

# A Novel Class of Autoantigens of Anti-neutrophil Cytoplasmic Antibodies in Necrotizing and Crescentic Glomerulonephritis: The Lysosomal Membrane Glycoprotein h-lamp-2 in Neutrophil Granulocytes and a Related Membrane Protein in Glomerular Endothelial Cells

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## Summary

Necrotizing and crescentic glomerulonephritis (NCGN) is frequently associated with circulating antineutrophil cytoplasmic autoantibodies (ANCA). It is established that ANCA are specific for soluble enzymes of granules of polymorphonuclear neutrophil granulocytes (PMN), such as myeloperoxidase (MPO) or protease 3 (PR3). The purpose of this study was to identify membrane proteins of PMNs, and/or glomerular cells, as additional autoantigenic ANCA targets. When membrane protein fractions were prepared from PMNs and isolated human glomeruli, and immunoblotted with ANCA sera of NCGN patients, two bands with apparent molecular masses of 170 and 80–110 kD (gp170/80–110) were labeled in PMNs, and a 130-kD glycoprotein (gp130) in glomeruli. Gp130 was purified, and monoclonal and rabbit antibodies (Abs) were produced which showed the same double specificity as the patient's ANCA. Using these probes, evidence was provided that gp170/80–110 is identical with human lysosomal-associated membrane protein 2 (h-lamp-2), because both proteins were immunologically cross-reactive and screening of a cDNA expression library from human promyelocytic leukemia cells with anti-gp130 Ab yielded a clone derived from h-lamp-2. Gp170/80–110 was localized primarily in granule membranes of resting PMNs, and was translocated to the cell surfaces by activation with FMLP. By contrast, gp130 was localized in the surface membranes of endothelial cells of human glomerular and renal interstitial capillaries, rather than in lysosomes, as found for h-lamp-2. Potential clinical relevance of autoantibodies to gp170/80–110 and gp130 was assessed in a preliminary trial, in which ANCA sera of patients ( $n = 16$ ) with NCGN were probed with purified or recombinant antigens. Specific reactivity was detected in ~90% of cases with active phases of NCGN, and frequently also in combination with autoantibodies specific for PR3 or MPO. Collectively, these data provide evidence that h-lamp-2 in PMNs and a different, structurally related 130-kD membrane protein on the cell surface of renal microvascular endothelial cells are autoantigenic targets for ANCA in patients with active NCGN.

Renal glomeruli are frequently involved in systemic vasculitis, particularly when small blood vessels are affected, such as microscopic polyangiitis and Wegener's granulomatosis (1). Diagnostic criteria for these diseases are their clinical course, histopathologic patterns in kidney biopsies (1–4), and serological markers, especially antineutrophil cytoplasmic autoantibodies (ANCA)<sup>1</sup> (5). In active stages glomerular le-

sions are characterized by necrosis of single capillary loops which are accompanied by extraglomerular deposition of fibrin, and by proliferation of epithelial cells of Bowman's capsule, causing capsular crescents. Glomerular lesions also occur without evidence for systemic disease, and are then designated as "idiopathic" (1).

Although the clinical course and histopathology of ANCA-associated diseases vary considerably, they have several features in common which suggest similar pathogenetic roots (6). For example, most diseases show involvement of small blood vessels (2), capillary loop necrosis, and crescent formation ("segmental and necrotizing glomerulonephritis [GN], crescentic GN") (7), rapid clinical progression ("rapid progres-

<sup>1</sup> Abbreviations used in this paper: ANCA, antineutrophil cytoplasmic autoantibodies; GN, glomerulonephritis; h-lamp-2, human lysosomal-associated membrane protein 2; MBP, maltose binding protein; MPO, myeloperoxidase; NCGN, necrotizing and crescentic glomerulonephritis; PLP, paraformaldehyde-lysine-periodate; PR3, protease 3.

sive GN") (7), and relative scarcity of immunoglobulins within the glomerular lesions ("pauci-immune GN") (2, 8). These recent insights have spurred attempts to develop classifications of ANCA-associated diseases, resulting in several provisional consensus nomenclatures (1, 9).

The discovery of ANCA (10) marks a new era in the study of vasculitis, and their serum titer is now an indispensable clinical parameter for detection, management, and monitoring of vasculitis and/or necrotizing and crescentic glomerulonephritis (NCGN) (3, 11–14). The major antigenic targets of ANCA were identified as soluble enzymes (15) of PMN granules, such as myeloperoxidase (6, 12), protease 3 (PR3, 16–18), leukocyte elastase (19), and others (20–22). In other studies, ANCA autoantibodies to endothelial cells were also recorded (23–26), however their precise antigen specificities are controversial. The purpose of this study was to identify membrane proteins of PMNs and/or glomerular endothelial cells as possible autoantigenic targets for ANCA in NCGN.

## Materials and Methods

**Selection of Patients and Characterization of ANCA Sera.** ANCA sera of 15 patients with histologically confirmed NCGN were collected at the time of kidney biopsy, including one transplanted patient with recurrence of disease. Classification of the diseases was performed according to reference 9. Grading of glomerular capillary loop necrosis, proliferative changes, sclerosis, and crescent formation was assessed by two reviewers. We excluded patients with vasculitis without kidney involvement, with Churg-Strauss syndrome, or Schoenlein-Henoch's purpura. As controls, sera from normal healthy subjects (four female, three male), and ANCA-negative patients with various glomerular and renal diseases (two membranous nephropathy, one hemolytic uremic syndrome, one focal sclerosis), as well as with NCGN associated with Goodpasture's syndrome (one primary disease, and another recurrent in a transplant) were tested. All sera were stored at 4°C, in the presence of 0.1% azide and 200 IE Aprotinin/ml (Trasylol®; Bayer, Leverkusen). The patient data are listed in Table 1.

Classification of ANCA patterns was performed by indirect immunofluorescence on commercially available test slides (BIOS, Munich, Germany). In parallel, PMNs were isolated by dextran fractionation (27, 28) from freshly drawn, heparinized blood, suspended in PBS ( $5 \times 10^3$  PMNs/ml), and attached onto slides (0.2-ml suspension/slide) by incubation for 5 min at 20°C, followed by fixation in 96% ethanol for 5 min at -20°C. An international c-ANCA standard IgG (obtained from the plasmapheresis fluid of a patient with Wegener's granulomatosis; Statens Serum Institut, Copenhagen, Denmark; 29) was similarly analyzed. Serial dilutions of sera (from 1:10 to 1:1280) were screened for Abs specific for glomerular endothelial cells, glomerular basement membrane, or nuclei by indirect immunofluorescence on unfixed 4- $\mu$ m cryostat sections of normal adult and fetal human kidneys, mouse kidney, and rat liver, using FITC-conjugated F(ab')<sub>2</sub> of rabbit anti-human IgG (DAKO A/S, Glostrup, Denmark). Antigen specificity and titer of ANCA-sera were determined by ELISA, using: (a) a commercial ANCA system, containing a PMN granule fraction (Anti-Neutrophil Zytosol Antikörper ELISA; BIOS), (b) neutrophil protease 3 (Anti-PR3 ELISA; Progen, Heidelberg); (c) myeloperoxidase (Anti-Myeloperoxidase Quantitativer ELISA; BIOS); (d) leukocyte elastase in some cases (kindly performed by Drs. E. Csernok and W. L. Gross, Rheumatological Department, Medical University, Lübeck, Germany).

**Membrane Protein Fractions and Antibodies.** Membrane protein fractions of isolated PMNs, glomeruli, or placenta were prepared by incubation in 200 mM Na<sub>2</sub>CO<sub>3</sub>, pH 10.5, and Triton X 114-phase partition (30). Murine mAbs were produced (31), using glomerular membrane protein fractions as antigen. Hybridoma supernatants were screened by immunoblot analysis using glomerular membrane proteins, and by indirect immunofluorescence on isolated PMNs and cryostat sections of normal human kidney. Hybridoma Ag11 was selected primarily by its similarities to ANCAs of NCGN patients, in particular by its ability to immunostain PMNs, as well as glomerular and placental endothelial cells. It was subcloned twice by limiting dilution, and maintained in Nutridoma medium (Boehringer Mannheim, Vienna, Austria). The IgG fraction was precipitated with 50% ammonium sulfate, and its IgG subclass was determined using an isotyping kit (Amersham International, Amersham, UK).

A polyclonal Ab was raised by intradermal immunization of a rabbit with 300  $\mu$ g of electroeluted gp130, suspended in CFA (Difco, Detroit, MI), followed by two boosts with 100  $\mu$ g antigen in IFA. Serum samples were collected before immunization, and in weekly intervals starting from week three after immunization. Purified gp130 was immobilized on CNBr-Sepharose 4B (300  $\mu$ g/ml; Pharmacia LKB, Uppsala, Sweden), and was used to affinity purify anti-gp130 IgG from rabbit and also ANCA sera.

The use of animals for experimental purposes was permitted by the Austrian Ministry of Science.

**Immunoblotting.** Membrane protein fractions of PMNs, glomeruli, or placenta were separated by 7% SDS-PAGE, and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Immunoblotting was performed as described (32) using the following Abs: (a) mAb Ag11 (50  $\mu$ g/ml); (b) rabbit anti-human myeloperoxidase IgG (Calbiochem-Novabiochem Corp., La Jolla, CA); (c) anti-human protease 3 (PR3) Ab WGM2 (kind gift from Drs. W. L. Gross and E. Csernok, 33); (d) anti-human lysosomal-associated membrane protein (h-lamp-2) monoclonal and polyclonal Abs (obtained from Dr. M. Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA); (e) anti-gp130 polyclonal Ab; (f) anti-gp130 polyclonal Ab which was affinity purified on the recombinant fusion protein of clone 1A1 (see below); (g) Sera from 15 ANCA-positive patients; (h) sera of 7 normal individuals and of 6 ANCA-negative patients; (i) commercial ANCA-standard IgG. Appropriate alkaline phosphatase-conjugated IgGs (Promega, Madison, WI) were used as secondary Abs and were detected by NBT-BCIP chromogenic substrate (Kierkegaard & Perry, Gaithersburg, MD). Duplicate blots were probed with secondary Abs alone as negative controls. Immunoblots using patient sera were analyzed by two reviewers and were scored 0 or +.

**Purification of Gp130 and Gp170/80–110.** Membrane protein fractions of PMNs, isolated glomeruli, and term placenta were solubilized in 1% SDS (0.1 mg protein/ml) at 20°C for 30 min with sonication, and adjusted to 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.9% sodium chloride, 20 mM Tris-HCl buffer, pH 7.2 (RIPA-buffer). Solubilized membrane protein fractions were sequentially incubated with mAb Ag11 (0.1 mg/ml) at 4°C for 1 h, followed by 300  $\mu$ l protein A-Sepharose 4B (Pharmacia LKB) for 30 min. The immune complexes were washed twice in Tris-buffered saline, pH 7.2, for 5 min, and the bound antigen was eluted with 1 M glycine-HCl buffer, pH 2.8, containing 1% Triton X-114, at 4°C. The eluate was neutralized with 1 M Tris-HCl buffer, pH 8.0, and concentrated by phase separation of Triton X-114 at 37°C. Proteins in the detergent phase were precipitated with acetone-ethanol at -20°C, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. In addition, gp130 was purified from

**Table 1. Histological Findings in Kidney Biopsies**

Patient (sex/age)	Biopsy (necrosis/proliferation/sclerosis)	ANCA (titer IF-pattern)	Patient (sex/age)	Biopsy (necrosis/proliferation/sclerosis)	ANCA (titer IF-pattern)
1 H.A. m/69	+ / + / + Cresc 50%	1:80 c + p	9 B.F. m/80	+ / + / + Cresc. 40%	1:80 c
2 S.A. f/85	+ / + / + Vasculitis	1:40 c + p	10 F.M. m/58	+ / + / + Cresc. 70%	1:160 c
3 W.E. f/66	+ / + / 0 Cresc. 40% granulom. vasculitis	1:80 c	11 K.A. f/79	+ / + / + Cresc. 35% granulom. vasculitis	1:160 c + p
4 L.J. f/66	+ / + / + Cresc. 30%	1:160 c	12 S.R. f/69	+ / + / + Cresc. 10% granulom. vasculitis	1:40 c
5 P.P. f/68	+ / + / + Cresc. 60% granulom. vasculitis	1:40 c + p	13 W.L. m/47	+ / + / +	1:80 c
6 K.S. m/35 TX	+ / + / 0 Cresc. 60%	1:40 c + p	14 S.E. f/78	+ / + / + Cresc. 40%	1:80 c
7 H.A. f/85	+ / + / + Cresc. 20%	1:80 c	15 G.F. m/81	+ / + / + Cresc. 40% vasculitis granulom.	1:40 c + p
8 W.F. m/72	+ / + / + Cresc. 80%	1:160 c	16 N.N. ANCA-stand.	Extra- cap. GN	1:80 c
Controls					
17 L.M. f/82	Membranous GN	neg.	20 D.B. f/48	Membranous GN	neg.
18 E.M. m/38	S-f-GN sclerosis	neg.	21 R.K. m/31 TX	S-f-GN Necrosis 10% Cresc.	AGBM 1:80
19 B.I. m/65	HUS	neg.	22 H.H. m/32	S-f-GN Necrosis 80% Cresc.	AGBM 1:40

*c/p*, type of immunofluorescence pattern (cytoplasmic or perinuclear); *Cresc.*, crescents are present (% of glomeruli affected); *granulom.*, granulomas are present; *HUS*, hemolytic uremic syndrome; *Necrosis*, necrosis of capillary loops; *Proliferation*, increase of glomerular cells; *S-f-GN*, segmental and focal glomerulonephritis; *Sclerosis*, increase of matrix; *TX*, transplantation; +/0, present/not present.

membrane protein fractions of human placenta by preparative SDS-PAGE and electroelution. Immunoblotting was performed with patient sera, control sera, and Abs, as described above.

*Molecular Cloning Nucleotide Sequencing of Gp170/80-110.* A  $\lambda$ g11-cDNA expression library from the human promyelocytic leukemia cell line HL 60 (HL 1020b; Clontech, Palo Alto, CA)

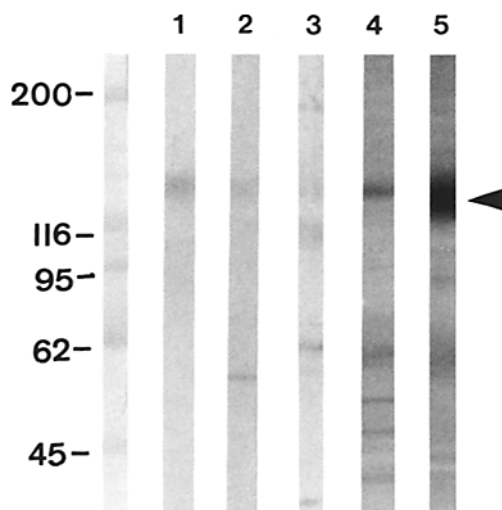
was screened ( $10^6$  PFU) with affinity-purified rabbit anti-gp130 Ab. A cDNA clone (1A1) was identified and sequenced (32, 34), and used to establish a recombinant fusion protein. Comparison of the nucleotide and amino acid sequences was performed by searching the EMBL data bank.

For expression of the recombinant fusion protein coded for by

clone 1A1 its cDNA was amplified by PCR from the  $\lambda$ gt11-cDNA template, using synthetic oligonucleotide primers with the sequences GGGAAATCTTCGGGCCATTGAATGACT (sense) and CTCGTCGACCTAAAATTGCTCATATCCA (antisense). The PCR product was subcloned into the pCR II cloning vector (TA Cloning System; InVitrogen, San Diego, CA), and its sequence was confirmed. The cDNA insert was excised using an in-frame EcoRI site at the 5' end, and subcloned into the maltose binding protein (MBP) vector pmal-c (New England Biolabs, Beverly, MA; 35). The orientation of the cloned cDNA and the integrity of the cloning site were confirmed by DNA sequencing. Synthesis of the fusion protein in transformed *Escherichia coli* DH5a was induced for 2 h with 0.3 mM isopropyl- $\beta$ -D-thiogalactopyranoside (Sigma Chemical Co., St. Louis, MO).

SDS-solubilized proteins of induced and noninduced bacteria, as well as electroeluted fusion protein were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with mAb Ag11, anti-gp130 polyclonal Ab, patient sera, ANCA standard serum, all ANCA-negative human sera, monoclonal and polyclonal rabbit anti-h-lamp-2 IgGs, and Abs to MBP (New England Biolabs). The recombinant fusion protein was also immobilized on CNBr-Sepharose 4B (Pharmacia LKB) and used for affinity purification of polyclonal rabbit anti-gp130 serum (32).

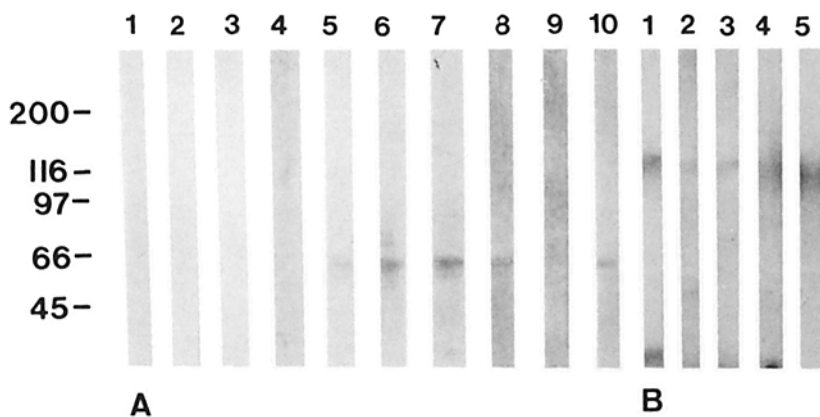
**Immunohistochemistry.** Unfixed 4- $\mu$ m-thick cryostat sections of surgical specimens of normal human kidney, lung, nasal and laryngeal mucosa, skin, liver, stomach, pancreas, small intestine, lymph node, spleen, term placenta, and umbilical cord were incubated with mAb Ag11 IgG (10  $\mu$ g/ml), followed by FITC-conjugated F(ab')<sub>2</sub> fragment rabbit anti-mouse IgG (Accurate Chemicals, Westbury, NY). 0.5- $\mu$ m frozen sections were cut from a paraformaldehyde-lysine-periodate (PLP)-fixed normal human kidney, and isolated PMNs, on a Reichert Ultracut ultramicrotome (Vienna, Austria) with F4 cryo-attachment (36). Binding of mAbs Ag11 or anti-h-lamp-2 was visualized by indirect immunofluorescence, using TRITC F(ab')<sub>2</sub> rabbit anti-mouse conjugates (Accurate Chemicals), or by avidin-biotin immunoperoxidase method (StrAvidGen Super Sensitive Universal Immuno-staining Kit; BioGenex Laboratories, San Ramon, CA). Slides with isolated PMNs were similarly stained by indirect immunofluorescence with the mAbs. HL 60 cells (American Type Culture Collection, Rockville, MD) before



**Figure 1.** Identification of gp130 as antigenic target of ANCA sera and of the mAb Ag11 in isolated human glomeruli. Fractions of glomerular membrane proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with a series of Abs. Lane 1, mAb Ag11; lane 2, international ANCA standard IgG obtained from the hemofiltrate of a patient with Wegener's granulomatosis; lane 3, serum of patient no. 9; lane 4, serum of patient no. 2; lane 5, serum of patient no. 15. The proteins labeled in lower intensity in lanes 2-5 besides gp130 were also observed in controls with ANCA-negative sera, and are therefore considered background labeling.

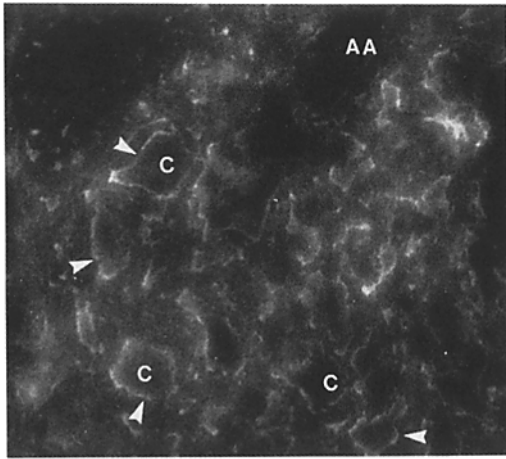
and after stimulation with 1.25% DMSO for 10 d (37), as well as bone marrow smears from normal individuals and from promyelocytic leukemia patients, were cytocentrifuged onto slides, fixed in 96% ethanol, and also processed by the avidin-biotin system.

Ultrathin frozen sections of PLP-fixed normal human kidneys or isolated, pelleted PMNs were prepared as described (38). Sections were incubated with mAb Ag11 (10  $\mu$ g/ml), a bridging affinity purified rabbit anti-mouse IgG, followed by goat anti-rabbit IgG-10 nm gold conjugate (1:20, Auroprobe; Amersham Corp.). In some experiments, freshly isolated PMNs (2  $\times$  10<sup>6</sup> cells/ml HBSS) were



**Figure 2.** Specific binding of ANCA sera to recombinant fusion protein 1A1 (derived from h-lamp-2), or to purified gp130. The fusion protein 1A1 was prepared from a clone derived from a  $\lambda$ gt11 expression library of human promyelocytic leukemia cells which showed sequence homology to h-lamp-2. Gp130 was affinity purified with mAb Ag11 from a membrane protein fraction of human placenta. Immunoblots of (A) recombinant MBP-fusion protein 1A1, and (B) purified endothelial 130-kD Ag11 membrane protein were incubated with the following primary Abs, and detected with alkaline phosphatase-conjugated secondary Abs: lanes A 1-3, negative controls, using (lane 1) sheep anti-human IgG-alkaline phosphatase conjugate only, (lane 2) sheep anti-rabbit IgG-alkaline phosphatase conjugate, and (lane 3) serum from a healthy human subject; lane A 4, mAb Ag11 as primary Ab; lane A 5, rabbit anti-MBP Ab; lane A 6, rabbit anti-gp130 IgG; lane A 7, ANCA standard IgG; lane A 8, serum of patient no. 15; lane A 9, serum of patient no. 2; lane A 10 rabbit anti-h-lamp-2 IgG; lane B 1, mAb Ag11; lane B 2, rabbit anti-gp130 IgG; lane B 3, ANCA standard IgG; lane B 4, serum of patient no. 2; lane B 5, serum of patient no. 15. The ~50-kD band at the bottom of the lanes is derived from mAb Ag11, and is also found in control experiments.

rabbit anti-gp130 IgG; lane A 7, ANCA standard IgG; lane A 8, serum of patient no. 15; lane A 9, serum of patient no. 2; lane A 10 rabbit anti-h-lamp-2 IgG; lane B 1, mAb Ag11; lane B 2, rabbit anti-gp130 IgG; lane B 3, ANCA standard IgG; lane B 4, serum of patient no. 2; lane B 5, serum of patient no. 15. The ~50-kD band at the bottom of the lanes is derived from mAb Ag11, and is also found in control experiments.



**Figure 3.** Glomerular endothelial staining pattern (arrowheads), obtained by indirect immunofluorescence with the ANCA serum of patient no. 9 (specific for gp130 only, see also Fig. 1, lane 3) in a cryostat section of a normal human kidney. (AA) Afferent arteriole; (C) capillary lumen.  $\times 400$ .

incubated in  $10^{-6}$ ,  $10^{-7}$ , and  $10^{-8}$  M FMLP (Sigma Chemical Co.) for 15 and 30 min at  $37^{\circ}\text{C}$  before fixation in PLP.

## Results

**Immunoblotting of Patient Sera.** When membrane protein fractions of glomeruli or placenta were immunoblotted with ANCA sera of 15 patients with NCGN and with ANCA standard IgG (listed in Table 1), two sera (no. 2 and 15) strongly reacted with a band of apparent molecular mass of 130 kD which was provisionally designated gp130. Other sera (no. 1, 3–9, and 11–13) and the ANCA-standard IgG (no. 16) showed weaker labeling, while two sera (no. 10 and 14) failed to bind gp130 (Fig. 1). All sera used, including ANCA-negative control sera, produced weak unspecific binding to several other variable proteins (Fig. 1). Affinity-purified gp130 was specifically labeled by two selected sera (no. 2 and 15), and by ANCA standard IgG (Fig. 2 B, lanes 3–5). Control sera from normal humans and patients with ANCA-unrelated renal diseases consistently failed to bind gp130 (not shown). When the sera of patients no. 2, 6, and 15 were fractionated on a gp130 affinity column, the resulting gp130-binding IgG fractions specifically immunoblotted to the recombinant fusion protein derived from cDNA clone 1A1 (not shown).

Immunoblots with ANCA sera on membrane protein fractions of isolated human PMNs labeled a sharp band at 170 kD and an additional broad band at 80–110 kD (provisionally designated gp170/80–110). However, they also showed a high background labeling of other variable proteins. Similar findings were obtained when immunoblotting was performed on enriched preparations of gp170/80–110 (not shown).

**ANCA, Antiendothelial Antibodies, and Reactivity with Gp130.** The ANCA pattern of sera from 15 patients with NCGN, and ANCA standard IgG were classified by indirect immunofluorescence on PMNs. ANCA sera were also characterized for their reactivity with endothelial cells (Fig. 3), and

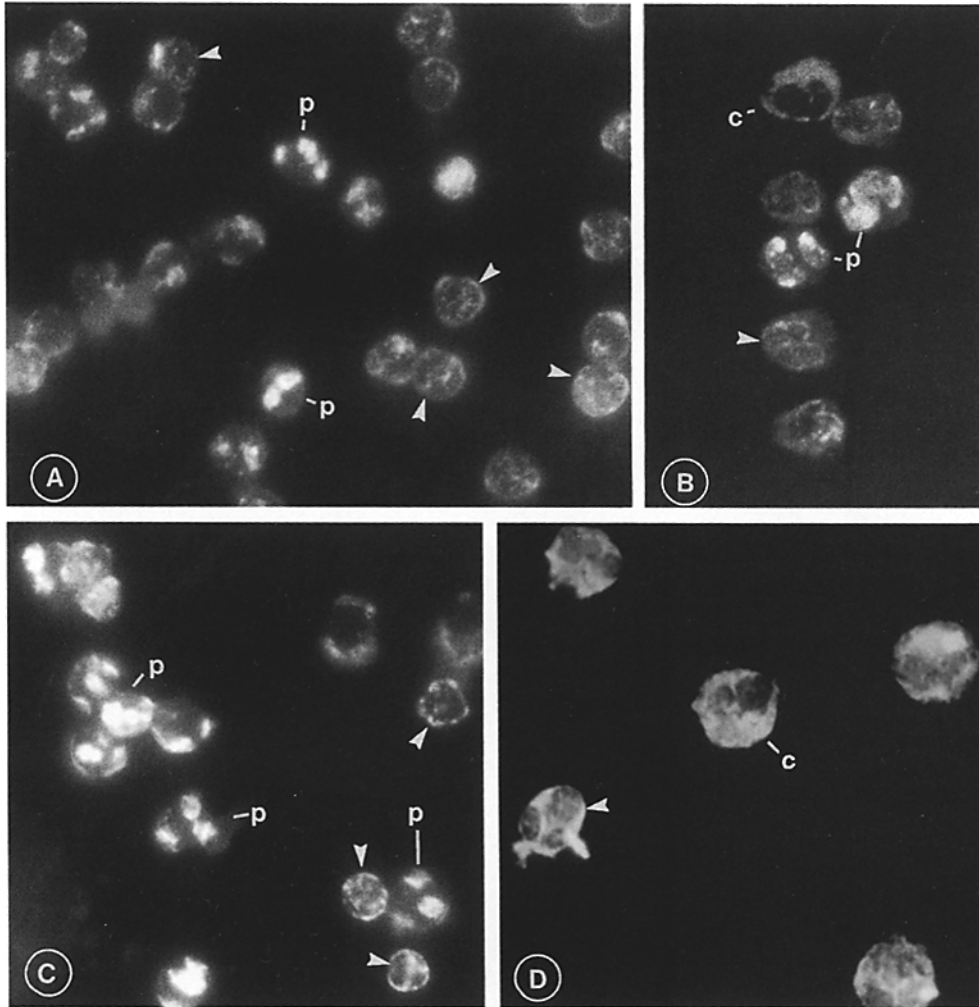
for their antigen specificity by immunoblotting and ELISA. A synopsis of the data is presented in Table 2. Collectively, ANCA sera were divided into four categories: (a) sera specific for gp130 only, with c-ANCA pattern, and mostly with reactivity with endothelial cells (25% of our patients); (b) sera with double specificity to gp130 plus to PR3, with c-ANCA pattern, and mostly labeling endothelial cells (25%); (c) sera with exclusive reactivity with PR3, expectedly showing a c-ANCA pattern and no reactivity with endothelium (12.5%); and (d) sera with mixed reactivity for gp130 and MPO, showing a “mixed c+p ANCA” pattern and mostly binding to endothelial cells (37.5%). Among the ANCA sera in this preliminary study no “pure” p-ANCA pattern was observed, and no serum was found which exclusively reacted with MPO (Table 2).

The mixed c+p ANCA pattern consisted of variable combinations of granular and speckled perinuclear, cytoplasmic, and/or cell surface labeling (Fig. 4, A and C), and was particularly obvious when PMNs were attached onto slides without cytocentrifugation. This mixed ANCA pattern was

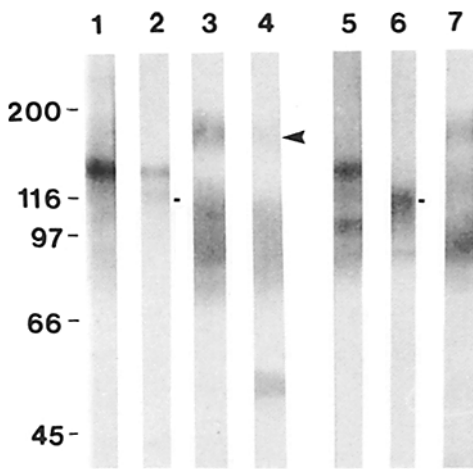
**Table 2.** Correlation of ANCA-pattern with Type of Antigen

Patients	c-ANCA	Mixed c + p ANCA
	AG11/ANCA/PR3/ MPO/endothelium	AG11/ANCA/PR3/ MPO/endothelium
<b>I</b>		
3 W.E.	+ / 0 / 0 / 0	—
7 H.A.	+ / 0 / 0 / 0 / +	—
8 W.F.	+ / 0 / 0 / 0 / +	—
9 B.F.	+ / 0 / 0 / 0 / +	—
<b>II</b>		
4 L.J.	+ / + / 0 / 0 / 0	—
12 S.R.	+ / + / + / 0 / +	—
13 W.L.	+ / + / 0 / 0 / +	—
16 X.Y.	+ / + / + / 0 / +	—
<b>III</b>		
10 F.M.	0 / + / + / 0 / 0	—
14 S.E.	0 / + / + / 0 / 0	—
<b>IV</b>		
1 H.A.	—	+ / 0 / 0 / + / +
2 S.A.	—	+ / 0 / 0 / + / +
5 P.P.	—	+ / 0 / 0 / + / ±
6 K.S.	—	+ / 0 / 0 / + / +
11 K.A.	—	+ / 0 / 0 / + / +
15 G.F.	—	+ / 0 / 0 / + / +

Categories of different patterns of ANCA staining: (I) Sera specific for gp130 only; (II) sera which contain antibodies to gp130 and in addition also to PR3, and/or are positive in the ANCA-ELISA; (III) sera which reacted exclusively with PR3; and (IV) sera with mixed reactivity with gp130 and MPO.



**Figure 4.** Effect of different methods of PMN preparation on the patterns of ANCA labeling on human PMNs by indirect immunofluorescence. PMNs were isolated by dextran fractionation, and were allowed to attach to microscopic slides (A and C), to be compared with PMNs attached by cytocentrifugation (B and D), before fixation with ethanol. Sera used were from patient no. 2, with double specificity for MPO and Ag11 antigenic proteins (A and B), and from patient no. 1, with the same double specificity (C and D). (A) A mixed staining pattern is observed which shows a typical perinuclear labeling, characteristic for the p-ANCA pattern (p), and speckled membrane staining (arrowheads), combined with a cytoplasmic staining resembling the c-ANCA pattern. There is great variability in the patterns among the PMNs. (B) A typical perinuclear p-ANCA pattern in high (p), or lower intensity (arrowhead) prevails among PMNs. (C) In this example a similar staining to A can be detected, whereas the same serum in D stains more in a c-ANCA pattern. Note, that sometimes the nucleus is covered by a "haze" of fluorescence (arrowheads), which is frequently found when Abs to MPO and Ag11 antigenic proteins are combined, but not in c-ANCA sera specific only for PR3 and/or Ag11 antigenic proteins. C, Eosinophilic granulocyte.  $\times 400$ .



**Figure 5.** Identification of membrane protein antigens of the mAbs Ag11 and anti-h-lamp-2 in several tissues, obtained by immunoblotting on membrane protein fractions of human glomeruli (lane 1), placenta (lane 2), PMNs (lane 3), and purified mAb Ag11 antigenic proteins from PMNs

similar to that observed when mAbs specific for gp130 and MPO were combined for indirect immunolabeling of PMNs (not shown).

**Characterization of Monoclonal Ag11 and Rabbit Anti-gp130 Antibodies.** mAb Ag11 (IgG<sub>2a</sub> subclass) was obtained from a mouse that was immunized with a preparation of glomerular membrane proteins. mAb Ag11 was selected because it showed the same specificity as natural ANCA sera, in that

(lane 4). This Ab is specific for a 130-kD protein (gp130) in glomeruli and placenta, and it binds to a 170 and a 80-110-kD proteins (gp170/80-110) in PMNs. (Lanes 5-7) Immunoblots with mAb anti-h-lamp-2, using the same protein preparations as in lanes 1-3. In glomeruli (lane 5) this mAb binds to gp130 and additionally to a 100-kD protein which is not recognized by the Ag11 Ab. In placenta (lane 6) the mAb anti-h-lamp-2 binds predominately to a ~110-kD protein, and in very low intensity also to the 130-kD protein. In PMNs (lane 7) essentially the same proteins are labeled as with the Ag11 Ab. The ~50-kD protein in lane 4 is derived from mAb Ag11 which was used to purify the antigen of PMNs.

**Table 3. Comparison of the Partial Sequences of h-lamp-2 and the AG11-antigen**

<b>EXON 4 (partial).</b>	
1	GAATTCGGGCGCATGAAATGACCTTTTATAGATGCAATAGTTTATCAACTTTTGAAAAGAAATGATGTTGTCACACTACTGGGATGTTCTTGTACAAGCTTTTGTCCAAAATGGCAGTGGACACAAATG 127
130	gAATTC...CCATTGAAATGACCTTTTATAGATGCAATAGTTTATCAACTTTTGAAAAGAAATGATGTTGTCACACTACTGGGATGTTCTTGTACAAGCTTTTGTCCAAAATGGCAGTGGACACAAATG 256
	GluPheGlyProLeuAenAepLeuPheArgCysAenSerLeuSerThrLeuGluLysAenAepValValGlnHisTyrTrpAepValLeuValGlnAlaPheValGlnAenGlyThrValSerThrAen ArgIle
<b>EXON 5</b>	
127	gAGTTCCTGTGTGATAAGGACAAAACCTCAACAGTGGCACCACCACATACACACCCTGTGCCATCTCTACTACAACACTACTCCAAAGGAAAAACCAGAAGCTGGAACCTATTCAAGTAAATAATGGCAATGACTTGT 267
229	gAGTTCCTGTGTGATAAGGACAAAACCTCAACAGTGGCACCACCACATACACACCCTGTGCCATCTCTACTACAACACTACTCCAAAGGAAAAACCAGAAGCTGGAACCTATTCAAGTAAATAATGGCAATGACTTGT 369
	GluPheLeuCysAepLysAepLysThrSerThrValAlaProThrIleHisThrThrValProSerProThrThrThrProThrProLysGluLysProGluAlaGlyThrTyrSerValAenAenGlyAenAepThrCys Pro
268	CTGCTGGCTACCATGGGCTGCAGCTGAACATCACTCAGGATAAG 312
370	CTGCGTGTACCATGGGCTGCAGCTGAACATCACTCAGGATAAG 414
	LeuLeuAlaThrMetGlyLeuGlnLeuAenIleThrGlnAepLys
<b>EXON 6</b>	
313	GTTGCTTCAGTTATTAACATCAACCCCAATAACAACCTCACTCCACAGGCAGCTGCCGTTCTCACACTGCTCTACTTAGACTCAATAGCAGCACCATTAAAGTATCTAGACTTTGTCTTGTCTGTG 435
175	GTTGCTTCAGTTATTAACATCAACCCCAATAACAACCTCACTCCACAGGCAGCTGCCGTTCTCACACTGCTCTACTTAGACTCAATAGCAGCACCATTAAAGTATCTAGACTTTGTCTTGTCTGTG 297
	ValAlaSerValIleAenIleAenProAenThrThrHisSerThrGlySerCysArgSerHisThrAlaLeuLeuArgLeuAenSerSerThrIleLysTyrLeuAepPheValPheAlaVal
<b>EXON 7</b>	
436	AAAAATGAAAACCGATTTTATCTGAAGGAAGTGAACATCAGCATGTATTGGTTAATGGCTCCG 499
203	AAAAATGAAAACCGATTTTATCTGAAGGAAGTGAACATCAGCATGTATTGGTTAATGGCTCCG 266
	LysAenGluAenArgPheTyrLeuLysGluValAenIleSerMetTyrLeuValAenGlySer
<b>EXON 8</b>	
499	gTTTTTCAGCATTCGAAATAACAATCTCAGCTACTGGGATGCCCCCTGGGAAGTTCCTATATGTGCAACAAAGAGCAGACTGTTTCAGTGTCTGGAGCATTTTCAGATAAATACCTTTGATCTAAAGGTTTCAGCCTTTCAAT 639
126	gTTTTTCAGCATTCGAAATAACAATCTCAGCTACTGGGATGCCCCCTGGGAAGTTCCTATATGTGCAACAAAGAGCAGACTGTTTCAGTGTCTGGAGCATTTTCAGATAAATACCTTTGATCTAAAGGTTTCAGCCTTTCAAT 266
	ValPheSerIleAlaAenAenAenLeuSerTyrTrpAepAlaProLeuGlySerSerTyrMetCysAenLysGluGlnThrValSerValSerGlyAlaPheGlnIleAenThrPheAepLeuArgValGlnProPheAen
640	GTGACACAAGAAAGTATTCTACAG 664
267	GTGACACAAGAAAGTATTCTACAG 291
	ValThrGlnGlyLysTyrSerThr
<b>EXON 9</b>	
664	gCTCAAGACTGCAGTGCAGATGACGACAACTTCTGTGCCATAGCCGTTGGGAGCTGCTTGGCAGGAGTACTTATTCTAGTGTGCTGGCTTATTTTATTTGGTCTCAAGCACCATTGCTGGATATGAGCAATTTTAG 804
188	gCTCAAGACTGCAGTGCAGATGACGACAACTTCTGTGCCATAGCCGTTGGGAGCTGCTTGGCAGGAGTACTTATTCTAGTGTGCTGGCTTATTTTATTTGGTCTCAAGCACCATTGCTGGATATGAGCAATTTTAG 328
	AlaGlnAepCysThrAlaAepAepAepAenPheLeuValProIleAlaValGlyAlaAlaLeuAlaGlyValLeuIleLeuValLeuLeuAlaTyrPheIleGlyLeuLysHisHisHisAlaGlyTyrGlnPhe* Ser

Upper lane: sequence of Ag11 antigen; lower lane: sequence of h-lamp-2. These sequence data are available from EMBL/GenBank/DBJ under accession numbers L09709-L09717.

it labeled specifically glomerular and placental gp130 by immunoblotting (Figs. 1, lane 1, 2 B, lane 1, and 5, lanes 1 and 2), and gp170/80-110 in PMNs (Fig. 5, lane 3). The antigen specificity of mAb Ag11 was apparently different from PR3 (molecular mass 29 kD), and MPO (~60 kD).

Polyclonal rabbit Ab obtained by immunization of a rabbit with purified gp130 showed the same characteristics as mAb Ag11 by immunoblotting (Fig. 2 B, lane 2), and by immunohistochemistry (not shown).

**Identification of Gp170/80-110 as h-lamp-2 of PMNs.** Screening of a  $\lambda$ gt11-cDNA expression library of HL60 cells with affinity-purified polyclonal gp130 IgG identified a clone, designated 1A1, with extensive homology to the human lysosomal membrane protein, h-lamp-2, both at the nucleotide and predicted amino acid levels (Table 3). cDNA of 1A1 coded for the entire cytoplasmic and transmembrane domains of h-lamp-2 and for 231 amino acids of the extracellular domain.

A 1A1-derived recombinant MBP fusion protein (37 amino acids of the cytoplasmic and transmembrane domains, and 231 amino acids of the extracellular domain) was expressed, which bound rabbit anti-gp130 IgG (Fig. 2 A, lane 6), but not mAb Ag11 (Fig. 2 A, lane 4). This fusion protein was specifically labeled by ANCA sera of patients no. 3, 6, 13, and 15, and by ANCA standard IgG. Weaker labeling was

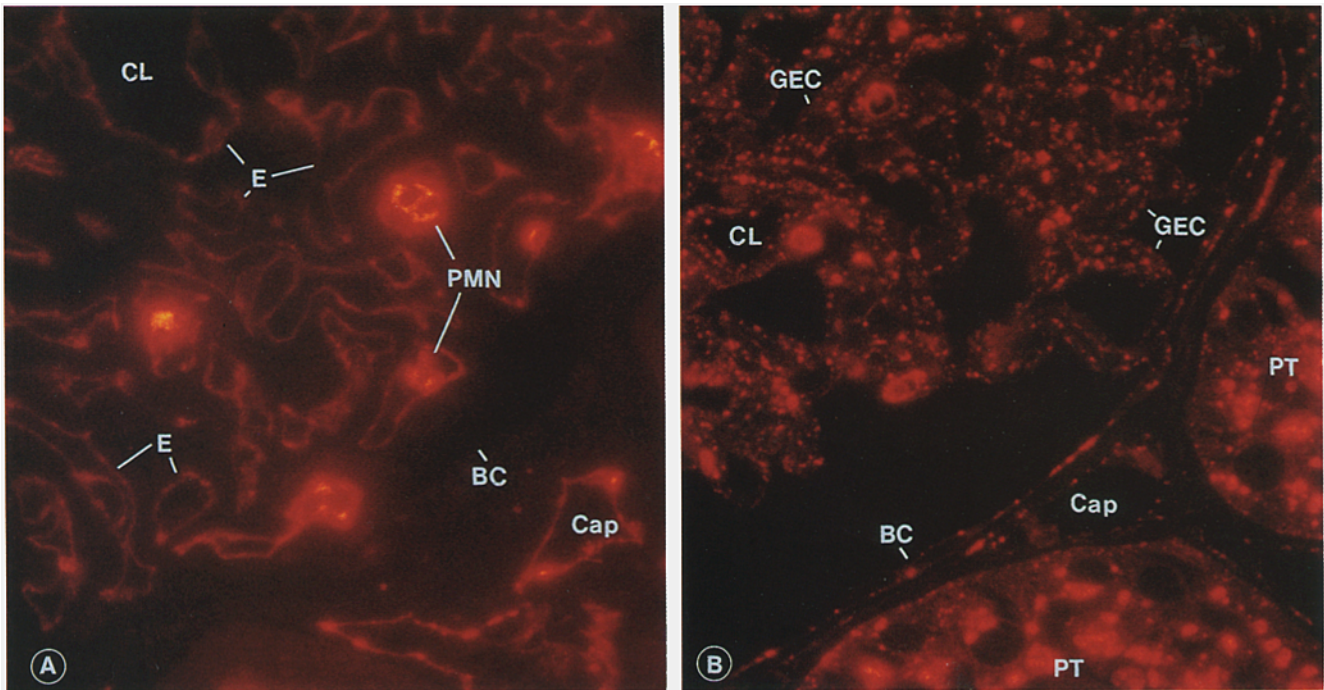
detected by sera of patients no. 1, 2, 4, 7, 8, and 11 (examples are given in Fig. 2 A, lanes 7-9).

When rabbit anti-gp130 Ab was affinity purified, using recombinant fusion protein of clone 1A1, it was found to immunolabel purified gp130 (not shown). Expectedly, 1A1 fusion protein was also labeled by rabbit Ab specific for h-lamp-2 (Fig. 2, A, lane 10). No binding was observed with control Abs (Fig. 2 A, lanes 1-3), and monoclonal anti-h-lamp-2 IgG.

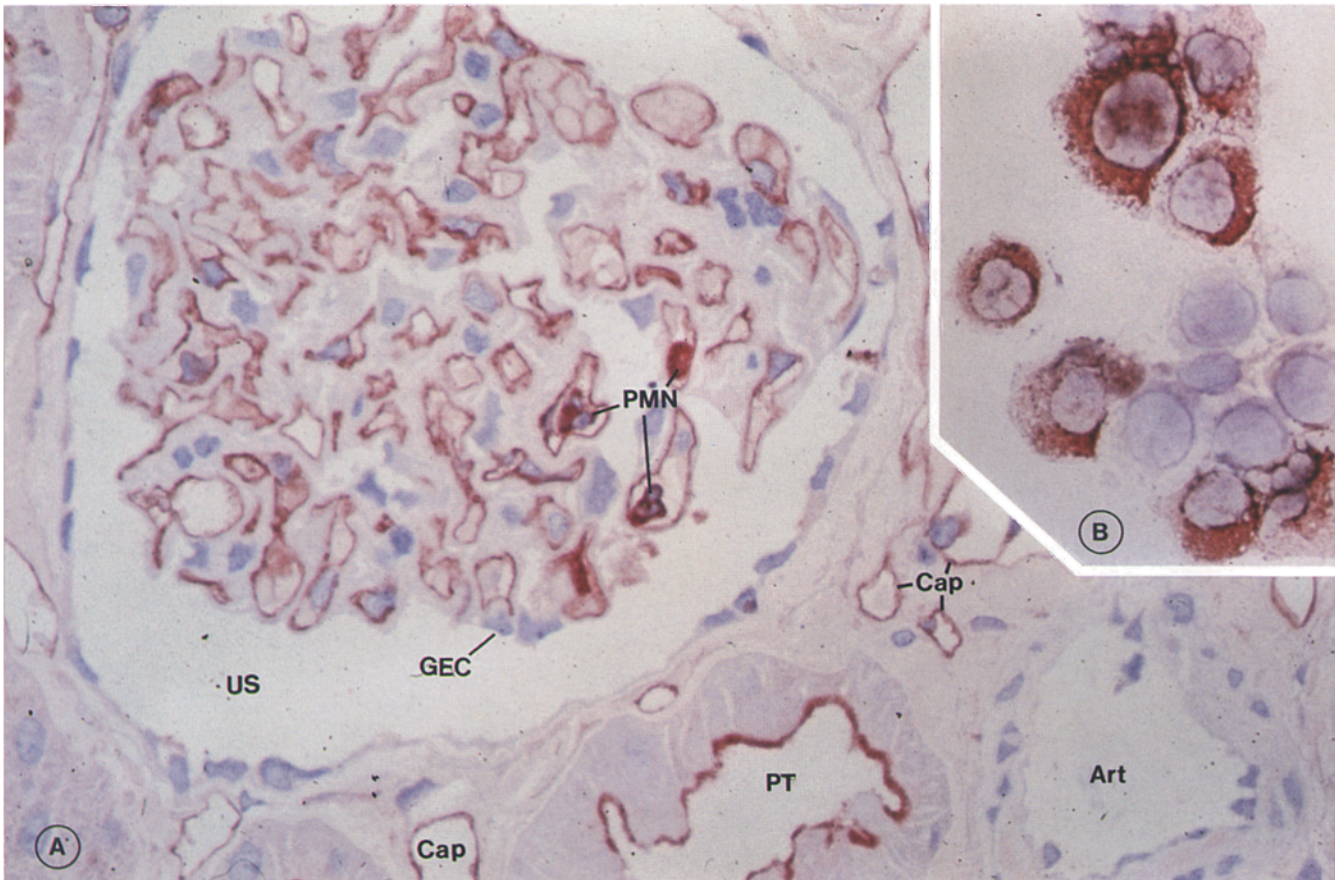
**Immunoblotting and Immunohistochemistry with Anti-h-lamp-2 Antibodies.** An anti-h-lamp-2 mAb specifically immunoblotted gp130 prepared from membrane protein fractions of human glomeruli, similar to mAb Ag11 (Fig. 5, lanes 1 and 5). In samples prepared from placenta, anti-h-lamp-2 Ab detected an additional protein with apparent molecular mass of 110 kD which was barely labeled by mAb Ag11 (Fig. 5, lanes 2 and 6). In preparations of PMN membrane proteins monoclonal h-lamp-2 Ab reacted with gp170/80-100, i.e., the same proteins as mAb Ag11 (Fig. 5, lanes 3 and 7).

In 0.5- $\mu$ m thick frozen sections of normal human kidney (Fig. 6, B) and isolated PMNs (not shown) anti-h-lamp-2 mAb labeled exclusively lysosomes of all cells in a granular pattern.

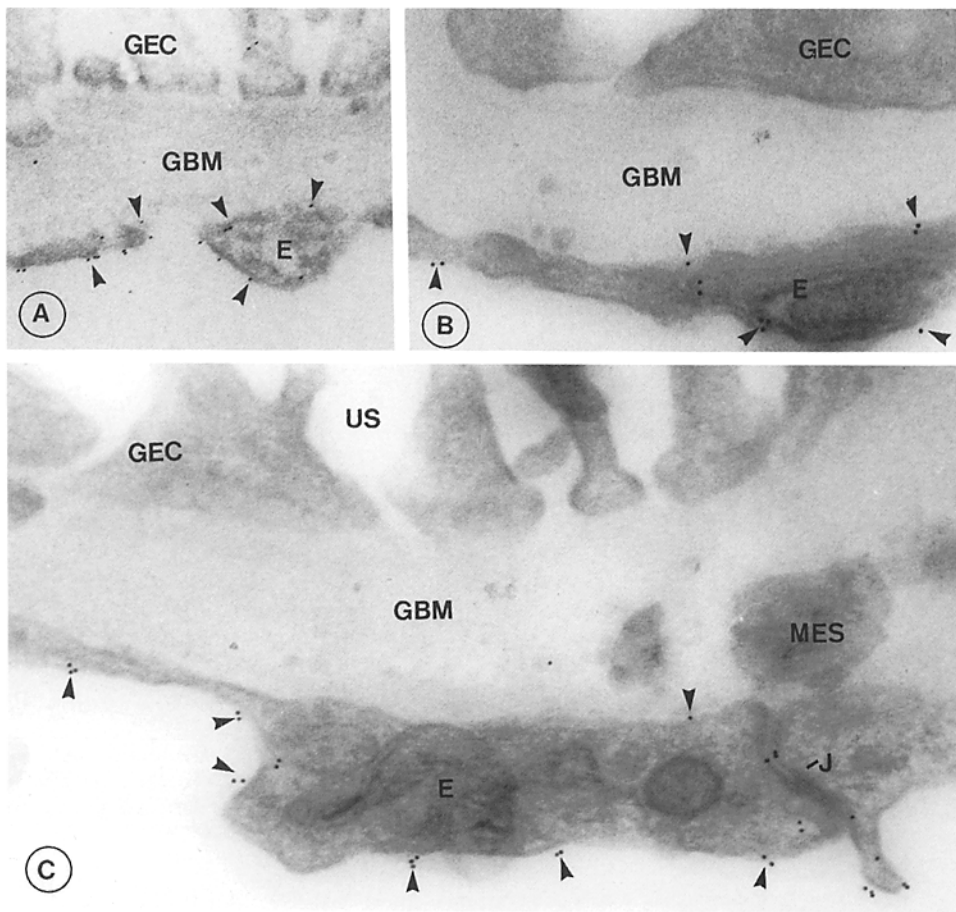
**Localization of Gp130 in Kidney and Other Tissues.** Endothelial cells of glomeruli and of interstitial capillaries, but not



**Figure 6.** Comparative localization of antigens of mAbs Ag11 (A) and anti-h-lamp-2 (B) by indirect immunofluorescence on consecutive 0.5- $\mu$ m-thick frozen sections of normal human kidney. (A) Ag11 labels surface membranes of endothelial cells in glomeruli (E), as well as interstitial capillaries (Cap), and also some trapped PMNs. (B) anti-h-lamp-2 mAb labels lysosomes of glomerular endothelial and epithelial cells (GEC), as well as of epithelia of Bowman's capsule, proximal tubules (PT), and interstitial capillary endothelium (Cap). (CL) capillary lumen; (BC) Bowman's capsule  $\times 400$ .







**Figure 8.** Localization of gp130 in human glomerular endothelial cells by immunogold labeling with mAb Ag11 on ultrathin frozen sections. (A, B, and C) Gold particles (arrowheads) bind exclusively to surfaces of glomerular endothelial cells (E), where they are concentrated at the luminal surfaces in a patchy pattern. Occasionally antigen is also detected at the abluminal side of endothelial cells, and close to intercellular junctions (J). There is no specific labeling of glomerular epithelial cells (GEC), of the basement membrane (GBM), or of extensions of a mesangial cell (MES) in C. (US) Urinary space.  $\times 18,000$ .

of arteries and arterioles, were specifically labeled by immunohistochemistry using mAb Ag11, as most clearly observed in 0.50- $\mu\text{m}$ -thick frozen sections of normal human kidneys (Figs. 6 A and 7 A). Specific and selective endothelial staining was also observed in the capillaries of placenta, and in the brush borders of kidney proximal tubules (Fig. 7 A). In addition, bile canaliculi, type I pneumocytes, and the epithelium of stomach and small intestine were labeled (not shown), but no other organs (heart, lymph node, umbilical cord, skin, skeletal muscle, laryngeal mucosa, and pancreas). Both mAb Ag11 and rabbit anti-gp130 Ab failed to label unstimulated and IL-1-treated cultured human umbilical cord endothelial cells, and they did not cross-react with several tissues from rat, mouse, and monkey (not shown).

By immunoelectron microscopy, mAb Ag11 exclusively labeled the endothelial cell membranes of human glomeruli, predominately on the luminal side, and, to a lesser extent,

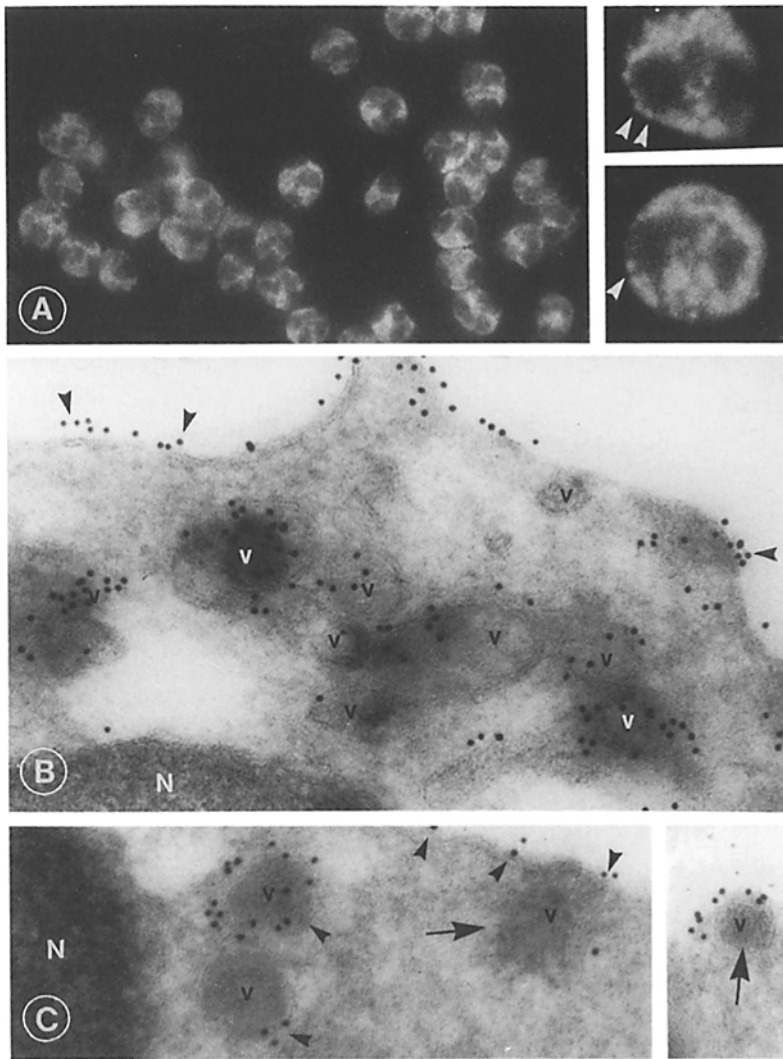
also on the abluminal plasmalemma (Fig. 8). Intracellular staining was not observed.

*Localization of Gp170/80-110 in PMNs and Related Cells.* PMNs which were entrapped within glomerular capillary loops intensely bound mAb Ag11 (Figs. 6 A and 7 A). mAb Ag11 produced a granular cytoplasmic c-ANCA pattern in isolated human PMNs by indirect immunofluorescence (Fig. 9 A).

By indirect immunogold electron microscopy on ultrathin frozen sections of unstimulated, freshly isolated PMNs, most gold particles were found on membranes of granules, but also in small patches on the cell surface. The intra- and extracellular distribution of gold particles was quantified (Table 4), and found to increase towards the surface localization by more than fourfold when isolated PMNs were stimulated by FMLP (Fig. 9, B and C).

In bone marrow smears, mAb Ag11 immunolabeled cells of the promyelocytic stage, but no earlier forms of the my-

**Figure 7.** Immunohistochemical localization gp130 in 0.5- $\mu\text{m}$ -thick frozen sections of a normal human kidney (A), and in DMSO-activated HL-60 cells (B), using mAb Ag11 and an avidin-biotin peroxidase method. (A) Within the glomerulus Ab labels exclusively endothelial cells, as well as some trapped PMNs. In the interstitium, endothelial cells of the capillaries (Cap), but not of a small artery (Art) are specifically labeled. There is also binding to the brush border region of proximal tubuli (PT). (B) The mAb Ag11 labels specifically granules of DMSO-activated HL 60-cells. (A)  $\times 400$ ; (B)  $\times 630$ .



**Figure 9.** Localization of the membrane protein gp170/80-110 on freshly isolated and activated human PMNs. (A) Indirect immunofluorescence on a 0.5- $\mu$ m-thick frozen section of isolated PMNs with mAb Ag11. Granular cytoplasmic staining (arrowheads) is resolved at high magnification. (B) Immunoelectron microscopy on ultrathin frozen sections of PMNs, 10 min after exposure to  $10^{-6}$  M FMLP. The number of gold particles is greatly enhanced when compared to nonactivated PMNs in C, as quantitated in Table 4. A granule is encountered in the process of exocytosis (arrow). Note the 10-nm gold particles both on the cell surface (arrowheads), and on granule membranes. (C) A granule (arrow) in the state of exocytosis which delivers Ag11 antigens at the cell surface. (A)  $\times 400$ ; (B and C)  $\times 18,000$ .

elopoietic lineage. In promyelocytic leukemia, intense granular staining of the tumor cells was observed (not shown), similar to the promyelocytic leukemia cell line HL-60, especially after induction of differentiation by DMSO (Fig. 7 B). Monocytes and lymphocytes were not labeled.

### Discussion

Necroses of single glomerular capillary loops and/or formation of capsular crescents are histopathologic hallmarks of NCGN. Development of these lesions involves, presumably, damage of glomerular endothelial cells and release of toxic products by activated immobilized PMNs, and could be related to the occurrence of ANCA (reviewed in reference 39). Since presentation of autoantigenic protein(s) on cell surfaces to circulating ANCA could be a central pathogenetic event (39-41) the question arises whether integral membrane proteins of PMNs and/or glomerular endothelial cells could serve as targets for ANCA. In this study we have identified gp170/80-110 in PMNs which specifically react with ANCA sera of patients with NCGN. Unexpectedly, these sera also

cross-reacted with an endothelial membrane protein of the renal microvasculature, provisionally designated as gp130. A mAb and rabbit polyclonal Ab with double specificities for gp130 and gp170/80-110 as the natural ANCA were used to further characterize the biochemical nature of the autoan- cross-reacted with an endothelial membrane protein of the

**Table 4.** Quantification of AG11-antigens on the Surface Membrane of Normal and Activated PMNs

	Number of PMN	$\mu$ m counted	Gold particles per $\mu$ m membrane
Not stimulated	22	130	$1.5 \pm 1.9$
FMLP stimulated	22	126	$5.4 \pm 2.8$

The difference of the number of gold particles per  $\mu$ m membrane between the control and the stimulated group is highly significant ( $p > 0.0001$ , Student's *t* test)

renal microvasculature, provisionally designated as gp130. A mAb and rabbit polyclonal Ab with double specificities for gp130 and gp170/80-110 as the natural ANCA were used to further characterize the biochemical nature of the autoantigenic membrane proteins, to define their localization, and to examine their possible clinical relevance.

Several results provided evidence that gp170/80-110 is identical with h-lamp-2 of PMNs. This conclusion was based on the findings that gp170/80-110 specifically reacted with a mAb to h-lamp-2, its amino acid sequence was identical with that of h-lamp-2, it was localized in the membranes of PMN granules, and anti-h-lamp-2 Ab and anti-gp130 IgG immunolabeled the same membrane proteins in HL-60 cells.

The major lysosomal membrane glycoproteins from human and other species (42-46) are transmembrane proteins with extensive and unique glycosylation, and they were thought to protect lysosomal membranes from degradation by enzymes. It is now established that h-lamp-2 is the prototype of a rapidly growing family of structurally related glycoproteins (leukosialin [47] lamp-1, lamp-3 [CD63], and limp II [reviewed in reference 45]) which shows a wide range of diverse functions, including pathological processes, such as formation of metastasis of colonic carcinoma (48) and increased PMN surface expression in scleroderma (49). Gp170/80-110 is primarily a resident of granule membranes in PMNs, but is inserted into the plasma membrane by exocytosis during PMN activation, as determined by quantitative immunocytochemistry. Similar relocations are known for other granule membrane proteins, such as Mac-1, LFA-1, and p150/95 (50) or lgp-A (51), and for h-lamp-2 in activated platelets (52). Thus, activation of PMNs could expose large amounts of gp170/80-110 on their surfaces, and make them available for binding of ANCA *in vivo* and *in vitro*. Since lamp-related glycoproteins were recently identified as counter-receptors for selectins which are essential for attachment of inflammatory cells to endothelium (45, 53), it is possible that ANCA sera which contain anti-h-lamp-2 Abs interfere with this process.

Current concepts of the pathogenesis of glomerular lesions in NCGN postulate exposure of ANCA antigens on the surfaces of PMNs and endothelial cells (39). A major obstacle for these hypotheses so far was lack of expression of respective antigens on endothelial cells *in vivo*. Here we report that the ANCA antigen gp130 is expressed selectively on microvascular endothelial cell surfaces in human kidneys *in vivo* (and *in vitro*, our unpublished data). This localization could account for the concentration of microvascular lesions to glomerular capillaries, and explain also the occurrence of anti-endothelial Abs in NCGN.

To establish a relation between gp130 and h-lamp, Abs against both proteins were tested for their cross-reactivity by immunopurification. Polyclonal anti-gp130 Ab, purified on recombinant fusion protein from clone 1A1 yielded an IgG fraction that immunostained the same structures as Abs against h-lamp-2. Since gp130 reacted with Abs to h-lamp-2 by immunoblotting, it was intriguing that it was localized at the endothelial cell surfaces rather than in lysosomal membranes where h-lamp-2 is located. One possible explanation is that the 130-kD antigen is structurally not identical with h-lamp-2, that it shares only one or several epitopes, and that it is directly targeted to the cell surfaces of endothelial cells. Another simple explanation is that the 130-kD membrane protein is an isoform of h-lamp-2 which differs primarily in its carbohydrate side chains, and that mAb Ag11 is specific for epitopes that are available on endothelial cell surface only. Distinction between these explanations will be possible when the complete amino acid sequence and structure of carbohydrate side chains of the 130-kD membrane protein will be known.

Close association of ANCA with diseases causing NCGN is now well established (3). Types of ANCA patterns obtained by indirect immunofluorescence on PMNs, and presence of Abs to MPO or PR3 were found to correlate with the type of the underlying disease, and with the activity of NCGN (3, 39, 40). In a preliminary trial we have assessed the frequency at which ANCA sera of patients with NCGN contained IgG specific for gp130 and gp170/80-110 and we found that 14 out of 16 contained such Abs. The presence of these Abs also correlated with the histopathology of kidney biopsies, in that they were exclusively associated with recent, necrotizing glomerular lesions. Selected ANCA sera were also purified on affinity columns containing gp130 and were shown to bind to the recombinant fusion protein of the h-lamp-2-derived clone 1A1, thus confirming specific reactivity of ANCA with h-lamp-2. In addition, the combined immunoblotting and ELISA findings indicated that a relatively large proportion of ANCA sera contained a mixture of Abs specific for gp170/80-110 and gp130, plus for MPO or PR3.

Collectively, the results of this study establish a novel role for h-lamp-2 as autoantigenic targets of ANCA in PMNs, and for a structurally related, immunologically cross-reactive 130-kD endothelial membrane protein in renal glomeruli. Location of these membrane proteins on the cell surface raises the intriguing possibility that they could contribute to cell activation by ANCA, and to direct cross-linking of PMNs to glomerular endothelial cells.

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