Expression of Rat Renal γ-Glutamyltranspeptidase in LLC-PK$_1$ Cells as a Model for Apical Targeting†

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ABSTRACT: In the rat, γ-glutamyltranspeptidase (γGT) is transcribed into four unique mRNAs from a single gene by use of at least three different promoters and alternative splicing. For the first time, two distinct full-length cDNAs encoding the protein for rat renal γ-glutamyltranspeptidase have now been isolated. Characterization by restriction enzyme mapping and nucleotide sequencing indicates that the two cDNAs, corresponding to transcripts I and II, differ *only* in the 5′ noncoding region. However, transcription from promoter I, most proximal to the coding sequence, apparently began 20 bases upstream from the major transcription start previously reported. Since in vitro transcription and translation of these two new γGT cDNAs were found to produce a full-length peptide (M_r ~62 000), both cDNAs were used to transfect LLC-PK$_1$ (porcine) cells, a polarized cell line most representative of the renal proximal tubule. Rat γGT was expressed in transfected cells as judged by immunofluorescence analysis, direct immunoprecipitation after metabolic labeling with [^{35}S]methionine, and an increase in γGT specific enzymatic activity (up to 5-fold). When clonal cell lines (I or II) were grown on Falcon filter inserts, the increased γGT activity was found only at the apical surface, consistent with polarized expression of the rat γGT. In contrast, transfection of the same cells with cDNA of human growth hormone resulted in both apical (70%) and basal lateral (30%) secretion of the expressed hormone. Altogether the data indicate that transfection of LLC-PK$_1$ cells with rat γGT and its derivatives should provide an alternative model system in which to characterize the cellular mechanisms involved in apical targeting.

The plasma membrane of epithelial cells is characterized by segregation of both lipids and proteins between the apical and basal lateral surfaces (Hubbard et al., 1989; Simons & Wandinger-Ness, 1990). While this polarity is enhanced by the presence of tight junctions between cells in a monolayer, the cellular mechanisms for the formation and maintenance of this distinct membrane content are under investigation.

The microvillar hydrolases of epithelial cells represent a relatively simple and well-characterized class of apical glycoproteins which function to degrade metabolites in the lumenal space of the respective organ. Three subclasses of these hydrolases can be defined on the basis of their synthesis, membrane attachment, and dimerization (Kenny & Maroux, 1982; Danielsen et al., 1984; Semenza, 1986; Hauri, 1988). The first subclass represents those proteins such as aminopeptidase N, which are synthesized as a single glycopeptide and self-associate posttranslationally to form a homodimer with both subunits retaining a membrane anchor. The second subclass represents those proteins such as sucrase–isomaltase and γ-glutamyltranspeptidase (γGT), which are synthesized as a single glycopeptide and are subsequently cleaved to yield a heterodimer. The subunits remain noncovalently associated, but only one of the subunits contains a membrane anchor. All the enzymes in these first two classes are anchored by noncleaved signal sequences at their amino terminus, resulting in cytoplasmic domains of only 4–29 residues, and relatively large glycosylated ectodomains (usually M_r ~100 000–200 000). Recent research on naturally occurring mutants of sucrase–isomaltase indicates that an apical targeting signal may be located in the amphipathic isomaltase subunit (Fransen et al., 1991) while anchor-minus forms of ApN (Vogel et al., 1992), neutral endopeptidase (Corbell et al., 1992), and dipeptidylpeptidase IV (Weisz et al., 1992) retain polarized expression consistent with the presence or a targeting signal within their ectodomains.

Proteins in the third subclass of microvillar hydrolases, such as alkaline phosphatase, are synthesized as a single peptide with a cleavable signal sequence, and are posttranslationally modified with glycosylphosphatidylinositol (GPI) at the carboxyl terminus (Hauri et al., 1988). It is now known that all GPI-anchored proteins are directly delivered to the apical surface of epithelial cells (Lisanti et al., 1988). Transfer of the carboxyl-terminal signal for GPI attachment to a basal lateral protein, by recombinant DNA techniques, results in apical delivery of the chimeric protein (Brown et al., 1989; Lisanti et al., 1989). Similarly, the GPI-anchored isoform of N-CAM is delivered to the apical surface of MDCK cells while the peptide-anchored isoform is expressed at the basal lateral surface (Powell et al., 1991b). However, a truncated form of Thy-1 lacking its GPI anchor is still apically expressed (secreted) in MDCK cells (Powell et al., 1991a).

The mechanism for apical delivery of non-GPI-modified proteins is *not* known. The cumulative data (Hopkins, 1991) resulting from studies of both apically expressed viral proteins (Brewer & Roth, 1991; Roth et al., 1987) and endogenous or transfected microvillar hydrolases (Low et al., 1991; Wessels et al., 1990; Matter et al., 1990; Bartles et al., 1987; Weisz et al., 1992), in various epthelial cells, indicate that there may be differential use of several mechanisms.

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γGT represents the smallest of the microvillar hydrolases and is found on the apical surface of all polarized cells where it functions in the catabolism of glutathione (Curthoys, 1987). The protein is encoded by 7 (Rajagopalan et al., 1990) or 13 (Goodspeed and Pitot, personal communication) exons in the rat gene and transcribed from at least 3 different promoters designated I, II, and III, in proximal to distal order, respectively (Griffiths & Manson, 1989; Chobert et al., 1990; Kurauchi et al., 1991). Alternative splicing of the transcripts results in three mRNAs which *apparently* differ in sequence only at the 5′ ends. Evidence, but not sequence data, for a fourth mRNA (IV) with a different 5′ noncoding region has now been reported (Darbouy et al., 1991).

γGT is synthesized as a propeptide of 62 000 Da and is N-glycosylated at five or six sites (Yamashita et al., 1983; Capraro & Hughey, 1983). In all tissues studied, it is found in a mature form as a heterodimer of ~50 000 and ~30 000 Da. However, cleavage of the peptide is not necessary for enzymatic activity (Tate & Galbraith, 1988). The larger subunit of the heterodimer contains the uncleaved signal sequence anchor, yielding a cytoplasmic domain of only four residues. A site for O-linked glycosylation is found adjacent to the membrane anchor (Blochberger et al., 1989), and six consensus sequences for N-linked glycosylation have been identified (Sakamuro et al., 1989). The small subunit is noncovalently associated with the large subunit and contains the γ-glutamyl binding site, two sites for N-linked glycosylation (Sakamuro et al., 1989), and one site for O-linked glycosylation (Blochberger et al., 1989). The relatively simple structure of the γGT makes it an ideal model for investigation of the targeting mechanisms which result in apical expression of this major class of microvillar proteins. Most notably, the γGT is significantly smaller than other microvillar hydrolases (glycosylated propeptide M_r ~75 000), and it does *not* form homodimers which could interfere with the release of any mutant or chimeric forms from the endoplasmic reticulum.

In order to develop this model system, it was necessary to isolate cDNAs representing the full-length γGT coding sequence. The amino acid sequence has been predicted from the nucleotide sequences of *overlapping and partial* cDNAs (Laperche et al., 1986; Coloma & Pitot, 1986). A preliminary report on our cloning of the full-length γGT cDNAs has been presented (Hughey et al., 1989). These cDNAs have been used for transfection of LLC-PK_1 cells, a porcine renal cell line which exhibits properties of the renal *proximal* tubule cells (Gstraunthaler et al., 1985) where γGT is localized in the kidney. While MDCK (canine kidney) cells have been used extensively in recent studies of polarized expression, biochemical characterization of MDCK cells has shown that they are most like the *distal* tubule cells (Gstraunthaler et al., 1985) of rat kidney. Since the cumulative data (Hopkins, 1991) resulting from studies with rat hepatocytes, MDCK cells, and human intestinal (Caco-2) cells indicate that there is cell specificity in the use of cellular mechanisms for delivery of apically targeted proteins, it is essential to characterize additional cell types in order to thoroughly understand the multitude of cellular mechanisms involved.

EXPERIMENTAL PROCEDURES

Materials. LLC-PK_1 cells were obtained from the American Type Culture Collection, and pig kidney cortex was obtained from Pel Freeze. Restriction endonucleases were purchased from Boehringer Mannheim, tissue culture media and G418 were purchased from GIBCO, and all electrophoresis reagents were from Bio-Rad. Filter inserts (25 mm, 0.45 μm) were from Falcon. T7 and SP6 polymerases and pGEM-4Z were from Promega, and pBLUESCRIPT-SK(-) was from Stratagene. Lipofectin was a product of GIBCO BRL, and the HGH transient gene expression assay system was made by Allégro, San Juan Capistrano, CA. [^{35}S]Methionine (1000 Ci mmol^{-1}) was purchased from the NEN division of Dupont, and dog pancreas membranes were purchased from Amersham. Film for fluorography (X-OMAT) was from Kodak. All other reagents were obtained from Sigma.

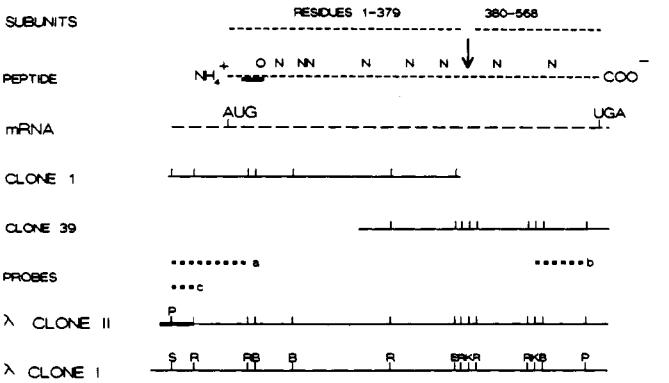

FIGURE 1: Linear map depicting rat γGT cDNAs, mRNA, and peptide structure. The 1.35-kb cDNA of clone 1 includes 277 bp of 5′ noncoding sequence, while the 1.05-kb cDNA of clone 39 includes 138 bp of 3′ noncoding sequence. On the basis of a common 450 bp sequence, the cDNA sequence predicts an mRNA encoding a reading frame of 1707 bp. Due to a posttranslational cleavage reaction (arrow), the propeptide usually yields two subunits. The larger subunit of 379 residues includes the noncleaved signal sequence (bar below the line) which acts as the membrane anchor. Six consensus sequences for N-linked glycosylation (N) are found in the subunit although only three of four are actually used. One site for O-linked (O) has been identified adjacent to the membrane anchor at the papain-sensitive cleavage site. The smaller subunit of 189 residues exhibits 2 sites for N-linked glycosylation and 1 O-linked oligosaccharide in an unknown position. See text for references. The 5′-specific probes were prepared by digestion of clone 1 with *Pst*I and *Bal*I (a) or *Pst*I and *Rsa*I (c), and a 3′-specific probe (b) was prepared by digestion of clone 39 with *Bal*I and *Pvu*II. These probes were used to screen a λgt10 rat renal cDNA library and characterize two full-length clones (I and II). These two new clones are identical by restriction mapping to the coding sequence represented by clone 1 and clone 39 [vertical lines are restriction sites for *Bal*I (B), *Eco*RI (E), *Kpn*I (K), *Pvu*II (P), *Rsa*I (R), and *Sac*I (S)]. The only difference between the cDNA sequences is found in the 5′ noncoding region, and is represented by a heavy line at the 5′ end of λ CLONE II.

Isolation and Subcloning of γGT cDNA. As shown in Figure 1, two overlapping cDNA clones (clone 1 and clone 39), representing the full-length rat transpeptidase mRNA coding sequence, were obtained from G. Guellaen and Y. Laperche (Laperche et al., 1986). A mixture of the two cloned cDNAs was used to initially screen a rat renal λgt10 cDNA library obtained from K. Lynch (Burnham et al., 1987). Forty positive clones were recovered and screened by duplicate plaque lifts using either the 5′ *Pst*I–*Bal*I fragment (a) from clone 1 or the 3′ *Bal*I–*Pvu*II fragment (b) from clone 39. Since these fragments are near either the start or the stop codons for the coding sequence, seven clones were considered to be full-length. In order to subclone full-length inserts of γGT cDNAs, the λgt10 DNA samples were treated with *Sac*I (nucleotide position −205) and *Nci*I (nucleotide position +1713) endonucleases. However, only one of the clones yielded a *Sac*I-sensitive insert (data not shown). When *Eco*RI digests of the six clones were analyzed by Southern blot analysis (Chomczynski & Qasba, 1984) using the smaller *Pst*I–*Rsa*I 5′ probe (c) shown in Figure 1, only the *Sac*I-sensitive cDNA reacted. More extensive restriction enzyme mapping (with *Bal*I, *Pvu*II, and *Rsa*I) of this one cDNA and the longest of the remaining

cDNAs (*Sac*I-insensitive) was consistent with the sequence predicted by the overlap of the clone 1 and clone 39 cDNAs. However, the differences at the 5′ end of these two cDNAs indicated that they represented the γGT mRNAs I and II (Chobert et al., 1990). This was subsequently confirmed by dideoxy sequencing (Sanger et al., 1977) of the two 5′ noncoding regions. The two λgt10-cloned cDNAs were partially digested with *Eco*RI, and full-length cDNA fragments (γGT I, 2150 bp; and γGT II, 2114 bp) were subcloned into the unique *Eco*RI sites of both pGEM-4Z and pBLUESCRIPT-SK(-), and the expression vector, pSFFVneo, obtained from D. Chaplin (Fuhlbrigge et al., 1988).

Cell Culture and Transfection. LLC-PK_1 cells were transfected with pSFFVneoγGT(I or II) by the method of Chen and Okayama (1987). Briefly, cells were incubated with a suspension of DNA (20 μg) overnight at 37 °C in 1.8% CO_2. The medium was changed the next day, and the incubator CO_2 was returned to normal at 5% CO_2. After 2 days, the aminoglycoside G418 was added to the medium at 0.5 mg mL^{-1} in order to select for cells expressing the transfected DNA carrying neomycin resistance (Fuhlbrigge et al., 1988). G418-resistant cells were evident after 7–10 days, and single colonies of resistant cells were isolated with cloning rings. Nonclonal cells were also maintained by combining all remaining colonies on a single plate.

In order to study the polarized expression of γGT, the clonal cells were plated on Falcon filter inserts and maintained in culture for 5 days. The formation of a confluent monolayer with tight junctions was tested by addition of [^{3}H]inulin (5×10^5 cpm) to the top chamber and measurement of radioactivity in the lower chamber over 30 min. Confluent plates of cells routinely showed no movement of label within the first 5 min. Therefore, γGT enzymatic activity on the apical and basolateral surfaces of these same cells was determined by addition of 1.0 mL of assay mix (Tate & Meister, 1974) to either the top or the bottom chamber at room tempeature while 1.0 mL of assay mix without substrate was present at the opposite surface. Aliquots of 0.75 mL were removed after 2 or 3 min, and the absorbance at 410 nm was determined. γGT activity was linear over this time (data not shown). Each filter was washed twice with phosphate-buffered saline (PBS) followed by 0.1 mL of extraction buffer (150 mM NaCl/10 mM HEPES, pH 7.2) containing 1% Triton X-100. Triton-insoluble material was removed by centrifugation for 10 min at 10000*g*, and γGT activity of these cell extracts was determined at room temperature as described previously (Tate & Meister, 1974). A unit of γGT enzymatic activity is 1 micromole per minute of product hydrolyzed. Protein concentrations were determined in cell extracts using the method of Lowry (Lowry et al., 1951).

Human growth hormone (hGH) cDNA in an expression vector (p22K) was a generous gift from Justus Cohen. Cells were transfected with the cDNA (10 μg) using lipofectin reagent (10 μg) as described by the manufacturer. The following day, cells were heavily plated onto Falcon filter inserts and tested for confluency after 2 days. Fresh medium (1 mL) was added to the top and bottom chambers, and the level of hGH was assessed by immunoassay as described by the manufacturer (Allégro).

In Vitro Transcription and Translation. RNA was synthesized from pGEM-4Z-γGT(I) and pGEM-4Z-γGT(II) using T7 RNA polymerase and SP6 RNA polymerase, respectively, as described by Promega, based on the method of Milton (1984). The pGEM-4Z-γGT(I) and -γGT(II) were first linearized by treatment with *Nde*I and *Hinc*II endonucleases, respectively. The transcripts were analyzed by agarose gel electrophoresis and the absorbance of 260 nm after extraction with phenol and chloroform, and ethanol precipitation. Translation of the RNA was carried out with an mRNA-dependent rabbit reticulocyte lysate prepared by R. Jagus (Jagus, 1987) using 0.1 μL of the crude transcription reaction. Dog pancreas microsomes were included in the translation mix where indicated, while translations without microsomes were substituted with KCl and $MgCl_2$ as suggested by the Amersham protocol. Reactions were carried out for 30 min at 30 °C and dialyzed on filters, and an aliquot was subjected to SDS–PAGE followed by fluorography of the dried gel.

Immunocytochemical Analysis. Polyclonal antibodies against the papain-purified rat γGT (Hughey et al., 1986) were raised in rabbits and showed significant cross-reactivity with the LLC-PK_1 cells. Rat γGT-specific antibodies were prepared by incubation of the antisera (0.5 mL, titer 175 units mL^{-1}) with 4 mL of renal brush border membrane vesicles (90 units of γGT activity) prepared by $CaCl_2$ differential precipitation from 40 g of pig kidney cortex (Stewart & Kenny, 1984). After overnight incubation of antibodies and membranes at 4 °C, the membranes were removed by centrifugation at 10000*g* for 30 min. Titer of the supernatant was 12 units mL^{-1}.

Cultured cells ($\sim 10^5$) were plated on polylysine-coated coverslips (12-mm circles) and allowed to adhere for 1 h before additional medium was added. The next day, the coverslips with cells were washed with PBS and then fixed with 4% paraformaldehyde. They were then blocked for 5 min in 1% BSA, 10% horse serum, 125 mM NaCl, and 50 mM Tris-HCl, pH 7.2. After being washed, the cells were incubated with rat γGT-specific antisera (diluted 1/100) for 90 min. After being washed 4 times with buffered saline, a goat anti-rabbit IgG antibody conjugated to fluorescein was added at 1/1000 dilution and incubated for 45 min. The slips were washed 4 more times with PBS before analysis with Zeiss standard microscope and photographed.

Immunoprecipitation of γGT. Cells were incubated overnight with 50 μCi of [^{35}S]methionine in 1 mL of modified Eagle's media without methionine, washed with PBS, scraped, and homogenized in a glass–Teflon vessel with extraction buffer (1% Triton X-100, 150 mM NaCl, and 10 mM HEPES, pH 7.2). Triton-insoluble material was removed by centrifugation at 100000*g* for 30 min. Rat γGT-specific or untreated antibodies were added to the supernatant for 1 h at 4 °C, followed by the addition of 15 μL of a 50% slurry of Protein A–Sepharose for at least 1 h. Immunoprecipitates were recovered by centrifugation and washed twice with extraction buffer and then twice with wash buffer (0.1% SDS, 150 mM NaCl, and 10 mM HEPES, pH 7.2). After the pellet was heated for 2 min at 90 °C in sample buffer (Laemmli, 1970), the eluted material was subjected to SDS–PAGE. The gel was subsequently soaked for 15 min in 1 M sodium salicylate and then dried, before analysis by fluorography at 4 °C using Kodak X-OMAT film.

RESULTS

Isolation and Characterization of cDNAs Representing the Full-Length γGT. In order to obtain a cDNA containing the full-length coding sequence of the rat γGT, a λgt10 rat renal library was screened as described under Experimental Procedures. In the course of subcloning the isolated cDNA clones, it became evident that one of the clones corresponded to γGT mRNA I while six corresponded to γGT mRNA II, described

previously (Chobert et al., 1990). The 5′ ends of the cDNA inserts from pBLUESCRIPT-SK(-)-γGT(I) and -γGT(II) were subsequently subjected to Sanger dideoxy sequencing. The results revealed 300 and 264 bp of 5′ noncoding sequence for the γGT I and γGT II cDNAs, respectively. There was identity of the 5′ noncoding region between −1 and −144 of cDNAs I and II. The 5′ noncoding sequence of cDNA I is identical to the sequence reported for clone 1 cDNA, but contains an additional 20 bp (5′TCCCTTCCCC-GGGC-AGCTCT3′) corresponding to the adjacent upstream sequence reported for the rat γGT gene (Rajagopalan et al., 1990; Kurauchi et al., 1991). Thus, γGT I cDNA represents mRNA resulting from transcription at a minor start site within the most proximal promoter of this gene. The unique portion of the 5′ noncoding sequence of the γGT II cDNA agrees with data subsequently published by Laperche (Chobert et al., 1990) for a second cDNA clone, which results from transcription from a more distal promoter. Transcription from the distal promoter apparently results in alternative splicing of the mRNA within the 5′ noncoding region (Rajagopalan et al., 1990). Transcription from a third promoter has been described (Griffiths & Manson, 1989) and also results in alternative splicing of the mRNA, producing ~400 bp of noncoding sequence. A fourth transcript including ~500 bp of noncoding sequence has been identified only in intestine and HTC cells and could result either from transcription at promoter III or from transcription at an unidentified fourth promoter (Darbouy et al., 1991). While most γGT transcripts in whole rat kidney result from similar use of both promoters I and II, the γGT II transcript has been reported only in the proximal tubule cells within the kidney (Chobert et al., 1990).

In Vitro Expression of Rat γGT from Cloned cDNAs. In order to be sure that the two cloned cDNAs encoded a full-length rat γGT peptide, pGEM-4Z-γGT(I) and pGEM-4Z-γGT(II) were subjected to endonuclease treatment with *Nde*I (a unique site in the plasmid) and *Hinc*II (in the multiple cloning site of the plasmid), respectively, to produce a linear form of each plasmid for in vitro transcription and translation reactions. RNA was transcribed using T7 and SP6 RNA polymerases, respectively. The transcripts were analyzed by agarose gel electrophoresis, and each produced a single band of the appropriate size by ethidium bromide staining. An aliquot of each transcript was used to carry out in vitro translation using [^{35}S]Met, either with or without microsomal membranes. The products were analyzed by fluorography of the dried gel after SDS–PAGE, as shown in Figure 2. Translation in the absence of membranes produced only products of M_r <20 000 apparently from translation of endogenous mRNA in the lysate. In the presence of membranes, a major band of M_r ~62 000 was produced, consistent with the full-length propeptide of 568 residues. In vitro translation of γGT from rat renal poly(A+) RNA is totally dependent on the presence of membranes (Nash & Tate, 1984). Apparently, the signal sequence of the nascent γGT binds the endogenous lysate signal recognition particle (SRP) which would specifically arrest γGT translation. Addition of membranes would result in docking of the SRP complex and renewal of translation due to cotranslational translocation into the lumen. Bands of slower mobility from M_r 65 000–75 000 are consistent with N-linked glycosylation of the propeptide by the membranes. Dog pancreas membranes do exhibit a very low level of the protease activity which cleaves the γGT propeptide (Nash & Tate, 1984). Thus, the bands at lower molecular weight could represent cleavage of the propeptide to the large (40 000–50 000 Da) and small

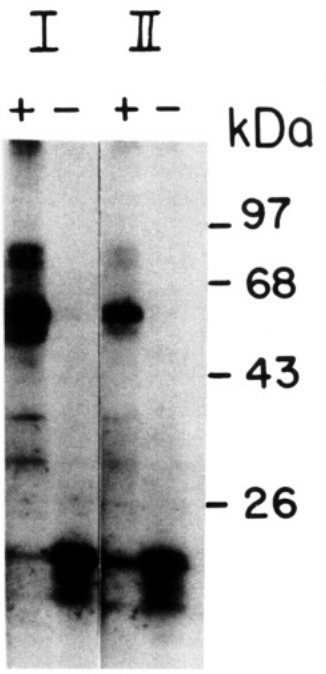

FIGURE 2: In vitro transcription and translation of pGEM-4Z-γGT-(I) and pGEM-4Z-γGT(II) cDNAs. Transcription and translation of the cDNAs were carried out as described under Experimental Procedures, and the resulting peptides were analyzed by fluorography of the gel after SDS–PAGE. Translations of the two transcripts (I and II) were carried out in either the presence (+) or absence (−) of dog pancreas microsomes. The numbers to the right indicate the molecular masses (kDa) of standard proteins at their respective mobilities.

(25 000–30 000 Da) subunits, both with and without N-linked glycosylation, respectively. All of these bands can be precipitated with specific antisera and could also result from premature termination of translation (data not shown). Translation of the γGT mRNA in the presence of membranes also reduces translation of the endogenous mRNA of the lysate (M_r <20 000). Despite the low level of peptide processing (cleavage and glycosylation), the results indicate that both the γGT I and γGT II cDNAs encode the full-length protein.

Expression of Rat γGT in LLC-PK$_1$ Cells. LLC-PK$_1$ cells were transfected with an expression vector (pSFFVneo) ligated with either of the two cloned γGT cDNAs. This vector (Fuhlbrigge et al., 1988) was initially constructed from pBLUESCRIPT-SK(-) by insertion of the Friend spleen focus forming virus LTR upstream from the unique *Eco*RI site of the multiple cloning site (MCS) and the SV40 early splice region and late polyadenylation signals from pSV2cat. The neomycin-resistance gene with the SV40 early region promoter, and splicing and polyadenylation signals, was also ligated into the downstream region of the MCS. The transfected cells were maintained in medium containing G418 to select for cells expressing the transfected cDNA. *Clonal* lines were prepared by selecting individual colonies with cloning rings.

In order to assess whether the rat γGT was being expressed in this pig kidney cell line, rat γGT-specific antibodies were prepared by preincubation with pig kidney brush border membrane vesicles. When this preadsorbed antiserum was incubated with transfected cells and control (nontransfected) cells, only the transfected cells showed immunofluorescence using a second antibody of goat anti-rabbit IgG conjugated to fluorescein (see Figure 3). While the nonclonal transfected cells showed a mixed reactivity with the antisera, the clonal lines were more consistently reactive (data not shown). The clonal cells transfected with pSFFVneo-γGT(I) exhibited a

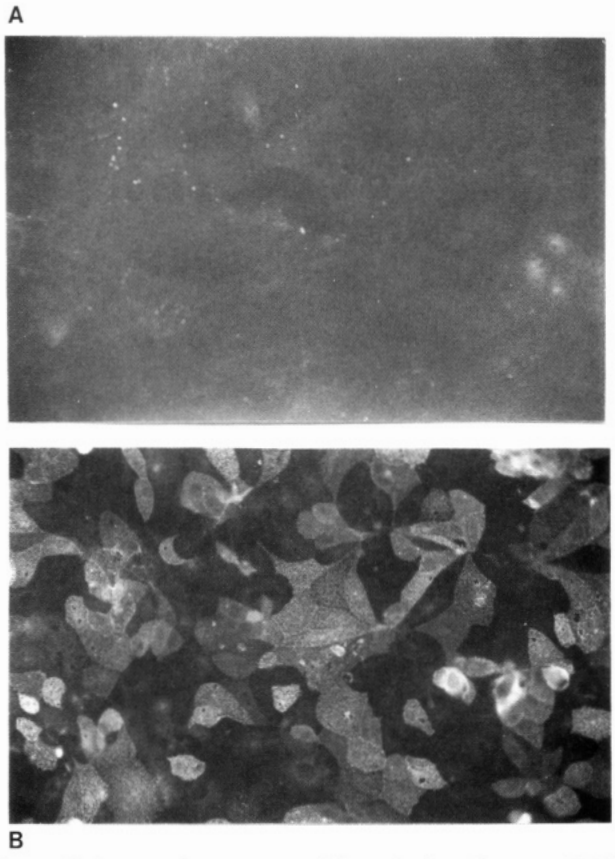

FIGURE 3: Immunofluorescence staining of cell-surface rat γGT in LLC-PK$_1$ cells. Either nontransfected cells or nonclonal lines of cells transfected with pSFFVneo-γGT(I) were plated and fixed on sterile coverslips as described under Experimental Procedures. Cells were incubated with rat γGT-specific rabbit antisera and then goat anti-rabbit IgG conjugated to fluorescein prior to photography. Fluorescence photographs are shown for (A) nontransfected cells and (B) cells transfected with γGT(I) cDNA.

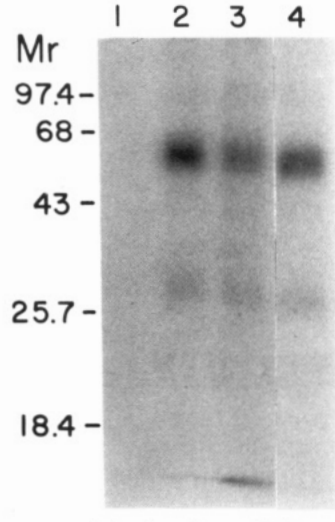

FIGURE 4: Immunoprecipitation of rat γGT from transfected LLC-PK$_1$ cells. Cells were metabolically labeled overnight with [^{35}S]-methionine, and the γGT was immunoprecipitated with rat γGT-specific antibodies as described under Experimental Procedures. Immunoprecipitates from nontransfected cells (lane 1), clonal cells transfected with γGT(II) cDNA (lane 2), nonclonal cells transfected with γGT(I) cDNA (lane 3), and clonal cells transfected with γGT-(I) cDNA (lane 4) were analyzed by fluorography after SDS–PAGE. Numbers to the left refer to the molecular weights ($M_r \times 10^{-3}$) of protein standards at their respective mobilities.

better reactivity than the clonal cells transfected with pSFFVneo-γGT(II), consistent with their specific γGT enzymatic activity shown in Table I (1.11 versus 0.74 unit mg^{-1}, respectively).

The cellular expression of the rat γGT was also studied by metabolic labeling of control and transfected cells overnight with [^{35}S]Met. Cells were subsequently washed and scraped from the plates and homogenized, and rat γGT was immunoprecipitated from the Triton X-100 solubilized cells using the rat γGT-specific antisera and Protein A–Sepharose. The immunoprecipitates were analyzed by fluorography of the dried gel after SDS–PAGE, and both clonal and nonclonal transfected cells exhibit the heterodimeric form of rat γGT observed in rat kidney. The broad bands at M_r ~30 000 and ~50 000 are consistent with both posttranslational cleavage of the propeptide and microheterogeneity in the processing of the N-linked oligosaccharides (Yamashita et al., 1983). The ratio of methionine to cysteine in the large to small subunits is 4:1, consistent with the intensity of the two subunits on the fluorogram. In contrast, no labeled proteins were immunoprecipitated from the control LLC-PK$_1$ cells, confirming the fact that the preabsorbed antiserum no longer reacts with the pig γGT and is really specific for the rat γGT.

When LLC-PK$_1$ control cells or clonal cells transfected with pSFFVneo-γGT(I or II) were plated on Falcon filter inserts at high density (2×10^6 cells), tight junctions were formed after 5 days in culture as determined by [^{3}H]inulin retention. As shown in Table I, nearly all the γGT enzymatic activity was found on the apical surface (93% and >97% for control and transfected cells, respectively). This represents a 4-fold increase in γGT activity at the apical surface and no increase at the basal lateral surface. Thus, the polarized expression of the rat γGT is as efficient as the expression of the endogenous pig γGT.

In contrast, transient transfection of LLC-PK$_1$ cells [transfected with pSFFVneo-γGT(I)] with the cDNA for human growth hormone resulted in ~70% secretion at the apical surface and ~30% secretion at the basal lateral surface. Similar data were observed after 7 and 24 h of accumulation, and in control experiments, hGH added to either chamber did not leak across the monolayer into the opposite chamber during the 24 h. Metabolic labeling of cells with [^{35}S]methionine (1 hour) also resulted in both apical and basal lateral accumulation (ratio ~2:1) of labeled hGH from chase times of 2–18 h when analyzed by fluorography after SDS–PAGE (data not shown). The secretion of rat growth hormone in MDCK cells (60–65% basal lateral) has been used previously (Gottlieb et al., 1986) to identify the default pathways for cell-surface delivery in polarized cells.

DISCUSSION

Hemagglutinin (HA) of influenza represents the most well characterized of the apically targeted proteins. Since HA retains its apical targeting even when its membrane anchor and cytoplasmic domain are deleted, the signal for targeting was thought to be within the ectodomain (Roth et al., 1987). Research in the area of endocytosis has revealed that the presence of a tyrosine residue within the cytoplasmic domain of cell-surface proteins is apparently essential for clustering in clathrin-coated pits for subsequent entry into endocytic vesicles. Since it was already known that most apical proteins are excluded from coated pits (Rodman et al., 1986), it was not surprising to find that addition of a tyrosine residue to the cytoplasmic domain of the HA (Brewer & Roth, 1991) resulted in endocytosis of this protein; unexpectedly, this alteration also resulted in a basal lateral localization for the HA. Along the same line, deletion of 14 residues (including a tyrosine)

Table I: Expression of γGT Activity and Human Growth Hormone in Polarized LLC-PK$_1$ Cells[a]

LLC-PK$_1$ cells	cell extracts		cells on filters (total units × 10^3)	
	total act (units × 10^3)	sp act (units mg^{-1})	apical	basolateral
(A) nontransfected, $n = 4$	25.6 (2.6)	0.22 (0.03)	19.3 (4.7)	1.4 (0.3)
(B) transfected (γGT I), $n = 5$	75.3 (13.0)	1.11 (0.21)	86.4 (5.0)	0.9 (0.1)
(C) transfected (γGT II), $n = 6$	75.0 (19.7)	0.74 (0.28)	71.8 (15.5)	1.8 (0.2)
(D) transfected (γGT I and hGH)				
filter 1			0.72[b]	0.32[b]
filter 2			0.49[b]	0.18[b]
filter 3			2.13[b]	0.83[b]

[a] Nontransfected cells (A) and cells transfected with (B) pSFFVneo-γGT(I) or (C) pSFFVneo-γGT(II) were plated on Falcon filter inserts and maintained in culture for 5 days to reach confluency. γGT enzymatic activity was determined for intact and Triton-extracted cells as described under Experimental Procedures. Data are presented as the mean values for multiple cultures. Standard deviations are in parentheses. (D) Three different dishes of cells already expressing γGT(I) were transiently transfected with the cDNA for human growth hormone (hGH) and and plated on filters. After confluency was reached, the accumulation of hGH in the top and bottom chambers was assessed after 24 h with fresh media. [b] hGH in media (ng mL^{-1}).

from the cytoplasmic domain of the polymeric immunoglobulin receptor (pIgR) results in delivery of the pIgR to the apical surface instead of the basal lateral surface of MDCK cells (Casanova et al., 1991). Replacement of the carboxyl-terminal GPI anchor of alkaline phosphatase with this 14-residue sequence results in delivery of the enzyme to the basal lateral surface. The addition of 47 residues to the cytoplasmic domain of the Fc receptor, by alternative splicing of the mRNA, changes the delivery of the receptor from the basal lateral surface to the apical surface and blocks its ability to enter endocytic vesicles (Hunziker & Mellman, 1989). Weisz et al. (1992) have now shown that depeptidylpeptidase IV (DPP IV) contains competing apical and basal lateral targeting information.

Together all these data present evidence for a very complex system that includes both positive and negative signals for polarized targeting, which may also be directly linked to signals for endocytic traffic. It appears that proteins which are expressed on the basal lateral surface can enter endocytic vesicles and even carry out transcytosis while most proteins which are expressed on the apical surface do not reenter this same intracellular membrane traffic. The one exception to this would be the recent finding of the human LDL receptor at the base of the apical microvilli *and* within coated pits of the kidney convoluted tubule cells of transgenic mice (Pathak et al., 1990).

There is also cell-specificity in the use of targeting mechanisms. For example, both of the microvillar hydrolases, DPP IV and aminopeptidase N (ApN), are delivered to the apical surface of rat hepatocytes by transcytosis from the basal lateral surface (Bartles et al., 1987; Bartles & Hubbard, 1988). However, transfection of MDCK cells (canine kidney) with cDNA for either human ApN (Wessels et al., 1990) or rat DPP IV (Low et al., 1991b) results in direct delivery of the newly made enzymes to the apical surface. While sucrase–isomaltase is delivered directly to the apical surface of the human intestinal cell line (Caco-2), both ApN and DPP IV were partially delivered by transcytosis from the basal lateral surface (Matter et al., 1990). While transfected MDCK cells deliver 62% of the bacterial endoglucanase E to their apical surface, transfected Caco-2 cells secrete 70% through their basal lateral surface (Soole et al., 1992). Thus, characterization of the targeting mechanism present in LLC-PK$_1$ cells will provide an additional model for comparison to the hepatocyte, MDCK, and Caco-2 systems. Already, Low et al. (1991a) have reported that DPP IV is delivered by direct and transcytotic pathways to the apical surface of transfected LLC-PK$_1$ cells. The ability to transfect and express the rat γGT in LLC-PK$_1$ cells will allow both a comparison to the targeting of the endogenous pig γGT and the ability to study alterd forms of this enzyme which is representative of a significant and important class of apical proteins.

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