

Glycoprotein II from adrenal chromaffin granules is also present in kidney lysosomes

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Glycoprotein II (GP II) is a protein found in the membranes of chromaffin granules from adrenal medulla. Immunoblotting (one- and two-dimensional) revealed that this antigen is also present in liver and in kidney. Subcellular fractionation of the latter organ indicated that GP II was present in lysosomes. This was confirmed by immunoelectron microscopy. The antiserum against GP II immunolabelled the membranes of organelles which could be identified as lysosomes by the labelling of their contents with an antiserum against cathepsin D. Thus GP II is an antigen common to secretory vesicles and lysosomes.

INTRODUCTION

The molecular composition of adrenal chromaffin granules, the catecholamine-storing organelles of this organ, has been defined in great detail (Winkler, 1976; Winkler *et al.*, 1986; Phillips, 1987). Major protein components of their membranes are the enzyme dopamine β -hydroxylase (Hörtnagl *et al.*, 1972) and cytochrome *b*-561 (Hörtnagl *et al.*, 1972; Apps *et al.*, 1980). Several glycoproteins have been identified and characterized (Huber *et al.*, 1979; Fischer-Colbrie *et al.*, 1984; Gavine *et al.*, 1984; Laslop *et al.*, 1986). One of them, named glycoprotein II (GP II), has a pI of 4.2–4.7 and an M_r of 100 000 (Obendorf *et al.*, 1988a). Immunological studies revealed that this protein had a widespread distribution in endocrine, but also exocrine, tissues (Obendorf *et al.*, 1988a). These results indicated that GP II was present both in endocrine and exocrine secretory vesicles. In the present paper we report that GP II is also found in liver and kidney. Subcellular fractionation and immunoelectron microscopy were used to establish the exact localization of GP II within the kidney. We demonstrate that GP II is found in the membranes of kidney lysosomes. Thus this glycoprotein is a common constituent of two organelles arising from the Golgi region, i.e. secretory vesicles and lysosomes.

MATERIALS AND METHODS

Materials

Bovine adrenal medulla, kidney and liver were collected from the local slaughterhouse. The anti-(bovine GP II) and anti-(cathepsin D) antibodies were obtained as described by Obendorf *et al.* (1988a). The antiserum against mitochondrial F_1 -ATPase was kindly provided by Dr. D. K. Apps, Department of Biochemistry, University of Edinburgh Medical School, Edinburgh, Scotland, U.K.

Biochemical assays

Proteins were measured by the Folin method using crystalline BSA as standard (Lowry *et al.*, 1951).

Immunological methods

Samples ('large granules' or density-gradient fractions) were subjected to SDS/PAGE in one- (Laemmli, 1970) and two-

dimensional (O'Farrell, 1975) systems, and immunoblotting was performed by the method of Burnette (1981) as described previously by Fischer-Colbrie & Frischenschlager (1985).

Subcellular fractionation

Purified chromaffin granules of bovine adrenal medulla were obtained by centrifugation through 1.8 M-sucrose (Smith & Winkler, 1967). Bovine kidney cortex was fractionated as described by Maunsbach (1966), with some modifications. After homogenization in 0.3 M-sucrose solution with a Potter-Elvehjem homogenizer, the homogenate was centrifuged at 500 g for 10 min. This first supernatant was centrifuged at 5000 g for 10 min to sediment a 'large-granule fraction'; the top layer of this fraction was washed away with 0.3 M-sucrose solution and the remaining pellet was resuspended and centrifuged at 5000 g for 5 min. Again the top layer of the pellet obtained was removed and the remaining pellet was resuspended in a small volume of 0.3 M-sucrose. This 'washed large-granule fraction' was then subjected to density-gradient centrifugation (Obendorf *et al.*, 1988b). The gradients, which consisted of sucrose solutions ranging from 1.3 to 2.0 M (see Smith & Winkler, 1966), were centrifuged for 90 min at 90 000 g. All fractions collected from the gradients (Smith & Winkler, 1966) were diluted with 0.3 M-sucrose (1:1, v/v; Obendorf *et al.*, 1988b) and spun for 60 min at 120 000 g. The sedimented particles were suspended in 5 mM-Tris/sodium succinate buffer, pH 5.9, and used for further analyses.

Immunoelectronmicroscopy

Small pieces of tissue (less than 2 mm³) from bovine kidney obtained approx. 10 min after slaughtering of the animals were transferred to 0.05% glutaraldehyde [EM (electron-microscopy) grade; Polysciences, Warrington, PA, U.S.A.] in phosphate-buffered saline (PBS; 0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.3) and fixed for 5 h at 4 °C. After three rinses (15 min each), tissue specimens were dehydrated in a regressive ethanol series using a progressive-lowering-of-temperature technique (Hobot, 1989). Subsequently the samples were embedded in Lowicryl K4M (Chem. Werke Lowi, Waldkraiburg, Germany) and polymerized by u.v. light for 24 h at -35 °C. Subcellular fractions from bovine kidney were fixed with 0.05% glutaraldehyde

Abbreviations used: GP II, glycoprotein II; PBS, phosphate-buffered saline.

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(Polysciences) in PBS, transferred to low-melting-point agarose (Sigma, St. Louis, MO, U.S.A.) and Lowicryl-embedded as described above.

Ultrathin sections were cut with a Reichert–Jung Ultracut microtome and mounted on Formvar-coated 200-mesh hex electron-microscopy grids (Polysciences).

For electron-microscopic immunostaining, ultrathin sections from both the bovine kidney and the gradient fractions were preincubated with ovalbumin (Sigma; 0.5%) in PBS (0.01 M, pH 7.3) for 5 min at room temperature, followed by an overnight incubation at 4 °C with polyclonal antisera against GP II and cathepsin D (both diluted 1:500). After two 5 min rinses with PBS (0.01 M, pH 7.3)/0.5% ovalbumin and 15 min in Tris/HCl (pH 8.2; 0.02 M) at room temperature sections were incubated at 37 °C for 1 h with a gold-conjugated goat anti-rabbit secondary antibody (20 nm; 1:25 in Tris/HCl containing 1% BSA (Sigma); Bioclin Immunogold Reagents, Cardiff, Wales, U.K.). The grids were finally rinsed in water and counterstained with uranyl acetate. As controls, sections were incubated either with a non-immune rabbit serum (diluted 1:250) or only with the secondary antibody with omission of the first antiserum.

For double immunostaining the selected surface immunostaining procedure described by Bendayan (1982) was applied on sections mounted on uncoated nickel grids (see Steiner *et al.*, 1990).

RESULTS

Immunoblotting for GP II

An antiserum against bovine GP II was used for immunoblotting of membranes of bovine kidney and liver. Fig. 1 demonstrates that, in both organs, an antigen was immunostained which migrated in one-dimensional electrophoresis like the adrenal one. In liver an additional faster-moving band was immunoreactive.

Two-dimensional immunoblotting of membranes from kidney, liver or chromaffin granules revealed (see Fig. 2) the presence of a double band (at a pI of 4.4). An analogous result was previously obtained (Obendorf *et al.*, 1988a) for the antigen in chromaffin granules.

Subcellular fractionation of kidney

Homogenates of bovine kidney were subjected to subcellular

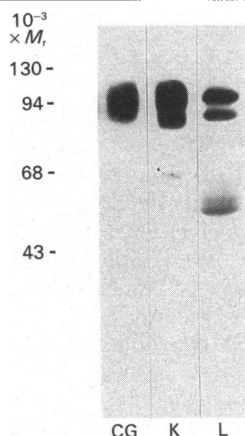


Fig. 1. Immunoblotting with an antiserum against GP II

Membranes of bovine adrenal chromaffin granules (CG; 40 µg of protein) and of large granules from bovine renal cortex (K; 270 µg of protein) and from bovine liver (L; 360 µg of protein) were subjected to one-dimensional electrophoresis followed by immunoblotting.

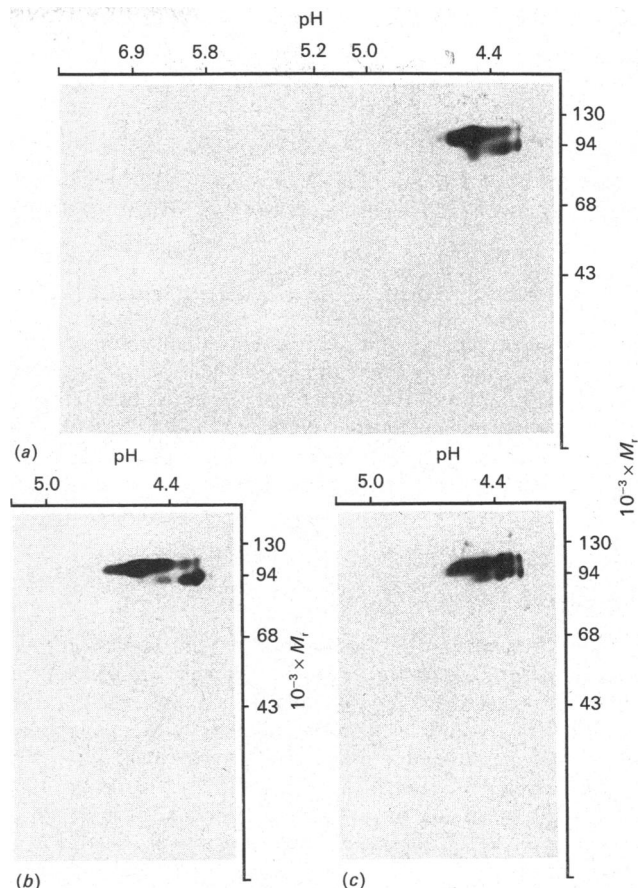


Fig. 2. Two-dimensional immunoblotting with an antiserum against GP II

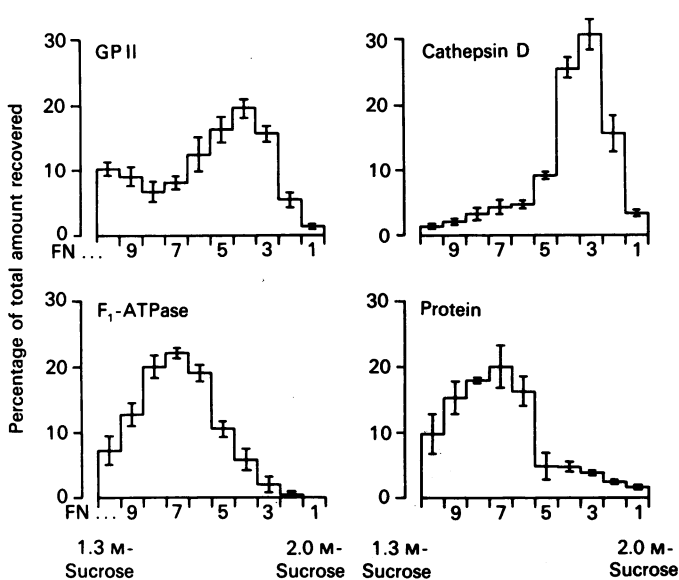
Membranes of large-granule fractions of bovine renal cortex (*a*; 540 µg of protein), of liver (*b*; 780 µg) and of bovine chromaffin granules (*c*; 80 µg) were subjected to two-dimensional electrophoresis followed by immunoblotting. For liver and chromaffin granules only the relevant parts of the two-dimensional blots are shown.

fractionation in order to define the localization of GP II in this organ (see Fig. 3). The distribution of mitochondria was determined by measuring the F_1 -ATPase as a marker. These organelles were concentrated in the lighter fractions of the gradient. Cathepsin D as a lysosomal marker was found in the denser regions of the gradient. The distribution of GP II did not exactly match that of cathepsin D, since the highest concentration of GP II was found in fractions 4 and 5, whereas cathepsin D peaked in fractions 3 and 4.

Immunohistochemical analysis

At the optical-microscopic level intense staining for GP II was present in the proximal tubules of the renal cortex (see Figs. 4a and 4b), whereas in the distal tubules of the renal medulla only a few cells appeared immunopositive (Figs. 4c and 4d).

The immunogold technique was used to immunostain for antigens at the ultrastructural level. In all of the proximal tubules the antiserum against cathepsin D immunostained the content of organelles (see Figs. 5a and 5c) which represent typical lysosomal structures. Their sizes were quite variable, and some of them appeared as heterogeneous phagolysosomes (see Maunsbach, 1969). After immunostaining with an antiserum against GP II, the gold label was found on the membranes of analogous organelles or at least close to them (see Figs. 5b and 5d). The specificity of the labelling can be seen from the absence of any background labelling. Furthermore, staining for GP II was



confined to membranes of lysosomal structures. Mitochondria, nuclear membranes and plasma membranes were not labelled (see Fig. 5b). There was also no evidence that membranes of the endoplasmic reticulum became labelled. In addition, fractions from the density-gradient experiments were also analysed at the ultrastructural level. In the top fractions (6-8) of the gradient mitochondria were concentrated. The anti-cathepsin antiserum labelled the content of organelles of about 2 μm diameter, whereas

Fig. 3. Subcellular fractionation of bovine kidney

A large-granule fraction from bovine renal cortex was subjected to density-gradient (1.3-2.0 M-sucrose solution) centrifugation. The fractions were analysed for protein, GP II, cathepsin D and mitochondrial F₁-ATPase. The columns from left to right correspond to the gradient fractions from the top to the bottom of the centrifuge tube. The abscissae are divided according to the volumes of the fractions. The ordinates give the percentages of the total amounts recovered/ml of fraction (FN). Two different gradient experiments were analysed. The results are presented as mean values ± S.E.M. The numbers of analyses were as follows: six for GP II; seven for cathepsin D; four for F₁-ATPase; and two for protein.

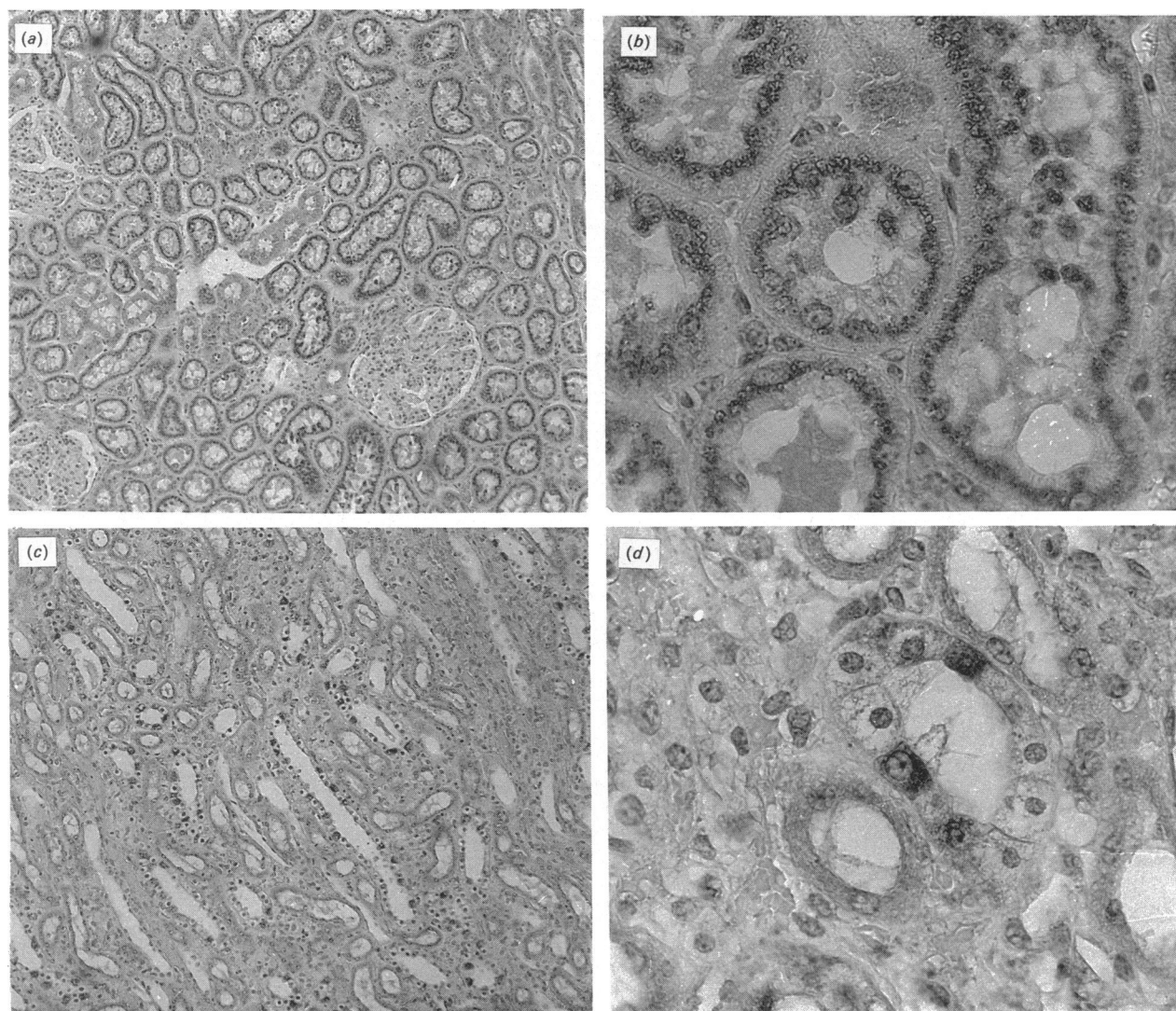


Fig. 4. Immunohistological localization of GP II in bovine kidney

(a and b) Immunoreactivity in renal cortex. A strong positive staining is seen in the cells of proximal tubules, whereas glomeruli are not stained. Magnifications: (a) 150 ×; (b) 600 ×. (c and d) In the renal medulla only a few cells of tubules are immunostained. Magnifications: (c) 150 ×; (d) 600 ×.

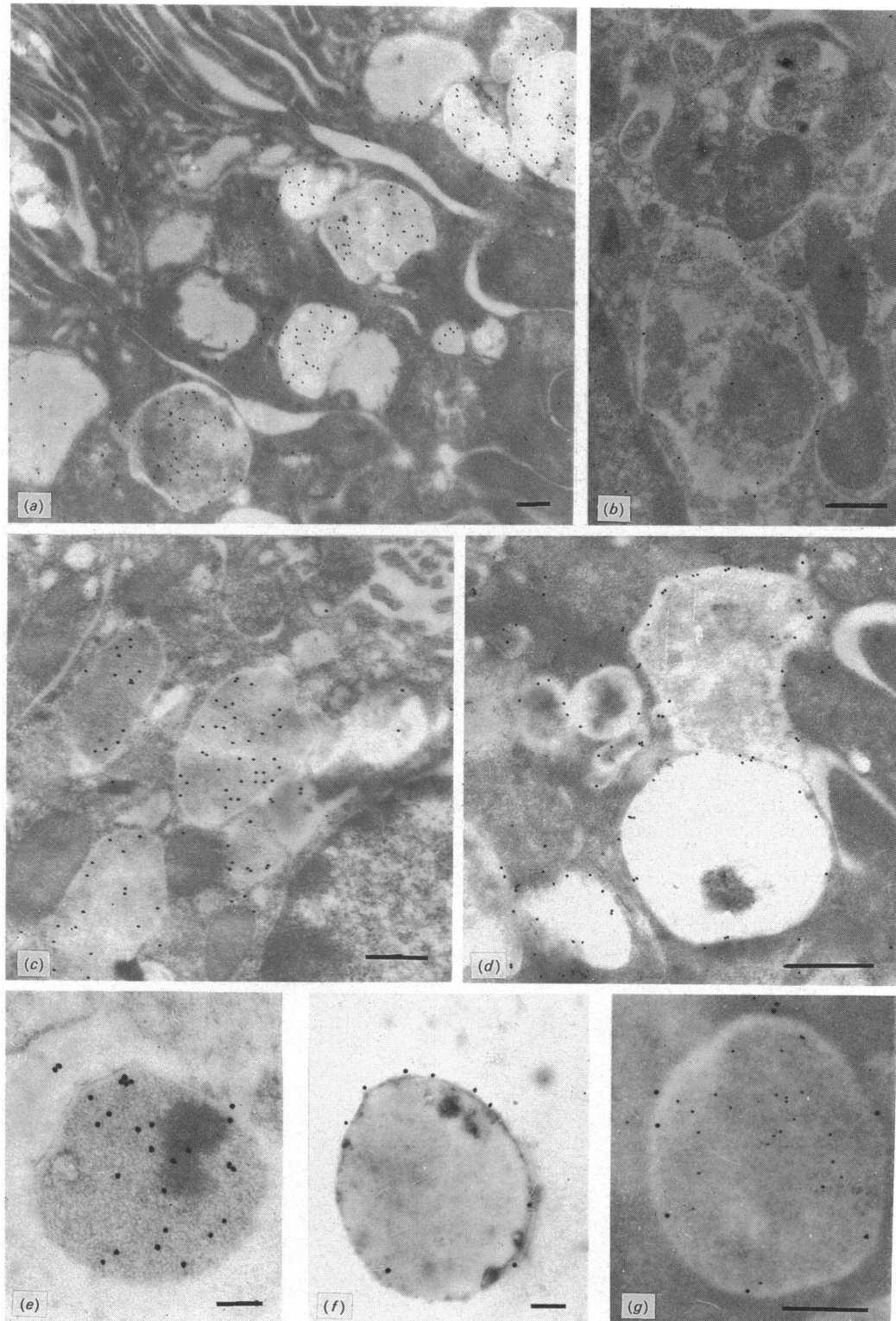


Fig. 5. Immunogold electron microscopy

For (a)–(d) ultrathin sections from the renal cortex were immunostained. Cathepsin D immunoreactivity is found in the content of vesicular structures (a and c), whereas the immunogold labelling for GP II is present on, or close to, the membranes of apparently analogous organelles (b and c). In (e) and (f) representative pictures of the immunostaining of organelles in density-gradient fraction 3 (see Fig. 3) are shown. The immunogold labelling for GP II is present on the membrane of a vesicle of diameter 2 μm , whereas that for cathepsin D is found in the content. For (g) an ultrathin section of renal cortex was sequentially immunostained on both sides of the same section (20 nm gold particles for GP II and 10 nm for cathepsin D). The bars represent 0.5 μm .

after the anti-(GP II) antiserum the gold label was found close to the membranes of organelles of similar sizes and structure. A representative picture from fraction 3 is shown in Figs. 5(e) and 5(f). There was no evidence in fractions 4–7 that the antiserum against GP II immunostained any membranes unrelated to lysosomal structures.

Finally, the lysosomal co-localization of GP II and cathepsin D was confirmed by sequential immunolabelling of both sides of the same ultrathin tissue sections (Bendayan, 1982). This result is shown in Fig. 5(g). The immunolabel for cathepsin D (10 nm gold particles) stains the content, whereas the GP II label (20 nm gold) is confined to the membrane of the same organelle.

DISCUSSION

GP II was originally isolated from the membranes of adrenal chromaffin granules (Obendorf *et al.*, 1988a). With immunoblotting this antigen was found in several endocrine tissues, but also in exocrine ones, e.g. the pancreas, where it appeared to be localized in the membranes of secretory vesicles (Obendorf *et al.*, 1988a). The present study was initiated when we discovered significant concentrations of GP II in liver and kidney. One- and two-dimensional immunoblotting revealed that we were dealing in this tissue with an antigen behaving in electrophoresis exactly like the adrenal one. To explain this finding we considered the possibility that GP II might be present in the lysosomes of these organs. Immunohistochemical staining of renal tissue was consistent with such a concept, since strong staining of tubule cells in the renal cortex, but a much more selective one in the renal medulla, was obtained. Previously an analogous result was obtained for typical lysosomal enzymes, i.e. cathepsins L, H and S (Rinne *et al.*, 1986; Kirschke *et al.*, 1989). These results have already indicated that the antiserum against GP II does not unselectively stain cellular membranes (e.g. mitochondria or endoplasmic reticulum), since these membranes are likely to be evenly distributed between proximal and distal tubules.

In subcellular-fractionation experiments, further evidence for a lysosomal localization of GP II could be obtained. This antigen had a gradient distribution quite different from a mitochondrial marker, but matched that of an established lysosomal enzyme, i.e. cathepsin D. However, the distribution of these two components in the gradient was not identical, since cathepsin D had its peak in a slightly denser position. This finding might be taken as evidence against a co-localization of these two components. However, in previous studies on the adrenal medulla we have already observed such slight dissociations of membrane components versus those of the content (see Obendorf *et al.*, 1988b). Such phenomena are likely to be due to the fact that larger vesicles, having relatively more content than the membrane, sediment to denser fractions, since the content has a higher density than the membranes. The slight difference in gradient distribution between GP II and cathepsin D found for the kidney may be caused by the same mechanism. In any case we have proved the lysosomal localization of GP II by quite an independent method, i.e. immunogold electron microscopy. In tissue sections the antiserum against cathepsin D labelled the content of vesicular, but quite heterogeneous, organelles of about 2 μm diameter, apparently representing lysosomes (Maunsbach, 1969). With the antiserum against GP II the membranes of these organelles could be immunolabelled. The labelling was quite specific, since other cellular membranes (mitochondrial, nuclear and plasma membranes) were not immunostained. Furthermore, when the isolated subcellular fractions were immunostained, a clear-cut GP II labelling of the membranes of organelles which were stained in their content for cathepsin D was obtained. Also

in these gradient fractions there was no evidence that membranes unrelated to lysosomes became labelled. Final proof of the GP II localization in lysosomes was obtained by a procedure (Bendayan, 1982) allowing double labelling of the same sections. Thus the combined biochemical and immunohistological results prove that GP II is present in the membranes of kidney lysosomes. Moreover, immunostaining at both the optical-microscopic and at the ultrastructural level indicate that GP II is apparently absent from other cellular membranes. Since in liver the antiserum reacted with an additional band, no further studies were performed in this organ.

In our previous study on adrenal medulla, no evidence was obtained that GP II, in addition to its presence in chromaffin granules, was also present in lysosomes (Obendorf *et al.*, 1988a). This was obviously due to the fact that this organ is extremely rich in chromaffin granules, but contains only a few lysosomes (Bradbury *et al.*, 1966); thus during subcellular fractionation, GP II in chromaffin granules overshadows any possible contribution by lysosomes.

GP II was the first defined antigen, being found in both endocrine and exocrine secretory vesicles. In the meantime a second antigen, a glycoprotein of 60 kDa, has been added (Yamashita *et al.*, 1989). GP II has now also been found in lysosomes. Other protein components for which one can postulate an analogous distribution are the subunits of the vacuolar type of the ATPase, which have been found in both lysosomes and endocrine vesicles; however, their subunits have molecular masses lower than that of GP II (Nelson & Taiz, 1989). Furthermore, a membrane antigen (110 kDa) of insulin secretory granules is also present in liver, as shown with a monoclonal antiserum (Grimaldi *et al.*, 1987). It is possible that further proteins will have this common distribution, and one should consider this when further proteins are isolated from these organelles. Two major lysosomal glycoproteins, namely LAMP1 and 2, have been characterized and their amino acid sequence has been elucidated (Chen *et al.*, 1985; Carlsson & Fukunde, 1989; Mane *et al.*, 1989). We are unaware of any studies reporting that these proteins are also present in endocrine vesicles. However, the possibility that GP II is related to these proteins now requires further investigation.

The finding of common antigens in endocrine and exocrine vesicles and lysosomes is intriguing. What is the function of such components? All these particles are derived from the Golgi complex and are targeted for fusion and fission with other membranes. Are proteins common to the organelles involved in such basic functions? Or are they involved in some still undefined transport processes, by analogy to the common proton transport provided by the vacuolar ATPase for all these various endo-membrane vesicles?

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