinity chromatography (34). Radiolabeling with Na¹²⁵I was performed using a modified chloramine T method (39).

Cells. U937 monocytoid cells were grown in RPMI 1640 (BioWhittaker, Walkersville, MD), supplemented with 7.5% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine (34). Neutrophils were isolated from human peripheral blood drawn into acid citrate dextrose [$1/7$ volume, 145 mM sodium citrate (pH 4.6) and 2% dextrose] from consenting volunteers. Isolation was performed using density centrifugation on Ficoll-Hypaque (Ficoll-Paque Plus, Amersham Biosciences, Piscataway, NJ), followed by dextran sedimentation of erythrocytes. Residual erythrocytes were subjected to hypotonic lysis, and the granulocytes were washed three times with Hanks' balanced salt solution

(HBSS) (1). The remaining cells were 98% granulocytes, of which >96% were neutrophils and 2% were eosinophils as determined by Wright staining. Viability was determined by trypan blue exclusion and lactic acid dehydrogenase (LDH) release. The amount of LDH was measured using the In Vitro Toxicology Assay Kit, Lactate Dehydrogenase Based, from Sigma Chemical Co. (St. Louis, MO), and percent viability is based on analysis of approximately 10 000 cells.

Cell Stimulation. U937 monocytoid cells ($1\text{--}1.8 \times 10^7$) were incubated under sterile conditions with or without 40–100 nM PMA (Sigma) in RPMI 1640, supplemented with 2 mM glutamine, 15 μ M MnCl₂, and 0.1% bovine serum albumin (BSA), which had been treated for 30 min at 4 °C with 5 mM DFP before being used. This incubation was performed in 100 mm Primaria tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) that had been precoated overnight with U937 medium and then washed three times with Dulbecco's phosphate-buffered saline (DPBS) prior to being used. These conditions lead to >50% cell adhesion at 2 h, a proportion that is necessary for the upregulation of plasminogen binding (34). After the 2 h incubation, nonadherent cells were harvested by decanting, and adherent cells were carefully dislodged by flushing with a pipet. Cells from the two stimulated populations were counted in a hemacytometer, and their viability was assessed by trypan blue exclusion and LDH release. To upregulate binding of plasminogen to neutrophils, cells were cultured for 18–20 h in 75 cm² flasks (Costar, Cambridge, MA) using Mo medium (34) [RPMI-1640, containing 100 units/mL penicillin G, 100 μ g/mL streptomycin, 1 mM sodium pyruvate, $5 \times 10^{-4}\%$ 2-mercaptoethanol, 25 mM HEPES (pH 7.4), and 5% FBS] in an incubator (humidified atmosphere with 5% CO₂) at 37 °C. In experiments where inhibitors were included, these were added at the initiation of the culturing condition. The cells were washed by centrifugation as previously described (38).

Ligand Binding Assays. Binding of radiolabeled plasminogen to U937 cells and neutrophils was assessed as previously described (5, 34). Briefly, cells were washed in HBSS containing 25 mM HEPES and resuspended in HBSS–HEPES containing 0.1% BSA (HBSS–BSA), supplemented with 1.2 mM CaCl₂ and 1.6 mM MgSO₄. Cells (U937, 4×10^6 mL⁻¹; neutrophils, 5×10^6 mL⁻¹) were incubated with 100 nM [¹²⁵I]plasminogen in the presence or absence of 100 mM EACA in a total volume of 200 μ L at 37 °C for 1 h, a time sufficient to reach equilibrium (1). Triplicate 50 μ L samples were layered over 300 μ L of 20% sucrose in HBSS–BSA and centrifuged for 2.5 min in a Beckman Microfuge 12 centrifuge (Beckman Instruments Inc., Fullerton, CA). The tube tips were amputated and counted in a gamma counter. Specific binding was determined to be the difference in radioactivity bound in the absence and presence of EACA [previously shown to be equivalent to the excess of nonlabeled plasminogen as an inhibitor of specific binding (1)] and converted to molecules of plasminogen bound per cell on the basis of the specific activity of the radiolabeled ligand. Routinely, ~90% of the binding was inhibited by 100 mM EACA. The conditions described above lead to optimal specific [¹²⁵I]plasminogen binding with no evidence of ligand internalization (28).

Cell Membrane Preparations. For U937 membranes, PMA-stimulated adherent and nonadherent cells (6×10^7

cells stimulated) and nonstimulated control cells (3×10^7) were harvested separately and washed three times with DPBS. Pelleted cells were resuspended in lysis buffer [10 mM Tris (pH 8.0) containing 5 mM DFP, 1.5 mM PMSF, 5 mM benzamidine, 100 kallikrein inhibitory units/mL aprotinin, 5 mM *o*-phenanthroline (Fluka Chemika, Buchs, Switzerland), 1 μ M leupeptin, 1 μ M pepstatin, and 0.02% NaN_3]. Cells were lysed by being repeatedly frozen and thawed, which was followed by ~ 10 up-and-down strokes in a small clearance Dounce tissue grinder (Wheaton Science Products, Millville, NJ) or by nitrogen cavitation (see below). Fractions enriched in cell membranes were prepared by differential centrifugation (modified from the procedures in refs 40 and 41). Cellular debris, nonlysed cells, and nuclei were pelleted by centrifugation at 800g for 10 min at 4 °C. The supernatant fraction was clarified by centrifugation at 16000g for 10 min, and cell membranes were pelleted by centrifugation at 100000g for 1 h at 4 °C. The supernatant of this last centrifugation step served as the cytosolic fraction. The membranes were resuspended in lysis buffer containing 2% SDS with brief sonication, and the protein content was measured (BCA Protein Assay, Pierce, Rockford, IL).

For neutrophil membrane preparations, at least 1×10^7 cells were used as starting material. Cells were suspended in cold relaxation buffer [10 mM PIPES, 100 mM KCl, 3 mM NaCl, 1 mM ATP, and 3.5 mM MgCl_2 (pH 7.3)] and disrupted by nitrogen cavitation in the presence or absence of Complete Protease Inhibitor Cocktail from Roche (Indianapolis, IN) at 350 psi for 20 min as described previously (42). EGTA (5 mM), PMSF (3 mM), and DNase (5 μ g/mL) were added, and then nuclei and intact cells were removed by centrifugation at 500g for 10 min at 4 °C. The supernatant was applied on top of a discontinuous Percoll (Amersham) density gradient and then centrifuged at 40000g for 25 min at 4 °C. The membrane fraction was collected and diluted in 10 mM Tris buffer. The membranes were pelleted by centrifugation at 100000g for 2 h at 4 °C, washed twice in Tris buffer at 4 °C, and then resuspended by a brief sonication in the presence of 2% SDS. The protein content was estimated spectrophotometrically at 280 nm.

For nuclear membrane preparations from both U937 cells and neutrophils, cells were treated with the Nuclei EZ Prep Nuclei Isolation Kit from Sigma, following the manufacturer's protocol. After isolation, the nuclei were washed in Tris buffer and then lysed following a modification of the protocol of Dignam et al. (43), in which the nuclei were incubated for 1 h at 4 °C with periodic agitation in HEPES buffer (pH 7.9) containing 0.4 M NaCl and EDTA, EGTA, DTT, and PMSF (1 mM each), with 5 μ g/mL DNase and Complete Protease Inhibitor Cocktail. After centrifugation at 500g for 5 min, the nuclear membrane pellet was solubilized in 2% SDS in Tris buffer.

Freedom of the plasma membrane preparations from nuclear membrane contamination was assessed by immunoblotting using 5-lipoxygenase as a nuclear membrane marker (44). Equal amounts of plasma and nuclear membrane proteins were loaded onto SDS-PAGE gels, and Western blotting was performed as indicated below. The intensity of the reaction of the membrane preparations with the 5-lipoxygenase antibody was determined by densitometric scanning of the blots (see below).

Gel Electrophoresis and Plasminogen Blotting. Sample buffer (45) was added to an equal volume of membranes or cytosolic fractions from each cell type, which had been adjusted to contain the same amount of protein on the basis of the absorbance at 280 nm. After being boiled for 3 min, samples were electrophoresed on 10–15% gradient acrylamide gels and then stained with Coomassie blue. Molecular masses were estimated relative to [^{14}C]methylated Rainbow prestained protein standards (Amersham). To detect plasminogen binding proteins in the cell membrane preparation, proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA) after SDS-PAGE. The transfer buffer was 10 mM CAPS and 10% methanol (by volume) (pH 11). After the transfer (300 mA, 2.5 h), the membrane was rinsed briefly in PBS and then blocked in PBS containing 3% BSA, 0.05% Tween 80, 200 kallikrein inhibitory units/mL aprotinin, 1.5 mM PMSF, and 0.02% NaN_3 for 30 min. The transfers were reacted with 40 nM [^{125}I]plasminogen for 3–5 h at 22 °C. Membranes were washed with PBS containing 0.05% Tween 80 for 2 h with frequent changes of the washing buffer. After drying, the membranes were subjected to autoradiography using Kodak BioMax MS film (Eastman Kodak Co., Rochester, NY). Band intensities were quantified using a densitometric scanner (Image version 1.57, National Institutes of Health, Bethesda, MD). The intensity of a specific band of an estimated molecular mass was determined as a percent of its contribution to the total intensity of the lane. This value was then compared to the values of the corresponding molecular mass bands in other lanes of gels. In specificity experiments, the [^{125}I]plasminogen was added together with 10 μ M nonlabeled plasminogen or 100 mM EACA. Alternatively, the membrane preparation was incubated with 50 units of PMSF-treated CpB (Worthington Biochemical Corp., Lakewood, NJ) for 1 h at 37 °C before SDS-PAGE. The percent residual reactivity was calculated from the intensities of the total lanes or the intensities of bands of the same mobilities from gels and autoradiograms developed under the same conditions.

Receptor Isolation and Sequencing. U937 cells (2×10^9) were washed in DPBS, and membrane preparations were prepared as described above. Approximately 14 mg of membrane proteins, solubilized with 2% SDS in Laemmli sample buffer, was applied to a columnar (38 mm diameter) SDS-PAGE system (PrepCell, Bio-Rad, Richmond, CA) with an acrylamide concentration of 12%. After the dye front eluted from the bottom of the gel, 1.6 mL fractions were collected. A preliminary analysis was performed via SDS-PAGE to identify protein peaks. In regions of interest, fractions were analyzed using [^{125}I]plasminogen ligand blotting. Active fractions were pooled and diluted 1:6 using 20 mM NH_4HCO_3 to reduce the SDS concentration to 0.02%. Affinity chromatography was used as a second purification step. Plasminogen was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Amersham). The coupled beads were packed into a column (5 cm \times 1 cm) and equilibrated with 20 mM NH_4HCO_3 containing 0.02% SDS. Diluted samples from SDS-PAGE (60–80 mL) were applied to the column and recirculated overnight at 4 °C (15 mL/h). After being washed with equilibration buffer at 22 °C, bound protein was eluted with 200 mM EACA. The eluted protein was dialyzed (5 kDa cutoff, Spectra Por,

Fisher Scientific, Pittsburgh, PA) against 20 mM NH_4HCO_3 for 2 h at 4 °C and subjected to SDS–PAGE on 15% acrylamide gels. The gels were stained with Coomassie blue, and the band of interest was excised. Tryptic digestion was performed; fragments were separated using high-performance liquid chromatography, and selected peaks were subjected to N-terminal sequence analysis in an Applied Biosystems model 475 or Procise gas-phase sequencer (Applied Biosystems, Foster City, CA). To increase the protein yield for sequencing, particularly from neutrophil membranes, the procedure described above was modified on occasion: membrane proteins were separated by SDS–PAGE, and then plasminogen binding was verified by ligand blot and autoradiography. The autoradiograph was aligned with a stained gel run simultaneously, and the protein band was excised and processed as described above for sequencing.

Preparation of Polyclonal Rabbit Antibodies to Plasminogen Receptor Peptides. Peptides of 8 and 19 amino acids, corresponding to the C-terminal and N-terminal sequences, respectively, of the isolated plasminogen receptor, were synthesized, coupled to keyhole limpet hemocyanin, and injected separately into rabbits (Quality Controlled Biochemicals, Inc., Hopkinton, MA). After a prolonged immunization, sera were collected from the animals, and immunoglobulin fractions were purified by sequential ammonium sulfate precipitation to 45% saturation followed by binding and elution from recombinant protein G–Sepharose (Zymed, South San Francisco, CA) columns. For Fab production, purified polyclonal antibodies were treated with immobilized papain, and Fc fragments were removed on a protein A column following the manufacturer's instructions (ImmunoPure Fab Preparation Kit, Pierce).

Flow Cytometry. Neutrophils ($2 \times 10^7 \text{ mL}^{-1}$) and U937 cells ($5 \times 10^6 \text{ mL}^{-1}$) were incubated in 50 μL of HBSS containing 10 μL of antibodies specific for histone H2B protein. The immunoglobulin fraction obtained from normal rabbit serum by ammonium sulfate precipitation served as a control. After a 30 min incubation on ice, the cells were pelleted by centrifugation and resuspended in 50 μL of HBSS containing fluorescent goat anti-rabbit IgG (Oregon Green 514, Invitrogen, Carlsbad, CA). Cells were further incubated on ice for 30 min, then washed and resuspended in HBSS, and fixed in 1% paraformaldehyde. Analysis was carried out by flow cytometry (FACScan, BD Biosciences, San Jose, CA) using the manufacturer's LYSIS II software.

Confocal Microscopy. Confocal microscopy was performed as previously described (46). Briefly, cells were washed in HBSS–HEPES buffer and then treated with 20 $\mu\text{g/mL}$ human γ -globulin as a blocking reagent for 30 min. Staining of histone H2B with the rabbit C-terminal peptide antibody (see above) and integrin $\alpha\text{M}\beta 2$ with mouse monoclonal antibody IB4 was performed using 2×10^5 cells for 30 min in 1.5% BSA–HBSS. Cells were washed with HBSS–HEPES and then stained with anti-rabbit Alexa-488 or anti-mouse Alexa-568 for an additional 30 min. Cells were washed, fixed in 2% paraformaldehyde, resuspended in mounting medium containing DAPI (4',6'-diamidino-2-phenylindole, Vector Laboratories, Burlingame, CA), and loaded onto Superfrost microscope slides (Cardinal Health, McGaw Park, IL) under coverslips. Images were captured with a Leica TCS-SP2 laser scanning confocal microscope (Leica Micro-systems GmbH, Heidelberg, Germany) at a

magnification of 40 \times . Images were collected sequentially to prevent cross-talk between the fluorophores. Intensities of fluorescence staining were analyzed using Image Pro plus (Meida Cybernetics, Silver Springs, MD). Green and red signals were manually thresholded from the treatment group giving the most intense staining (PMA-stimulated, nonadherent U937 cells or cultured neutrophils). These parameters were then kept constant and applied to quantify the staining of the other cells in the group (PMA-stimulated, adherent and nonstimulated U937 cells or to freshly isolated neutrophils). Several fields, each containing multiple cells, were analyzed for two to three different slides in this manner, and an average integrated optical density was determined for each stain.

Western Blotting. Membrane proteins were transferred to PVDF membranes as described above. After blocking (3% BSA, 0.5% bovine γ -globulins, 10% normal goat serum, and 0.01% NaN_3 in Tris-buffered saline), the primary antibody was added for 60 min. After washing, a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody was added and colorimetric detection was performed according to the manufacturer's instructions (Opti-4CN detection kit, Bio-Rad). Molecular mass markers were unstained Precision Protein Standards developed with StrepTactin–horseradish peroxidase conjugate (Bio-Rad).

Statistical Analyses. Results are presented as means \pm the standard error of the mean (SEM). Comparisons were made using paired *t*-tests. A *p* value of <0.05 was considered to be statistically significant.

Reagents. Leupeptin, 2-guanidinoethylmercaptosuccinic acid, bovine and human γ -globulins, HEPES, and CAPS were from Calbiochem. Aprotinin was obtained from Miles Inc. (Kankakee, IL) or Calbiochem. Pepstatin A, benzamidine, PIPES, ATP, Tris, SBTI (cell culture-tested), DFP, DNase, and BSA (fraction V) were from Sigma Chemical Co. HBSS, sodium pyruvate, and trypan blue were purchased from GIBCO-BRL (Grand Island, NY). The monoclonal mouse anti-annexin II antibody was from Zymed. Human neutrophil elastase inhibitor (HNE/CMK) and cathepsin G inhibitor (CG/CMK) were from Enzyme Systems Products (Livermore, CA). Rabbit polyclonal antiserum to 5-lipoxygenase was from Cayman Chemical (Ann Arbor, MI). Normal goat and rabbit sera and rabbit anti-ubiquitin polyclonal antibodies were from Chemicon International, Inc. (Temecula, CA). Purified HeLa cell nuclear histone H2B was a generous gift from S. Sanker (Department of Biochemistry, Case Western Reserve University, Cleveland, OH).

RESULTS

Modulation of the Plasminogen Binding Proteins on U937 Monocytoid Cells. The U937 monocytoid cell line was chosen as an initial model for investigating the molecular basis for the modulation of plasminogen binding capacity. These cells (10^7) were stimulated with 40–100 nM PMA, and an adherent population and a nonadherent population were separated after 2 h. The viability of the cells after incubation, as determined by trypan blue exclusion, was 94% for unstimulated cells and 98 and 95% for PMA-stimulated adherent and nonadherent cells, respectively. The LDH assays indicated that cell viability was $>99\%$ for both

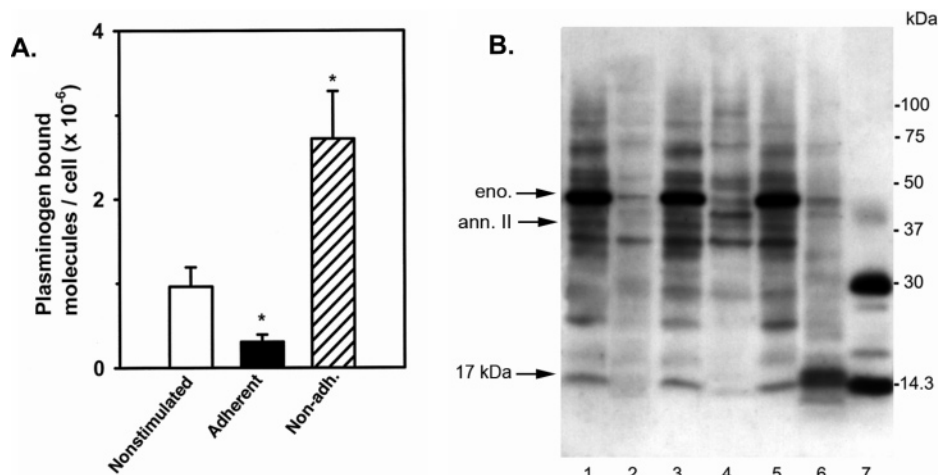


FIGURE 1: Modulation of binding of plasminogen to U937 cells. (A) The U937 cells (1×10^7) were stimulated with 40–100 nM PMA, and adherent and nonadherent cells were separated after 2 h. Specific binding of [125 I]plasminogen (100 nM) to the cell populations was assessed after 1 h at 37 °C. The results are the means \pm SEM of 13 separate experiments, and the differences between the adherent and nonadherent relative to the control cells are statistically significant ($*p < 0.001$). (B) Representative [125 I]plasminogen ligand blots of membrane and cytosolic fractions of various U937 cell populations: lanes 1, 3, and 5, cytosolic fractions; and lanes 2, 4, and 6, membranes. Lanes 1 and 2 are from nonstimulated cells; lanes 3 and 4 are from adherent PMA-stimulated cells, and lanes 5 and 6 are from nonadherent PMA-stimulated cells. The final lane contains 14 C standards (the high intensity of the 30 and 14.3 kDa bands is due to binding of plasminogen to these standards). The ligand blots are transfers of SDS–PAGE (10–15% acrylamide, nonreducing conditions). The positioning of α -enolase (eno) and annexin II (ann) is based on Western blots. The 17 kDa protein band also is identified.

stimulated and unstimulated cells. The average adhesion measured in 15 different experiments was 58%. The plasminogen binding capacity of the two populations was assessed and compared to that of nonstimulated control cells. As shown in Figure 1A, binding of plasminogen to the nonadherent cells was markedly enhanced (2.8-fold relative to nonstimulated cells and 9.1-fold relative to the adherent cells) but was attenuated in the adherent cells (3.2-fold relative to the nonstimulated cells). All comparisons were statistically significant ($p < 0.001$). Previous studies have shown that in these cell populations, changes are due to differences in the number but not the affinity of the plasminogen binding sites (34). From each of the three cell populations, control, adherent, and nonadherent cells, membrane and cytosolic fractions were prepared by differential ultracentrifugation after lysis of the cells by repeated freezing and thawing or by nitrogen cavitation (similar results were obtained with both methods of cell disruption). Immunoblotting after SDS–PAGE of the cytosolic and membrane fractions was performed with an antibody to glyceraldehyde-3-phosphate dehydrogenase. This protein is primarily cytosolic but is also found in membrane and nuclear fractions (47, 48). Densitometric scanning of the gels indicated the same relative distribution of glyceraldehyde-3-phosphate dehydrogenase ($\sim 75\%$ cytosolic) was observed for cytosolic and membrane fractions derived from nonstimulated and PMA-stimulated adherent and nonadherent U937 cells. Thus, there was no evidence of selective recovery or contamination of proteins within the subcellular fractions derived from the different cell populations.

The plasminogen binding proteins in the subcellular fractions were analyzed by ligand blotting transfers from 10–15% gradient nonreduced SDS–PAGE with [125 I]plasminogen. A typical ligand blot of membrane and cytosolic fractions from the three different cell populations is shown for U937 cells in Figure 1B and is representative of three such experiments. On average, there were 15 bands in the

ligand blots of the cytosolic fractions. The band patterns were very similar. No systematic differences in the number and intensity of the bands as determined by densitometry in the three cytosolic fractions were observed. The most intense band in the cytosolic fraction of all three cell types was at ~ 48 kDa, which corresponds to α -enolase as determined by Western blotting. In the membrane preparations, there were at least 55 readily discernible protein bands in Coomassie-stained gels (not shown). Some bands in the corresponding [125 I]plasminogen ligand blots were common to all three membrane preparations. Of note in this regard, the α -enolase band at 48 kDa was similar in intensity (7–10% of total [125 I]plasminogen reactivity) in the three membrane preparations. A band at 40 kDa, the molecular mass of the annexin II subunit, was detected variably in the membrane preparations, and its intensity did not increase in the membranes from nonadherent cells. A band at ~ 35 kDa was present in the membranes of control and adherent cells but not in the membranes of nonadherent cells. However, the most striking difference among the membrane fractions was a band of 17 kDa in the nonadherent cells. This band contributed only 2.0–2.4% of the plasminogen reactivity in the membrane preparations of the control and adherent cells but 21% of the plasminogen reactivity with the membranes from the nonadherent cells. A band of this mobility was present in the cytosolic fractions of all three cell populations, but its intensity was similar in the membrane preparations from the adherent and control cells. Thus, of the various bands reactive with [125 I]plasminogen, only the intensity of the 17 kDa band was substantially greater in the membranes of the nonadherent than the adherent or controls cells and was enriched in the membrane versus the cytosolic fractions of the nonadherent cells.

To verify the specificity of the reactivity of the various bands with plasminogen in ligand blots in general and that of the 17 kDa band specifically, the transferred proteins in the membrane fraction were reacted with [125 I]plasminogen

Table 1: Specificity of [125 I]Plasminogen Ligand Blots from U937 Monocytoid Cells

	residual intensity (%) ^a		
	nonlabeled plasminogen ^b	EACA ^b	CpB ^b
total lane intensity	15.8	9.6	50
α -enolase	7.9	6.3	51
annexin II	4.4	8.4	ND ^c
17 kDa band	3.8	5.0	48

^a Calculated from densitometric scans of gels and expressed as a percent of the total lane intensities or the intensities of the identified protein bands in the absence of an inhibitor. ^b The conditions of treatments were as follows: 10 μ M nonlabeled plasminogen, 100 mM EACA, treatment with 50 units of CpB for 1 h at 37 °C prior to SDS-PAGE. ^c Not discernible. [125 I]Plasminogen reacts with CpB in such a way that the annexin II band could not be distinguished.

in the absence or presence of (a) a 250-fold excess of nonlabeled plasminogen or (b) the C-terminal lysine analogue EACA or (c) after treatment with CpB to remove the C-terminal basic amino acid from plasminogen binding proteins. The gels were subjected to densitometric scanning, and the changes in total intensities and that of the α -enolase, annexin II, and the 17 kDa bands were quantified and are expressed as a percent of the untreated samples (see Table 1). By this evaluation, the 17 kDa band represented a specific interaction with [125 I]plasminogen (inhibited by nonlabeled plasminogen) and involved a C-terminal lysine (inhibited by both EACA and CpB treatment). With each treatment, the reduction in the level of binding of plasminogen to the 17 kDa band closely matched the reduction in the rate of the reaction with all plasminogen binding proteins as well as with previously identified plasminogen receptors, α -enolase and annexin II. The effectiveness of the CpB treatment strongly suggests that the 17 kDa protein recognizes plasminogen via a carboxy-terminal lysine.

Modulation of Plasminogen Binding Proteins on Human Neutrophils. We have previously reported that culturing of neutrophils leads to an upregulation of their plasminogen binding capacity (34, 38, 49). This upregulation is dependent upon proteolytic modification of the cell surface by neutrophil enzymes, including elastase and cathepsin G, indirectly leading to the exposure of new carboxy-terminal lysines which serve as plasminogen binding sites. As shown in Figure 2A, the upregulation of binding of plasminogen to cultured versus freshly isolated neutrophils is dramatic (22-fold), and addition of SBTI or more selective inhibitors of individual neutrophil proteases to culture media abolishes this upregulation. Viability of the cultured cells, with or without SBTI, was >97%, as determined by LDH release. Membrane preparations of freshly isolated and cultured (without and with enzyme inhibitors) neutrophils were separated by SDS-PAGE, blotted on PVDF membranes, and reacted with [125 I]plasminogen (Figure 2B). There were ~15 plasminogen reactive bands of at least 35 clearly discernible protein bands on Coomassie-stained membrane preparations. As seen in Figure 2B, a prominent plasminogen binding protein at ~17 kDa was induced by culture, and its appearance was suppressed by SBTI and the more specific elastase and cathepsin G inhibitors. Thus, the intensity of this band correlated with the plasminogen binding capacity of the intact neutrophils. A band at ~12–14 kDa also was enhanced in the cultured neutrophils (see lane 2 of Figure 2B). The intensity of this 12–14 kDa band was variable;

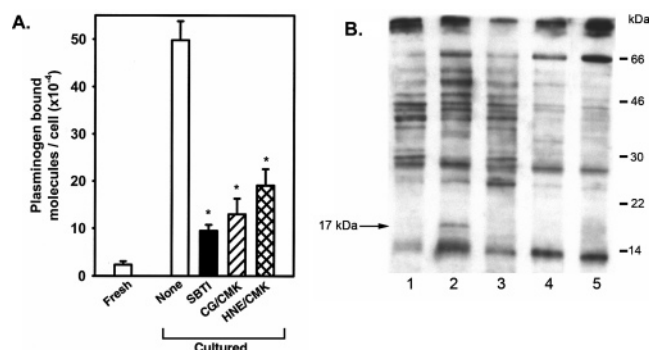


FIGURE 2: Modulation of binding of plasminogen to human neutrophils. Neutrophils (1×10^7) were either freshly isolated (control cells) or cultured for 20 h without (none) or with the following protease inhibitors: SBTI or inhibitors of cathepsin G (CG/CMK) or neutrophil elastase (HNE/CMK). (A) Specific [125 I]-plasminogen binding was assessed as described in the legend of Figure 1. The results are the means \pm SEM of five separate experiments. An asterisk denotes a p of <0.05 via a paired Student's t -test comparing binding in the presence of each inhibitor with the noninhibited (none) cultured cells. (B) [125 I]Plasminogen ligand blot of membrane preparations of human neutrophils (1×10^8 cells). SDS-PAGE was carried out on 14% acrylamide gels run under reducing conditions. The amount of protein loaded per lane was adjusted to the same absorbance at 280 nm: lane 1, freshly isolated cells; lane 2, neutrophils after being cultured for 20 h without enzyme inhibitors; lane 3, cells cultured in the presence of 1 mg/mL SBTI; lane 4, cells cultured in the presence of cathepsin G inhibitor CG/CMK at 2 μ M; and lane 5, cells cultured in the presence of elastase inhibitor HNE/CMK at 40 μ M. The position of the 17 kDa protein is indicated.

when the 17 kDa band was most prominent, the 12–14 kDa band was less prominent and vice versa. The relationship between these bands became more apparent in subsequent studies. Other bands, noticeably those in the 40–60 kDa range, were also upregulated in the cultured neutrophils. However, since a 17 kDa plasminogen binding band was observed with nonadherent U937 cells and cultured neutrophils, we focused on identifying this protein.

Identification of the 17 kDa Plasminogen Binding Protein. Since a 17 kDa plasminogen binding protein(s) was regulated in both neutrophils and U937 cells, we sought to determine its identity. To isolate this protein from U937 cells, a membrane fraction was prepared from 2×10^9 cells which yielded a total of ~14 mg of protein. This fraction was subjected to preparative SDS-PAGE, and the eluted fractions were tested for reactivity with [125 I]plasminogen in ligand blots after SDS-PAGE on analytical gels. The fractions harboring plasminogen binding proteins of 17 kDa were pooled, diluted to reduce their detergent content, trace radiolabeled with 125 I to facilitate detection, and then subjected to affinity chromatography on a plasminogen–Sephacrose column. Fractions were collected after elution with EACA. After dialysis, the proteins were again analyzed by ligand blotting from SDS-PAGE, which verified that a plasminogen binding protein of 17 kDa had been isolated. Initial attempts at direct N-terminal sequencing of the 17 kDa protein failed. Consequently, a tryptic digestion was performed on excised gel bands, and the resulting peptides were separated by HPLC. The N-terminal sequences of two peptides yielded unambiguous results and were obtained from two different membrane preparations (see Table 2).

To isolate the 17 kDa protein from neutrophils, the cells were lysed by nitrogen cavitation, and a membrane fraction

Table 2: Alignment of Internal Sequences of the 17 kDa Protein from U937 Monocytoid Cells and Human Neutrophils with Histone H2B

U937 Cells	
Fragment 1	L L L P G E L A K
Histone H2B ^a	(99)R L L L P G E L A K H
Fragment 2	X T S A K
Histone H2B ^a	(120)K Y T S S K
Neutrophils	
Fragment 3	A V T K A Q K K D G
Histone H2B ^a	(16)K A V T K A Q K K D G K
Fragment 4	K D G K K R K R S R
Histone H2B ^a	(23)K K D G K K R K R S R K

^a The amino acid sequences of histone H2B (SwissProt accession number P62807) are from ref 51. The residue number in histone H2B is given in parentheses.

was obtained by differential ultracentrifugation. The membrane proteins were separated by SDS-PAGE and transferred onto PVDF membranes, and the presence of the 17 kDa plasminogen binding protein was verified by reaction with [¹²⁵I]plasminogen in a ligand blotting format. N-Terminal sequencing was then performed on tryptic peptides generated from excised gel bands. Again, two different peptide sequences were obtained (Table 2), which were compared to those of proteins in the Swiss Protein Database. Each of the two sequences from each of the two cell types was consistent with a single protein set; the 17 kDa plasminogen binding protein was histone H2B (Table 2). There are multiple genes on different chromosomes that encode histone H2B proteins that are very highly homologous (many >98%) (50). The sequences obtained from the 17 kDa band matched the first histone H2B sequence determined by direct protein sequencing (51) except for the presence of a Ser instead of the Ala at position 124, which we detected. However, several of the histone H2B sequences in the database do have an Ala at this position. In addition, each sequence in the 17 kDa protein is preceded by a trypsin sensitive lysyl or arginyl peptide bond in the histone H2B sequence determined by Ohe et al. (51). The identification of the 17 kDa protein as a histone H2B is also supported by isoelectric focusing; the 17 kDa protein exhibited a pI of ≥10, consistent with the reported pI of histone H2B (52). Histone H2B sequenced by Ohe et al. (51) consists of 125 amino acids, lacks an obvious transmembrane domain, and, importantly, does have a lysine as its C-terminus.

Histone H2B is primarily a nuclear protein, but its presence on cell surfaces, including on leukocytes, has been reported (53–55). To verify that the histone H2B detected in the plasma membrane preparations from nonadherent U937 cells and cultured neutrophils was not a consequence of nuclear membrane contamination, we measured the content of 5-lipoxygenase, a nuclear membrane protein (44), in the plasma membrane preparations by Western blotting using a nuclear membrane preparation as a positive control. Similar amounts of total protein were loaded onto the gels, and the Western blots were scanned. For PMA-stimulated, nonad-

herent U937 cells, only 4.2% of the 5-lipoxygenase was in the plasma membrane preparation and the remaining 95.8% was in the nuclear membranes. These values were similar for membranes prepared from nonstimulated U937 cells. For cultured neutrophils, only 1.3% of the 5-lipoxygenase was in the plasma membrane, and this value was similar (5.1%) when the membranes were isolated from cells cultured in the presence of SBTI when cell surface expression of histone H2B was inhibited.

Expression of Histone H2B on U937 Cells. To assist in the characterization of histone H2B as a plasminogen receptor, an antiserum was raised in rabbits to an eight-amino acid peptide corresponding to its C-terminal sequence, VT KYTSSK (plus a six-carbon spacer added to its N-terminus). Western blots were performed with this antiserum to test its reactivity with membranes derived from control and PMA-stimulated adherent and nonadherent U937 cells. This antiserum showed little reactivity with the control or adherent cell membranes (Figure 3A). In contrast, a major 17 kDa band was detected in the membranes of nonadherent cells. The antiserum also reacted with purified histone H2B, which exhibited the same mobility as the 17 kDa band within the membranes of the nonadherent cells.

Recognizing that proteins, including histone H2B, which associate with the membrane fraction and can bind plasminogen in ligand blots may not be oriented appropriately for binding of ligand to intact cells, we isolated the immunoglobulin fraction from the antiserum to the C-terminal peptide of histone H2B and used it to detect cell surface expression. The reactivity of the antibody with control U937 cells or the nonadherent cells obtained after stimulation with PMA was examined by flow cytometry (not shown), and an increase in the reactivity of the antibody with the nonadherent cells was observed. To examine changes in surface expression of histone H2B expression more quantitatively, confocal microscopy was performed. Integrin $\alpha M\beta 2$ was used as a positive control for surface expression. As shown in Figure 3B, under the staining conditions that were used, histone H2B was not detected on the surface of nonstimulated control cells or on PMA-stimulated adherent cells, although surface expression of $\alpha M\beta 2$ and nuclear staining with DAPI were detected. In contrast, H2B expression was detected on the surface of the PMA-stimulated, nonadherent cells. The intensities of the histone H2B and $\alpha M\beta 2$ surface expression on the cell populations were quantified (Figure 3C). Using image analysis programs setting the threshold on the basis of the staining of histone H2B on the PMA-stimulated, nonadherent U937 cells, staining of the PMA-stimulated, adherent cells and nonstimulated cells was not detectable. The level of histone H2B expression on the nonadherent cells was ~1400-fold greater than on the other cell populations. $\alpha M\beta 2$ staining was similar on all cell types.

Expression of Histone H2B on Neutrophils. Western blots for histone H2B were performed with the membrane fractions of neutrophils with and without culture. As seen in Figure 4A, a 17 kDa form of histone 2B was not present in the membrane fraction of freshly isolated neutrophils (lane 1) but was present in the membranes of cultured neutrophils (lane 2). Culturing the neutrophils in the presence of SBTI (lane 3) or the cathepsin G inhibitor CG/CMK (lane 4) decreased the association of histone H2B with the membrane fraction. The presence of histone H2B in the membranes of

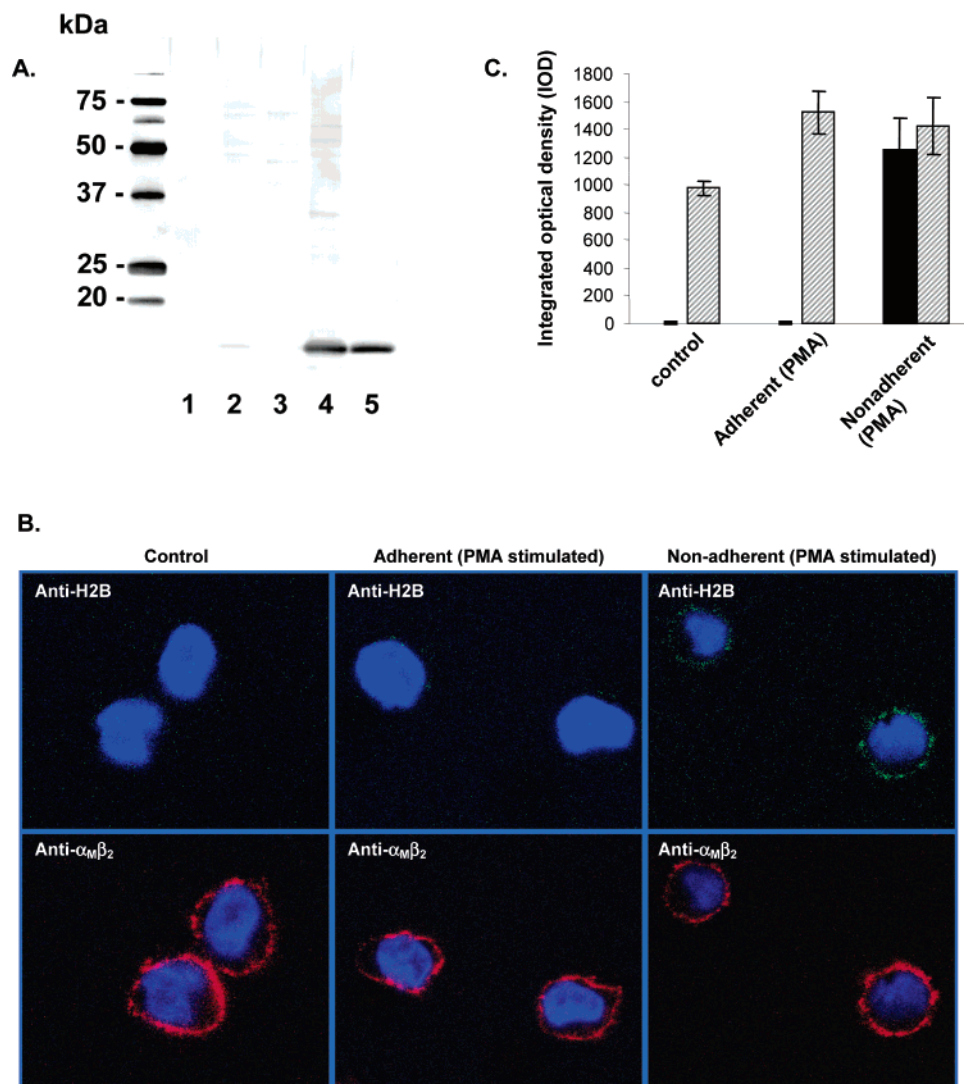


FIGURE 3: Expression of histone H2B by U937 cells. (A) Membranes from U937 cells were subjected to SDS-PAGE (10–15% acrylamide, 2.2% cross-linker, run under nonreduced conditions), and electrophoretic transfers were probed by Western blotting with the antibody raised to the C-terminal peptide of histone H2B: lane 1, untreated and nonincubated cells; lane 2, control (incubated without PMA) cells; lane 3, PMA-stimulated adherent cells; lane 4, PMA-stimulated nonadherent cells; and lane 5, purified histone H2B from HeLa cell nuclei. (B) Confocal microscopic images of control, PMA-stimulated and adherent, or PMA-stimulated and nonadherent U937 cells. Cells were stained with DAPI (blue) to identify nuclei and with antibody either to histone H2B (C-terminal peptide antibody) followed by an Alexa-488-labeled secondary antibody or to integrin $\alpha M\beta 2$ (control for cell surface staining) followed by an Alexa-568-labeled secondary antibody. The images were taken at a magnification of 40 \times and are representative of multiple areas on multiple slides. (C) Quantitation of the staining intensities of histone H2B or $\alpha M\beta 2$ from confocal microscopy. Images like those shown in panel B were analyzed using Image Pro plus. The maximum intensity readings of H2B or $\alpha M\beta 2$ antibodies were determined manually for the PMA-stimulated, nonadherent U937 cells, and then the intensities of the two fluorophores on the PMA-stimulated adherent and nonstimulated control U937 cells were determined using these thresholds as described in Experimental Procedures to obtain the average integrated optical density for each stain. Bars for histone H2B staining are black, and bars for $\alpha M\beta 2$ staining are striped.

cultured neutrophils was detected with antiserum raised to the C-terminal peptide of the protein. As noted above, on higher-percentage gels, plasminogen binding protein bands in the range of 12–14 kDa were variably detected (e.g., see lane 2 of Figure 2). We observed that inclusion of large amounts of protease inhibitors during preparation of the neutrophil membranes reduced the intensity of the 12–14 kDa bands while increasing the relative intensity of the 17 kDa histone H2B band. The relationship between these bands was resolved by testing their reactivity with antiserum raised to the C-terminal and N-terminal peptide of histone H2B (a 19-amino acid peptide). The antiserum to the C-terminal peptide reacted with the 17 kDa and the 12–14 kDa bands, whereas the antiserum to the N-terminal peptide reacted with only the 17 kDa (not shown). This pattern suggests that the

12–14 kDa band is likely to be a proteolytic product of H2B. Also, the antiserum to the C-terminal fragment reacted variably with an ~ 30 kDa band in several of the membrane preparations. An antibody to ubiquitin also reacted with proteins of this mobility, and it may represent the reported complex of histone H2B and ubiquitin (56, 57). Finally, some additional bands noted at a higher molecular mass (~ 70 kDa) were nonspecific reactions of the second antibody.

Changes in cell surface expression of histone H2B were evaluated on fresh and cultured neutrophils by confocal microscopy using the immunoglobulin fraction of the antibody raised to the C-terminal peptide of histone H2B (Figure 4B). Histone H2B expression was detected rarely on freshly isolated neutrophils and, when present, was sparse on the surface (Figure 4B, left panel). On cultured neutro-

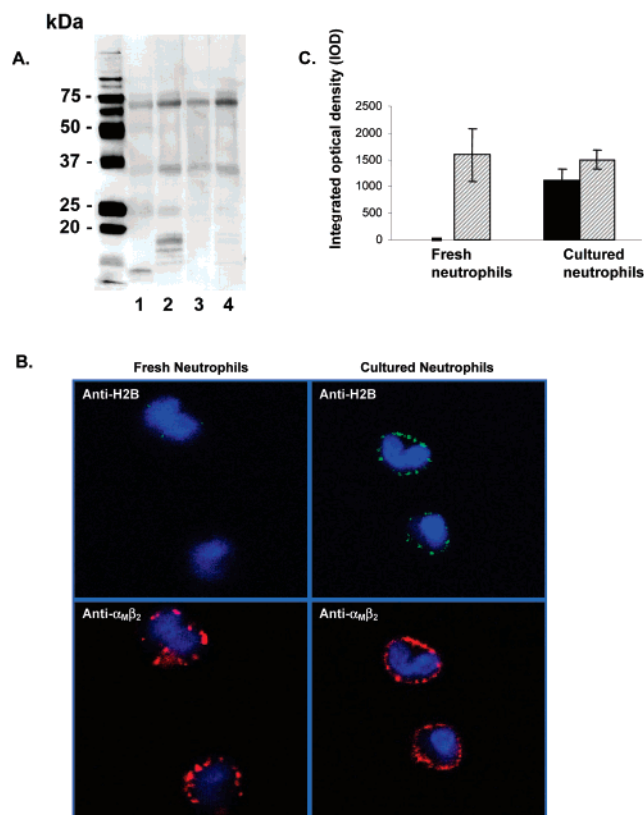


FIGURE 4: Expression of histone H2B by neutrophils. (A) Membranes from neutrophils were subjected to SDS-PAGE on a gradient gel with 10–15% acrylamide and 2.2% cross-linker, run under nonreduced conditions. Electrophoretic transfers were probed by Western blotting with the antibody raised to the C-terminal peptide of histone H2B: lane 1, freshly isolated neutrophils; lane 2, neutrophils incubated for 18 h without inhibitors; lane 3, neutrophils incubated for 18 h with 1 mg/mL SBTI; and lane 4, neutrophils incubated for 18 h with 2 μ M cathepsin G inhibitor CG/CMK. The noticeable band at \sim 70 kDa results from nonspecific interaction with the secondary (HRP conjugate) antibody. (B) Confocal microscopic images of freshly isolated or cultured (18 h) neutrophils stained with DAPI (blue) to identify nuclei and with antibody either to the C-terminal peptide of histone H2B (top panels) followed by an Alexa-488-labeled secondary antibody or to integrin α M β 2 (bottom panels) followed by an Alexa-568-labeled secondary antibody. The images were taken at a magnification of 40 \times and are representative of multiple areas on two separate slides. (C) Quantitation of the staining intensities of histone H2B or α M β 2 from confocal microscopy. Images like those shown in panel B were analyzed by using Image Pro plus by setting the maximum intensity readings of H2B or α M β 2 antibodies to the reactivity with the cultured neutrophils and then quantifying the intensities of the two fluorophores on the freshly isolated neutrophils to obtain the average integrated optical density for each stain. Bars for Histone H2B staining are black, and bars for α M β 2 staining are striped.

phils, histone H2B was detected on the surface of most cells, displaying a punctate distribution (Figure 4B, right panel). Quantitation of the intensity of staining indicated a more than 1000-fold difference in the fluorescence of the cultured versus the freshly isolated cells (Figure 4C). Nevertheless, integrin α M β 2 staining was similar on the surfaces of the two cell populations.

Function of Histone H2B as a Plasminogen Receptor on U937 Cells and Neutrophils. To assess the contribution of histone H2B to the plasminogen binding capacity of the cells, the influence of the IgG fraction of the antiserum raised against the carboxy-terminal peptide of histone H2B was

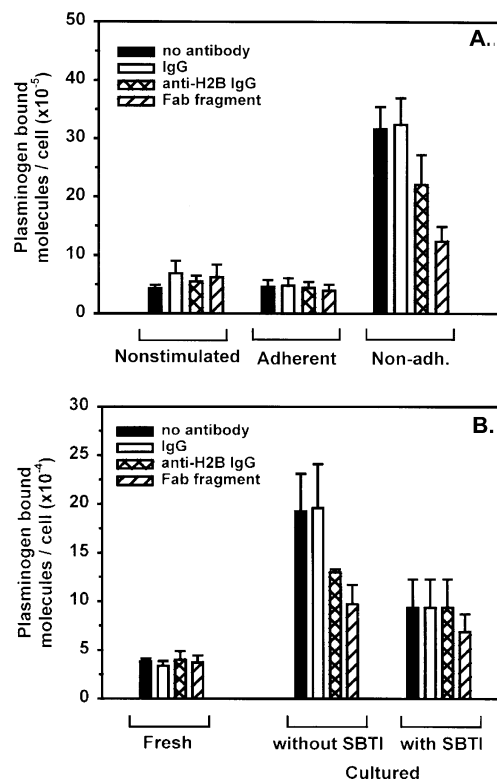


FIGURE 5: Role of histone H2B in binding of plasminogen to cells. (A) U937 cells (1.8×10^7) were stimulated with 40 nM PMA for 2 h, and specific [125 I]plasminogen binding was assessed for nonstimulated (control) cells and the adherent and nonadherent PMA-stimulated cell populations. The effects of rabbit IgG (50 μ g), anti-histone IgG (50 μ g), Fab fragments of anti-histone H2B IgG (16 μ g), or no IgG, each added simultaneously with the radiolabeled plasminogen, are shown. The decreases in the level of plasminogen binding induced by anti-histone H2B IgG and Fab fragments were statistically significant ($p < 0.05$). (B) Neutrophils (4×10^7) were cultured for 20 h in media with and without added SBTI (1 mg/mL). Binding of [125 I]plasminogen to the cells was assessed without addition of IgG, with addition of irrelevant rabbit IgG, with addition of the IgG fraction of the antiserum to the C-terminal peptide of histone H2B, or with its Fab fragments. The decreases in the level of binding of plasminogen to the neutrophils cultured without SBTI by anti-histone H2B or its Fab fragments are statistically significant ($p < 0.05$, $n = 4$).

assessed. As shown in Figure 5A, when this antibody was added, it decreased the level of binding of plasminogen to PMA-stimulated nonadherent U937 cells. The extent of the decrease was significant ($p < 0.05$) and extensive (30%). Fab fragments were generated from the same IgG fraction. These also produced a substantial reduction in the level of plasminogen binding, causing a 61% inhibition. The effect of the histone H2B antibody was specific for binding of plasminogen to the nonadherent cells; it did not inhibit binding of plasminogen to the PMA-stimulated adherent cells and unstimulated control U937 cells (Figure 5A).

The role of histone H2B in binding of plasminogen to neutrophils also was evaluated. With cultured neutrophils, the histone H2B antibody reduced the level of plasminogen binding by 33% and the Fab fragment reduced the level of binding by 50% (Figure 5B). The antibody had no significant effect on binding of plasminogen to freshly isolated neutrophils or neutrophils cultured in the presence of SBTI (Figure 5B). Thus, the inhibitory effects of the histone H2B antibodies were consistent with its pattern of expression in neutrophil membranes observed in Figures 3 and 4.

DISCUSSION

As previously reported (34, 37) and verified in this study, stimulation of U937 monocytoid cells with PMA or culture of human neutrophils leads to a substantial upregulation in the plasminogen binding capacity of these cells. For both cell types, this modulation is a consequence of an increase in the number and not the affinity of the plasminogen binding sites on the cells (34). In the study presented here, we sought to identify a molecular basis for these alterations. Ligand blots of membranes prepared from cells expressing either relatively low or relatively high plasminogen binding capacities were compared. A single major difference in the plasminogen binding proteins was observed between the two cell populations. This was the appearance of a 17 kDa plasminogen binding protein in the membrane fraction of the nonadherent U937 cells and the cultured neutrophils. The 17 kDa plasminogen binding protein was identified as histone H2B by amino acid sequencing from both cell types. Histone H2B has a lysine as its C-terminus, compatible with it being a plasminogen receptor (5) and consistent with the reduction in the level of binding of plasminogen to ligand blots of the protein upon treatment with EACA or carboxypeptidase B. Furthermore, antibodies or Fab fragments reactive with the C-terminal peptide of histone H2B showed enhanced reactivity with the surface of PMA-stimulated, nonadherent U937 cells and cultured neutrophils by confocal microscopy. Finally, these same reagents caused a substantial reduction in the level of binding of plasminogen to these intact cells. Hence, histone H2B may represent a regulated plasminogen receptor in both U937 cells and neutrophils.

Histone H2B is a well-characterized constituent of the cell nucleus, organizing DNA into nucleosomes (58), where it is involved in transcription (56). However, under certain conditions, cell surface expression of histone H2B has been reported. Relevant to our focus on leukocytes is the fact that surface localization of histone H2B has been noted on activated T lymphocytes and cultured monocytes (54, 59). Human lung carcinoma LX-1 cells were also reported to constitutively express histone H2B on their surface (60). In HIV-infected patients, autoantibodies directed against histone H2B on CD4⁺ T lymphocytes have been correlated with disease activity (61). It is interesting to speculate that autoantibodies directed at histone H2B may influence its function as a plasminogen receptor. A reduction in the potential of these cells to use plasminogen to support inflammatory cell migration might contribute to the increased susceptibility of HIV patients to infectious diseases. Autoantibodies to histone H2B also have been reported in as many as 70% of patients with autoimmune diseases, such as systemic lupus erythematosus (62). The decreased level of generation of fibrinolytic potential on the surface of cells may help to explain the increased risk for thromboembolic disorders in patients with systemic lupus erythematosus and other immunological disorders.

In the two cellular models analyzed in our study, histone H2B not only is a regulated plasminogen receptor but also plays a prominent role in mediating the observed increases in plasminogen binding. Antibodies to its C-terminal sequence inhibited the increment in plasminogen binding induced by the culture conditions by 30–60%. Under these culture conditions, antibodies reported to inhibit binding of

plasminogen to α -enolase (63) and annexin II (13) reduced the level of plasminogen binding by less than 10% (unpublished). These observations do not preclude the possibility that these and other plasminogen receptors may contribute to the basal level of plasminogen binding to the cells, which is substantial and was unaffected by the histone H2B antibodies or that collectively they may account for the 40–70% of plasminogen binding that is insensitive to histone H2B antibodies. The role of these other plasminogen receptors may become dominant under other circumstances in which plasminogen binding to cells is modulated. Indeed, it is surprising how effective the histone H2B antibodies were in inhibiting plasminogen binding in view of the marked quantitative upregulation of binding of plasminogen to the nonadherent U937 cells and the cultured neutrophils. Conceivably, in addition to directly binding plasminogen, histone H2B or antibodies to it may influence the function of other plasminogen receptors on the cell surface.

Histone H2B lacks a transmembrane domain, and hence, the mechanism regulating its cell surface expression is unclear but is presumably similar to that which can lead to expression of other plasminogen receptors without transmembrane domains, α -enolase (5), annexin II (10), TIP49a (15), and cytokeratin 8 (64). In general, enhanced plasminogen binding and plasminogen receptor expression appear to occur in association with cellular responses that perturb and remodel the cell surface. Adhesion is one such event that remodels the cell surface and alters plasminogen binding capacity (37, 65), and apoptosis is another response associated with significant changes in cell surface (66–68) and marked increases in plasminogen binding (69, 70). Of note, activated T lymphocytes express histone H2B on their surface as an early event of apoptosis (55). We have previously shown that the increase in the plasminogen binding capacity of cultured neutrophils depends on the activity of proteolytic enzymes associated with the cells, including cathepsin G and elastase (38). Since these enzymes do not cleave substrates to generate new C-terminal lysines (71, 72), it is likely that they remodel the cell surface to induce and optimize surface expression of histone H2B. This remodeling, rather than the insertion of novel proteins, also may apply to other candidate plasminogen receptors and explain why the differences in the number and intensity of plasminogen blotting bands in membranes from the adherent versus nonadherent U937 cells and from fresh and cultured neutrophils were very similar (with the exception of histone H2B) despite the marked differences in binding of plasminogen to the intact cells. Each of the newly exposed plasminogen receptors on the remodeled cell membrane must remain tightly associated with the cell surface. In the case of histone H2B, anchorage may occur through its highly polar N-terminus to the negatively charged glycosaminoglycan, proteoglycan, or phosphatidylserine moieties (73). There are 15 positively charged residues in the first 35 residues of the protein, and even with limited proteolysis to reduce the size from 17 to 12–14 kDa, a polar amino-terminal aspect would remain. It should be noted that these size estimates are based on the electrophoretic mobility of histone H2B relative to protein standards. The mobility of histone H2B with SDS–PAGE is known to be anomalous (74). Indeed, the amino acid sequence predicts a molecular mass of intact histone H2B of ~14 kDa.

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