

# Kringle 4 of human apolipoprotein[a] shares a linear antigenic site with human catalase

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**Abstract** Monoclonal antibody (mab) 1A2, directed against human apolipoprotein[a] (apo[a]), revealed a strong reaction with peroxisomes as shown by immuno-gold labeled cryosections of human liver biopsies. This reactivity was not due to the presence of apo[a] in peroxisomes but to a cross-reactivity of mab 1A2. Immunoblot analysis of peroxisomal fractions and purified human catalase demonstrated that mab 1A2 reacts with catalase. Conversely, an anti-catalase antibody also recognized apo[a]. By sequence comparison we identified a 4-amino acid motif (YY-P-N) that is shared between the highly repetitive kringle 4 motif of apo[a] and the carboxy-terminal third of the peroxisomal marker enzyme catalase. No other identical sequences were identified in these proteins. Results from the following experiments indicated that 1A2 recognizes this short linear epitope. *i*) Mab 1A2 reacted only with the 4 amino acid peptide sequence in a pin-ELISA using immobilized overlapping peptides. *ii*) A synthetic peptide including this sequence completely inhibited the 1A2 immunoreactivity to apo[a] and catalase. *iii*) A recombinant fusion protein tagged with the putative epitope was recognized by mab 1A2. Our findings demonstrate that unknown linear epitopes in native proteins can be identified by sequence comparison between known proteins. The practical implication is that antibodies against apo[a] must be controlled for this cross-reactivity before using them for immunohistochemical studies of intracellular apo[a] in tissues or cells. — Dieplinger, H., G. Gruber, K. Krasznai, S. Reschauer, C. Seidel, G. Burns, H.-J. Müller, A. Császár, W. Vogel, H. Robenek, and G. Utermann. Kringle 4 of human apolipoprotein[a] shares a linear site with human catalase. *J. Lipid Res.* 1995. 36: 813–822.

**Supplementary key words** monoclonal antibody • cross-reaction • epitope • sequence alignment

Monoclonal antibodies (mabs) have been extensively used for immunochemical analysis of antigens. The exact epitope recognized by a mab on a native protein is difficult to characterize as most mabs obtained from immunizations with the whole antigen bind conformational epitopes (for a review, see ref. 1). Recently, X-ray diffrac-

tion analysis of crystallized complexes of antibodies bound to their antigens revealed the exact and complete structure of epitopes in a few cases (2, 3). These epitopes occupied extensive areas comprising 15–22 amino acids from different parts of the protein. Epitope mapping studies with large panels of synthetic peptides and monoclonal antibodies supported the concept of conformational, discontinuous epitopes (4). In contrast, there is still a widespread conception in the literature that epitopes on native proteins consist of linear segments of about 6 amino acids that can be mimicked by synthetic peptides of similar length (5). The studies that have revealed this concept have been criticized because immunochemical reactions of peptides were compared with those of the whole protein without the guarantee of investigating the protein in its native form (e.g., by means of ELISA analysis). It has been claimed that antibodies that recognize peptides react with denatured rather than native proteins (1). If true, it is therefore often impossible to define precisely the native structure of an antigenic epitope without crystallographic analysis.

In the present report we describe a monoclonal antibody (mab) raised against human plasma apolipoprotein[a] (apo[a]) that cross-reacts with the intracellular enzyme catalase, a marker protein for peroxisomes. Apo[a] is the specific, high molecular weight protein component of the atherogenic plasma lipoprotein[a] (Lp[a]) (for a review

Abbreviations: apo, apolipoprotein; mab, monoclonal antibody; GBP, galactose binding protein.

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see ref. 6). It consists of tandemly repeated kringle structures, each stabilized by three intramolecular disulfide bridges, and of a protease domain. For sake of clarity, we used the nomenclature of Guevara et al. (7) to describe the primary structure of apo[a]. According to these authors, the repetitive kringle 4 domain of apo[a] is formed by ten basic kringles deemed kringle 4 type 1 through 10 with the type 2 being present in multiple copies. Detailed characterization revealed an identical linear epitope of four continuous amino acid residues on both antigens. Our findings demonstrate the occurrence of a strongly antigenic linear epitope on two native proteins.

## METHODS

### Antibodies

Mab 1A2 was obtained by immunizing mice with apo[a] (phenotype S2) which was prepared from Lp[a] by reductive cleavage with dithiothreitol, as described elsewhere (8). The same apo[a] preparation was used to produce a polyclonal rabbit antiserum. Polyclonal antisera against human and bovine catalase were produced by immunizing rabbits with the respective commercially available proteins (Sigma, St. Louis, MO). Anti-bovine-catalase reacts specifically with human catalase of HepG2 cells as shown by immunoblotting and immunohistochemistry (M. Hüttinger, personal communication).

### Electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis under reducing conditions was performed according to Neville (9). Acrylamide concentrations, 6.6% or 10%, were chosen in the separation gels for analysis of apo[a] or catalase, respectively. Electrophoresis of liver homogenates and subcellular fractions was carried out using gradient gels with 6.6–15% polyacrylamide concentrations. Immunoblotting was performed according to Towbin, Staehlin, and Gordon (10) using mab 1A2 and polyclonal anti-catalase and anti-apo[a] antibodies for detection of the respective antigens. Alternatively, electrophoresis was performed on non-denaturing or SDS-containing gels using the PHAST system of Pharmacia (Uppsala, Sweden). This system was chosen to investigate the immunoreactivity of mab 1A2 to apo[a] in its native (Lp[a]-associated) form. Samples of purified Lp[a] were separated on 4–15% native or SDS-containing PHAST gels under reducing or nonreducing conditions according to the manufacturer's protocol. Electrophoretic transfer was performed by semi-dry blotting in a TE 70 SemiPhor Semi-Dry Transfer Unit (Hofer Scientific Instruments, San Francisco, CA) (11) after the small PHAST gels had been removed from their plastic support with a 0.25-mm thin metal wire. Immunodetection by mab 1A2 was performed as described previously.

As electrophoresis on PHAST gels allows a comparatively lower protein loading, the more sensitive chemiluminescence ECL detection system (Amersham Life Science) was used instead of the peroxidase substrate chloro-naphthol.

### Quantification of Lp[a] by ELISA

This analysis was carried out as previously described (12), with modifications. Briefly, a two-site enzymeimmuno-metric technique was applied using affinity-purified rabbit anti-apo[a] as capture antibody followed by incubation with mab 1A2 and detection with peroxidase-labeled rabbit anti-mouse antibody (Dako A/S, Denmark) for detection. A commercially available Lp[a]-positive plasma (Immuno, Vienna, Austria) served as standard.

### Immunocytochemistry

Immunolabeling of ultrathin frozen sections from human liver biopsies was performed as described by Griffiths et al. (13). Briefly, liver tissue pieces of 1 mm<sup>3</sup> size were fixed with 8% formaldehyde immediately after needle biopsies were taken. After cryoprotection with 2.3 M sucrose, the specimens were mounted on copper blocks and cut into 50–100 nm thick sections with a diamond knife in a cryo ultramicrotome (Ultracut, Reichert, Vienna, Austria). Sections were then transferred to Formvar-coated copper grids for immunogold labeling using mab 1A2 against apo[a] and a polyclonal rabbit antibody against catalase which were detected and visualized by Protein A-gold (14) in the electron microscope. The incubation with mab 1A2 was followed by treatment with a rabbit antibody to mouse immunoglobulins (Cappel, Organon Teknika, Turnhout, Belgium) to be recognized by Protein A.

### Homogenization and subcellular fractionation of human liver tissue

One g of normal human liver tissue was obtained from resections of liver tumor operations in accordance with the faculty's ethics commission. Tissue was homogenized in 250 mM sucrose, 3 mM imidazole, pH 7.4, with two strokes in a Teflon-piston homogenizer. Homogenates were subjected to density gradient ultracentrifugation on a 15–40% metrazimide gradient (in homogenization buffer) at 30,000 rpm and 4°C in a VTi 65.1 vertical rotor (Beckman Instruments, Palo Alto, CA) (15). Marker enzymes for peroxisomes, endoplasmic reticulum, and Golgi complex (catalase, NADPH-cytochrome c-reductase, and UDP-galactose:glycoprotein galactosyltransferase) were measured by standard techniques in subcellular fractions (16–18).

### Sequence analysis

Published sequences of human apolipoprotein[a], human catalase, rhesus apolipoprotein[a], and human plasminogen were compared by sequence alignment (19–22).

Analysis of the antigenic properties of the kringle 4 motif (Kyte-Doolittle hydrophilicity index, surface probability, flexibility, Jameson-Wolf antigenic index) was performed with the Sequence Analysis Software Package from GCG Inc. using the programs "Peptide Structure" and "Plot-Structure" (23).

### Epitope analysis

i) *Pin-ELISA*: The epitope on apo[a] recognized by mab 1A2 was defined by ELISA using overlapping dodecapeptides covalently bound to polyacrylic acid-coated polyethylene rods (pin-ELISA) (24). Peptides spanning the whole kringle 4 type 2 sequence as well as peptides from kringle 4 type 9 and 10, kringle 4 and the protease domain differing only by one amino acid (therefore overlapping 11 residues) were bound to the rods. The pin-ELISA was carried out by Dr. R. Melen, Centraal Diergeneeskundig Instituut (CDI-DLO), 8200 AB Lelystad, Holland.

ii) *Inhibition studies with synthetic peptides*: Synthetic dodecapeptides encompassing the sequence identical between apo[a] and catalase were purchased from Research Genetics (Huntsville, AL). Peptide 1 consisted of residues 44–55 from kringle 4 type 2 of apo[a] (H-S-R-T-P-E-Y-Y-P-N-A-G), peptide 2 consisted of the respective sequence 389–400 from plasminogen (H-Q-K-T-P-E-N-Y-P-N-A-G). The inhibitory potency of these two peptides on 1A2 was investigated by the ELISA and immunoblotting techniques described above. In the ELISA, Lp[a] concentration was measured in five different plasma samples. Antibody 1A2 was preincubated at different protein ratios (1:1, 1:10, 1:50, 1:100) with the two peptides for 30 min at room temperature prior to its use in the ELISA and immunoblot. Incubations without preincubated peptides served as positive controls.

iii) *Expression and characterization of recombinant fusion peptides*: Standard cloning techniques (25) were used to construct derivatives of plasmid pVB2 (26) that encode galactose binding protein (GBP) fusion peptides differing by the presence (p-GBP-apo[a]) or absence (pGBP) of the putative apo[a] epitope YY-P-N at the carboxy-terminal end. For the construction of pGBP-apo[a], synthetic oligonucleotides encoding 11 amino acids from the apo[a] sequence were introduced at the 3' end of the GBP-encoding *mgIB* gene in plasmid pGBP. After transformation into *Escherichia coli* strain RM-82 (27), bacterial clones containing the plasmids were grown overnight at 30°C in DYT medium. Periplasmatic proteins were released from the cells by osmotic shock (28) prior to analysis by SDS-PAGE and immunoblotting using monoclonal antibody 1A2.

## RESULTS

### Reactivity of mab 1A2 with plasma proteins

This antibody of the IgG 1 subtype has previously been shown in immunoblots to be specific for apo[a] from

whole plasma (29). In particular, 1A2 does not cross-react with plasminogen, in contrast to another mab against apo[a] (2A1) that was obtained by immunization with the same apo[a] (not shown). 1A2 also recognizes apo[a] in plasma samples from rhesus monkeys (not shown). Furthermore, this antibody recognizes apo[a] from plasma and Lp[a] preparations not only in the native (i.e., as Lp[a]) and denatured forms, but also in free and apoB-associated forms (Fig. 1). We have, therefore, been using this antibody in our laboratory for phenotyping apo[a] by immunoblotting and for quantifying apo[a] by ELISA or DELFIA (12, 29, 30).

### Immunocytochemistry

Immunolabeling of ultrathin sections of human liver tissue with mab 1A2 was initially performed to study the intracellular localization and metabolism of apo[a]/Lp[a]. Surprisingly, this resulted in a massive labeling of peroxisomes whereas no significant labeling was observed in other subcellular compartments (Fig. 2A). In a control experiment, comparable labeling was achieved by a polyclonal antibody against catalase, the marker enzyme of peroxisomes (Fig. 2B). Peroxisome-specific labeling was further demonstrated by comparison with labeling of liver sections from a patient with the peroxisome-deficient Zellweger syndrome (Fig. 2C, D). We found no peroxisome-specific labeling with either mab 1A2 or anti-catalase on these sections; label was distributed over the cytosolic areas. This distribution was comparable to the immunolocalization of peroxisomal matrix proteins (e.g.,

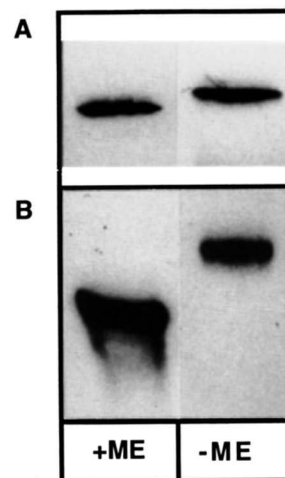
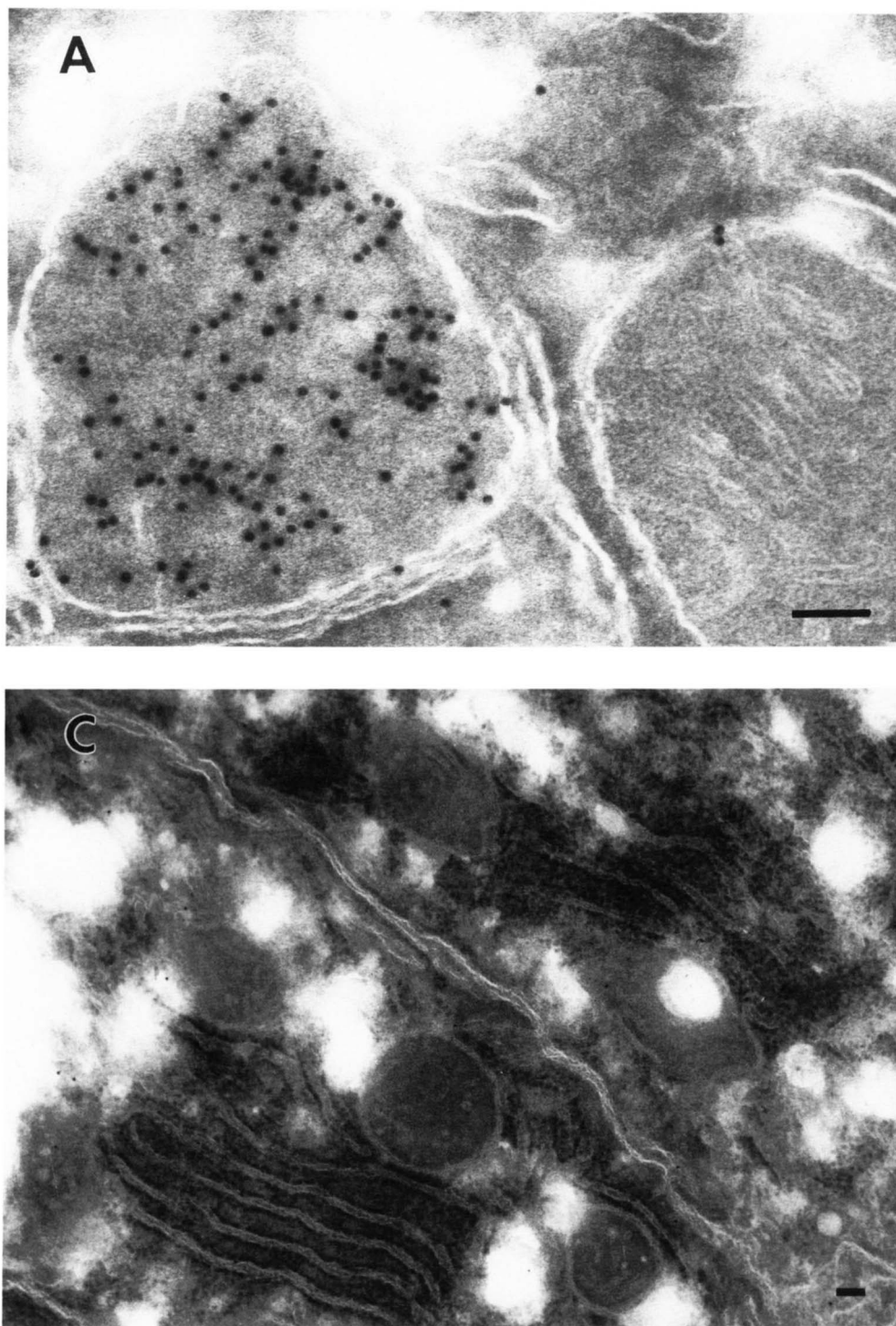
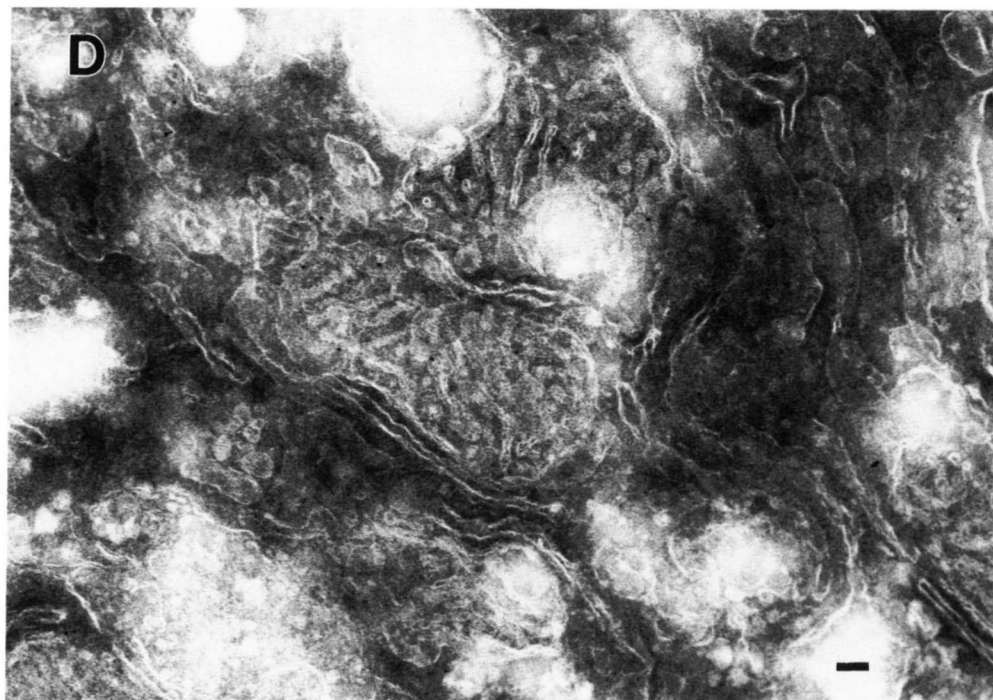
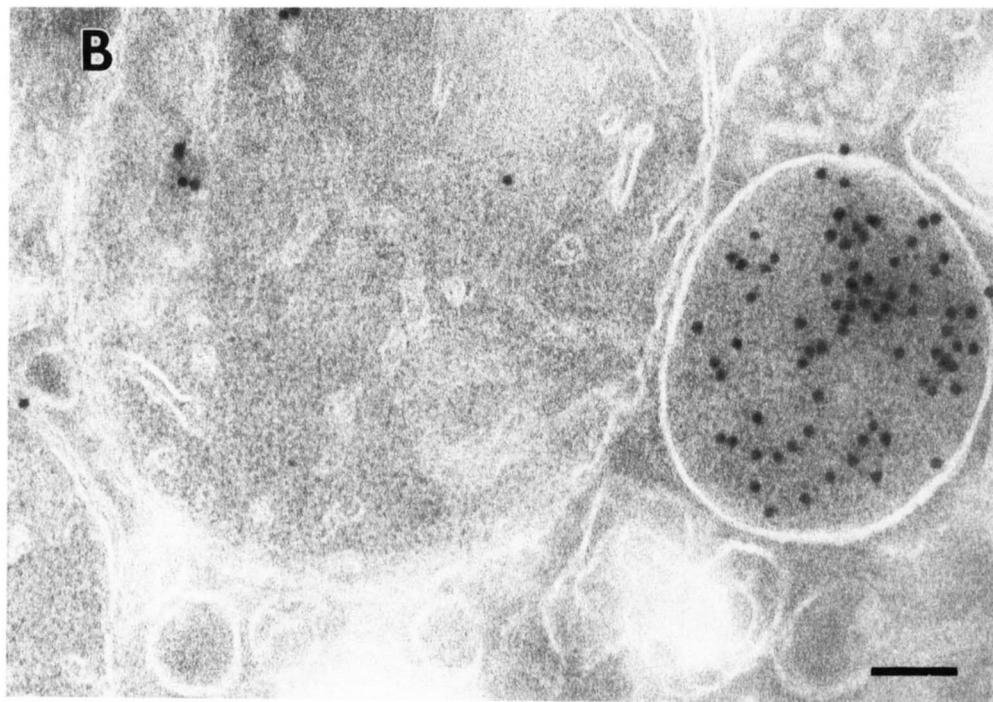


Fig. 1. Immunoreactivity of mab 1A2 to apo[a] under non-denaturing or denaturing conditions. Purified Lp[a] was subjected to PHAST polyacrylamide gel electrophoresis under non-denaturing (panel A) and SDS-containing (denaturing) conditions (panel B). Samples were either non-reduced (-ME) or had been reduced by  $\beta$ -mercaptoethanol prior to electrophoresis (+ME). Apo[a] bands were separated with different sizes under denaturing and, to a lesser extent, under non-reducing conditions because apo[a] is complexed to apoB under non-reducing conditions.



**Fig. 2.** Immunogold labeling of ultrathin frozen sections of human liver tissue. Ultrathin frozen sections of a liver biopsy from a healthy donor (A, B) and a patient with Zellweger syndrome (C, D) were incubated with mab 1A2 (A, C). Alternatively, sections were incubated with polyclonal rabbit anti-human catalase antibody (B, D) to identify peroxisomal compartments. After incubation with Protein A-gold the sections were examined in the electron microscope (14). Bar, 100 nm.



catalase) in liver sections from patients with Zellweger syndrome and controls (31) and also suggested that the immunoreactivity was localized in peroxisomes.

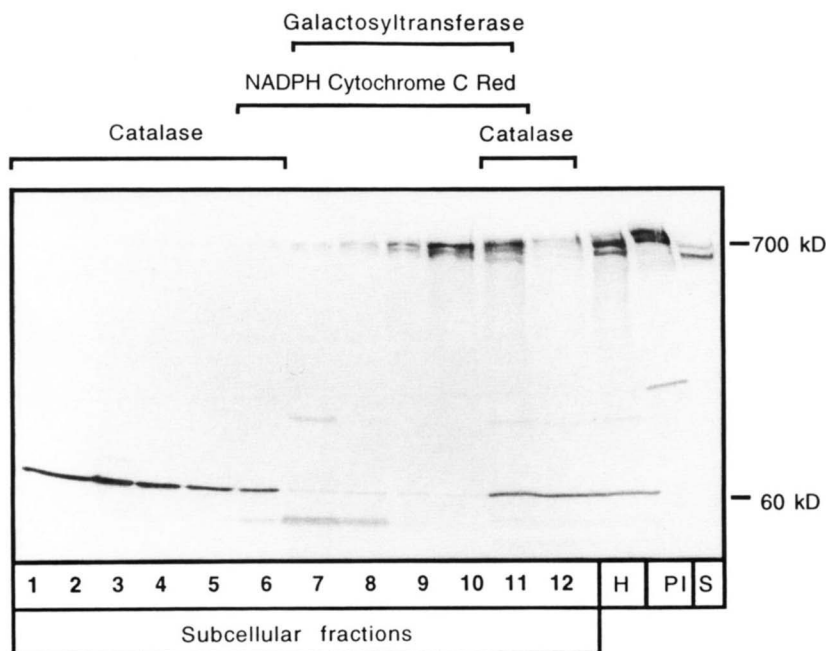
### Cell fractionation, electrophoresis, and immunoblotting

In order to identify the peroxisomal protein(s) recognized by mab 1A2, homogenates and subcellular fractions of human liver were analyzed by SDS-PAGE and immunoblotting with mab 1A2. In homogenates, 1A2 detected proteins in the molecular mass range of apo[a] as well as a protein with a mass of approximately 60 kDa which reacted strongly on the blot (Fig. 3). In the size-range of apo[a], two bands were observed, one representing the intracellular apo[a] precursor and the other the mature protein. This observation is in agreement with similar findings in a transfected mammalian cell line expressing a recombinant form of apo[a] (32). We then separated the cell homogenate into subcellular density fractions and discovered the immunoreactive 60 kDa protein predominantly in the dense fractions which contain peroxisomes as characterized by catalase assay. Because the size of the immunoreactive band corresponded to the known mass of catalase, we tested apo[a]- and human catalase-containing immunoblots with antibodies against

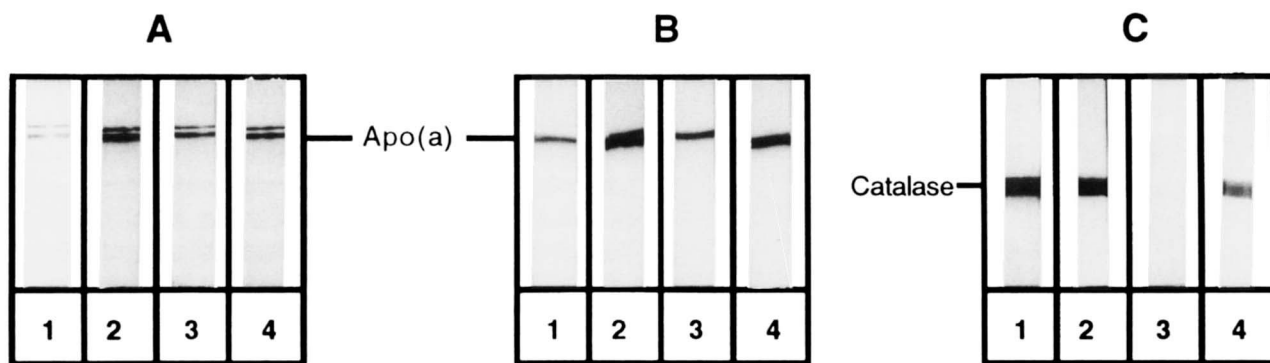
human catalase and apo[a] (Fig. 4). Anti-catalase reacted with the same 60 kDa protein previously detected by mab 1A2 as well as with the high mass apo[a] protein bands. To further confirm a possible cross-reactivity between apo[a] and catalase, we performed immunoblots of commercially available catalase with anti-catalase, mab 1A2, and a polyclonal antibody against apo[a]. Mab 2A1 served as a negative control. Fig. 4C shows strong reactivities of anti-catalase, 1A2, and the polyclonal antibody with catalase whereas mab 2A1 did not react with the enzyme. The experiments shown in Figs. 3 and 4 were repeated by using anti-bovine instead of anti-human catalase; the same cross-reactivity between 1A2 and anti-catalase was observed (not shown).

### Sequence comparison

We then aligned the amino acid sequences of the apo[a] and catalase molecules (19, 20) to see whether they share a linear sequence that could explain the cross-reactivity of mab 1A2. The result of this alignment is shown in Fig. 5: within 12 amino acids a linear stretch of 4 amino acids (YYPN) was found to occur in both sequences. This tetrapeptide is located on kringles 4 type 2, 3, 4, 6, 7, and 8 of human apo[a] (amino acids 50-53) and the carboxy-terminal third of human catalase (amino acids 403-406).



**Fig. 3.** Immunoreactivity of mab 1A2 with intracellular proteins of human liver tissue. Tissue from a human liver biopsy sample was homogenized and fractionated into subcellular fractions by density ultracentrifugation. Fractions containing peroxisomes, endoplasmic reticulum, and Golgi complex were identified by their respective marker enzymes (catalase, NADPH-cytochrome c-reductase, and UDP-galactose:glycoprotein galactosyltransferase, respectively). Subcellular fractions (1-12, from dense bottom to light top fractions), the postnuclear supernatant of homogenized (H) human liver tissue, a plasma sample from the donor of the liver (PI), and a human plasma standard with apo[a] phenotype SIS2 (S) were subjected to 6.6-15% SDS-PAGE under reducing conditions and immunoblotted with mab 1A2.



**Fig. 4.** Cross-reactivity of mab 1A2 and anti-human catalase. Human Lp[a]-positive plasma of apo[a] phenotype S1S2 (panel A) and purified Lp[a] from an Lp[a]-positive donor of the S1 phenotype (panel B) were separated by 6.6% SDS-PAGE. Purified human catalase was subjected to 10% SDS-PAGE (panel C, all of them under reducing conditions). After electrophoretic transfer nitrocellulose, blots were incubated with a polyclonal antibody to catalase (lanes 1), mab 1A2 (lanes 2), mab 2A1 (lanes 3), and a polyclonal antibody to apo[a] (lanes 4). 2A1 does not react with catalase. This monoclonal antibody cross-reacts with plasminogen (Fig. 1) and is therefore directed against an epitope different from the epitope recognized by 1A2.

One neighboring additional amino acid (P, amino acid 48 in apo[a]) is also identical in both proteins.

As mab 1A2 also recognizes apo[a] of rhesus monkeys but does not recognize human plasminogen (Figs. 1, 2), we also compared the respective sequences of rhesus apo[a] and human plasminogen in the same box of 12 amino acids (Fig. 5) (21, 22). One of the kringle 4 domains (K34) of rhesus apo[a] includes the YYPN motif, whereas in most other kringle 4 domains this sequence is changed to NYPN. This latter 4 amino acid motif is identical to the respective sequence of human plasminogen. When comparing human apo[a] and plasminogen, only one within 10 amino acids is different (Y50 in apo[a] versus N395 in plasminogen).

Computer-assisted structure prediction revealed that the identified sequence stretch on kringle 4 exerts high hydrophilicity, surface probability, and antigenicity which suggests a highly immunoreactive site.

### Epitope mapping

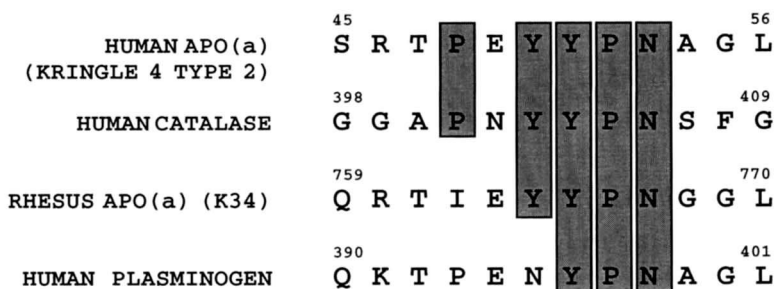
These experiments were performed to directly establish that the shared sequence represents the linear epitope recognized by mab 1A2. 9 overlapping dodecapeptides reacted with mab 1A2 in the pin-ELISA (data not shown), indicating that the reactive epitope is formed by the

4-residue stretch YYPN that is shared between apo[a] and catalase.

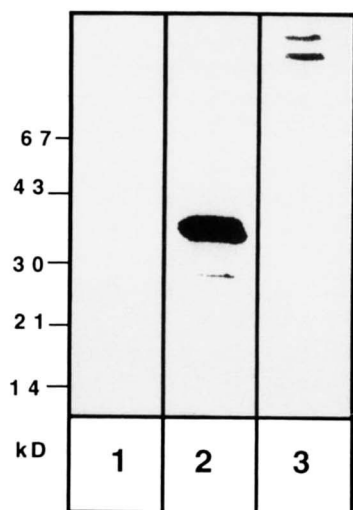
Two synthetic peptides containing the cross-reactive epitope of apo[a] (peptide 1: H-S-R-T-P-E-YYP-N-A-G), and the comparable sequence of plasminogen (peptide 2: H-Q-K-T-P-E-NYP-N-A-G) were used in inhibition studies. 1A2 immunoreactivity was inhibited by peptide 1, but not by peptide 2 in immunoblots of apo[a] from plasma and purified Lp[a] and of catalase (data not shown). Peptide 1 but not peptide 2 could totally inhibit also the immunoreactivity in ELISA-based Lp[a] quantification of five different plasma samples (data not shown). These experiments revealed that recognition of apo[a] in plasma samples by antibody 1A2 could be totally inhibited by peptide 1 irrespective of the antigen presentation in denatured (immunoblots) or native (ELISA) form.

### Expression and characterization of a fusion protein including the putative epitope

Galactose binding protein (GBP) and a fusion peptide containing 11 amino acids (S-R-T-P-E-YYP-N-A-G) of apo[a] at the carboxy-terminus of GBP (GBP-apo[a]) were expressed into the periplasma of bacteria. No significant difference was observed in the expression level of GBP and the GBP-apo[a] fusion peptide (data not



**Fig. 5.** Sequence alignment of human apo[a], human catalase, rhesus monkey apo[a], and human plasminogen. The term type 2 classifies kringle 4 repeats with completely identical amino acid sequence.



**Fig. 6.** Immunoreactivity of mab 1A2 with a fusion protein containing the putative epitope. Periplasmatic proteins from pGBP (lane 1) and pGBP-apo[a] (lane 2) transformed cells were separated on a 10% SDS-PAGE under reducing conditions. A human plasma sample (lane 3) was included as a control. After transfer to nitrocellulose filters, mab 1A2 was used for immunodetection.

shown). Immunoblotting with antibody 1A2 did not reveal immunoreactivity of GBP (**Fig. 6**, lane 1). In contrast, the 36 kDa GBP-apo[a] fusion peptide (lane 2) reacted strongly with antibody 1A2.

## DISCUSSION

In the present study we have identified a linear epitope of 4 continuous amino acids on two otherwise unrelated human proteins (apolipoprotein[a] and catalase) which was discovered by cross-reactivity of a monoclonal antibody (mab 1A2) raised against apo[a]. The linearity of the epitope was demonstrated by sequence alignment, pin-ELISA, the ability of the synthetic peptide (H-S-R-T-P-E-YY-P-N-A-G) to inhibit the 1A2 antibody immunoreaction and by immunochemical recognition of a fusion protein containing the cross-reactive epitope.

Mab 1A2 also reacts with apo[a] from rhesus monkeys which contains the same YYPN motif on one of the kringle 4 repeats (K34). This finding, together with the data from pin-ELISA analysis further indicate the small size of this epitope. While occurring as a “unique epitope” in the published sequence of rhesus apo[a], the sequence occurs several times on kringle 4 structures: in addition to being present on the multiple kringle 4 type 2, it is also found on types 3, 4, 6, 7 and 8 of human apo[a].

Comparison of the cross-reactive sequence with the sequence of human plasminogen revealed a surprising detail: within 10 amino acids both sequences are completely identical with the exception of residue 50 on apo[a]: Y is exchanged for N in plasminogen. This single nonconser-

vative exchange obviously renders the epitope on the plasminogen molecule into a conformation that is no longer recognized by mab 1A2. This single residue might therefore be considered an “energetically critical residue” (1).

The identified sequence most likely has a high surface probability which is a prerequisite for a strong antigenic site. The surface localization of the sequence is indirectly confirmed by crystallographic analysis of kringle 4 of human plasminogen (33). Unfortunately, such data are not yet available for apo[a] or its kringle subunits. Kringles 4 from apo[a] and plasminogen are 78–88% homologous in amino acid sequence. In particular, the six cysteine residues that form the three intrachain disulfide bonds and stabilize the kringle structure are conserved. It is therefore likely that the tertiary structures of kringle 4 from apo[a] and plasminogen are very similar. As a consequence, the published crystal structure of plasminogen kringle 4 allows the conclusion that the 1A2-epitope is located on an accessible surface stretch of the molecule (33). This conclusion is also supported by the reactivity of mab 1A2 with unreduced Lp[a], which leaves the intrachain disulfide bonds intact and provides a “native” condition for the kringle structure (**Fig. 1**). Structure prediction analysis of the published repetitive kringle 4 revealed that the position of the discovered epitope most likely corresponds to a highly antigenic area of the protein. Presumably, apo[a] kringle 4 has no alpha-helices and only a few beta sheets that are distant from the epitope. There are, however, several beta turns, typical for highly antigenic sites, flanking the epitope.

Crystallographic data obtained from beef liver catalase suggest that the epitope described here is also located on the hydrophilic surface of the human molecule (34). Native catalase has a molecular mass of 240 kDa and is composed of four identical, non-covalently linked subunits, each containing a heme group. The whole molecule forms several alpha-helical and beta-sheet structures, especially in domains involved in binding of the heme group. There exists, however, a long “wrapping domain” from residues 366–420, forming an outer layer to each subunit. This domain, which contains a long stretch of polypeptide chain lacking any discernible secondary structure, contains the cross-reactive epitope recognized by mab 1A2 (residues 403–406).

Cross-reactivities of monoclonal antibodies with various proteins are a frequent phenomenon and are well established in the literature (35, 36). They are generally discovered by an antibody raised against a known protein cross-reacting with unknown other proteins, thus occasionally leading to the characterization of the unknown protein (37). In most cases, the cross-reactive epitope remains unknown (38). Our discovery of an immunological cross-reactivity on apo[a] and catalase led to the detection of a short immunodominant linear epitope on two well-known human proteins.



Our study has two important practical implications. *i*) Any antibody (poly- or monoclonal) against apolipoprotein[a] that is intended to be used in immunocytochemical studies of apo[a] has to be tested with respect to cross-reactivity with catalase. This cross-reactivity would otherwise lead to false positive recognition signals. *ii*) The identified cross-reactive sequence could be used for epitope tagging in order to identify or purify recombinant proteins. ■

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