Epithelial sorting of a glycosylphosphatidylinositol-anchored bacterial protein expressed in polarized renal MDCK and intestinal Caco-2 cells

Kathleen L. Soole¹,², Mark A. Jepson¹, Geoffrey P. Hazlewood³, Harry J. Gilbert² and Barry H. Hirst¹,*

¹Department of Physiological Sciences, University of Newcastle upon Tyne, Medical School, Newcastle upon Tyne NE2 4HH, UK
²Department of Biological and Nutritional Sciences, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, UK
³Department of Cellular Physiology, The Babraham Institute, Babraham, Cambridge CB2 4AT, UK

*Author for correspondence

SUMMARY

To evaluate whether a glycosylphosphatidylinositol (GPI) anchor can function as a protein sorting signal in polarized intestinal epithelial cells, the GPI-attachment sequence from Thy-1 was fused to bacterial endoglucanase E⁺ (EGE⁺) from Clostridium thermocellum and polarity of secretion of the chimeric EGE⁺-GPI protein was evaluated. The chimeric EGE⁺-GPI protein was shown to be associated with a GPI anchor by TX-114 phase-partitioning and susceptibility to phosphoinositol-specific phospholipase C. In polarized MDCK cells, EGE⁺ was localized almost exclusively to the apical cell surface, while in polarized intestinal Caco-2 cells, although 80% of the extracellular form of the enzyme was routed through the apical membrane over a 24 hour period, EGE⁺ was also detected at the basolateral membrane. Rates of delivery of EGE⁺-GPI to the two membrane domains in Caco-2 cells, as determined with a biotinylation protocol, revealed apical delivery was approximately 2.5 times that of basolateral. EGE⁺ delivered to the basolateral cell surface was transcytosed to the apical surface. These data indicate that a GPI anchor does not represent a dominant apical sorting signal in intestinal epithelial cells. However, the mis-sorting of a proportion of EGE⁺GPI to the basolateral surface of Caco-2 cells provides an explanation for additional sorting signals in the ectodomain of some endogenous GPI-anchored proteins.

Key words: Caco-2, MDCK, protein sorting, epithelial cell, endoglucanase, glycosylphosphatidylinositol anchor

INTRODUCTION

In polarized epithelial cells newly synthesized membrane or secreted proteins are probably sorted in either the trans-Golgi network or the post-Golgi into different vesicle populations to be delivered to the different membrane domains (Simons and Wandinger-Ness, 1990; Rodriguez-Boulan and Powell, 1992). Signals involved in sorting proteins to and through either membrane domain have been examined, particularly in Madin-Darby canine kidney (MDCK) cells. Proteins that do not contain sorting signals (apart from an N-terminal signal peptide that directs proteins into the endoplasmic reticulum) follow the constitutive or default pathway for secretion, which may be defined by studying the pathway taken by heterologous proteins normally expressed in epithelial cells. Thus, the secretory pathway taken by a bacterial protein, endoglucanase E⁺ (EGE⁺) from Clostridium thermocellum, defines the default pathway when transfected into MDCK and human intestinal Caco-2 epithelial cells; apical:basal, 60:40 and 30:70, respectively (Soole et al., 1992).

From studies of the expression of heterologous and endogenous proteins in MDCK cells, in particular, it has become clear that positive sorting signals exist that target proteins to either the apical or basolateral plasma membrane. Basolateral signals have been identified in the cytoplasmic domain of several proteins, including the ligand-receptors for nerve growth factor, low density lipoprotein, poly(IgA) and poly(Ig)Fc (for review, see Wollner and Nelson, 1992). Additionally, a basolateral signal has been identified in the membrane-spanning region and cytoplasmic domain of vesicular stomatitis virus glycoprotein (VSV G). Fusion of the VSV G protein cytoplasmic domain to placental alkaline phosphatase (PLAP), normally an apical protein, resulted in the re-directing of PLAP to the basolateral surface (Brown et al., 1989).

The glycosylphosphatidylinositol (GPI) moiety that links the C-terminal of some proteins to the outer leaflet of the membrane lipid bilayer has been identified as an apical targeting signal in MDCK cells (Lisanti et al., 1988, 1989; Brown et al., 1989). The GPI recognition sequence comprises of a C-terminal hydrophobic region that signals the attachment of a GPI anchor to the protein soon after translation (Ferguson et al., 1986). Chimeric proteins produced in transfected MDCK cells have demonstrated that the GPI anchor signal is a dominant apical sorting signal in MDCK cells, as the fusion of the signal to proteins normally located at the basolateral membrane redirects the polypeptides to the apical surface (Brown et al., 1989; Lisanti et al., 1989, 1990a; Powell et al., 1991a). However, recent evidence suggests that many endogenous GPI-anchored proteins contain additional apical sorting information, recognised in MDCK cells, in their ectodomains.
(Brown et al., 1989; Lisanti et al., 1989; Casanova et al., 1991; Powell et al., 1991b).

At least five endogenous GPI-anchored proteins are associated exclusively with the apical surface of Caco-2 cells, as shown by their removal from the cell surface by PI-specific phospholipase C (Lisanti et al., 1990b). However, it remains to be established whether the lipid simply anchors the protein to the cell surface or, in addition, also acts as an apical targeting signal in these intestinal cells. In contrast to findings in MDCK, endogenous GPI-anchored proteins and chimeric proteins such as gD-1-DAF, which has the decay-accelerating factor (DAF) GPI anchor signal sequence fused to the C-terminal end of herpes simplex gD-1, are localized at the basolateral surface in polarized Fischer rat thyroid epithelial cells (Zurzolo et al., 1993).

From the foregoing discussion, it is apparent that the sorting mechanism varies considerably between different epithelial cell types and it cannot, therefore, be assumed that a GPI anchor is a dominant apical sorting signal in Caco-2 cells.

In this study, we have addressed two questions. First, can a GPI anchor sequence, attached to a bacterial protein, target the chimeric protein to the plasma membrane of transfected mammalian cells? Second, does this GPI anchor result in specific sorting of the protein to the apical membrane in the intestinal cell line Caco-2? We fused the GPI anchor sequence from Thy-1 to the C-terminal end of EGE. We demonstrate that the GPI anchor sequence from Thy-1 can redirect EGE to the apical membrane in transfected MDCK and Caco-2 cells, where it is readily released into the medium by the action of either proteases or phospholipases. Although endogenous GPI-anchored protein was predominantly delivered to the apical surface of Caco-2 cells, a low level of EGE was detectable on the basolateral plasma membrane surface. The basolaterally located EGE may represent a low level of mis-reading of this signal in Caco-2 cells, providing an explanation for the existence of additional apical sorting signals in the ectodomain of some GPI-anchored proteins.

MATERIALS AND METHODS

Plasmid construction

Truncated celE, celE′, coding for residues 4-367 of mature EGE in which an internal EcoRI site has been removed by site-directed mutagenesis (Hall et al., 1988) was excised from pHGB2 on a HindIII/SstI fragment. This fragment was cloned into a recombinant plasmid where the human growth hormone (hGH) signal sequence had been cloned into pH1.22 (Chambers et al., 1988) and in phase with the NotI site of the multiple cloning site of pMTL22. Using PCR, a KpnI/ClaI fragment coding for residues 489-703 of the Thy-1.2 sequence (Ingraham et al., 1986) was created. This fragment codes for the GPI anchor signal sequence and attachment site of Thy-1.2. This fragment was cloned into the 3′ end of celE′ such that the sequence was also in phase with the NotI site. From this recombinant plasmid, the truncated celE′-Thy-1.2 was excised on a 1.5 kb BamHI/BglII restriction fragment and cloned into the eukaryotic expression vector, pSRe, digested with BamHI/BglII as described previously (Soole et al., 1992), to create the plasmid pSReEGETHY1.

Cell culture and selection

Culture and filter seeding conditions for MDCK strain I and Caco-2 cells were as described previously (Soole et al., 1992). MDCK and Caco-2 cells were transfected with pSReEGETHY1 by lipofection and electroporation, respectively, and stable transfectants were selected using G418 (Geneticin; Gibco BRL) and pooled as described previously (Soole et al., 1992). The resulting transfected cell lines were designated, MDCK(EGE-GPI) and Caco-2(EGE-GPI). MDCK(EGE-GPI) and Caco-2(EGE-GPI) cells were routinely grown on Anocell (Nunc) and Transwell (Costar) filter inserts, respectively. When seeded onto the porous supports, the transfected lines behaved similarly to the wild-type cells with respect to morphology of the monolayer; however, as found for other transfected cell lines, the transepithelial electrical resistances (Rt) were consistently higher (Soole et al., 1992).

Detection of EGE′ expression

Expression of EGE′ was determined either enzymically or by indirect immunofluorescence (see below). EGE′ catalytic activity was determined in cell lysates or media using a specific, sensitive fluorimetric assay with 4-methylumbelliferyl-β-D-cellobiose (MUC) as the substrate (Soole et al., 1992).

Histochemistry and immunofluorescence

MDCK(EGE-GPI) and Caco-2(EGE-GPI) cells, grown on polycarbonate filters, were washed in phosphate-buffered saline (PBS; pH 7.4) and fixed in 3% (v/v) paraformaldehyde in PBS either for 2 hours at room temperature or overnight at 4°C. Fixed cells were stained with EGE-affinity-purified polyclonal antiserum raised against bacterial EGE′. Neat antiserum was applied to apical, basal or both surfaces as indicated for 16 hours at 4°C. Filters were washed several times in PBS and bound anti-EGE′-antibodies were detected using a FITC-labelled goat anti-rabbit IgG second antibody (1:50 dilution; Sigma) in the presence of 30% (v/v) goat serum for 1 hour at room temperature.

Monolayers of Caco-2 and Caco-2(EGE-GPI) cells were stained for dipetidylpeptidase IV (DPPIV) and membrane dipeptidase essentially as described by Howell et al. (1992) and for sucrase-isomaltase as described by Hughson et al. (1989). Alkaline phosphatase expression was visualised histochemically following the protocol previously described (Soole et al., 1992). Monolayers of MDCK and MDCK(EGE-GPI) were stained with monoclonal antibodies against a 114 kDa apical (4.6.5a) or a 58 kDa basolateral (6.23.3a) membrane protein as described previously (Balcara-Øverdahl et al., 1984; Soole et al., 1992).

After final washes, layers were mounted in Vectashield (Vector, Burlingame) before examination on a Nikon Diaphot microscope fitted with a Bio-Rad MRC-600 confocal laser scanning system.

TX-114 extraction, partitioning and PI-PLC digestion

Hydrophobic and hydrophilic proteins may be partitioned in TX-114 by a temperature-induced phase separation (Bordier, 1981). Confluent monolayers of cells were extracted in TX-114 as described by Bordier (1981) and adapted by Lisanti et al. (1988). Cell layers were washed in PBS, filters cut out, placed in microfuge tubes and extracted in 1% TX-114, 0.1 mM PMSF, 20 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml antipain and 0.2% (v/v) Phenol Red in Tris-buffered saline (TBS; pH 7.4) for 1 hour at 4°C. After vigorous vortexing, the cell suspension was centrifuged for 10 minutes at 13,000 rpm. The supernatant was collected, reextracted and the resultant supernatant loaded onto a 0.3 M sucrose cushion, incubated for 5 minutes at 37°C and centrifuged for 5 minutes at 6,000 rpm. The aqueous phase (red in colour) was easily separated from the detergent phase; volumes were recorded and each phase assayed for EGE′ activity.

To investigate the attachment of a GPI anchor to EGE′-GPI, the detergent phase was separated into two fractions and incubated with or without 8 units/ml of protease-free phosphoinositide-specific phospholipase C (PI-PLC) from Bacillus thuringiensis (Low, 1987) for 4 hours at 37°C. An equal volume of TX-114 extraction buffer was added to the suspension, mixed and left on ice for 30 minutes. The aqueous and detergent phases were separated as described above.
Trypsin and PI-PLC release of cell surface EGE′

Confluent monolayers of MDCK(EGE-GPI) and Caco-2(EGE-GPI) cells were washed several times with normal serum-free growth medium. Medium containing 0.1% trypsin was added to either the apical or basal compartment in a total volume of 1 mL. Serum-free medium was placed in the contralateral compartment. After 1 hour at 37°C, with occasional gentle mixing, the RΩ of the monolayers was recorded and the medium collected, spun and assayed for EGE′ activity. The filters were washed in ice-cold PBS and fixed in 3% paraformaldehyde in PBS for immunofluorescence analysis.

PI-PLC. 5 units/mL, was added to either the apical or basal surface of Caco-2(EGE-GPI) cells (12 days after seeding, RΩ 683-862 Ω·cm²) and the amount of EGE′ released was quantified after 1 hour at 37°C in serum-free medium.

Biotinylation of cell surface and transcytosis of EGE′ in Caco-2(EGE-GPI)

Monolayers of Caco-2(EGE-GPI) displaying RΩ > 600 Ω·cm² were surface biotinylated following the procedure of Sargiacomo et al. (1989), with modifications. Preliminary experiments indicated that EGE′ was optimally biotinylated in a buffer containing (in mM) 10 triethanolamine, 137 NaCl and 3 KCl (TNK; pH 9.0) with 0.5 mg/ml of biotin-LC-NHS; triethanolamine, 137 NaCl and 3 KCl (TNK; pH 9.0) with 0.5 mg/ml and used immediately. Both surfaces were labelled by the addition of 750 µl of biotin solution was replaced with fresh solution. After another 20 minutes of gentle shaking, the filters were washed 3×5 ml in ice-cold serum-free medium. A 1 ml portion of pre-warmed serum-free medium was added to each filter compartment and placed in a 37°C, humidified CO₂-incubator. Immediately after biotinylation and at various times thereafter, trypsin was added to either the apical or basal medium to a final concentration of 0.1% (w/v) and incubated for 1 hour further at 37°C to release cell surface EGE′. For each filter, the medium in the contralateral compartment to the trypsin was collected and assayed, and compared with a control filter to check the monolayer integrity with respect to EGE′ passage. Transepithelial resistances were monitored throughout the experiment; if a decrease was observed due to the monolayer becoming disrupted, that filter was discarded.

To all medium samples, 0.2% soybean trypsin inhibitor (Sigma) was added and mixed vigorously. Streptavidin-agarose beads (75 µl of 1 mg/ml slurry; Pierce) were added and samples gently mixed for 16-18 hours at 4°C. Medium samples plus beads were centrifuged at 9,000 g for 5 minutes, the supernatant removed and recentrifuged, and the final supernatant assayed for EGE′ activity. The polarity of the streptavidin-agrose beads in precipitating all of the biotinylated EGE′ was confirmed in two ways: (1) addition of more beads did not decrease the EGE′ activity measured in the supernatants. (2) Streptavidin-horseradish peroxidase conjugates did not bind to an ELISA plate coated with samples of the final supernatant, whereas binding was observed when plates were coated with medium samples prior to the streptavidin-agrose bead incubation step.

RESULTS

Localization of EGE′-GPI in transfected MDCK and Caco-2 cells

MDCK and Caco-2 cells were transfected with plasmid pSRαEGETHY1, which encodes EGE′ fused to the Thy-1 GPI anchor attachment sequence (EGE′-GPI). Expression and localization of EGE′ was determined by indirect immunofluorescence. Polyclonal antiserum specific for EGE′ was applied to either the apical (A) or basal (B) surface of the cell layer and confocal optical sections were imaged at the apical (A) or basal (B) aspects of the cells. Confocal laser scanning microscope transverse (x-z) optical sections of the fluorescence when anti-EGE′ antiserum was applied to both cell surfaces (C) showed that EGE′ was expressed almost exclusively on the apical surface of the cells. EGE′ staining was absent from untransfected MDCK cells (D). (E) Autoimmunofluorescence (sensitivity increased) of the untransfected MDCK cells shown in (D). Arrows mark the base of the filter. Bar, 25 µm.

Fig. 1. Cell surface distribution of EGE′ in cells containing pSRαEGETHY1 (MDCK(EGE-GPI)) and untransfected MDCK cells. Confluent monolayers of cells grown for 5 days on Anopore culture inserts were fixed and labelled for expression of EGE′ by indirect immunofluorescence. Antiserum specific for bacterial EGE′ was applied to either the apical (A) or basal (B) surface of the cell monolayer and confocal optical sections were imaged at the apical (A) or basal (B) aspects of the cells. Confocal laser scanning microscope transverse (x-z) optical sections of the fluorescence when anti-EGE′ antiserum was applied to both cell surfaces (C) showed that EGE′ was expressed almost exclusively on the apical surface of the cells. EGE′ staining was absent from untransfected MDCK cells (D).

Using immunofluorescent staining to examine the expression of EGE′ in the Caco-2(EGE-GPI) cell line, it was found that a high level of expression could be detected on the apical surface of these cells (Fig. 2a). However, basolateral EGE′ staining was also noted when the antiserum was applied to the basolateral surface (Fig. 2b). Confocal optical sectioning of these monolayers confirmed the localization of EGE′ on both surfaces of the Caco-2(EGE-GPI) monolayers (Fig. 2c). The polarity of the transfected Caco-2 cells was compared with that of the parent cell line by comparison of the staining pattern of endogenous GPI-anchored (alkaline phosphatase and membrane dipeptidase) and transmembrane, non-GPI-anchored (sucrase-isomaltase and DPP IV) proteins. Alkaline phosphatase (Pinto et al., 1983), sucrase-isomaltase (Mattler et
al., 1990) membrane-dipeptidase and DPPIV (Howell et al., 1992) have all been characterized as predominantly brush-border apical membrane proteins. Comparison of their histochemical or immunofluorescent staining patterns in wild-type Caco-2 with transfected Caco-2(EGE-GPI) cells indicated that the transfected cells are polarized with respect to all of their endogenous apical protein expression (Fig. 3).

**EGE′ is associated with GPI anchor in MDCK(EGE-GPI) and Caco-2(EGE-GPI)**

There are two structural properties of GPI anchors that can be exploited to demonstrate their association with a protein: (1) the attachment of a GPI anchor to a protein confers hydrophobicity and hence a membrane location on that protein; and (2) the susceptibility of the GPI membrane anchor to cleavage by PI-PLC. The addition of PI-PLC to a mixture of hydrophobic proteins will induce in a GPI-linked protein a transition from a hydrophobic to a hydrophilic state by the release of the detergent binding domain (Lisanti et al., 1988).

Monolayers of MDCK(EGE-GPI) and Caco-2(EGE-GPI) were extracted from polycarbonate filters with the detergent TX-114 and the aqueous and detergent phases were partitioned. The majority of EGE′ activity was detected in the detergent phase (Fig. 4), in agreement with the enzyme from both these cells lines having a hydrophobic nature consistent with the membrane location of EGE′ detected by immunofluorescence microscopy. In contrast, EGE′ in TX-114 extracts from the previously described MDCK-EGE and Caco-2-EGE cell lines expressing the secreted (non-GPI-anchored) form of EGE′ (Soole et al., 1992) was found to partition completely (95-98%) into the aqueous phase (data not shown). When the detergent phase containing putative EGE′-GPI was incubated with PI-PLC, 80-90% of the EGE′ re-partitioned in the aqueous phase (Fig. 4), consistent with the removal of the GPI-anchor from EGE′.

PI-PLC was also able to release EGE′ from the surface of Caco-2(EGE-GPI) cells. Under control conditions, with no PI-PLC, 1.62 ± 0.08 and 0.061 ± 0.009 nmol MUC (mean ± range (n=2)) of EGE′ activity was released during a 1 hour incubation from the apical and basolateral surfaces, respectively. In the presence of apical PI-PLC, apical release over 1 hour was increased to 4.13 ± 0.02 nmol MUC. Similarly, basal PI-PLC increased basal release of EGE′ to 3.65 ± 0.06 nmol MUC.

**EGE′ is associated with apical and basolateral membrane domains**

EGE′ was detectable in the medium bathing both...
Table 1. EGE′ associated with apical and basolateral membrane surfaces in confluent monolayers of MDCK(EGE-GPI) and Caco-2(EGE-GPI) cells

<table>
<thead>
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| Cells were grown on either Transwell inserts for 12 days (Caco-2(EGE-GPI)) or Anopore inserts for 5 days (MDCK(EGE-GPI)). Monolayers were washed with PBS twice and then once with serum-free growth medium. Serum-free medium (1 ml) was added to each compartment in a series of filters and 0.1% (w/v) trypsin was added to either the apical or basolateral medium as indicated. After 1 hour of incubation at 37°C, cell layer integrity was confirmed by determining the Rτ for each filter. Medium was collected only for intact cell layers and assayed for EGE′ activity. Results are presented as total EGE′ activity in each medium sample (n=4).

*Medium bathing either apical or basolateral (basal) membrane surface in which EGE′ activity was determined.
†After 1 hour of incubation with fresh medium containing 0.1% trypsin.

GE′ delivery to apical and basolateral membrane domains in Caco-2(EGE-GPI)

Two biogenic routes for apical proteins have been described in Caco-2 cells; direct and indirect. Biosynthesis of the apical membrane proteins, sucrase-isomaltase and DPPIV involves delivery of the protein to the basolateral membrane and then transcytosis to the apical membrane (Mattler et al., 1990; Le Bivif et al., 1990). The detection of low levels of cell-associated EGE′ on the basolateral surface of Caco-2(EGE-GPI) cells raises the question of whether the basolateral membrane is involved in the biosynthetic pathway of EGE′-GPI. To test this hypothesis, we devised a quantitative assay to estimate the delivery of newly synthesized EGE′ to each cell surface. The approach involved biotinylation of both cell surfaces to give a

MDCK(EGE-GPI) and Caco-2(EGE-GPI) cells. This release into the bathing medium may indicate either endogenous peptidase- or phospholipase-mediated liberation from the plasma membrane. EGE′ release from the cells was greatest from the apical cell surfaces (Table 1), perhaps reflecting polarized distribution of endogenous peptidases/phospholipases. The release of EGE′ was not a consequence of membrane shedding as other apically located proteins (alkaline phosphatase and sucrase isomaltase) were not detected in the bathing media. To evaluate the total quantity of extracytoplasmic EGE′ associated with each membrane domain, it was necessary to determine the amounts of both the EGE′ secreted into the medium and the enzyme associated with each membrane. pSRαEGETHY1 was designed to encode the catalytic domain of EGE (EGE′) attached via a 15 residue hydroxyamino acid-rich linker sequence to the GPI anchor signal sequence; full-length EGE contains an N-terminal catalytic domain linked via hydroxyamino acid-rich sequences to a non-catalytic domain (Hall et al., 1988). The linker sequence will be particularly sensitive to proteolytic cleavage (Hall et al., 1988), while EGE′ activity is refractory to protease action (Hall et al., 1993). Thus EGE′ can be released from the membrane by addition of trypsin without affecting its activity. Trypsin, 0.1%, treatment released all surface EGE′ as judged by immunofluorescence of both MDCK(EGE-GPI) and Caco-2(EGE-GPI) cell layers after treatment (Fig. 5). Using this trypsin assay to quantify the steady-state EGE′ activity associated with each membrane surface, revealed 96.5% and 42.5% of the plasma membrane-bound EGE′ to be associated with the apical membrane of MDCK(EGE-GPI) and Caco-2(EGE-GPI) cells, respectively. During treatment with trypsin, epithelial integrity was maintained as illustrated by the ability of trypsin to remove EGE′ only from the surface to which it was added (Table 1) and by the maintenance of Rτ (data not shown).

This trypsin treatment procedure was then used to quantify the time-course of delivery of EGE′ to each membrane surface in Caco-2(EGE-GPI) cells; and the sum of EGE′ in each medium and on each cell surface. There was a time-dependent increase in EGE′ associated with the apical membrane (Fig. 6). In contrast, EGE′ associated with the basolateral membrane remained relatively constant with time (Fig. 6). Over a 24 hour period the majority, approximately 80%, of the EGE′ was associated with the apical membrane compartment (Fig. 6). In contrast, release of lactate dehydrogenase is minimal into both membrane compartments (Soole et al., 1992), suggesting little cell lysis during this period.

Table 1. EGE′ associated with apical and basolateral membrane surfaces in confluent monolayers of MDCK(EGE-GPI) and Caco-2(EGE-GPI) cells

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Cells were grown on either Transwell inserts for 12 days (Caco-2(EGE-GPI)) or Anopore inserts for 5 days (MDCK(EGE-GPI)). Monolayers were washed with PBS twice and then once with serum-free growth medium. Serum-free medium (1 ml) was added to each compartment in a series of filters and 0.1% (w/v) trypsin was added to either the apical or basolateral medium as indicated. After 1 hour of incubation at 37°C, cell layer integrity was confirmed by determining the Rτ for each filter. Medium was collected only for intact cell layers and assayed for EGE′ activity. Results are presented as total EGE′ activity in each medium sample (n=4).

*Medium bathing either apical or basolateral (basal) membrane surface in which EGE′ activity was determined.
†After 1 hour of incubation with fresh medium containing 0.1% trypsin.
and at various times later, the medium bathing both membranes would be non-biotinylated. Immediately after biotinylation, GPI) cells, polycarbonate filters with confluent monolayers of Caco-2(EGE-GPI) and MDCK(EGE-GPI) monolayers, respectively, prior to trypsin incubation. Similarly low levels of basal EGE' were detected after basal trypsin treatment (data not shown). All images were collected under identical conditions. Bar, 25 μm.

Fig. 5. Trypsin removal of cell surface EGE’ from confluent monolayers of MDCK(EGE-GPI) and Caco-2(EGE-GPI) cells. After incubation with trypsin as described in Table 1, cell layers were fixed and labelled for EGE’ as described for Figs 1 and 2 and visualized by confocal laser scanning microscopy. (A and B) Confocal optical sections imaged at the apical aspects of the cells, represent immunofluorescent staining specific for EGE’ with apical application of the antiserum, for Caco-2(EGE-GPI) and MDCK(EGE-GPI) monolayers, respectively. (C and D) Reveal barely detectable levels of apical EGE’ in identical images of MDCK(EGE-GPI) and Caco-2(EGE-GPI) monolayers, respectively, after trypsin treatment. Similarly low levels of basal EGE’ were detected after basal trypsin treatment (data not shown). All images were collected under identical conditions. Bar, 25 μm.

Fig. 6. Time course of appearance of EGE’ associated with both apical and basolateral cell surfaces and corresponding bathing medium for Caco-2(EGE-GPI) monolayers. A series of polycarbonate filters with confluent monolayers of Caco-2(EGE-GPI) cells, RT > 600 Ω. cm², were washed with PBS and serum-free medium and for all filters 1 ml of serum-free growth medium was placed above and below filters, t=0. At various times, 0.1% trypsin was added to the apical and basal bathing media and after incubation for 1 hour at 37°C, the media were collected, assayed for EGE’ activity to give total apical (○) and basolateral (□) delivered EGE’. These samples represent the EGE’ associated with each cell surface. Values represent means, with error bars of ± 1 s.e.m., from four separate filters.

Fig. 7. Cell surface delivery of EGE’ in Caco-2(EGE-GPI) monolayers. Confluent monolayers of Caco-2(EGE-GPI) cells, grown on Transwell polycarbonate filters for 14 days, were surface biotinylated as described in the text. After biotinylation, any filters where resistance had fallen were discarded. Pre-warmed serum-free medium was placed above and below filters and at various time intervals 0.1% (w/v) trypsin was added to the apical and basal medium. After a further incubation at 37°C for 1 hour, the medium was collected. When trypsin was present, the medium sample represents all EGE’ associated with that plasma membrane surface during that time period. Biotinylated EGE’ was removed from each sample by incubation with streptavidin-agarose beads and centrifugation. Non-biotinylated EGE’ was determined by assaying the supernatant for EGE’ activity. Results are presented as total non-biotinylated EGE’ delivered to the apical (■) and basal (○) cell surface and represent means, with error bars of ± 1 s.e.m., of six observations.

Transcytosis of EGE’ in Caco-2(EGE-GPI)

To investigate for transcytosis of EGE’, both cell surfaces were biotinylated, excess biotin removed and then the biotinylated-EGE’ (total of EGE’ in media and cell surface-related EGE’ released by trypsin) at each cell surface was determined over time. EGE’ activity was determined before and after treatment with streptavidin-linked beads, the difference giving the biotin-EGE’.

Initially, greater amounts of EGE’ were accessible to zero time measurement. Biotin was then removed from the medium and thus, after time zero, newly synthesized EGE’ would be non-biotinylated. Immediately after biotinylation, and at various times later, the medium bathing both membranes of the cells was collected and the surface EGE’ released by trypsin treatment. Epithelial integrity was maintained during trypsin treatment, similarly to that described above. The combined media and surface samples were incubated with streptavidin-linked beads, which bound all of the EGE’ that was biotinylated at the beginning of the experiment. Therefore, the increase in EGE’ activity remaining in the supernatant, compared to the zero time value after the removal of the streptavidin beads, represented EGE’ that was not biotinylated at time zero and was thus delivered to the respective membrane after biotinylation. Delivery of newly synthesized EGE’ to the apical membrane was approximately 2.5 times greater than that to the basolateral membrane (Fig. 7).

The appearance of EGE’ at the basolateral surface could be a consequence of mis-sorting of EGE’ to the basolateral membrane, or EGE’ could, in addition to direct delivery to the apical surface, be redirected to this membrane domain via transcytosis from the basolateral surface. These questions are addressed in the next experiments.
biotinylation at the basolateral as compared with the apical cell surface, consistent with the greater expression of EGE’ on the basolateral surface of Caco-2(EGE-GPI) cells (Table 1). The biotin-EGE’ associated with the basolateral cell surface declined with time (y = −0.165x + 4.71 nmol MUC h⁻¹; r² = 0.940) and this was mirrored by an equivalent increase (y = 0.213x + 2.35 nmol MUC h⁻¹; r² = 0.914) in biotin-EGE’ at the apical cell surface (Fig. 8). These data indicate basolateral to apical transcytosis of EGE’.

**DISCUSSION**

It has been previously reported that a protein of bacterial origin, such as EGE’ from *C. thermocellum*, can be expressed as a functional, glycosylated protein in transfected MDCK and Caco-2 cells (Soole et al., 1992). The presence of an N-terminal eukaryotic signal peptide directed secretion of EGE’ from both cell lines and, due to the absence of eukaryotic sorting signals within the mature bacterial protein, defined the default pathway of secretion from these cells. Data presented here demonstrate that it is possible to direct the attachment of a GPI anchor to a bacterial protein by the addition of the appropriate amino acid sequence at the C-terminal end of the bacterial protein. In this study, the sequence used was that appropriate amino acid sequence at the C-terminal end of the protein anchor sequence. (Conzelmann et al., 1986). EGE’-GPI, expressed by transfected MDCK and Caco-2 cells was shown to be associated with a GPI anchor by demonstrating the membrane location of cell-associated EGE’, the partitioning of EGE’ from these cells into the hydrophobic phase and the resumption of hydrophilic characteristics after treatment with a PI-specific phospholipase C, which cleaves the linkage to the diacylglycerol moiety (Low, 1987).

For MDCK cells it has been well documented that proteins that are linked to a GPI anchor sequence are specifically targeted to the apical plasma membrane. Data presented here for MDCK(EGE-GPI) cells indicate that EGE’ is exclusively expressed on the apical surface of these cells when attached to a GPI anchor, consistent with the GPI anchor being a dominant apical targeting signal in these cells. Alternatively, it might be argued that since the cytoplasmic tail of several basolateral proteins is necessary for basolateral targeting (e.g. see Wollner and Nelson, 1992), the membrane-bound form of EGE’ is following the apical default pathway in MDCK cells. Such an argument ignores the evidence for the dominant effect of GPI anchor signals (Brown et al., 1989; Lisanti et al., 1989, 1990a; Powell et al., 1991a).

In Caco-2 cells, several endogenous GPI-anchored proteins have been shown to be located on the apical plasma membrane. These include alkaline phosphatase, carcinoembryonic antigen and 5'-nucleotidase (Lisanti and Rodriguez-Boulan, 1992; present study). However, at least some apical GPI-anchored proteins, such as DAF, Thy-1.2. and PLAP, contain apical sorting signals in their ectodomains (Brown et al., 1989; Lisanti et al., 1989; Casanova et al., 1991; Powell et al., 1991b). Signals that may be present in the ectodomain of the endogenous GPI-anchored proteins of the Caco-2 apical membrane have not been examined and thus a definitive statement concerning the role of the GPI anchor as an apical sorting signal in these cells cannot be made. To address the role of the GPI anchor in Caco-2 cells we have investigated the sorting of EGE’-GPI. When EGE’-GPI was expressed in Caco-2 cells, the rate of delivery of the bacterial protein to the apical membrane was approximately 2.5 times that to the basolateral membrane (Fig. 7). In addition, over a 24 hour period 80% of the extracellular EGE’ activity had passed through or was on the apical membrane. As non-GPI-anchored EGE’ is secreted predominantly through the basolateral membrane (70%; Soole et al., 1992), the GPI anchor represents a dominant sorting signal for this protein.

Owing to the hydrophobic nature of the GPI anchor sequence, N-terminal to the cleavage site, it would be reasonable to argue that EGE’-GPI could be attached to the basolateral plasma membrane via the Thy-1 membrane-spanning region of the fusion protein and as such its presence on the basolateral surface represents a mis-processing of the GPI-anchor attachment. Examination of the membrane attachment of Thy-1 in two classes of Thy-1 negative mutant lymphoma cell lines revealed that the protein was, in fact, not attached to the membrane by a GPI anchor but was a transmembrane protein (Conzelmann et al., 1986). However, as EGE’ could be removed from the basolateral surface of tight monolayers of Caco-2(EGE-GPI) by incubation with PI-PLC it is unlikely that EGE’ is attached to the basolateral membrane by the GPI protein anchor sequence.

The presence of EGE’-GPI on the basolateral surface may implicate a transcytotic biosynthetic pathway in the protein’s delivery to the apical surface as demonstrated for sucrase-isomaltase and DPPIV (Mattler et al., 1990; Le Bivic et al., 1990).
Indeed, direct analysis of biotin-labelled EGE’ demonstrated transcytosis from the basolateral to apical cell surfaces of Caco-2 (EGE-GPI) cells. Low levels of endogenous GPI-anchored alkaline phosphatase have also been noted on the basolateral membrane of Caco-2 cells in some studies, although no evidence for subsequent transcytosis to the apical membrane was observed (Le Bivic et al., 1990; Ellis et al., 1992). In the present study, there was no evidence for basolateral staining of alkaline phosphatase or DPP IV. GPI-anchored proteins, such as Thy-1 (Bretsch et al., 1980), are excluded from clathrin-coated pits, suggesting their exclusion from normal transcytotic pathways. However, a recent report has localized PLAP in coated pits of vesicles prepared from human placenta (Makiya et al., 1992), suggesting a possible route for the transcytosis of some GPI-anchored proteins.

In summary, GPI-anchoring can re-direct a bacterial protein in both intestinal and kidney epithelial cells. However, the efficiency was lower in the intestinal cell line. The so-called redundant apical targeting information reported for the ectodomain of several GPI-anchored proteins (Brown et al., 1989; Lisanti et al., 1989; Casanova et al., 1991; Powell et al., 1991b) may in fact be essential for the efficient sorting in Caco-2 cells. This may be analogous with apical targeting signals in the ectodomains of transmembrane proteins such as aminopeptidase N and endopeptidase-24.11 (Vogel et al., 1991b) may in fact be essential for the efficient sorting in Caco-2 cells. The ability of a GPI anchor sequence to target, by both direct and indirect routes, heterologous proteins in model entocytes indicates that selective delivery of such proteins to the lumen of the intestine in transgenic animals (Hall et al., 1993) is a realistic goal.

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