Carboxypeptidase M, a Glycosylphosphatidylinositol-anchored Protein, Is Localized on Both the Apical and Basolateral Domains of Polarized Madin-Darby Canine Kidney Cells*

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Carboxypeptidase M, a glycosylphosphatidylinositol-anchored membrane glycoprotein, is highly expressed in Madin-Darby canine kidney (MDCK) cells, where it was previously shown that the glycosylphosphatidylinositol anchor and N-linked carbohydrate are apical targeting signals. Here, we show that carboxypeptidase M has an unusual, non-polarized distribution, with up to 44% on the basolateral domain of polarized MDCK cells grown on semipermeable inserts. Alkaline phosphatase, as well as five other glycosylphosphatidylinositol-anchored proteins, and transmembrane γ-glutamyl transpeptidase exhibited the expected apical localization. Basolateral carboxypeptidase M was readily released by exogenous phosphatidylinositol-specific phospholipase C, showing it is glycosylphosphatidylinositol-anchored, whereas apical carboxypeptidase M was more resistant to release. In contrast, the spontaneous release of carboxypeptidase M into the medium was much higher on the apical than the basolateral domain. In pulse-chase studies, newly synthesized carboxypeptidase M arrived in equal amounts within 30 min on both domains, indicating direct sorting. After 4–8 h of chase, the steady-state distribution was attained, possibly due to transcytosis from the basolateral to the apical domain. These data suggest the presence of a unique basolateral targeting signal in carboxypeptidase M that competes with its apical targeting signals, resulting in a non-polarized distribution in MDCK cells.

Regulatory B-type carboxypeptidases play important roles by specifically cleaving C-terminal Arg or Lys residues from peptides and proteins (1). Carboxypeptidase M (CPM), a member of this family of enzymes, is a glycosylphosphatidylinositol (GPI)-anchored plasma membrane enzyme, widely distributed in human tissues (1–5) and often highly expressed in epithelial cells (1, 5), including Madin-Darby canine kidney (MDCK) cells (6). The MDCK cell line is speculated to have originated from distal renal tubular epithelial cells (7, 8) and has been used extensively as a model of polarized renal tubular epithelium. These cells were also used to show that all GPI-anchored surface proteins are specifically localized to the apical surface (9). Additional studies on the sorting of GPI-anchored proteins revealed their apical localization to be a conserved feature of polarized epithelial cells from other tissues and species (10). These data, together with studies using genetically engineered GPI fusion proteins (11, 12), resulted in the classification of the GPI anchor as a dominant apical sorting signal (13, 14). More recently, the MDCK cell line was used as a model system to show that N-linked carbohydrate is an additional apical targeting signal (13, 15).

CPM activity and mRNA are found in human kidney (5, 16), which also secretes CPM into urine (17, 18). Although the roles of CPM in kidney function have not been clearly defined, erythropoietin, bradykinin, and epidermal growth factor (EGF) are potential CPM substrates that are generated in the kidney and excreted into urine. Kinetic studies with bradykinin (19) and EGF (17) showed that these peptides are good substrates of CPM in vitro. Bradykinin induces natriuresis, diuresis, and prostaglandin synthesis in the kidney; thus, inactivation by CPM could play a role in the regulation of salt and water balance (1, 20). The biological role of CPM in renal tubular epithelium will depend on its apical or basolateral localization because its endogenous peptide substrates and corresponding receptors can also have polarized distributions. For example, CPM is responsible for the initial metabolism of EGF to des-Arg9–EGF at the surface of MDCK cells (17). Because the EGF receptor is predominantly expressed on the basolateral domain of these cells (21), the functioning of CPM in this pathway would be possible only if it was expressed on the same domain. CPM was positively identified on the apical surface of MDCK cells (6), but a possible basolateral localization could not be determined because of the inaccessibility of the antibodies to this surface in the techniques that were employed. However, the fact that CPM is both GPI-anchored and N-glycosylated would argue against a substantial basolateral distribution. In this study, the cell-surface distribution and sorting of CPM were investigated. We show that CPM is present on both the apical and basolateral domains of MDCK cells, in contrast to the apical localization reported for other GPI-anchored proteins.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum (FBS) was from Atlanta Biologicals, Inc. Dulbecco’s modified Eagle’s medium (DMEM), Ham’s nutrient mixture F-12, Hank’s balanced salt solution (HBSS), phosphate-buffered saline (PBS), reduced glutathione, iodoacetamide, Triton X-100, and

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† The abbreviations used are: CPM, carboxypeptidase M; GPI, glycosylphosphatidylinositol; MDCK, Madin-Darby canine kidney; EGF, epidermal growth factor; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; HBSS, Hank’s balanced salt solution; PBS, phosphate-buffered saline; sulfo-NHS-biotin, sulfo-N-hydroxysuccinimidobiotin; dansyl-Ala-Arg, 5-dimethylaminonaphthalene-1-sulfonyl-l-alanyl-l-arginine; PI-PLC, phosphatidylinositol-specific phospholipase C; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.
Localization of Carboxypeptidase M in MDCK Cells

Triton X-114 were from Sigma. Sulfo-N-hydroxysuccinimido-LC-biotin (sulfo-NHS-LC-biotin) and sulfo-NHS-ss-biotin were from Pierce. Immobilized streptavidin was from Roche Molecular Biochemicals. 5-Iodo-1-D-methyl-1-deoxynonaphthalene-4-sulfo-L-tyrosine (dansyl-Ala-Arg) was synthesized and purified as described (22). The ProLong antifade kit and Fluorescein isothiocyanate-conjugated goat anti-rabbit (Arg) was synthesized and purified as described (22). The Pro-Long antifade kit (Molecular Probes, Inc., Eugene, OR). Redivue Pro-mix I-[35S] in vitro labeling mixture was from Amersham Pharmacia Biotech. Phosphatidylinositol-specific phospholipase C (PI-PLC) from Bacillus thuringiensis was from ICN or Oxford Glycosystems.

Most other chemicals were from Fisher.

Cells and Cell Culture—MDCK cells (CCL-34) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM containing 4.7 g/liter sodium bicarbonate, 25 mM Hepes, 100 units/liter penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated FBS.

Determination of Enzyme Activity—CPM activity was determined in a fluorometric assay with dansyl-Ala-Arg as the substrate as previously described (24, 25).

Verification of MDCK Cell Monolayer Integrity—MDCK cells (5 × 10^4) were seeded into 12- or 24.5-mm Transwell culture inserts, respectively, and grown for 5–7 days, after which experiments were performed. The integrity and tightness of the MDCK monolayers were routinely determined by transepithelial electrical resistance and occasionally by [3H]methoxyamin (5000 Da) diffusion. Cells used for experiments had a transepithelial electrical resistance of 1 MΩ cm². The product was extracted, and the fluorescence was measured in the supernatant. Activities in samples were compared with the control side. Cells were incubated at 37 °C in 5% CO₂ for 0.5–1 h, and the basolateral to 12-mm (0.5 ml) or 24.5-mm (1 ml) inserts. An equal volume of 275 μl of sample in a total reaction volume of 275 μl. γ-Glutamyl transpeptidase was measured in a colorimetric end-point assay with γ-glutamyl-p-nitroanilide as the substrate essentially as described (24, 25).

Determination of Protein Concentration—Protein concentrations were measured as described (26) using BSA as the standard.

Verification of MDCK Cell Monolayer Integrity—MDCK cells (5 × 10⁴ or 2.5 × 10⁵ cells) were seeded into 12- or 24.5-mm Transwell culture inserts, respectively, and grown for 5–7 days, after which experiments were performed. The integrity and tightness of the MDCK monolayers were routinely determined by transepithelial electrical resistance and occasionally by [3H]methoxyamin (5000 Da) diffusion. Cells used for experiments had a transepithelial electrical resistance of >400 Ω cm² and an apical-to-basolateral [3H]methoxyamin (1 μCi/ml) diffusion of <2%.

Domain-selective Biotinylation—Cell monolayers in 24.5-mm inserts were biotinylated apically or basolaterally as described (9) with some modifications. All treatments were performed on ice. Cells were washed five times with ice-cold PBS containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS-CM), and 1 ml of sulfo-NHS-LC-biotin (0.5 mg/ml) in PBS-CM was added (50 μl containing 50 μl of sample of each treatment). The filters were removed, and insoluble matter was removed by a 1-h centrifugation at 100,000 × g. An either equal or double volume of immobilized streptavidin slurry (50%) was added to the lysates, and the mixture was incubated at 4 °C overnight. The streptavidin was removed by a 10-min centrifugation in a microcentrifuge, and CPM, γ-glutamyl transpeptidase, and alkaline phosphatase activities were measured in the supernatant. Activities in samples were compared with controls treated identically except for omission of the biotinylation reagent. The amount of each enzyme on the apical and basolateral domains was taken as the amount of activity precipitated by streptavidin.

Identification of Biotinylated CPM by Immunoprecipitation, SDS-PAGE, and Electrobolting—Membranes containing biotinylated cell monolayers were excised, and the cells were solubilized in 20 mM potassium phosphate buffer, pH 7.5, containing 0.15 M NaCl, 1% Triton X-100, and 60 mM n-octyl glucoside for 2–12 h at 4 °C with rotation. The filters were removed, and insoluble material was removed by a 1-h centrifugation at 100,000 × g. An either equal or double volume of immobilized streptavidin slurry (50%) was added to the lysates, and the mixture was incubated at 4 °C overnight. The streptavidin was removed by a 10-min centrifugation in a microcentrifuge, and CPM, γ-glutamyl transpeptidase, and alkaline phosphatase activities were measured in the supernatant. Activities in samples were compared with controls treated identically except for omission of the biotinylation reagent. The amount of each enzyme on the apical and basolateral domains was taken as the amount of activity precipitated by streptavidin. Monolayers containing biotinylated cell monolayers were excised, and the cells were solubilized in 1 ml of cell lysis buffer (25 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, 2.5% Triton X-100, 60 mM n-octyl glucoside, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 1 μM pepstatin A, and 10 μM E-64) and centrifuged at 100,000 × g. An either equal or double volume of a 1:3 protein A-Sepharose slurry. The precipitates were washed four times with mixed micelle buffer (25 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl, 5 mM EDTA, 8% sucrose, 1% Triton X-100, 0.2% SDS, and 0.2 mM phenylmethylsulfonyl fluoride) and then once with the same buffer without detergents. Bound protein was eluted in Laemmi buffer containing 0.1% dithiothreitol and resolved by SDS-PAGE (8% gel). Proteins were electroblotted onto Immobilon-P and detected with streptavidin-horseradish peroxidase followed by chemiluminescence using an ECL kit (Amersham Pharmacia Biotech).

Immunofluorescence Microscopy—Cells were grown for 5 days on 6.25-mm diameter Falcon P.E.T. membrane cell culture inserts. The cells were rinsed twice with HBSS-BH and then fixed in 1% formaldehyde (depolymerized from paraformaldehyde) in the same buffer.

Release of CPM by PI-PLC—Confluent monolayers in 12-mm inserts were rinsed three times with HBSS-BH. Then, 0.5 ml of the same buffer, containing various concentrations of PI-PLC (0.002–0.5 units/ml) and 0.1% heat-inactivated BSA (56 °C for 30 min), was added to the apical or basolateral side of the inserts, and the buffer without PI-PLC was added to the opposite side. After incubation for 4 h at 37 °C, the buffer containing all cells was removed by a 10-min centrifugation in a microcentrifuge; and the released CPM activity was determined in the supernatant. The basal spontaneous release of CPM was measured in control cells treated identically except that buffer without PI-PLC was added instead.

To measure the release of CPM by PI-PLC from membrane fractions, cells were rinsed with PBS, scraped off, pelleted; resuspended in fractionation buffer (50 mM Hepes, pH 7.5, and 0.25 M sucrose) containing 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 1 μM pepstatin A, and 10 μM E-64; lysed by sonication for 3 × 10 s, and fractionated by sequential centrifugation as described (4). The final P₃ membrane fraction was washed once, resuspended in fractionation buffer, and incubated with or without PI-PLC (0.5 units/ml final concentration) at 37 °C for 1 h. One-half of the volume was removed from each reaction, and the soluble and cell membrane-bound enzymes were separated by a 1-h centrifugation at 100,000 × g. New PI-PLC (0.5 units/ml, 1 unit/ml final concentration) or buffer was added to the remaining reaction mixtures; and the incubation was continued for an additional hour, followed by centrifugation as described above. CPM and alkaline phosphatase were measured in both the high speed sediments and supernatants.

Plasma Membrane Distribution of GPI-anchored Proteins—The surface distribution of GPI-anchored proteins in MDCK cells was determined by domain-specific biotinylation, Triton X-114 extraction, and PI-PLC release essentially as described (9). Samples were separated by SDS-PAGE (7.5% gel) and electroblotted onto Immobilon-P. Biotinylated proteins were detected by alkaline phosphatase-coupled streptavidin, and bands were quantitated with a Protein Design Institute scanning densitometer.

Metabolic Labeling—Confluent monolayers of MDCK cells in 24.5-mm inserts were washed twice with HBSS-BH and then incubated for 30 min in Cys- and Met-deficient DMEM containing 5% dialyzed FBS (1000-Da cutoff). Cells were metabolically labeled for 30 min in 1 μCi/ml [35S]Met/[35S]Cys in the deficient medium. The labeling me-
the apical and basolateral domains contained approximately two-thirds and one-third of the extracellular CPM activity, respectively (Table I). A negligible amount (<2%) of hydrolyzed substrate was detected on the apical side when dansyl-Ala-Arg was added to the basolateral side and vice versa (Table I, Footnote c), indicating that the monolayer was impermeable to the substrate. The carboxypeptidase activity in MDCK cell membranes had previously been identified as CPM by means of enzymatic properties and reactivity with specific antiserum to purified CPM on Western blots (6). However, it could not be ruled out that another, minor carboxypeptidase was also present on these cells and if, expressed solely on one side of the cells, it could affect the distribution determined for CPM. To exclude this possibility, we specifically immunoprecipitated CPM activity from either the apical or basolateral side by first removing either the apical or basolateral activity with domain-specific biotinylation and streptavidin precipitation, followed by immunoprecipitation of the activity remaining in the supernatant with antiserum specific for CPM. More than 95% of the remaining activity on either the apical or basolateral side was immunoprecipitated by specific anti-CPM antiserum (data not shown), eliminating the possibility of a significant contribution by another peptidase to the activity being measured.

The relatively non-polarized distribution of CPM was also assessed by measuring CPM activity precipitable by streptavidin after domain-selective biotinylation of the cells. The distribution of CPM was even less polarized (56% apical and 44% basolateral) in these experiments (Table I), which may more accurately reflect its true distribution. This is because cells growing on the plastic wall of the inserts are excluded when the filters are excised (see “Experimental Procedures” for details), whereas when substrate is added to each domain, the cells on the substrate contribute to the apical (but not the basolateral) activity. Similar results were obtained when apically or basolaterally biotinylated CPM was subjected to SDS-PAGE and visualized with streptavidin-horseradish peroxidase and chemiluminescence (Fig. 1). Quantification of the bands by densitometry showed the apical and basolateral distribution to be 66 and 34%, respectively (average of two experiments). The 52–53-kDa protein detected on both domains is consistent with the previously published molecular mass (54 kDa) of MDCK

### Table I: Extracellular distribution of CPM in MDCK cells

| Domain-selective biotinylation | Dansyl-Ala-Arg hydrolysis |
|-------------------------------|--------------------------|
| Apical                        | 56 ± 7                   |
| Basolateral                   | 44 ± 7                   |

a Results are the means ± S.E. (n = 6).

b Percent of total extracellular CPM activity precipitated by streptavidin after either apical or basolateral biotinylation. Total extracellular CPM activity is defined as the combined activity precipitated by streptavidin after biotinylation on both sides. Of the total cellular CPM activity, 45 ± 9 and 35 ± 4% were precipitated after apical and basolateral biotinylation, respectively. The nonprecipitable activity is presumed to be intracellular.

c Percent of total extracellular CPM activity measured by addition of dansyl-Ala-Arg substrate to either the apical or basolateral side. Total extracellular CPM activity is defined as the combined apical and basolateral hydrolysis of dansyl-Ala-Arg. The amount of product (dansyl-Ala) detected on the opposite side was 0.72 ± 0.47% and 1.93 ± 1.20% of that on the test side when dansyl-Ala-Arg was added to the basolateral and apical sides, respectively.
cell CPM (6). Combined, these results show that CPM has a relatively non-polarized distribution in MDCK cells and that there is no difference in the molecular mass of CPM on the apical or basolateral domain.

**Immunofluorescent Staining of CPM**—When MDCK cells were stained for CPM, intense labeling was found at the apical surface of the epithelium, as revealed by confocal optical sectioning (Fig. 2). The somewhat punctate staining pattern observed probably reflects clustering of CPM induced by antibody crosslinking, as has been reported for other GPI-anchored proteins. The lateral surface was also reactive with anti-CPM antibody, whereas the basal surface was marked to a lesser extent. Occasionally, some cells showed a more intense staining at the basal surface (Fig. 2, d and e). These data are consistent with the above studies demonstrating CPM on both the apical and basolateral domains.

**PI-PLC Release of CPM**—Previous studies showed CPM to be attached to the plasma membrane of MDCK cells by a GPI anchor (6). However, in these experiments, 28% of the activity in a P2 membrane fraction remained membrane-bound after treatment with 0.03 units/ml PI-PLC for 2 h at 37 °C (6). To rule out incomplete digestion as the cause, P2 membrane fractions were treated with higher amounts of PI-PLC. After treatment for 1 h with 0.5 units/ml PI-PLC, 39% of the CPM activity remained in the membrane fraction, and an additional hour of treatment with new PI-PLC resulted only in an additional 6% release (average values from two experiments). As a comparison, a much smaller fraction of GPI-anchored alkaline phosphatase (13%) was resistant to release by PI-PLC in the same samples.

In light of these results, the possibility existed that a fraction of CPM might be attached to membranes by a non-GPI anchor, which consequently could account for the CPM present on the basolateral side. However, when intact cell monolayers in inserts were treated with PI-PLC on the basolateral side, a dose-dependent release of CPM activity into the basolateral buffer was observed (Fig. 3). There was also a time-dependent release of CPM from the basolateral surface by a single concentration of PI-PLC (data not shown). Surprisingly, CPM on the apical surface was more resistant to release by PI-PLC (Fig. 3). In contrast, although in control cells treated with only buffer, the spontaneous release of CPM over 4 h was relatively low, it was much greater from the apical domain (1.15 ± 0.25 nmol/h/insert, n = 6) than from the basolateral side (0.005 ± 0.008 nmol/h/insert, n = 6). These data show that basolateral CPM is GPI-anchored and that the spontaneous release of CPM from MDCK cells, which we previously showed was due to an endogenous phospholipase (29), comes primarily from the apical domain.

**Distribution of Other GPI-anchored Proteins in MDCK Cells**—The presence of a substantial portion of CPM on the basolateral domain of MDCK cells stands in contrast to the predominantly apical distribution of GPI-linked proteins that has been reported (9, 10). To rule out the possibilities that the non-polarized distribution of CPM was due to either an aberration in the protein sorting pathway in these cells or the use of a different technique for determining its distribution, the cellular distribution of GPI-anchored proteins was investigated following the same protocol used in previous studies (9). Following biotinylation, Triton X-114 extraction, and PI-PLC treatment, 10 proteins were detected in these cells of which nine were apparently GPI-anchored (Fig. 4). Of these, five were found either exclusively or predominantly on the apical side; one was exclusively on the basolateral side; and three had a relatively non-polarized distribution (between 37 and 58% apical and 42 and 63% basolateral) (Fig. 4 and Table II). Western analysis of a parallel blot with anti-CPM antiserum revealed that band 5 is CPM (data not shown). (The slightly higher molecular mass of 57 kDa calculated for CPM in this experiment was due to the use of different molecular mass standards.) The distribution of CPM (band 5) calculated by this method (58% apical and 42% basolateral) is consistent with the results from the other methods used. As additional controls, the distribution of GPI-anchored alkaline phosphatase as well as the apical transmembrane-anchored enzyme, γ-glutamyl transpeptidase, was determined by domain-selective biontinylation and streptavidin precipitation. Both proteins exhibited the expected apical localization (81–89%) (Table III), providing further evidence that the protein sorting pathway was not unusual in these cells. The non-polarized distribution of CPM was confirmed in the same samples (Table III). Together, these data suggest that no generalized aberration of the protein sorting pathway exists in these cells.

**Apical and Basolateral Sorting of Newly Synthesized CPM**—To determine if the surface distribution of CPM arises from non-polarized sorting of newly synthesized enzyme, pulse-chase studies were carried out. Metabolically labeled CPM appeared in approximately equal amounts at both the apical and basolateral sides of cells within 30 min, suggesting that CPM is initially sorted directly to both domains (Fig. 5). The total amount of labeled CPM on the cell surface peaked between 2 and 4 h on both domains and then slowly declined on the basolateral side and remained stable on the apical side up to 8 h. Substantial amounts remained on both domains even after an 8-h chase. A slight enrichment of CPM on the apical domain was observed over time, with the distribution approximately equaling the steady-state distribution between 4 and 8 h (Fig. 5B).

To investigate whether the non-polarized distribution of CPM could be due to predominant apical sorting, but faster degradation at the apical surface, the rate of elimination of apically or basolaterally biontinylated CPM from the cells was determined (Fig. 6). The elimination pattern indicated a first-order process with approximately equal rates of loss of CPM from either the apical or basolateral domain. The calculated half-lives of apical and basolateral CPM were 9.5 and 8.1 h, respectively. Thus, a difference in the rate of loss of CPM from the two domains does not explain the unusual distribution of CPM.

As the spontaneous release of CPM occurs mainly from the apical domain (see above), the slow loss of newly synthesized CPM from the basolateral domain and the maintenance of steady levels of CPM on the apical domain (Fig. 5B) might be due to transcytosis from the basolateral to the apical domain, followed by release from the apical surface. To investigate this possibility, basolateral CPM was labeled with biotin containing a cleavable disulfide linkage. At time 0 or after various times of incubation at 37 °C up to 20 h, glutathione was added to either the apical or basolateral side to remove the biotin from cell-surface CPM. Cells that were not treated with glutathione served as controls for the total amount of remaining cell-associated biontinylated CPM at a given time point. As expected, glutathione added to the basolateral side removed the biotin from essentially all of the labeled CPM at time 0, whereas no biotin was released from CPM by glutathione added to the apical side (Fig. 7). However, 1 h after biontinylation, ~30% of the biontinylated CPM was unavailable to glutathione added to the basolateral side, indicating it had been endocytosed. Furthermore, at 1 h, a small portion of the CPM that had been biontinylated on the basolateral side was available to glutathione added to the apical side and even more at 4 h (Fig. 7). This indicates that the CPM, originally biontinylated on the basolateral side, had been transcytosed to the apical side. After 20 h,
the amount of biotinylated CPM remaining in the cells after glutathione treatment on either side equaled the total amount of biotinylated CPM in the control cells, indicating that the small amount of remaining CPM was intracellular, possibly destined for degradation (Fig. 7).

**DISCUSSION**

Sorting of newly synthesized and internalized membrane proteins in polarized epithelial cells occurs by means of targeting signals present in the proteins, leading to their sequestration in specific transport vesicles in the trans-Golgi network (13–15, 30). All GPI-anchored proteins studied to date are apically sorted, and the addition of a GPI anchor to normally non-polarized or basolateral proteins targets them to the apical surface (9–11, 13, 14, 30). N-Linked carbohydrates on glycoproteins are also signals for apical targeting (13, 15).

The finding in this study of a relatively non-polarized distribution of CPM in MDCK cells is quite surprising. This is because CPM contains two dominant apical targeting signals: N-linked carbohydrate and a GPI anchor (6, 16, 19). This sorting pattern is unlikely to be due to an unusual clonal population of cells because the same distribution was found in cells obtained from the American Type Culture Collection on two different occasions, and the cells formed tight junctions as shown by a transepithelial electrical resistance of >400 ohms cm² and an apical-to-basolateral [³H]methoxyinulin diffusion of <2%. The distribution of alkaline phosphatase and γ-glutamyl transpeptidase was predominantly (81–89%) apical as reported (31). Following a method originally used to show that GPI-anchored proteins are apically sorted (9), five other
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Fig. 3. Dose-dependent release of CPM from MDCK cells by PI-PLC. The apical or basolateral side of MDCK cell monolayers was incubated with various doses of PI-PLC for 4 h. Buffer from the treated side was removed and assayed for CPM activity with dansyl-Ala-Arg substrate. Values are given as the means ± S.D. (n = 3; error bars smaller than the symbols are not shown).

Fig. 4. Identification of the apical and basolateral distribution of GPI-anchored proteins in MDCK cells. Filter-grown MDCK cell monolayers were selectively biotinylated on the apical or basolateral side and solubilized in buffer containing 1% Triton X-114. GPI-anchored proteins, extracted into the detergent phase, were digested with PI-PLC (+), followed by extraction into an aqueous buffer, and finally precipitated with sodium deoxycholate and trichloroacetic acid. Control samples (−) were treated identically except without the addition of PI-PLC. Samples were separated by SDS-PAGE (7.5%) under reducing conditions and electroblotted onto Immobilon-P. Biotinylated proteins were detected with streptavidin-alkaline phosphatase. Numbered bands correspond to those listed in Table II. The positions of molecular mass markers are shown on the right. K, kilodaltons.

GPI-anchored proteins also had a predominant apical distribution. Two other GPI-anchored proteins had relatively non-polarized distributions, whereas one appeared to be exclusively basolateral. However, caution must be used in concluding from these data that other GPI-anchored proteins have substantial basolateral distributions because this technique relies only on molecular masses of the bands to identify proteins on the apical and basolateral membranes. Additional specific assays would be needed to confirm the distribution of the other proteins, as we did for CPM. Nevertheless, the distribution of CPM determined with this technique was consistent with the specific assays used.

It is unlikely that the non-polarized distribution is due to a non-GPI-anchored form of CPM that is sorted to the basolateral surface. First, basolateral CPM was readily released by PI-PLC, confirming that CPM is attached by a GPI anchor on this domain. Second, the C-terminal sequence of CPM contains all of the required features necessary for GPI anchoring (16), including a mildly hydrophobic region preceded by a more polar "hinge region" and the most probable residue at the attachment site (Ser406, the so-called "ω" site) as well as the preferred residues at the ω + 1 and ω + 2 sites (32). Although 31% of the total CPM activity in the P3 membrane fraction was resistant to release by PI-PLC, it is not uncommon for some portion of GPI-anchored proteins to remain membrane-associated after PI-PLC treatment (33, 34). Possible explanations include sequestration into plasma membrane invaginations, localization within a lipid environment that prevents the PI-PLC access to the anchor structure, membrane attachment of CPM by a GPI anchor isoform resistant to cleavage by PI-PLC, and attachment to membranes by an alternative mechanism. In any case, the higher level of release of CPM from the basolateral surface by exogenous PI-PLC would indicate that the resistant fraction

### Table II

| Band No. | Mass (kDa) | Specific density (g) | Distribution (%) |
|----------|------------|---------------------|-----------------|
| 1        | 108        | 0.143               | 0               | 100             |
| 2        | 95         | 0.076               | 0               | 100             |
| 3        | 90         | 0.101               | 28              | 72              |
| 4        | 68         | 0.254               | 42              | 58              |
| 5        | 57         | 0.883               | 47              | 53              |
| 6        | 47         | 0.172               | 100             |                 |
| 7        | 37         | 0.883               | 56              | 44              |
| 8        | 34         | 0.577               | 63              | 37              |
| 9        | 30         | 0.299               | 0               | 100             |
| 10       | 27         | 0.525               | 100             | 0               |

* Protein bands in apically or basolaterally biotinylated cells were detected by alkaline phosphatase-conjugated streptavidin and quantitated by densitometry. Specific density (i.e. density of GPI-anchored proteins) is the band density of PI-PLC-treated samples minus the band density of untreated controls. There are no values for band 2 because PI-PLC treatment did not result in an increase in band density, indicating that this protein is not GPI-anchored.

* The apical and basolateral distribution of GPI-anchored proteins based on specific band density.

### Table III

| Activity precipitated | AP | GGT | CPM |
|----------------------|----|-----|-----|
| % of extracellular   |    |     |     |
| Basolateral          | 81 ± 6 | 89 ± 5 | 56 ± 2 | 44 ± 2 |
| Apical               | 19 ± 6 | 11 ± 5 |        |

* Enzyme activity precipitated by streptavidin after biotinylation of the apical or basolateral domain. Extracellular is the added apical and basolateral activities precipitated from the cell lysate. The extracellular activity represents 97% (alkaline phosphatase (AP)), 60% (γ-glutamyl transpeptidase (GGT)), and 85% (CPM) of the total activity in measured in cell lysates before streptavidin precipitation. Values for CPM are given for comparison and were determined in the same samples used to measure alkaline phosphatase and γ-glutamyl transpeptidase. Results are the means ± S.E. (n = 3).
derives from apical (not basolateral) membranes. In this regard, it is also possible that the GPI anchor of CPM is modified during transcytosis to make it resistant to exogenous PI-PLC once it reaches the apical membrane, although the apparent direct targeting of CPM to both domains makes this unlikely.

The relatively non-polarized distribution of CPM in these cells results from the direct sorting of CPM to both the apical and basolateral domains, although the mechanism of the basolateral targeting of GPI-anchored CPM remains unclear. All basolateral targeting signals identified to date reside on the cytoplasmic domain of membrane proteins and can be classified into three groups (13): 1) a tyrosine-based motif that has the general consensus sequence YXXF, where F is a bulky hydrophobic amino acid; 2) a dileucine motif (either Leu-Leu or Leu-Ile); and 3) specific sequences, such as the recently described 23-residue cytoplasmic juxtamembrane sequence on the EGF receptor (35). In some cases, the basolateral targeting motifs also serve as endocytic signals (13). The fact that CPM has a GPI anchor means that it has no cytoplasmic domain and therefore cannot have any of the basolateral targeting signals that have been established for other proteins. The C-terminal sequence of CPM contains both a potential tyrosine-based (YXXF) basolateral targeting signal as well as a dileucine motif; however, the Tyr399-Arg400-Asn401-Leu402 sequence is in the extracellular domain, and the Leu420-Leu421 motif is in the membrane anchor signal region (16). Neither of these motifs has been shown to function as a basolateral targeting signal within these contexts. Additionally, removal of the C-terminal membrane anchor signal and GPI attachment occur in the endoplasmic reticulum, early after synthesis of the protein (33). Consequently, the dileucine motif would not be present at the level of the trans-Golgi where sorting occurs. Retention of the C-terminal hydrophobic anchor signal sequence cannot be an explanation for its basolateral localization as our data show that basolateral CPM is GPI-anchored. In addition, others have shown that proteins with uncleaved signals for GPI anchoring are retained in the endoplasmic reticulum and degraded (36, 37). The most logical explanation is that the extracellular domain of CPM contains a positive basolateral targeting signal that competes with the glycan and GPI apical targeting signals, resulting in a relatively non-polarized distribution. Alternatively, the CPM C-terminal hydrophobic signal anchor sequence or the GPI anchor itself might possess unique features that result in basolateral targeting.

The transcytosis of CPM from the basolateral to the apical domain is consistent with the transcytosis reported for another GPI-anchored protein, GP2, in MDCK cells (38). The biological role of the transcytosis pathway is not entirely clear. It may be...
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The regulation of peptide activity on the basolateral side might regulate kinin activity on both the apical and basolateral sides. Thus, the non-polarized distribution of CPM may be of physiological importance as high levels of kinins have been detected in tubular as well as interstitial renal fluid (39). Because bradykinin is believed to act in a paracrine or autocrine fashion, proteolytic cleavage on the luminal side of tubular epithelium is unlikely to affect bradykinin activity on the serous side and vice versa. Thus, the non-polarized distribution of CPM in MDCK cells raises the possibility that it might separately regulate kinin activity on both the apical and basolateral sides. The regulation of peptide activity on the basolateral side might be more significant as other kininases (e.g., neutral endopeptidase or angiotensin-converting enzyme) are primarily found on luminal brush border membranes of renal tubules (39, 40). Immunohistochemical studies have confirmed the presence of bradykinin B2 receptors on both the apical and basolateral sides of collecting duct epithelial cells as well as in distal straight tubules and connecting tubules (41). We found direct evidence for the ability of CPM to inhibit a bradykinin-mediated B2 receptor response on the basolateral side of MDCK cells. In these experiments, a specific inhibitor of CPM potentiated the release of arachidonic acid from MDCK cells stimulated by bradykinin applied to the basolateral side.  

Another peptide that is active in the renal system is EGF. Previously, we found CPM to be the only protease that metabolizes EGF on the surface of MDCK cells (17). The function of this metabolism is not clear as the generated metabolite, des-Arg53-EGF, had equal mitogenic potency on MDCK cells (17). The novel finding that CPM is present on the basolateral side of MDCK cells co-localizes it with the basolaterally localized EGF receptor. Whether removal of the C-terminal Arg could alter other activities or intracellular transport of EGF is not known. For example, in MDCK cells, 5–30% of the EGF bound to basolateral receptors is transcytosed to the apical side without the receptor (21, 42), during which time (90–120 min) it would be expected that removal of the C-terminal Arg would take place (43, 44). Thus, the initial processing of EGF may serve as a signal or, alternatively, remove a signal for targeting of EGF to different extra- or intracellular locations, where it could either exhibit additional activities or be further degraded. Metabolism of EGF by CPM on the basolateral side may thus have consequences not observed with apically applied EGF.

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