MACROPHAGE COMPONENT gp160, A MAJOR TRYPSIN-SENSITIVE SURFACE GLYCOPROTEIN*

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Macrophages act as vital effector cells in immune defense against bacteria, viruses (2), and tumor cells (3), and as accessory cells to lymphocytes, e.g., in presentation of certain antigens (4). Macrophages also execute diverse clearance functions, including removal of oponsized particles (5), aged and damaged cells (6), and resorption of collagen (7).

Recent findings document the prominence and diversity of proteases associated with macrophages. These include a protease(s) that activates plasminogen (8), the enzymes elastase (9) and collagenase (10), the complement components C1 (11), C2 (12), and factors B (13) and D (14), which are proteases or zymogen forms, and also proteases that cleave the cell coat of fibroblasts (15), myelin (16), serum amyloid (17), macrophage migration inhibition factor (18), and immunoglobulins (19). Many of these proteases are serine active site esterases (e.g., 8, 17, 18, 20). The extracellular activities of several proteases are increased in stimulated and activated macrophages (e.g., 8, 10, 21).

Cumulatively, these findings suggest that the macrophage surface, which is vital for the cell's varied recognition and uptake functions, is regularly exposed to active proteolytic enzymes. This communication describes a guinea pig peritoneal macrophage surface glycoprotein gp160,[1] unique among major surface components in its sensitivity to mild trypsin treatment.

Materials and Methods

Galactose oxidase, soybean trypsin inhibitor, β-galactosidase, and L-(1-tosyl-amido-2-phenyl)ethylchloromethyl ketone (TPCK) (Worthington Biochemical Corp., Freehold, N. J.); neuraminidase (*Vibrio cholerae*) and lactoperoxidase (Calbiochem Behring Corp., American Hoechst Corp., San Diego, Calif.); DEAE-cellulose (DE32), Whatman Inc., Clinton, N. J.); NP40 (BDH Chemicals, Ltd., Poole, England); Coomassie brilliant blue (Schwarz/Mann Div. Becton, Dickinson & Co., Orangeburg, N. J.); 2,5-diphenyloxazole, sodium [^{125}I]iodide and potassium [^{3}H]borohydride (New England Nuclear, Boston, Mass.); α-methyl-D-mannoside, phosphorylase a, deoxyribonuclease I, albumin, carbonic anhydrase, 2-mercaptoethanol, diisopropylfluorophosphate (DFP), Hepes, sodium dodecyl sulfate (SDS), sodium periodate (Sigma Chemical Co., St. Louis, Mo.); sodium caseinate (practical grade), acrylamide and X-ray film

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[1] *Abbreviations used in this paper are:* DFP, diisopropylfluorophosphate; gp160, glycoprotein of ~160,000 daltons present at the surface of guinea pig peritoneal macrophages and cleaved on mild trypsinization; HBSS, Hanks' balanced salt solution; PMN, polymorphonuclear leukocyte(s); SDS, sodium dodecyl sulfate; LcH, *Lens culinaris* lectin; TPCK, L-(1-tosyl-amido-2-phenyl)ethylchloromethyl ketone.

(Eastman Kodak Co., Rochester, N. Y.); creatine kinase (Boehringer Manheim Biochemicals, Indianapolis, Ind.); Hanks' balanced salt solution (HBSS) (Microbiological Associates, Walkersville, Md.); iodoacetamide and cyanogen bromide (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and Sepharose-6B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) were obtained as indicated.

Three times crystallized bovine trypsin (Worthington Biochemicals Corp., 180 U/mg) was pretreated with TPCK (22). Myosin was a gift of Dr. K. Fujiwara, Harvard Medical School, Boston, Mass. *Lens culinaris* lectin (LcH) purified from lentil beans (Peak brand, D and D Bean Co., Greeley, Co.) (23) was coupled at 2.5 mg/ml in 0.1 M NaHCO3, pH 8.4, 0.1 M α-methyl-D-mannoside to Sepharose-6B activated by CNBr (24).

Cells. Peritoneal macrophage preparations obtained from Hartley guinea pigs 5 d after intraperitoneal injection of 30 ml 1% sodium caseinate (25) were washed twice with cold HBSS (300 g × 5 min). The cells consisted of 92% macrophages and 8% polymorphonuclear leukocytes (PMN) and were ≥98% intact (trypan blue exclusion). Guinea pig PMN were collected 16 h after intraperitoneal injection of 25 ml 10% sodium caseinate (25).

Radiolabeling of Surface Carbohydrate Moieties. Using a modified method (26), 2×10^7 peritoneal macrophages/ml in HBSS without glucose with 5 mM Hepes were incubated with 50 U/ml neuraminidase and 25 μg/ml galactose oxidase for 30 min at room temperature, and washed once at 4°C. The cells were resuspended at 2×10^7/ml and [^{3}H]KBH_4 (60 μg/ml with 400 μCi in 5 μl 0.01 M NaOH) added in two increments 2 min apart. After 15-min incubation at room temperature, the radiolabeled cells were washed by pelleting in cold HBSS. The specificity of macrophage glycoprotein labeling was shown by the total lack of reaction when galactose oxidase was omitted.

Radioiodination of Surface Protein Moieties. Using a modified method (27) cells ($4–5 \times 10^7$/ml) were suspended in HBSS with 8–20 μM $Na^{125}I$ (50–150 μCi/ml) and 9 μg/ml lactoperoxidase at room temperature. H_2O_2 was added in 10 portions (each 13 μl of 0.03%) over 10 min. The reaction was terminated in cold HBSS (5–10 vol) containing 0.1 mM NaI, and the cells washed by pelleting.

Trypsinization of Cells. Radiolabeled cells at 4×10^7/ml were incubated at 37°C with 1–600 μg TPCK-treated trypsin/ml in HBSS with 10 μg/ml deoxyribonuclease I. The reaction was terminated with a threefold molar ratio of soybean trypsin inhibitor or 1 mM DFP, and the cells washed twice in cold HBSS.

Polyacrylamide Gel Electrophoresis. Conditions for SDS-electrophoresis (28) were as described (20) with exponential gradients of 7.5 to 14% polyacrylamide. The standard proteins myosin, β-galactosidase, phosphorylase a, albumin, creatine kinase, carbonic anhydrase, and soybean trypsin inhibitor indicated 200,000, 130,000, 94,000, 68,000, 40,000, 29,000 and 22,000 mol wt, respectively.

Analytical Techniques. Polyacrylamide gels with ^{3}H-samples were impregnated with 2,5-diphenyloxazole and subjected to fluorography (29). Protein concentration was determined with the Folin method (30) on samples precipitated with 5% trichloroacetic acid, washed with 0.5 M perchloric acid, and solublized with 0.5 M NaOH.

Purification of gp160. Radiolabeled control macrophages or radiolabeled macrophages treated with 6 μg/ml trypsin were extracted with 0.5% NP-40 in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DFP, 3 mM iodoacetamide (1 ml/2.4×10^7 cells) for 5 min at room temperature and 10 min at 4°C. Insoluble material was removed at 12,000 g_{max} × 15 min and the extract applied to LcH-Sepharose (4–5 ml/ml resin) equilibrated with 0.3% NP-40, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (0.3% N.T.S.) at room temperature. After washing with 0.3% N.T.S., specifically bound proteins were eluted with 0.1 M α-methyl-D-mannoside in 0.3% N.T.S. (LcH eluate, 3–4 ml/ml resin).

LcH eluates were dialyzed at 4°C against 0.3% NP-40, 10 mM Tris-HCl, pH 7.4 (0.3% N.T.), and applied at a ratio of 1.5 ml/ml resin to DEAE-cellulose equilibrated with 0.3% N.T. After washing with 0.3% N.T. the columns were eluted with 60 mM NaCl in 0.3% N.T. and then with 500 mM NaCl in 0.3% N.T. Fraction A comprised the nonadherent and wash material, Fraction B the 60 mM NaCl eluate, and Fraction C the 500 mM NaCl eluate.

Results

Radiolabeling Carbohydrate Moieties of Macrophage Surface Glycoproteins. Treatment of freshly isolated macrophages with neuraminidase-galactose oxidase and reaction with [^{3}H]KBH_4 revealed multiple ^{3}H-labeled macrophage surface glycopeptides (Fig. 1 A); the most prominent is a glycopeptide of apparent 160,000 mol wt. No reaction with [^{3}H]KBH_4 occurs when neuraminidase is omitted, suggesting minimal terminal galactosyl/*N*-acetylgalactosaminyl residues on macrophage surface glycoproteins.

Radiolabeling Protein Moieties of the Macrophage Surface. On radioiodination of macrophages, 15 major surface polypeptides are identified; those with 200,000, 160,000, 93,000, 86,000 and 59–63,000 mol wt are intensely iodinated (Fig. 1 B).[2]

Effects of Trypsin on Surface Components. Treatment of ^{3}H-labeled macrophages with low concentrations of trypsin (6 μg/ml) causes cleavage of one prominent ^{3}H glycopeptide of 160,000 mol wt, which is referred to as gp160 (Fig. 2 A). Under these conditions, cleavage of gp160 is almost complete; milder conditions (less trypsin or shorter times) cause less complete cleavage (data not shown). The remainder of the major ^{3}H-surface glycopeptides are resistant to trypsin at 6 μg/ml, and to a great extent at 60 μg/ml.

The selectivity of cleavage is also observed on trypsin treatment of macrophages labeled in protein moieties with ^{125}I. Trypsin at 6 μg/ml cleaves predominantly one ^{125}I-labeled surface polypeptide of apparent 160,000 mol wt (Fig. 2 B). ^{125}I-labeled material that does not enter 7.5% polyacrylamide and is trypsin-sensitive (possibly fibrin) was not further investigated.

The Role of Disulfide Bonds in gp160. When electrophoretic analysis is done without reduction of disulfide bonds, ^{125}I-gp160 of trypsinized and control cells both migrate as 160,000 mol wt polypeptides (shown later), i.e., fragmentation of trypsinized gp160 does not occur until disulfide bonds are broken. Thus, native gp160 is a single polypeptide molecule with intrachain disulfide bond(s), and gp160 from trypsinized cells remains on the membrane as a multi-chain molecule with interchain disulfide bond(s). The finding that trypsinized gp160 remains assembled in the membrane suggested that cleavage occurs at a limited number of sites.

Purification of gp160. To purify gp160 and examine its proteolytic fragments, ^{125}I-macrophages were extracted with NP-40 and the extracts chromatographed on LcH-Sepharose. Specific ^{125}I-surface proteins are recovered in the α-methyl-mannoside eluate. LcH-affinity chromatography can be used to purify a subfraction of macrophage surface proteins. The specific eluate contains ^{125}I-gp160 in almost quantitative yield; purification (based on Folin determination) is 35-fold.

DEAE-cellulose chromatography further purifies gp160, which is recovered in the fraction eluting with 60 mM NaCl, whereas the bulk of protein elutes with 500 mM NaCl. The 60-mM NaCl fraction consists to ~30% of gp160 (based on Coomassie

[2] Control experiments indicating that the radioiodinated components represent macrophage surface polypeptides include: (*a*) In the absence of added H_2O_2, no reaction occurs. (*b*) At 4°C where pinocytosis is greatly reduced, the same components are labeled. (*c*) Iodination of monolayer macrophages produces a similar ^{125}I-polypeptide pattern, thus eliminating contaminant nonadherent and dead cells as the source of ^{125}I-polypeptides. (*d*) ^{125}I-labeled macrophage preparations (>90% macrophages) further purified on Ficoll density centrifugation showed a similar labeling pattern. (*e*) Comparison with ^{125}I-labeled peritoneal PMN indicates that none of the major ^{125}I-polypeptides of macrophage preparations are due solely to contaminating PMN.

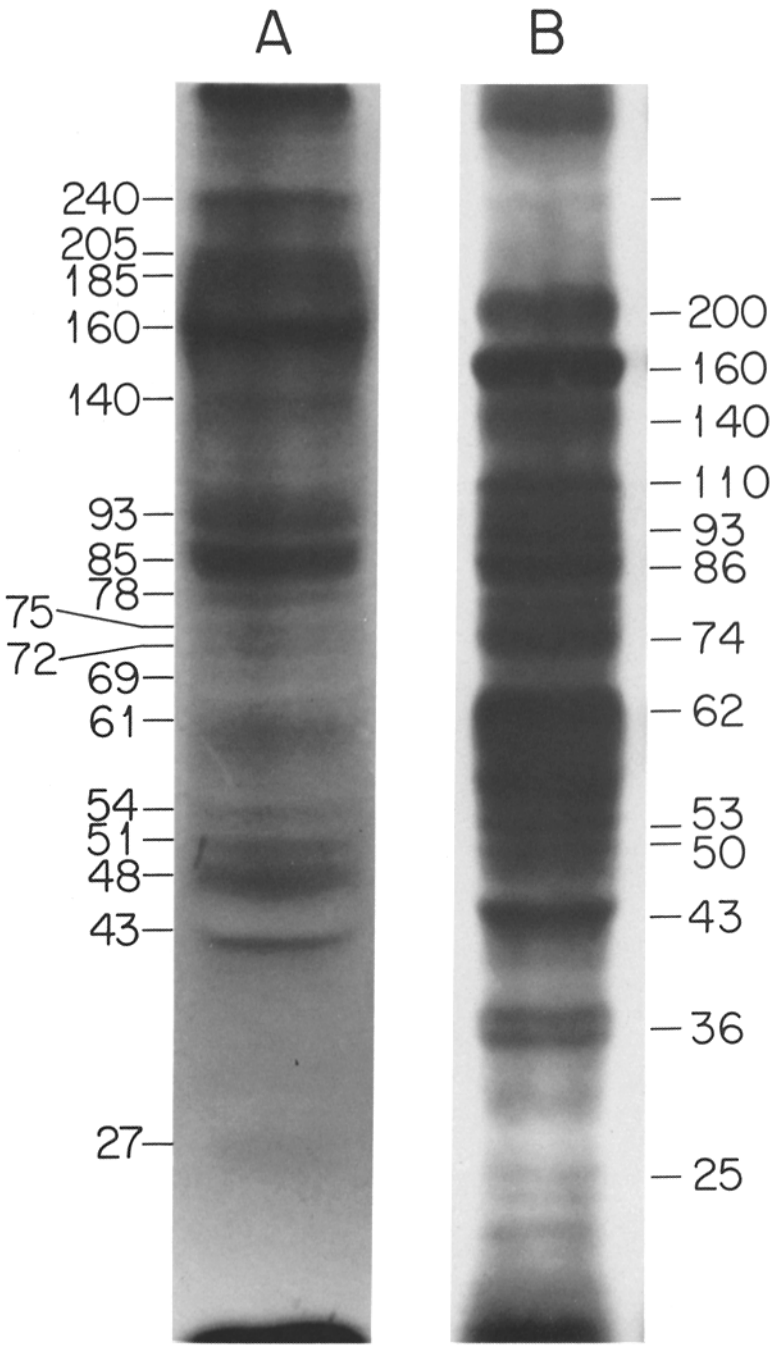

FIG. 1. A. Fluorograph of ^{3}H-labeled surface glycopeptides of peritoneal macrophages. Intact neuraminidase/galactose oxidase-treated cells were labeled with [^{3}H]KBH_4, solubilized with SDS and 2-mercaptoethanol, and electrophoresed. Apparent molecular weight of prominent glycopeptides is indicated. B. Autoradiograph of ^{125}I-labeled macrophage surface polypeptides. Cells were radioiodinated, solubilized with SDS and 2-mercaptoethanol, and electrophoresed. Note the extensive but not complete overlap of components in A (carbohydrate labeling) and in B (protein labeling).

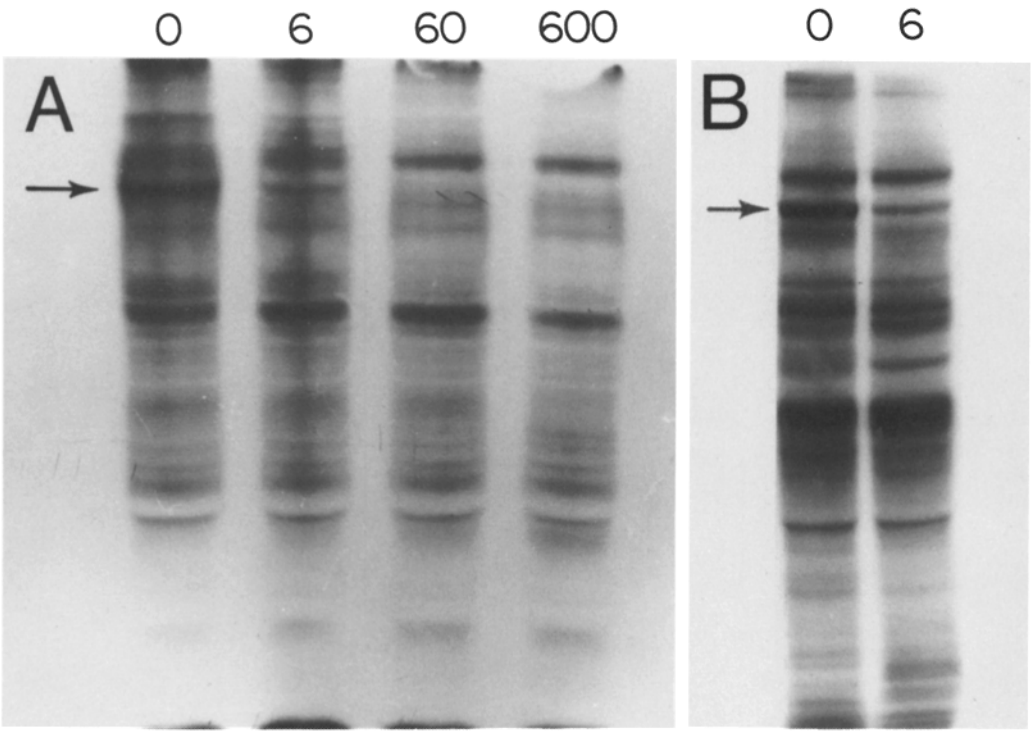

FIG. 2. A. ^{3}H-labeled surface glycopeptides of control and trypsin-treated macrophages. Cells labeled using neuraminidase, galactose oxidase, and [^{3}H]KBH_4 were treated as indicated with 0, 6, 60, or 600 μg trypsin/ml for 20 min, solubilized with SDS and 2-mercaptoethanol, and electrophoresed. Arrow indicates gp160. B. ^{125}I-surface polypeptides of control and trypsin-treated macrophages. Radioiodinated cells were treated as indicated with 0 or 6 μg trypsin/ml for 20 min, and solublized with SDS and 2-mercaptoethanol. Arrow indicates gp160.

blue-staining of gels); it contains two contaminant ^{125}I-polypeptides (shown below). Overall purification is estimated at 300- to 400-fold.

Analysis of Tryptic Fragments of gp160. Under nonreducing conditions, purified ^{125}I-fractions from trypsinized cells appear identical to the corresponding fraction from control cells (Fig. 3, left lanes). All nonreduced purified fractions contain ^{125}I-gp160 and two contaminant ^{125}I-polypeptides, i.e., trypsinized gp160 can be purified by the procedure developed for native gp160.

On exposure of macrophages to trypsin and subsequent reduction of the purified fractions, a time-dependent loss of the 160,000 mol wt ^{125}I-polypeptide is observed concurrent with the simultaneous appearance of two proteolytic ^{125}I-labeled fragments (Fig. 3, right lanes). Based on electrophoretic mobility, the molecular weights of the gp160 fragments are 85,000 and 71,000.[3] A time-dependent loss of a faintly-staining 160,000-mol wt band, and the appearance of the two tryptic fragments can be seen in the corresponding Coomassie blue-stained gel (data not shown). Thus, treatment of macrophages with low concentrations of trypsin cleaves the prominent surface glycoprotein gp160 into two major chains of 85,000 and 71,000 mol wt. Because both

[3] The possibility that other gp160 fragments of low molecular weight escaped detection cannot be eliminated.

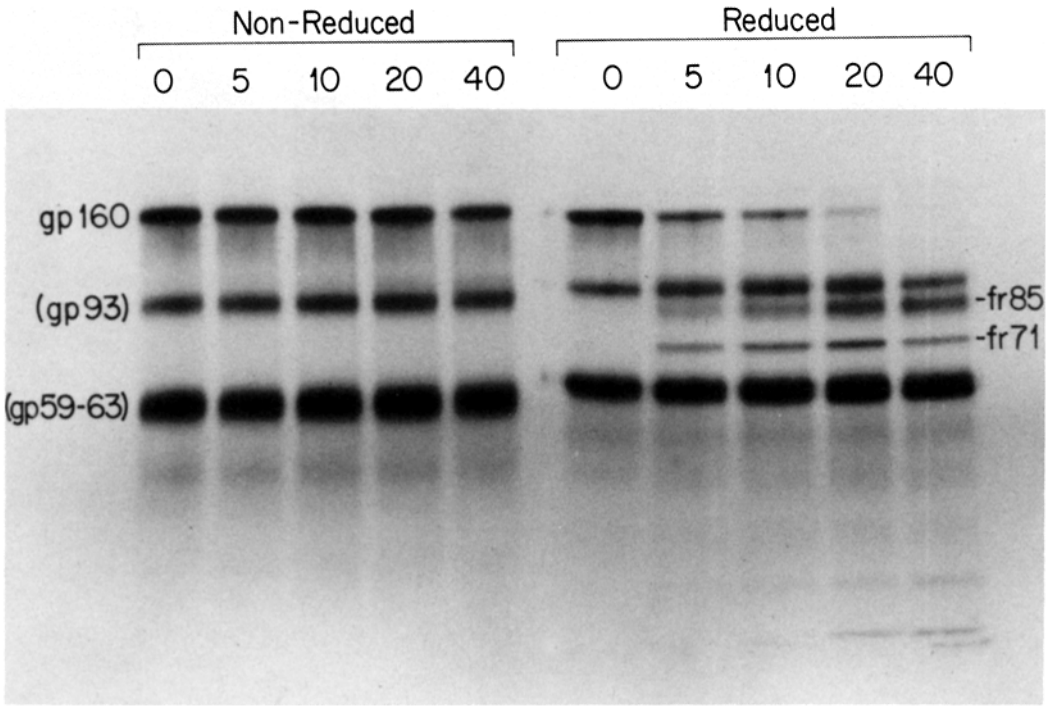

FIG. 3. Purified ^{125}I-labeled fractions of control and trypsinized macrophages. Radioiodinated macrophages were incubated with 6 μg trypsin/ml for 0, 5, 10, 20, or 40 min (as indicated). gp160-containing fractions were purified and electrophoresed under nonreducing conditions and after reduction with 2-mercaptoethanol. Tryptic fragments of gp160 are indicated in the autoradiograph (fr 85, fr 71).

fragments are accessible to lactoperoxidase and trypsin, they are exposed, at least partially, on the outer surface of the membrane.

Location of the Carbohydrate Site on gp160. To locate the carbohydrate site(s) on gp160, macrophages labeled with neuraminidase, galactose oxidase, and [^{3}H]KBH_4 were trypsinized and ^{3}H-gp160 purified. On nonreducing electrophoresis, the purified fractions from trypsinized and control cells appear identical; both contain a ^{3}H-labeled 160,000 mol wt glycopeptide (Fig. 4, left lanes). Reducing SDS-electrophoresis of the purified fraction from trypsinized cells reveals the loss of the ^{3}H-labeled 160,000 mol wt glycopeptide and the concomitant appearance of a single ^{3}H-labeled 85,000 mol wt glycopeptide (Fig. 4, right lanes). Thus, the carbohydrate site(s) of gp160, which interact(s) with neuraminidase/galactose oxidase, is (are) located on the 85,000-mol wt fragment.

Discussion

A major macrophage surface component, gp160, a glycoprotein with approximately 160,000 mol wt, is shown to be cleaved on mild trypsinization of intact cells. The tryptic fragments remain assembled in the membrane as a disulfide-bridged molecule. gp160 was purified ~300- to 400-fold to 30% purity using detergent solubilization, lectin affinity chromatography, and ion exchange chromatography. gp160 from trypsinized cells could be purified by the same process. On reduction of disulfide

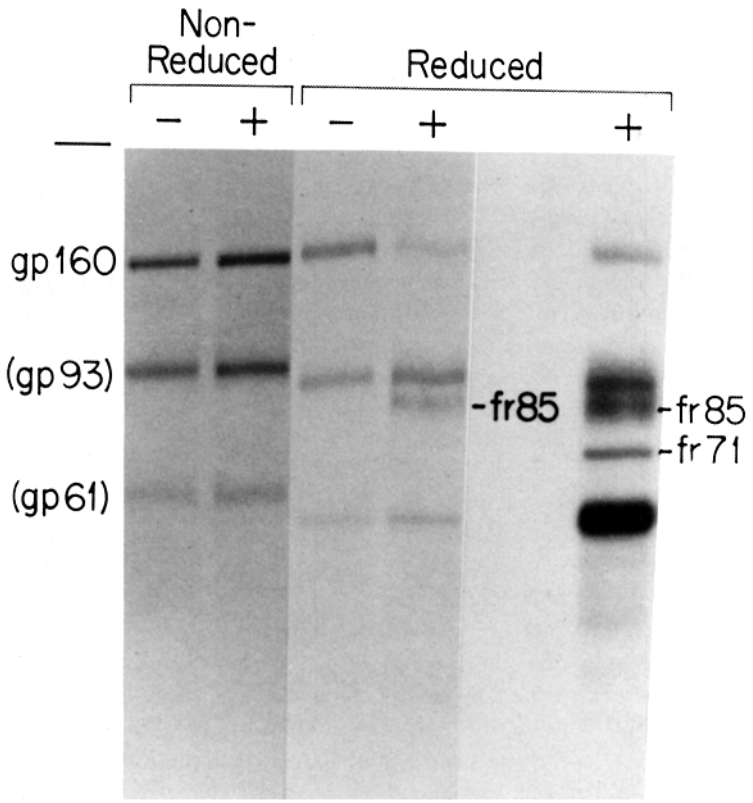

FIG. 4. Purified ^{3}H-labeled fractions of control and trypsinized macrophages. Neuraminidase, galactose oxidase, and [^{3}H]KBH_4 treated cells were incubated without (−) and with (+) 6 μg trypsin/ml for 20 min. gp160-containing fractions were purified and electrophoresed without reduction (two left lanes) and after reduction with 2-mercaptoethanol (next two lanes). To compare the ^{3}H-tryptic glycopeptide fragment (fr 85) with the ^{125}I tryptic-fragments, a reduced purified fraction from trypsinized ^{125}I-labeled cells was electrophoresed on the same gel (far right lane).

bonds, trypsinized gp160 solubilized with SDS is split into two fragments of 85,000 and 71,000 mol wt. Both fragments are labeled in intact cells by lactoperoxidase-catalyzed iodination; they are thus both exposed on the outer surface of the cell. The reactive carbohydrate site is located on the 85,000-mol wt fragment because only this fragment is labeled in intact cells by neuraminidase, galactose oxidase, and [^{3}H]-KBH_4.

Consideration of the effects of mild proteolytic conditions on macrophage surface components is important because proteolytic conditions approximate the in vivo environment of these cells. Macrophages accumulate, persist, and function at sites of inflammation and under these conditions secrete a variety of active proteases (31). Macrophage surface proteins may also be exposed to proteases released by living or dead PMN or by tumor cells. Indeed, Pearlstein et al. (32) hypothesized that surface resistance to proteolysis is a requirement for macrophages to function. The majority of the prominent radiolabeled surface components of guinea pig macrophages identified in this study are resistant to mild trypsin treatment; gp160 is unique by virtue of its trypsin-sensitivity.

In mouse peritoneal macrophages a surface component of ~160,000 mol wt has been detected (33). Pearlstein et al. (32), however, found no ^{125}I-mouse macrophage surface component sensitive to mild trypsinization. This lack of a trypsin-sensitive analog of gp160 on mouse macrophages may be due to specie difference or differences in experimental details.[4]

Experiments to determine whether gp160 is present on guinea pig PMN were ambiguous. A ^{125}I-labeled 160,000 mol wt polypeptide, which adheres to LcH-Sepharose, is less prominent on PMN. Whether the PMN component is identical to macrophage gp160 is not clear. The 160,000-dalton PMN component is cleaved by mild trypsinization (6 μg/ml for 20 min), but several other PMN surface components are also cleaved, i.e., sensitivity to mild trypsin treatment is not a distinguishing characteristic for a PMN surface component.

No evidence was found for a ^{125}I- or ^{3}H-labeled macrophage surface component with a subunit structure similar to fibronectin. The latter, a particularly trypsin-sensitive surface glycoprotein of fibroblasts, is a disulfide-bonded dimer of a 220,000 mol wt subunit (34). Macrophage gp160 does not resemble fibroblast fibronectin in subunit structure or in function, i.e., in adhesion to substratum because trypsin treatment of macrophages (6, 60, 600 μg/ml × 20 min) did not alter their ability to adhere to culture dishes (data not shown).

The function of gp160 is not yet known. gp160 is the only prominent surface protein cleaved when macrophages are subjected to mild trypsinization. The cleavage reaction is also specific in that only two fragments were found indicating hydrolysis of one or a limited number of polypeptide bonds. This specificity suggests an important functional correlate, possibly an activation reaction. Other single polypeptide molecules transformed by specific proteolytic cleavage into active disulfide-bonded multichain molecules include Factor VII (35), prekallikrein (36), and the complement precursors proC4 (37) and proC3 (38). Alternatively, gp160 might be a protease inhibitor with a reaction mechanism similar to α_2-macroglobulin (39).

Summary

Macrophages secrete a large number of proteases, implying in vivo exposure of the cell surface to proteolytic conditions. Mild trypsin treatment of ^{125}I-labeled guinea pig peritoneal macrophages preferentially cleaves one surface component of apparent 160,000 mol wt. Similar trypsin treatment of macrophages with ^{3}H-labeled carbohydrate surface moieties also cleaves a single ^{3}H-labeled 160,000 mol wt glycoprotein, referred to as gp160. Nonreducing sodium dodecyl sulfate (SDS)-electrophoresis established that gp160 of trypsinized cells remains assembled in the membrane as a multichain disulfide-bonded molecule. gp160 was purified by detergent extraction, *L. culinaris* lectin affinity chromatography and DEAE-cellulose chromatography. The corresponding molecule from trypsinized cells was purified by the same procedure. Reducing SDS-electrophoresis of purified trypsinized ^{125}I-labeled gp160 revealed two proteolytic fragments with apparent molecular weights of 85,000 and 71,000. Thus, mild trypsin treatment of macrophages preferentially cleaves a single surface protein, possibly at a single site. Because the two fragments of gp160 are accessible to lactoperoxidase and trypsin, both must be exposed on the outer membrane surface.

[4] In a recent study on mouse cells, Austyn and Gordon used a monoclonal antibody to identify a macrophage-specific antigen with properties similar to gp160 (Gordon. Personal communication.).

The reactive carbohydrate site was found on the 85,000 mol wt fragment, which alone contains the ^{3}H-label introduced into intact cells by neuraminidase, galactose, oxidase, and [^{3}H]KBH_4.

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