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Identification of High Molecular Weight Antigens Structurally Related to Gamma-Glutamyl Transferase in Epithelial Tissues

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Summary. Heterologous antibodies to gamma-glutamyl transferase (γGT), an ectoenzyme associated with the apical surface of many types of epithelial cells involved in secretion and transport, have been used to identify and partially characterize the spectrum of antigens in a series of epithelial tissues that exhibit a range of enzyme activities. In addition to antigens corresponding to the subunits of the active enzyme (mol wt 55K, 30K), antigens of mol wt ~85–≥95K have been detected using an antibody raised against the enzyme purified in nonionic detergent. The latter species are shown to share antigenic determinants with and to be structurally related to the enzyme subunits; however, they do not bind significantly to antibodies raised to protease-solubilized γGT. Further, they constitute the major antigens in tissues that exhibit relatively low levels of enzyme activity. These polypeptides are apparently larger than a recently characterized biosynthetic precursor of the γGT subunits. Although they do not have γGT activity themselves and their function is undefined, the possibility that they may represent highly glycosylated polypeptides related either to γGT precursors (that persist without processing) or to the large enzyme subunit merits consideration.

Key Words γ-glutamyl transferase · membrane glycoproteins · epithelia · apical plasma membrane · radioiodination

Introduction

Gamma-glutamyl transferase (γGT; EC 2.3.2.2,) is an enzyme that catalyzes the cleavage of the γ-glutamyl residue of glutathione (GSH) and effects its transfer to water or under appropriate conditions to an acceptor amino acid or dipeptide [reviewed in 10, 33]. Although γGT is present at low levels as a plasma membrane-associated ectoenzyme in a wide variety of cell types [33], the early cytochemical studies of Glenner et al. [16] have shown that it is highly concentrated on the apical surface of epithelial cells, notably some of those that mediate exchanges between external compartments and the internal milieu through secretory and resorptive processes (e.g., cells forming the kidney proximal tubule, the intestinal villar surface, the exocrine pancreas, the proximal portion of the epididymis, the choroid plexus, and the ciliary epithelium of the retina).

The purified active enzyme is a heterodimeric glycoprotein in which the large subunit (55–58K)[1] provides a plasmalemmal anchor presumably by a membrane-spanning segment located near the amino terminus [31] and the small subunit (30K)[1] apparently contains the active site [21, 51], probably near to the region of intersubunit contact [14]. Antibodies to γGT have been raised primarily to enzyme purified following solubilization from the membrane by proteolysis using papain or bromelain ([22, 34]; referred to as PγGT). These antibodies have been used to show that the enzyme is synthesized as a monomeric precursor that is processed to subunits by endoproteolytic cleavage [3, 7, 25, 32, 36, 37]. Further, immunocytochemical studies conducted principally in kidney have indicated that antigens are localized primarily on apical membranes of proximal tubule cells where active enzyme is known to be concentrated [28, 44].

The studies reported in this paper have resulted from a general interest in probing the movement of membrane polypeptides into and out of the surfaces of polarized epithelial cells and by the specific observation that γGT activity marks in common secretion granule membranes and plasma membranes in acinar cells of the parotid gland [1, 2, 6]. In order to investigate this compositional overlap using an immunochemical approach, we have raised separate heterologous antibodies to γGT purified from renal cortex (the richest known source of active enzyme)

[1] Apparent mol wt reported in the literature for γGT subunits characterized in rat tissues vary from 46–55K for the large subunit and 22–30K for the small subunit. Extensive glycosylation is likely to contribute to the variability of reported values as well as to the diffuse appearance of polypeptide bands on gel electrophoretograms. Values cited in the text are taken from the present study.

in both its detergent (Triton X-100)-soluble ([20]; referred to as TγGT) and protease-solubilized forms. Although both types of antibodies bind to the subunits of the active enzyme, only anti-TγGT antibodies recognize additional polypeptides of mol wt ~85–≥95K that in kidney are shown to be antigenically and structurally related to the enzyme subunits. These polypeptides are minor components of the surface membranes of epithelial cells (e.g., kidney proximal tubule and pancreatic acinar cells) that contain substantial enzyme activity. However, they are the prevailing antigens in cell types (e.g., parotid acinar cell and hepatocyte) where enzyme activity is greatly reduced. At present the function of these high mol wt antigens is not defined.

Materials and Methods

General Assays

γGT activity was determined at 25°C and pH 7.5 by following enzyme-catalyzed transpeptidation using 2 mM L-γ-glutamyl-*p*-nitroanilide and 20 mM glycylglycine, respectively, as donor and acceptor substrates [49].

Total protein was assayed according to Markwell et al. [30] using bovine plasma albumin as standard.

Purification of γGT from Rat Kidney

The membrane form of γGT solubilized in Triton X-100 (TγGT) was purified from rat kidney according to the procedure of Hughey and Curthoys [20]. Protease-solubilized forms of γGT (PγGT) were purified by a procedure involving bromelain digestion [50].

Antibody Preparations

Rabbit antibodies have been prepared against purified TγGT and PγGT, the latter reacted with 17 mM glutaraldehyde [29]. Antigens were each emulsified in Freund's adjuvant and injection and bleeding of rabbits were carried out using a protocol modified from Papermaster et al. [39].

For some experiments anti-TγGT antiserum was subfractionated by passage over an affinity column containing PγGT covalently bound to Sepharose 4B (prepared by reacting purified PγGT with cyanogen bromide-activated Sepharose as specified by Pharmacia, Inc.). Serum containing antibodies not bound to PγGT was collected as a "flow through" fraction. The column was washed with pH 8 until OD_{280} was <0.02, and affinity purified antibody was eluted with 200 mM glycine at pH 2.2 [38] immediately neutralized with 2 M Tris base and stored in the presence of 1 mg/ml albumin and 0.02% sodium azide.

Immunofluorescent Localization of γGT Antigens and Cytochemical Detection of γGT Activity

Rat tissues used for localization of γGT by indirect immunofluorescence were fixed by *in situ* perfusion with phosphate-buffered (0.1 M, pH 7.3) 3% formaldehyde with or without 0.25% glutaraldehyde. Fixation was continued on small pieces of excised tissue for 3 hr at 4°C and subsequently the pieces were washed with phosphate buffer and infiltrated with sucrose (up to 18% wt/vol) in phosphate buffer. 6–8 μm thick sections were cut on a cryomicrotome from blocks of tissue frozen by immersion in 2-methylbutane cooled by liquid nitrogen.

Immunolocalization procedures were carried out at room temperature except for incubation with the primary anti-γGT antibody which was done at 37°C. Sections were quenched initially with 0.1 M Tris (40 min) followed by 0.5% $NaBH_4$ (10 min), washed with PBS (10 mM Na phosphate, 0.15 M NaCl, 40 min) and then high salt PBS (20 mM Na phosphate, 0.5 M NaCl, 15 min). Primary antibody staining (1 : 50 dilution of immune serum diluted with 15% goat serum in high salt PBS) was performed for 3 hr in a humidified chamber. Specimens were then washed with high salt PBS and reacted with secondary antibody (Rhodamine-conjugated goat anti-rabbit IgG initially freed of highly charged IgG [4] and diluted with goat serum in high salt PBS ($\sim 1 \times 10^{-2}$ mg/ml) for 1 hr in a dark, humidified chamber. Finally the sections were washed in succession with high salt PBS and PBS and mounted in glycerol-PBS (9 : 1 (wt/wt)). Controls in which anti-γGT was replaced by preimmune serum were processed in parallel. A Zeiss photomicroscope equipped with epifluorescence illumination was used to view the results.

Enzyme cytochemical studies were conducted on cryostat sections of epithelial tissues that had been fixed by perfusion with 3% formaldehyde with or without 0.05% picric acid in 0.1 M phosphate buffer at pH 7.4 and cut after cryoprotecting and freezing in phosphate-buffered 18% sucrose. The specimens mounted on gelatin-coated slides were processed using either the procedure of Glenner et al. [16] for pancreas, parotid, and liver or the procedure of Seligman et al. [42] for kidney. Reaction times at room temperature were: 3 min for kidney and pancreas, 15 min for liver, and 25 min for parotid. Following incubation the slides were washed with physiologic saline, coated with glycerol-PBS, and immediately examined in a Zeiss photomicroscope. Parallel samples of fixed and unfixed pancreatic tissue were weighed, homogenized at the same concentration in phosphate-buffered sucrose, and assayed for γGT biochemically. Fixed tissue was found to retain ~60% of the activity of unfixed tissue.

Isolation of Plasmalemmal Fractions Used for Immunoprecipitation

Plasma membrane-containing fractions have been prepared from each of the rat epithelial tissues studied. Brush border membranes from kidneys perfused *in situ* with PBS containing 1 μg/ml antipain, 1 μg/ml pepstatin, 0.5% vol/vol Trasylol®, and 0.4 mM phenylmethylsulfonyl fluoride (PMSF), were obtained using the calcium precipitation procedure of Malathi et al. [27] in which mannitol solutions were supplemented with the same spectrum of proteolytic inhibitors. Hepatocyte plasma membranes were prepared according to the procedure of Hubbard et

al. [19] from livers of rats perfused as above. Plasmalemmal fractions of exocrine pancreatic and parotid glands were prepared as previously described [2], modified in the case of the pancreas by addition of 1 mM benzamidine.

Radioiodination of Purified TγGT and Plasmalemmal Fractions

In all cases purified TγGT and isolated membrane fractions (0.1–1 mg protein) were radiolabeled using 1 mCi $Na^{125}I$ by the chloramine T procedure [17] conducted for 4 min at 0°C. After quenching with $Na_2S_2O_5$, ^{125}I-TγGT was separated from free iodide by gel filtration on Biogel P6 in 50 mM NH_4HCO_3. Labeled membrane fractions were either washed by centrifugation in 20 mM sodium phosphate + 50 mM potassium iodide (pH 7.4) or subjected to gel filtration on agarose 0.5-M in the same phosphate-iodide buffer. Membranes were finally resuspended and stored at −20°C in 20 mM sodium phosphate.

Immunochemical Analyses

Antibody specificity and titer were judged based on the selectivity and extent to which γGT enzymatic activity could be removed from detergent-solubilized membrane preparations in a two-step complexation and sedimentation procedure. Kidney brush border membranes dissolved in either 1% NP-40, 50 mM phosphate (pH 7.2), 1 mM EDTA (γGT activity 100% retained) or the radioimmunoprecipitation (RIPA) buffer of Brugge and Erikson ([5]; 80% retention of enzyme activity) were mixed with a series of antibody dilutions; following adequate time for antigen-antibody interaction to occur (≥90% in >20 hr), an excess of *Staphylococcus aureus* Cowan I strain (10% wt/vol in the same buffer) was added to complex IgG [23]. After 60 min with occasional mixing, *S. aureus*-immune complexes were removed by centrifugation and supernatant solutions were assayed for enzyme activity. Controls containing comparable dilutions of preimmune serum or no antibody were processed and assayed in parallel.

Immunoprecipitation of radioiodinated membranes for electrophoretic analyses were carried out on samples solubilized initially in 1% SDS, immersed immediately thereafter for 5 min in a boiling water bath (as a precaution against latent protease activity intrinsic to γGT-containing fractions [13]) and diluted to reconstitute RIPA buffer [5] supplemented with 0.5 M NaCl, 1 μg/ml antipain, 1 μg/ml pepstatin, 1% (vol/vol) Trasylol®. The samples were centrifuged ≥ 4×10^6 $g_{av} \times$ min, and supernates were adjusted to a final volume of 1.0–1.4 ml, mixed with antibody, and incubated 20 hr at 4° with constant gentle agitation. Antigen-antibody complexes were bound to an excess of *S. aureus* added in RIPA buffer containing 2 mg/ml bovine plasma albumin (BPA) and sedimented by centrifugation 5 min at 10,000 × *g*. The bacterial cell-immune complexes were washed four times in RIPA buffer (initially containing 2 mg/ml BPA) and resuspended in 4% SDS, 0.06 M Tris, 20% glycerol at pH 6.8. Following addition of 20–25 mM dithiothreitol, incubation 5 min at 100°C and centrifugation (5 min, 10,000 × *g*), the supernatant fraction produced was prepared for SDS polyacrylamide gel electrophoresis.

Immunoblotting and lectin (^{125}I-concanavalin A) blotting on nitrocellulose replicas of SDS-polyacrylamide gel electrophoretograms was carried out essentially according to Gershoni and Palade [15]. 15 mg/ml hemoglobin in PBS was used to reduce nonspecific adsorption to the nitrocellulose transfers during washing steps as well as during incubation with immune reagents. Specifically adsorbed primary antibody was detected using ^{125}I-labeled goat-anti-rabbit IgG.

In cases where primary antibodies bound to selected antigen bands on the nitrocellulose transfers were to be recovered for reblotting onto other transfers, small segments of transfers processed through ^{125}I-goat-anti-rabbit IgG as described above provided templates. Desorption of antibody was achieved using 200 mM glycine buffer, pH 2.8 [38] and was immediately followed by neutralization using 0.5 M Tris, pH 9.

Polyacrylamide Gel Electrophoresis; Further Processing of Individual Polypeptide Bands and Peptide Mapping

Polyacrylamide gel electrophoresis in the presence of SDS was carried out on reduced (20–25 mM dithiothreitol, 5 min, 100°C) and alkylated (80–100 mM iodoacetamide, 30 min, room temperature) samples using the Laemmli procedure [26]. Gels fixed in isopropanol-acetic acid [46] were stained either in 0.08% Coomassie blue R250 or with the silver staining procedure of Merril and coworkers [35]. ^{125}I-labeled polypeptides were detected by autoradiography of dried gels performed at −70°C using a duPont Lightning Plus® image-intensifying screen.

In cases where individual antigenic bands were to be examined further by peptide mapping, the gels of iodinated immunoprecipitates were soaked in 10% isopropanol prior to drying. Following autoradiography, individual bands were cut from dried gels using the autoradiogram as a template and were swollen in 0.75 ml 50 mM ammonium bicarbonate containing 50 μg/ml proteolytic enzyme (most frequently, α-chymotrypsin). Digestion was carried out for 24 hr at 37°C. Subsequently, the pieces of acrylamide were removed and the digests were lyophilized, spotted on cellulose thin-layer plates and subjected in series to first-dimension electrophoresis in acetic acid/formic acid/water and second dimension chromatography in butanol/pyridine/acetic acid/water supplemented with 7% (wt/vol) 2,5-diphenyloxazole [11]. Fluorograms were exposed at −70°C in the presence of an image-intensifying screen.

Materials

Male Sprague-Dawley rats used in all experiments were obtained from Charles River Suppliers, Wilmington, MA. Protease inhibitors were obtained as follows: Trasylol® (FBA Pharmaceuticals); antipain, pepstatin, phenylmethylsulfonyl fluoride (Sigma); benzamidine (Calbiochem). Rhodamine-conjugated goat-antirabbit $F(ab)_2'$ was obtained from Cooper Biomedical. Nitrocellulose used for immunoblotting was obtained from Schleicher and Schuell. Cyanogen bromide-activated sepharose and concanavalin A were from Pharmacia. *Staphylococcus aureus* Cowan I strain was grown and processed according to Kessler [23]. $Na^{125}I$ used for radioiodination was obtained from Amersham. All other reagents were obtained from general suppliers.

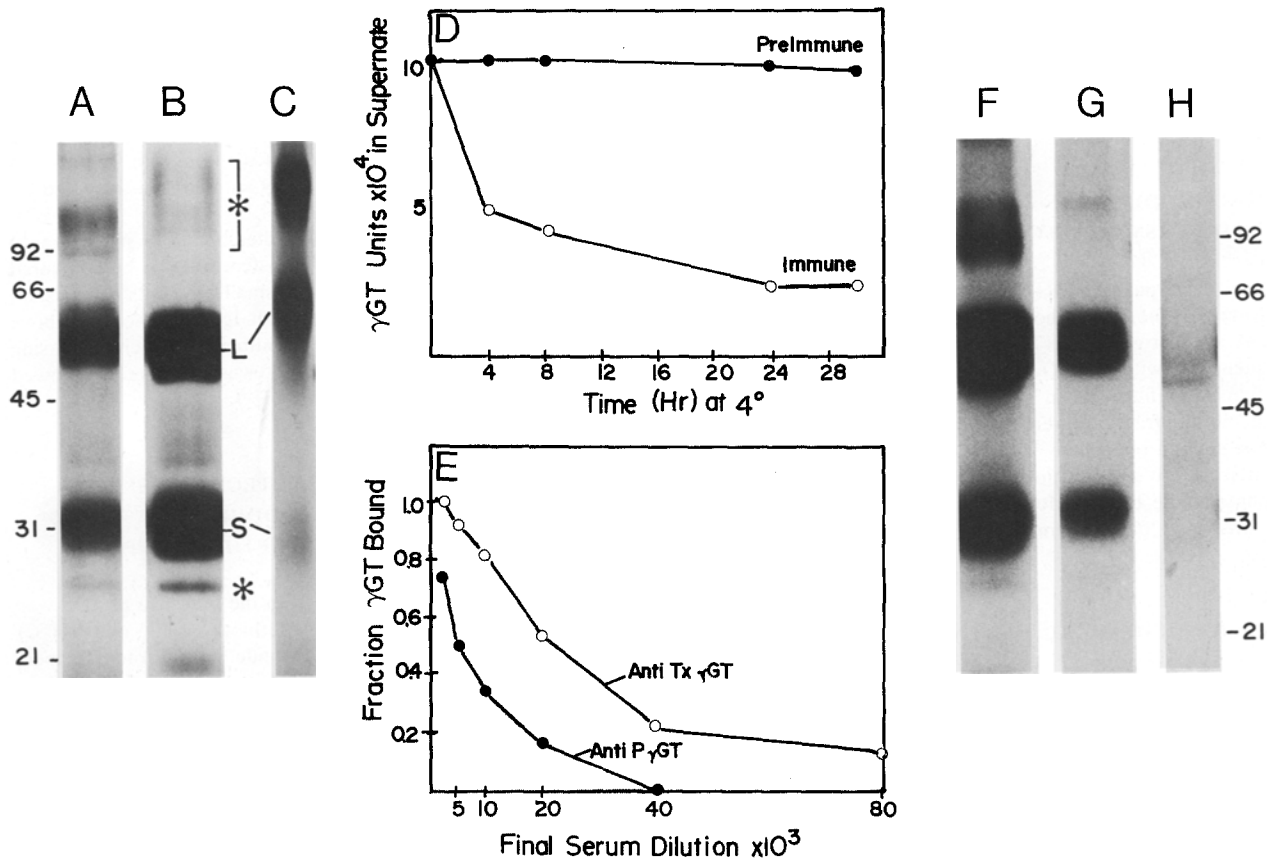


Fig. 1. Characterization of γGT antigen and heterologous rabbit anti-γGT sera. (*A*, *B*) Silver-stained SDS polyacrylamide gel electrophoretogram showing the polypeptide bands comprising TγGT and PγGT, respectively. The large (55–58K) and small (~30K) subunits of the enzyme are marked *L* and *S*, respectively; high mol wt polypeptides barely visible are indicated by asterisks. (*C*) Autoradiograph of a [^{125}I] concanavalin A overlay on purified TγGT electrotransferred from a polyacrylamide gel to nitrocellulose. The 85–95K species are intensely labeled, suggesting extensive glycosylation. Timecourse of the binding of γGT solubilized from kidney brush borders in the presence of immune and preimmune sera. The fraction of γGT activity remaining in solution after incubation in the presence of limiting amounts of antibody and subsequent sedimentation of immune complexes bound to *S. aureus* is shown. (*E*) Comparison of the titers of comparable antiTγGT and antiPγGT sera. Fractional γGT activity remaining in solution (determined as in *D*) is plotted as a function of serum dilution. (*F–H*) Autoradiogram of antigens precipitated from detergent-solubilized ^{125}I-labeled kidney brush border membranes by antiTγGT, antiPγGT, and preimmune serum, respectively. Note that antigens of mol wt ~95K are prevalent in relation to *L*, *S* with antiTγGT but are barely detected, if at all, with antiPγGT

Results

Initial Characterization of γGT Antigen and AntiγGT Antibodies

Preparations of γGT purified from rat kidney as a part of this study exhibit enzyme-specific activities that compare favorably to values reported in the original procedures (TγGT, 508 units/mg protein; PγGT, ≥790 units/mg protein). When these prospective antigens were examined on silver-stained SDS polyacrylamide gel electrophoretograms (Fig. 1*A*, *B*), the principal bands observed (~55K and ~30K) corresponded to the large and small subunits of the enzyme [20, 51]; however, additional minor species were detected (~25K and 85–95K). The minor bands were not stained by Coomassie blue; however, they have a substantial binding capacity for concanavalin A (Fig. 1*C*), a lectin known to bind to both subunits of γGT [36]. Both features suggest prominent glycosylation. Because of the presence of these minor bands (and the consequent possibility of contamination) we chose initially to prepare immune sera to the denatured large subunit excised from unfixed SDS polyacrylamide gels. However, these antibodies exhibited low avidity when tested for their ability to complex γGT from detergent-solubilized kidney brush border membranes. Further, they were localized successfully by immunocytochemical techniques only in kidney where antigen concentrations are highest. Consequently, we raised heterologous antibodies directly to the

purified TγGT and PγGT preparations with the intention of evaluating the contributions of the minor polypeptides as antigens.

The initial characterization of the antisera was performed using purified kidney brush border membranes (the direct source of antigen) as well as the purified antigen. Figure 1D–H presents a characterization of early bleed antisera obtained three weeks after initiating injections of TγGT and PγGT. γGT activity can be complexed by IgG of immune serum and removed from solution by subsequent binding to excess *S. aureus* [23]. At activity levels typically encountered in many of the preparations studied, antigen-antibody interaction can be demonstrated readily; complete reaction of limiting amounts of antibody requires 24 hr (Fig. 1*D*). Further, as shown in Fig. 1*E*, the titer of antiTγGT (~930 units soluble γGT bound/ml serum) is substantially higher than that of antiPγGT (~160 units soluble γGT bound/ml serum) when a series of dilutions of each antiserum is compared. Neither antibody, however, caused detectable inhibition of enzyme activity, when tested following incubation of antiserum with limiting quantities of antigen for 24 hr at 4°C. Finally, the results of immunoprecipitations from solubilized ^{125}I-labeled brush border membranes using antiTγGT, antiPγGT and preimmune sera are shown in Fig. 1*F–H*. Enzyme subunits account for >~95% of the total radiolabeled antigen specifically complexed by antibodies in the immune sera. The most notable difference between the two immunoprecipitates is the presence with antiTγGT of two bands at apparent mol wt ~85, 95K, constituting about 5% of the labeled polypeptides recovered and corresponding in mobility to the faint silver-stained species observed in Fig. 1*A*.

Antigenic and Primary Structural Relationships between the High Mol Wt (~85, 95K) Species and Enzyme Subunits

Two approaches were used to evaluate whether the polypeptides of high apparent mol wt recognized in the antiTγGT immunoprecipitate constitute species antigenically related to the γGT subunits or independent contaminants of the original purified enzyme preparation. First, samples of purified TγGT and solubilized kidney brush border fractions were subjected to SDS polyacrylamide gel electrophoresis, electro-transferred to nitrocellulose, and reacted with antiTγGT serum. Bound antibodies were located and desorbed at low pH from individual antigen bands on the nitrocellulose blot as specified under Materials and Methods and incubated with a second strip of nitrocellulose which contained all of the resolved antigens but had not been exposed previously to primary antibody. Cross-reactivities were visualized by subsequent treatment with ^{125}I-goat-antirabbit IgG and autoradiography. Results obtained using this blot → reblot procedure [38] are shown in Fig. 2. Strip *A* is an autoradiogram of the original blot of purified TγGT reacted directly with ^{125}I-secondary antibody and identifies three major bands corresponding to the small subunit (*S*), the large subunit (*L*) and 85–95K polypeptides[2]. Antibodies desorbed individually from *S*, *L*, and 85–95K species and reblotted on resolved antigens yield the autoradiograms shown on strips *B*, *C*, and *D*, respectively. Anti*S* rebinds preferentially to *S* (and to a minor band of higher electrophoretic mobility) but binding is also detected to *L* and, to a lesser extent, on the higher mobility portion of the 85–95K region. Anti*L* rebinds preferentially to *L* but also to *S* and

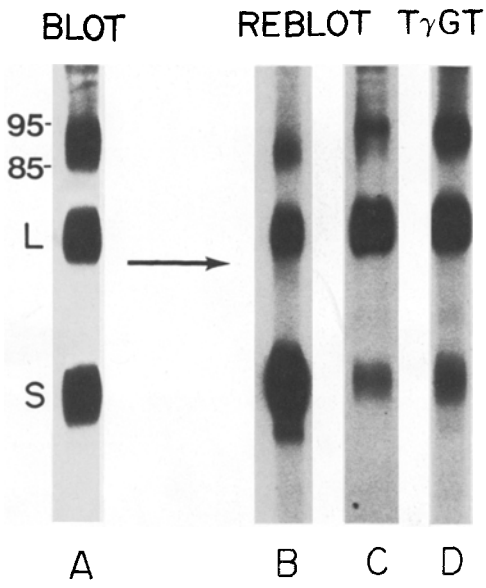


Fig. 2. Blot → reblot experiment in which antibodies specifically bound to individual bands comprising purified TγGT on nitrocellulose have been desorbed individually and rebound to nitrocellulose strips containing the original antigen. In all cases bound antibody has been detected by 2nd step interaction with ^{125}I-goat antirabbit F(ab)$_2$. (*A*) The original spectrum of antigenic bands characterized by antibody binding *without* subsequent desorption and reblotting prior to detection with the second-step antibody. (*B*) autoradiogram showing pattern for antibody desorbed from *S* of the original blot and reblotted back onto total antigen; (*C* and *D*) Comparable autoradiograms for antibodies desorbed, respectively, from *L* and 85–95K bands of the original blots and reblotted back onto total antigen

[2] Comparison of Fig. 1*A* and Strip *A* of Fig. 2 indicates that the 95K antigen band is considerably more prevalent in the latter preparation. This apparently reflects its selective retention relative to the enzyme subunits during electrotransfer to nitrocellulose.

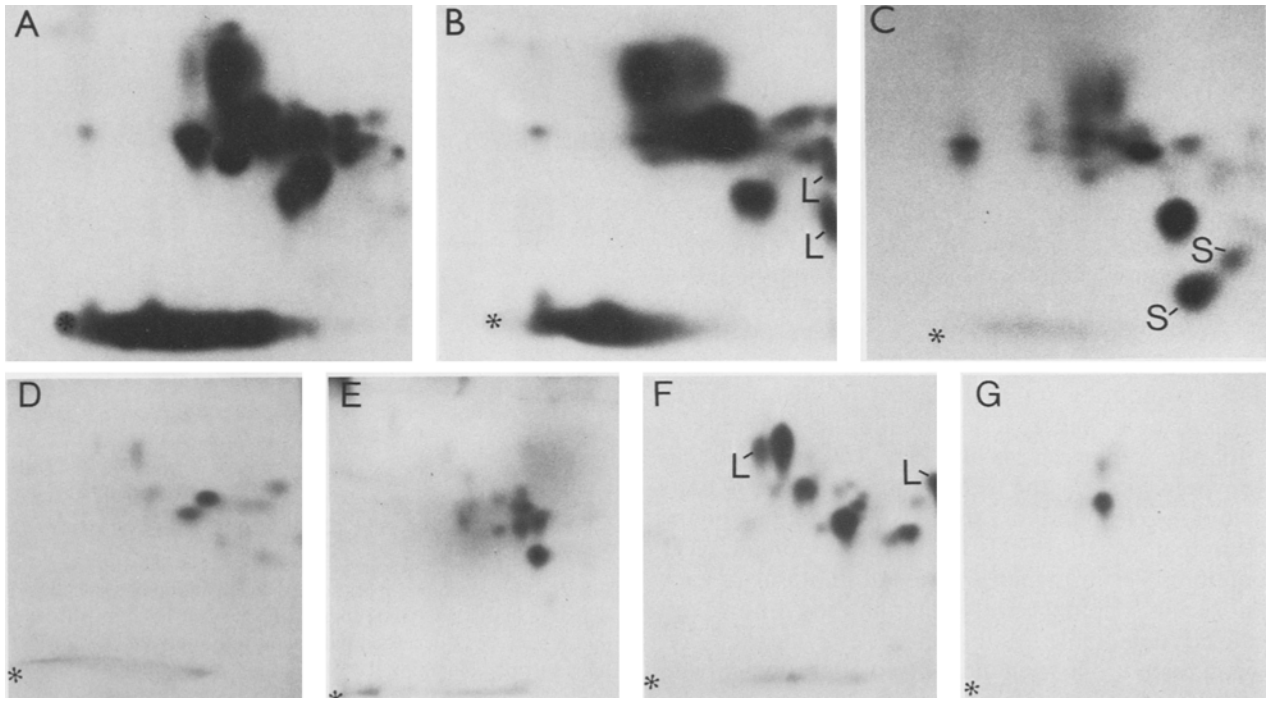


Fig. 3. Fluorograms of chymotryptic maps of radioiodinated polypeptides constituting TγGT (*A–C*) and antigens immunoprecipitated from ^{125}I-labeled kidney brush border membranes (*D–G*). Resolution in two dimensions by electrophoresis (horizontal) and chromatography (vertical) is shown for polypeptides comprising 85–95K species (*A*), large subunit (*B*), small subunit (*C*), and antigens corresponding to 95K (*D*), 85K (*E*), large subunit (*F*), and small subunit (*G*) species. Labeled peptides present in maps of the large and small subunits that do not have an obvious equivalent in other maps are designated *L*- and *S*-specific by the letters *L* and *S*, respectively. When the antigens mapped in *D–G* are compared, *D* and *E* exhibit overlap that includes only a few of the labeled peptides in *D*. However, *D* and *E* considered together exhibit extensive resemblance to *F* and *G* considered together, although differences in mobility and especially quantity of individual peptides are apparent. *: origin of maps

to the 85–95K antigens, especially at the level of 95K. Interestingly, anti85–95K rebinds preferentially to *L* and to both *S* and polypeptides throughout the 85–95K region. Thus, antigenic cross-reactivities exist between all polypeptides detected in purified TγGT (Fig. 1*A*). It should be emphasized, however, based on the amount of 85–95K antigen identified in strip *A* and the relative amount detected in the reblots with anti*S* (strip *B*) and anti*L* (strip *C*), that cross-reaction does not occur to the extent that antigen is present and may indicate only partial structural similarity. Identical results have been obtained using purified kidney brush border preparations as antigen (*not shown*).

A second approach involving peptide mapping of individual radioiodinated polypeptide bands comprising either TγGT or antiTγGT immunoprecipitates was used to evaluate whether the antigenic cross-reactivities observed above are likely to result from homologies in primary sequence. Chymotryptic hydrolysates of labeled bands excised from dried gels were resolved on cellulose thin layers by electrophoresis followed by chromatography. Figure 3*A–C* shows maps prepared from purified TγGT where individual polypeptides are expected to be heavily iodinated. Figure 3*D–G* shows maps obtained from individual bands comprising immunoprecipitates from radioiodinated kidney brush border membranes. In this case iodination of γGT-related polypeptides is anticipated to be less extensive; however, the entire spectrum of antigens should be represented in each molecular weight range rather than a possible subset constituting the purified enzyme (obtained in low yield [20]). For purified TγGT comparative examination indicates that there is extensive similarity in pattern but not complete overlap of iodinated peptides between the 85–95K species (Fig. 3*A*) and the large subunit (Fig. 3*B*). Further, several but not all of the peptides observed in the pattern for the small subunit (Fig. 3*C*) co-migrate with peptides present in the digest from the 85–95K region and, to a more limited extent, with those derived from the large subunit. Extensive, but not complete, overlap has also been obtained for polypeptides comprising TγGT following hydrolysis with either *S. aureus* protease or chymo-

trypsin and subsequent resolution on one-dimensional polyacrylamide gels according to the procedure of Cleveland et al. ([9]; *not shown*).

The maps in Fig. 3*D–G* show fewer iodinated peptides, as expected, especially for *S* (Fig. 3*G*). Interestingly, the pattern obtained for *L* (Fig. 3*F*) is similar to the summed patterns of the individual 85K (Fig. 3*E*) and ~95K (Fig. 3*D*) polypeptides. Again, however, the comparison emphasizes similarity but not identity between *L* and the high mol wt antigens.

Comparative Localization of γGT-Related Antigens and Enzyme Activity in Epithelial Tissues by Indirect Immunofluorescence and Cytochemical Staining

The Table presents the γGT enzyme activities (normalized to total protein) measured for four different epithelial tissues from a 150-g rat and identifies a range of values that extends over more than three orders of magnitude from kidney to liver. We decided to compare the cellular distribution of antiTγGT antigens, antiPγGT antigens, and enzyme activity in these same tissues by both indirect immunofluorescence and cytochemistry since no contaminating polypeptides structurally unrelated to γGT were identified in characterizing kidney antigens. The results obtained using 6 μm thick cryostat sections are shown in Figs. 4 and 5. In Fig. 4*b*, *d*, *f*, *h* (antiTγGT) the rhodamine-conjugated secondary antibody identifies a high antigen concentration on apical epithelial surfaces. Staining is particularly apparent on kidney proximal tubules (Fig. 4*b*) where enzyme is known to be associated with the highly amplified microvillar surface (Fig. 4*a*). For exocrine pancreas, both antigen (Fig. 4*d*) and enzyme reaction product (Fig. 4*c*) are prominent along the border of the apical secretory surface, but they also mark the apical cytoplasm diffusely where accumulated zymogen granules are located. In addition, discrete accumulations of antigen (and possibly reaction product) along the basolateral surface of pancreatic acinar cells are more prominent than in any of the other epithelial cell types examined. For parotid acinar cells antigenic staining is unusually prominent (Fig. 4*f*) when considered in relation to the modest level of enzyme reaction product (Fig. 4*e*) obtained only after extended incubation. Both markers are concentrated along the highly branched apical secretory surface.

A major discrepancy in the localization of antiTγGT antibodies and enzyme activity is observed in liver. Although activity is restricted principally to cells lining the bile ducts (Fig. 4*g*), the bulk of the antigen staining was concentrated along the apical, bile canalicular surface of hepatocytes (Fig. 4*h*). Overall, antigen staining was less prominent than in the other epithelial tissues examined.

Table. Specific activities of γGT in selected rat tissues

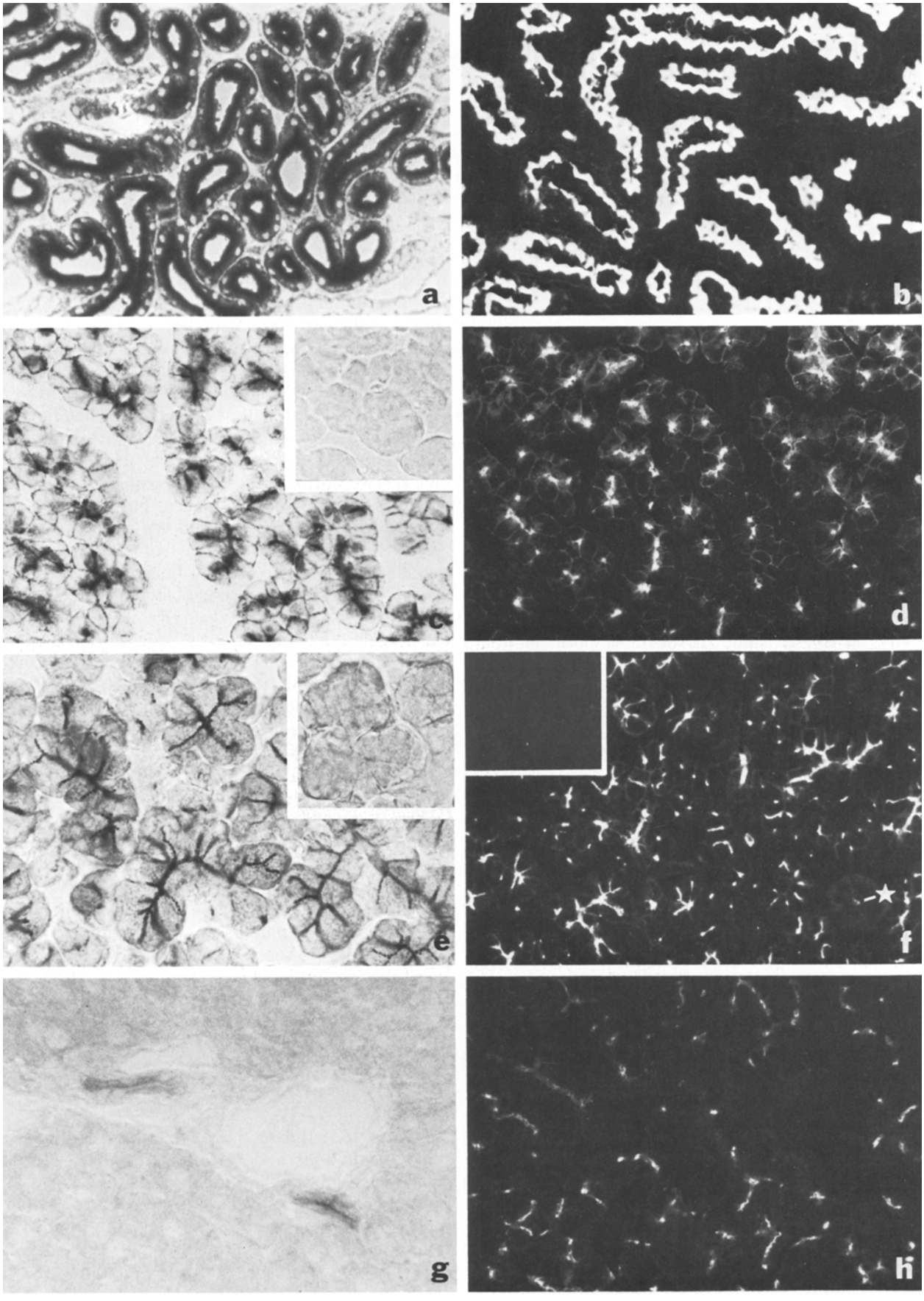
Tissue	μmol/min/mg/protein
Kidney	1.87
Pancreas	0.12
Parotid	0.024
Liver	0.0007

When kidney and liver tissue (which respectively exhibit the highest and lowest enzyme specific activities of the tissues studied) were examined by immunocytochemical staining using antiPγGT the results shown in Fig. 5 were obtained. In kidney (Fig. 5*a*) the immunofluorescent pattern paralleled the distribution of enzyme activity (Fig. 4*a*); it coincided with the apical surfaces of proximal tubule profiles shown in the paired phase contrast image (Fig. 5*b*) and did not extend detectably to the distal tubule or capillary network. In liver, antiPγGT stains bile duct profiles at the level of portal triads (Fig. 5*d* and *e*) where enzyme activity is located (Fig. 4*g*) and exhibited only highly restricted staining of the hepatocyte bile canalicular surface in the near vicinity (Fig. 5*c*).

In the case of pancreas, the immunofluorescent patterns obtained using antiPγGT and antiTγGT closely resembled one another (*not shown*), whereas in parotid very little staining was detected using antiPγGT. These results clearly suggest that γGT antigens and enzyme activity co-localize qualitatively and quantitatively only in tissues that contain high levels of enzyme activity. Prominent immunostaining in epithelial tissue with low activities indicates the presence of structurally related antigens that are probably enzymatically inactive.

Immunochemical Characterization of Plasmalemmal Antigens in Different Epithelia

In order to estimate the relative contributions of enzyme subunits and γGT-related antigens to the observed immunofluorescent staining, plasma membrane fractions isolated from each of the four epithelial tissues were radioiodinated, solubilized and immunoprecipitated with antiTγGT or antiPγGT. The immunoprecipitates were subjected to SDS polyacrylamide gel electrophoresis followed


a
b
c
d
e
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h

Fig. 4. (*facing page*). Comparative localization of γGT activity (by enzyme cytochemistry; *a, c, e,* and *g*) and antigens (by indirect immunofluorescence; *b, d, f,* and *h*) in cryostat sections of epithelial tissues. (*a, b*) Kidney cortex showing very heavy staining of the brush border of proximal tubule cells with almost no staining at other levels of the nephron. *a, b* × 240. (*c, d*) Exocrine pancreas showing activity and antigen highly concentrated on the apical secretory surface of acinar cells with lower levels observed over accumulated zymogen granules. The inset in *c* shows the absence of cytochemical staining when glycylglycine is omitted from the reaction mixture. *c* × 290; *d* × 280. (*e, f*) Parotid gland showing the intensely stained secretory canaliculi on acinar cells and fainter staining occasionally observed on the apical surface of secretory ducts (white ★). Insets in *e* and *f* show, respectively, a cytochemical control analogous to that shown in *c* (inset) and the absence of immunostaining (characteristic of all tissues) when antiTγGT is replaced by preimmune serum. *e* × 450; *f* × 350. (*g, h*) Liver, showing enzyme activity only over bile duct profiles located in a portal triad but general immunostaining of the bile canalicular surface of hepatocytes. *g* × 450; *h* × 440. In all four epithelial tissues faint immunostaining is observed along the basal plasmalemma of parenchymal cells. It is most prominent in pancreas where punctate accumulations can be seen

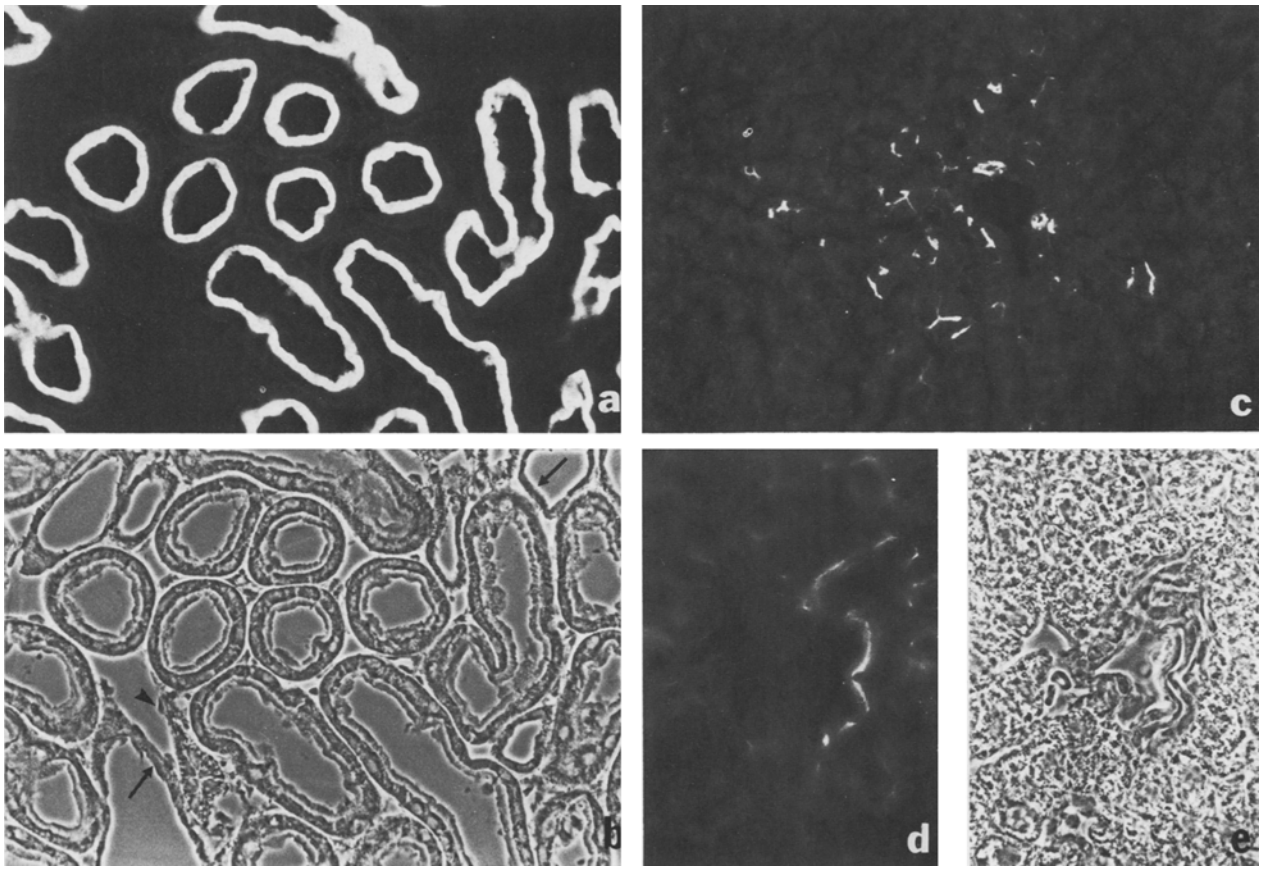

Fig. 5. Indirect immunofluorescent staining of cryostat sections of kidney and liver using antiPγGT. The immunofluorescent (*a*) and paired phase contrast (*b*) images of kidney cortex indicate clearly that staining is restricted to the apical surface of the proximal tubule and is absent over profiles of distal tubule (arrow in *b*) and blood vessels (arrowheads in *b*). *a, b* × 260. *c* shows that antiPγGT stains the bile canalicular surface of hepatocytes only in regions near the transition to bile ductules near the portal triad. Most of the parenchyma is unstained. The reactivity of bile ductules is emphasized in the paired immunofluorescent and phase images shown in *d* and *e*, respectively. *c* × 190; *d, e* × 370

by autoradiography and the results obtained with antiTγGT are shown in Fig. 6. Evidently plasma membrane fractions derived from tissues exhibiting high specific activities of γGT (Table) are generally enriched in enzyme subunits in relation to high mol wt γGT-related antigens. By contrast, polypeptides ≥95K constitute the principal antigens in tissues such as parotid and liver which have low enzyme activities. This observation explains both the quantitative discrepancy between prominent fluorescent staining yet low enzyme activity noted above for parotid (Fig. 4*e* and *f*) and the qualitative discrep-

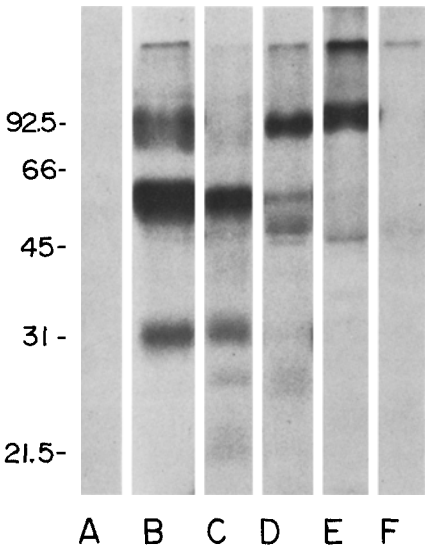

Fig. 6. Immunoprecipitates of ^{125}I-labeled plasma membrane fractions subjected to polyacrylamide gel electrophoresis and autoradiography. Purified fractions were iodinated, solubilized, and subjected to immunoprecipitation with either preimmune serum (*A* and *F*) or antiTγGT (*B–E*). The samples—kidney brush border membranes (*A* and *B*), pancreatic plasma membranes (*C*), parotid plasma membranes (*D*) and liver plasma membranes (*E*)—are displayed in decreasing order of γGT specific activity. The progressive decrease in relative prevalence of antigens corresponding to the large (55–58K) and small (25–30K) subunits and increase in prevalence (as well as apparent mol wt) of the 85–≥95K antigens from *B* to *E* is evident

ancy between immuno- and enzymatically-reactive cell types identified in liver (Fig. 4*g* and *h*).

Immunoprecipitation of isolated parotid or liver plasma membranes with antiPγGT (the same serum used to obtain Fig. 1*A*) did not yield bands distinct from the faint species detected using preimmune serum. Apparently either the degree of antigen radiolabeling is not high enough or the avidity of antiPγGT is insufficient to complex the low concentration of antigen (active enzyme) present in these solubilized membrane preparations.

Comparison of the high mol wt antigens (Fig. 6) among the tissues examined indicates differences in electrophoretic mobility such that the least mobile species derive from tissues that exhibit the lowest levels of active enzyme. One further study was conducted using kidney and liver membranes to judge further the enzymatic inactivity of these polypeptides. First, an affinity column of immobilized PγGT was used to resolve antiTγGT into bound (affinity purified) and unbound (flow through) antibody fractions. When titered for ability to complex active enzymes from solubilized brush border membranes, the affinity purified fraction bound 960 units/ml, whereas the flow through fraction bound 0.06 units/ml. Each fraction was used for immune overlay on kidney brush border membranes and liver plasma membranes that had been blotted onto nitrocellulose following SDS polyacrylamide gel electrophoresis. The results are shown in Fig. 7. Striking differences are observed in the antigen spectrum recognized by each antibody fraction. Affinity purified antibody binds strongly to both enzyme subunits and to a much more limited extent to 85–95K antigens in kidney membranes (Fig. 7*A*). In each case binding is diffuse and covers the whole range of antigen sizes. Binding to liver membranes (only at >95K) is detected after prolonged exposure of the autoradiogram (Fig. 7*C*). By contrast, flow-through antibody binds prominently to subsets of kidney antigens (Fig. 7*B*). Almost nothing is seen at *S*; for *L* and 85–95K antigens, binding favors species having higher apparent mol wt. Figure 7*D* shows that the flow-through fraction readily binds to high mol wt liver plasmalemmal antigens and even detects a band at the level of *L*. These results argue strongly for the enzymatic inactivity of the bulk of high mol wt antigens and even suggest that a fraction of *L* antigens are not part of active enzyme (since the titers of affinity and flow through fractions were measured without denaturation). Although separate experiments indicate that antibodies desorbed from either liver or kidney high mol wt antigens bind interchangeably to the high mol wt antigens in the other type of membrane (*not shown*), further detailed analysis will be required to define the degree to which electrophoretic mobility differences reflect distinct polypeptide primary structure or extents and patterns of glycosylation.

Discussion

We have developed antibodies to γGT for two principal reasons: (1) to evaluate structural relationships between forms of a membrane polypeptide known to be associated with both the cell surface and internal membranes and (2) to evaluate dynamic relationships between such forms, especially in exocrine secretory systems where exocytosis brings antigen-containing compartments (granule- and plasma membranes) into transient continuity. Antigen (enzyme) purification and initial antibody characterization have been carried out using the membranes of renal cortical tissue. The important findings reported in this paper stem from the presence of minor polypeptides of apparent mol wt ~85–≥95K present in purified TγGT but apparently not PγGT. These polypeptides are prominent antigens and blot → reblot and peptide mapping studies have shown that they are, in part, structurally re-

lated to γGT and thus are not merely unrelated contaminants. Further, using an immunochemical approach these or analogous polypeptides of similar apparent mol wt have been found to constitute the principal antigens in tissues exhibiting prominent immunofluorescent staining but only low levels of active enzyme.

The apparent absence of γGT activity associated with the high mol wt species is supported by the different locations of antigen recognized by antiTγGT and cytochemical activity (as well as antigen recognized by antiPγGT) observed in liver tissue. Even though only ~60% of the total enzyme activity of the tissue is detected by the cytochemical procedure, we are confident that the principal liver antigen found on hepatocytes is enzymatically inactive because it selectively binds antibodies having a negligible titer for active enzyme (Fig. 7). These observations coupled with the inability of antiPγGT to detect high mol wt antigens (especially in kidney membranes) and the clear lack of complete overlap observed in comparing antigens by peptide mapping (Fig. 3) suggest that there are structural as well as possible functional distinctions between the high mol wt antigens and the enzyme subunits. Thus, the possibility that high mol wt antigens represent aggregates of subunits arising as an artifact during preparation of samples for electrophoretic analysis [12, 41] can be discounted. However, the extent of structural and functional interrelationships remain to be defined.

Several recent studies of γGT in rat kidney have identified a monomeric kinetic precursor of the subunits of the active enzyme [3, 7, 25, 32, 36, 37]. Proteolytic processing of this transient species to subunits apparently can occur both preceding [3, 7, 37] and following [25, 32, 36] its appearance in cellular compartments involved in complex glycosylation. Although the structural basis (if any) for these options has not been defined, the apparent mol wts of the polypeptides involved (≤78K) are lower than the range characterized in the present study for the kinetically stable high mol wt antigens. Detailed studies of the carbohydrate composition of PγGT purified from several rat tissues and related transformed cells have indicated not only that oligosaccharide chains are exclusively asparagine-linked and structurally very heterogeneous [52–55] but also that the total number of chains per enzyme molecule may vary considerably either between different tissues [53, 54] or within the same tissue in normal, neonatal, and neoplastic states [48, 53, 55]. Although we cannot attribute enzyme activity to the high mol wt antigens characterized in this study, their size is comparable to that reported for combined subunits of the forms of γGT found to be

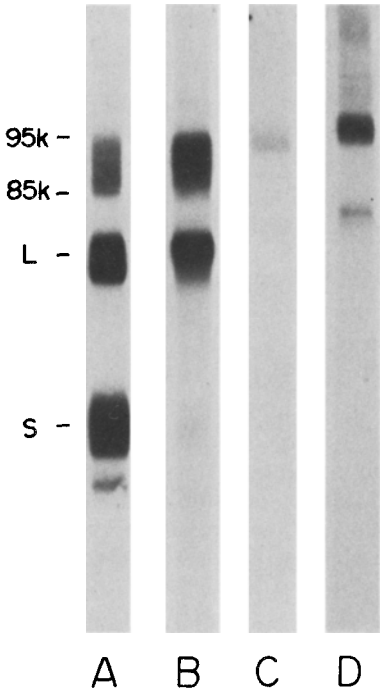

Fig. 7. Immunoblots of affinity purified and flow-through fractions of antiTγGT (separated on immunobilize PγGT) on kidney brush border membranes and liver plasma membranes. Bound antibodies were detected by autoradiography after incubation with ^{125}I-labeled goat anti-rabbit IgG. (*A* and *B*) Kidney membranes exposed, respectively, to affinity purified and flow-through fractions. (*C* and *D*) Liver membranes exposed, respectively, to affinity purified and flow-through antibody fractions. 85–95K polypeptides and enzyme large (*L*) and small (*S*) subunits are identified

glycosylated to uncharacteristically high levels [55]. Thus, in view of their avid lectin binding, it is possible that they represent γGT precursors that are glycosylated to a proportionately much greater extent than the principal precursor characterized in biosynthetic studies of kidney tissue [37] and cultured HTC cells [3]. Alternatively, since the flow-through fraction of antiTγGT recognizes *L*-sized, and not *S*-sized, polypeptides selectively, additional glycosylation could involve *L* stripped of *S* (in a manner not yet defined) following early cleavage of the primary translation product. In either case the possibility that O-linked oligosaccharides may be present cannot be discounted since previous characterizations of the carbohydrate structure of γGT have focused exclusively on the protease-soluble portion of the enzyme molecule. Additional oligosaccharides may preclude normal processing, thereby endowing the high mol wt antigen with stability. Interestingly, and potentially relevant to these considerations, the same γGT biosynthetic studies cited above [3, 37] provide evidence for 95–100K antigens which in

one case [3] have been shown to have an extended half-life relative to the direct enzyme precursor[3]. Coupled with the following observations: (1) selective binding of anti*S* to ~85K polypeptides and anti*L* to ≥95K polypeptides in blot → reblot experiments (Fig. 2); (2) progressively decreased electrophoretic mobilities of high mol wt antigens in tissues exhibiting lower γGT activity (Fig. 5, Table); and (3) recent demonstrations of inducible changes in the level of protein glycosylation [8, 24], these findings provide a basis for testing in future studies whether the extent of glycosylation and the level of γGT activity are modulatable *in situ* and can lead to selective activation (or refractoriness to activation) among multiple forms of γGT-related polypeptides (or precursors). Such regulation may introduce a new variable for consideration in studies of changes in antiPγGT immunocytochemical reactivity and γGT activity in neoplastic transformation [45, 47, 55].

In addition to the suggestion that the high mol wt antigens are proportionately more highly glycosylated than the direct precursor of γGT or the enzyme subunits, the possibility should be tested that these polypeptides represent distinct gene products and that primary structure contributes to the differential processing. The existence of more than a single γGT-related gene seems appropriate for consideration since even among PγGT preparations purified to comparable levels from different rat tissues (kidney and pancreas) there are significant differences in amino acid composition [47].

Finally, a comment should be made in relation to the low levels of specific immunofluorescent staining observed on the basolateral surfaces of epithelial cells, especially pancreatic acinar cells, shown in Fig. 4. Such staining has been reported previously in kidney using a relatively uncharacterized antiPγGT antibody [44]. In the present case we have not established which antigens are responsible for the observed staining or whether its punctate quality reflects concentration with some membrane-associated structural specialization (plasmalemmal foldings?). However, identification on the basolateral front clearly suggests that γGT-related polypeptides may not represent completely polarized markers restricted in location to the epithelial apical surface. Further studies are needed to establish if antigen present on the basolateral plasmalemma reflects membrane traffic between the distinct surface domains [18, 40, 43].

[3] Both biosynthetic studies were conducted using antiPγGT. If these species and the ~95K antigens we have characterized prove to be identical polypeptides, then antiPγGT may be able to detect this class of antigens only under restricted circumstances, for example, when complete glycosylation and processing to the final conformation does not occur.

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