A protein targeting signal that functions in polarized epithelial cells *in vivo*

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Eukaryotic membrane-associated polypeptides often contain a glycosylphosphatidylinositol (GPI) anchor that signals the attachment of GPI lipids to these proteins. The GPI anchor can function as a basolateral or apical targeting signal in mammalian cells cultured *in vitro*, although the function of the GPI anchor *in vivo* remains to be elucidated. In this study we have evaluated the effect of fusing a GPI anchor sequence to a prokaryotic reporter protein on the cellular location of the polypeptide in

polarized epithelial cells of transgenic mice. The bacterial enzyme, when fused to a eukaryotic signal peptide, was secreted through the basolateral membrane of small-intestinal enterocytes; however, when the enzyme was linked to the GPI anchor sequence the polypeptide was redirected to the apical surface of the epithelial cells. These data provide the first direct evidence that the GPI anchor functions as an apical membrane protein sorting signal in polarized epithelial cells *in vivo*.

INTRODUCTION

The targeting of proteins to specific membrane domains in polarized mammalian cells is critical for differentiation and the maintenance of normal cellular function [1]. Polypeptides that are sorted to extracytoplasmic locations contain N-terminal signal peptides that have been the focus of numerous studies [2]. In addition, many proteins that are located at the apical surface of polarized cells contain a glycosylphosphatidylinositol (GPI) anchor [3,4]. In Madin–Darby canine kidney (MDCK) cells the GPI anchor seems to function as an apical sorting signal. Several studies have shown that viral glycoproteins normally located at the basolateral surface of MDCK cells are redirected to the apical membrane when fused to a GPI anchor [5]. Similarly, in polarized intestinal Caco-2 cells, 70 % of a bacterial protein was secreted through the basolateral membrane [6]. However, when the prokaryotic polypeptide was linked to a GPI anchor, it was transported to the apical surface of these cells [7]. The view that the GPI anchor functions as an apical targeting signal is also supported by the observation that several GPI-linked proteins, endogenous to MDCK and Caco-2 cells, are associated exclusively with the apical surface [3]. However, as GPI-anchored proteins can also contain apical sorting signals in their ectodomains [8–11], it is possible that the primary role of the GPI anchor in these polypeptides is to facilitate membrane attachment.

Although GPI-anchored proteins generally exhibit apical targeting, the GPI-anchored protein p137 displays bidirectional transectosis in Caco-2 cells, although it is not clear whether the GPI anchor itself mediates this process [12]. In contrast with MDCK and Caco-2 cells, endogenous GPI-anchored proteins, and a chimaeric polypeptide in which the GPI anchor was fused to herpes simplex gD-1, were localized at the basolateral surface of Fischer rat thyroid epithelial cells [13]. Polarized primary thyrocytes, however, contain GPI-linked polypeptides on their apical membranes [14].

From the foregoing discussion it is apparent that the membrane location of GPI-anchored proteins varies between different mammalian cell lines, and that protein sorting data *in vitro* do

not always reflect the trafficking of proteins *in vivo*. To evaluate whether the GPI anchor functions as an apical sorting signal in polarized intestinal cells *in vivo*, we have expressed two forms of a bacterial protein in the small-intestinal enterocytes of transgenic mice. The protein, when fused to a eukaryotic signal peptide, was secreted primarily through the basolateral membrane into the bloodstream. In contrast, the prokaryotic polypeptide, when linked to a mammalian signal peptide and a GPI anchor sequence, was located on the apical surface of the intestinal enterocytes and in the small-intestinal lumen. These results provide direct evidence that the GPI anchor functions as an apical membrane protein sorting signal in polarized cells *in vivo*.

MATERIALS AND METHODS

Plasmid construction

The bacterial genes used in this study are derivatives of *celE*, a gene that encodes endoglucanase E (EGE) from *Clostridium thermocellum* [15]. The two derivatives of *celE* that were used to generate transgenic mice were constructed as follows. The promoter of the gene encoding the rat intestinal fatty acid binding protein, extending from nucleotides –1178 to +8 [16], derived from pHGG1, was inserted into pMTL20p [17] on a 1.2 kb *KpnI*/*Bam*HI restriction fragment to generate pSA3. The DNA sequences encoding the catalytic domain of EGE fused to the human growth hormone signal peptide [18] and Thy-1.2 GPI anchor (EGE'; [19]), and the catalytic domain of EGE fused to the human growth hormone signal peptide (EGE'), were excised from pSRαEGETHY1 [7] and pCELEE [18], respectively, on 3.3 kb and 3.1 kb *Bam*HI/*Stu*I restriction fragments and cloned into *Bam*HI/*Stu*I-digested pSA3 to create pSA2 and pSA1, respectively (Figure 1).

Generation of transgenic mice

The *celE*-containing DNA inserts in pSA1 and pSA2 were excised on 4.2 and 4.5 kb *Xho*I restriction fragments respectively.

Abbreviations used: EGE, endoglucanase E; EGE', catalytic domain of endoglucanase E linked to a eukaryotic signal peptide; EGE'', catalytic domain of EGE fused to a eukaryotic signal peptide and a GPI anchor attachment sequence; FITC, fluorescein isothiocyanate; GPI, glycosylphosphatidylinositol; MCR, multiple cloning regions; MDCK, Madin–Darby canine kidney; TEM, transmission electron microscopy.

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Figure 1 Physical map of recombinant plasmids encoding EGE' and EGE''

The restriction sites are designated as follows: B, *Bam*HI; Bg, *Bgl*II; Cl, *Cl*AI; C, *Eco*RV; R, *Eco*RI; H, *Hind*III; S, *Sal*I. The triangles represent the following multiple cloning regions (MCR): 1, the MCR of pMTL20p between the *Eco*RI and *Kpn*I sites and the MCR of pBluescript KS⁻ between the *Eco*RI and *Kpn*I sites; 2, the MCR of pMTL22 between the *Sst*I and *Cl*AI sites in pSA1 and between the *Sst*I and *Kpn*I sites in pSA2; 3, the MCR of pMTL20 [17] between the *Xho*I and *Hind*III sites. The recombinant plasmid pSA1 (upper panel) consists of the promoter region of the gene encoding rat intestinal fatty acid binding protein (*fabp*; hatching at left end), a synthetic DNA sequence encoding the human growth hormone signal peptide (*sp*; white areas), truncated *celE* (*celE'*), encoding residues 4–367 of mature EGE, which constitutes the catalytic domain of the enzyme, followed by a translational stop codon (cross-hatching) and the polyadenylation signal of the rabbit β -globin gene (*polyA*; hatching at right end). The plasmid pSA2 (lower panel) is identical with pSA1 except that the *celE'* sequence does not contain a translational stop signal after codon 367 and, 3' of the endoglucanase gene, a DNA sequence that encodes the GPI anchor sequence [17] of Thy 1.2 (*thy1.2*; black area) was inserted. Both pSA1 and pSA2 are derived from the vector pMTL20p.

The DNA fragments were purified by agarose gel electrophoresis and NACS column chromatography. The DNA was diluted in 10 mM Tris/HCl buffer, pH 7.4, containing 0.1 mM EDTA, to a concentration of 4 ng/ml and injected into the pronuclei of freshly fertilized eggs derived from the progeny of a mating between [C57BL6 $\times$ CBA] F₁ mice. Approximately 700 eggs were injected with each construct. Embryos were cultured overnight in M16 medium, and viable embryos were transferred to the infundibulum of the fallopian tubes of pseudopregnant mice.

Approximately 30 eggs were implanted per mouse. Live-born mice, at 3 weeks of age, were anaesthetized with halothane, before removing a tail biopsy for screening for the presence of the transgenes by probing Southern blots of *Sst*I-digested tail DNA with ³²P-labelled *celE'*. Founder animals were mated with [C57BL6 $\times$ CBA] F1 mice to establish pedigrees hemizygous for the transgenes.

Quantification of EGE'

Transgenic mice were anaesthetized with halothane, bled and then killed by cervical dislocation. Intestines were divided into five segments defined as the duodenum, jejunum, ileum, caecum and colon. The tissues were resuspended in 1 ml of 50 mM Tris/HCl buffer, pH 7.5, containing 250 mM sucrose (buffer A), and treated in one of two ways. (1) The tissues were washed in buffer A, homogenized and the cellular debris was pelleted by centrifugation at 13000 *g* for 20 min at 4 °C; the cleared homogenate was assayed for EGE' antigen. (2) The tissue segments were cut open longitudinally and vortex-mixed in buffer A. The intestinal tissue was removed and the luminal contents were centrifuged for 10 min at 4 °C; the supernatant was assayed for EGE' antigen. To analyse EGE' in the systemic circulation, blood was allowed to clot and serum was separated by centrifugation. EGE' in the various samples was quantified by competitive inhibition ELISA as follows: micro-ELISA plates were coated for 15 h at 4 °C with EGE' at 0.1 μ g/ml in PBS. The

plates were then washed four times with PBS containing 0.1 % Tween-20 and 0.1 % BSA. Soluble antigen standards (1–500 ng/ml) and appropriate samples (50 μ l) were added to individual wells followed by 25 μ l of primary antibody diluted 1:1000 in PBS containing 0.1 % Tween-20 and 0.2 % BSA (PTB buffer). After incubation at room temperature for 2 h, the plates were washed four times in PTB buffer. Goat anti-rabbit IgG coupled to hydrogen peroxidase, diluted 1:1000 in PTB buffer, was added to each well, and after incubation for 1 h at 22 °C, excess secondary antibody was removed by washing the plates four times with PTB buffer. Bound antibody conjugate was detected by the addition of 100 mM sodium acetate buffer, pH 6.0, containing 0.1 mg/ml 3,3',5,5'-tetramethyl benzidine and 0.06 mg/ml hydrogen peroxide. The enzyme reaction was terminated after 15 min by the addition of 20 % (v/v) sulphuric acid, and the *A*₄₅₀ was measured with a Dynatech MR5000 microplate reader. The concentration of EGE' in the samples was determined from a standard curve of log(EGE' concentration) against *A*₄₅₀.

Transmission electron microscopy (TEM)

Adult mice were killed by cervical dislocation, the abdomen was incised and 1 cm lengths of jejunum were removed. The excised tissues were cut open along their mesenteric edges and pinned flat, mucosal surface uppermost, on cork boards. The tissues were washed thoroughly with PBS and immersed in 100 mM sodium phosphate buffer, pH 7.3, containing 2 % (w/v) glutaraldehyde, for 5 h at 4 °C. After washing with PBS, the cells were permeabilized with 0.1 % Triton X-100 at room temperature for 30 min. Endogenous peroxidase activity was blocked by immersing the tissues in 0.3 % hydrogen peroxide solution in 100 % (v/v) methanol for 15 min at room temperature. After rinsing with PBS, the tissues were incubated with a 1:100 dilution (diluted in PBS) of rabbit anti-EGE' antiserum at 4 °C for 16 h. After washing with PBS, the tissues were incubated with goat serum, diluted 1:3 (v/v) in PBS, for 30 min at room temperature, and then with a 1:400 dilution of goat anti-rabbit IgG antibodies conjugated to hydrogen peroxidase for 1 h at room temperature. The tissues were then washed in PBS and bound second antibody conjugate was detected by immersing the tissue for 15 min in 2.8 mM 3,3'-diaminobenzidine tetrahydrochloride containing 0.02 % hydrogen peroxide. Tissues were then processed for TEM by immersion in an aqueous solution of 1 % OsO₄ for 1 h at 4 °C, followed by washing in 100 mM sodium phosphate buffer, pH 7.3. Samples were dehydrated successively in 25, 50, 75 and 100 % (v/v) acetone, then immersed successively for 1 h in 1:4, 1:3 and 1:2 acetone/epoxy resin and then embedded in epoxy resin at 60 °C for 24 h. Sections (70 nm thick) were cut, transferred to a 100-mesh Formvar-coated copper grid, stained with 4 % (w/v) uranyl acetate for 1 h at 20 °C and saturated lead citrate for 30 min at 20 °C and examined with a Phillips CM100 electron microscope.

Confocal microscopy

Samples were removed and immediately frozen in OCT embedding compound on to cork squares in liquid-nitrogen-cooled isopentane. Sections, 8 μ m thick, of frozen tissue were dried on poly-L-lysine-coated slides and fixed in methanol at –20 °C for 20 min. Sections were then incubated with anti-EGE' antiserum, diluted 1:100 in PBS, for 13 h at 4 °C. Unbound antibody was removed by washing the tissues in PBS which were then incubated with goat serum, diluted 1:3 in PBS, for 30 min at room temperature. EGE'–antibody complexes were

detected by incubating the tissues with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG antibodies (diluted 1:80 in PBS) for 1 h at 22 °C. Unbound fluorescent antibody was removed by washing with PBS. To locate alkaline phosphatase, antibody-stained sections were washed with water and PBS, and were then immersed in 200 mM Tris/HCl buffer, pH 8.5, containing 0.52 mM naphthol AS-MX phosphate, 1 mg/ml Fast Red TR salt and 1 mM levamisole for 5 min at room temperature. The tissues were washed in PBS and water, dehydrated in ethanol and mounted in Vectashield mounting medium. Fluorescent images were collected with a Bio-Rad MRC600 confocal laser scanning image system, with an argon ion laser attached to a Nikon Diaphot microscope.

RESULTS

To determine the secretory route of a prokaryotic endoglucanase in polarized mammalian epithelial cells *in vivo*, a derivative of *celE* [15] that encodes EGE from *C. thermocellum* was expressed in transgenic mice. A derivative of the gene (*celE'*) encoding EGE' was fused to a small-intestinal-specific promoter and a mammalian polyadenylation signal (Figure 1), and the DNA was used to generate six transgenic mouse lines. As endoglucanase activity is absent from mammalian cells, EGE' is

Figure 2 Small-intestinal expression of EGE'

Tissues from the duodenum, jejunum, ileum, caecum and colon of four transgenic lines that contain the insert from pSA1 (upper panel) and six transgenic lines that contain the insert from pSA2 (lower panel) were assayed for EGE' antigen and protein. Bars with the same pattern in either the upper or lower panels denote data from the same transgenic line. The standard errors for each determination are shown.

Figure 3 EGE' in the intestinal lumen and blood of transgenic mice

The upper panel shows the quantity of EGE' in the blood and small-intestinal lumen of four transgenic lines that express EGE' (contains the insert from pSA1). The lower panel displays the level of EGE' in the blood and small-intestinal lumen of six transgenic lines that synthesize EGE' (contain the insert from pSA2). Bars with the same pattern in either the upper or lower panels denote data from the same transgenic line. The standard errors for each determination are shown.

a sensitive reporter for studying protein secretory pathways. Analysis of tissues from the transgenic mice revealed that in four of the lines EGE' was expressed primarily in small-intestinal enterocytes located in the jejunum and ileum (Figure 2, upper panel). No endoglucanase activity was detected in the stomach, liver, lung, heart or spleen (results not shown), consistent with the reported tissue specificity of the promoter [16]. The other two transgenic lines did not synthesize EGE'. To determine whether EGE' was secreted from the small-intestinal epithelia and, if so, whether the enzyme was exported through the basolateral and/or apical membrane, the small-intestinal lumen and blood were analysed for EGE' antigen. The data, presented in Figure 3 (upper panel), showed that endoglucanase in the blood and small-intestinal lumen was in the ratio 8:1, indicating that the enzyme was secreted primarily through the basolateral membrane.

To evaluate whether the GPI anchor can act as a dominant apical sorting signal in small-intestinal enterocytes *in vivo*, transgenic mice were generated containing a derivative of *celE* (*celE''*) transcribed from a small-intestine-specific promoter that encodes EGE' fused to a GPI anchor attachment sequence (EGE''; Figure 1). Analysis of the 15 transgenic lines generated revealed six lines that expressed EGE'' in small-intestinal tissue (Figure 2; lower panel), but not in the other

Figure 4 Immunohistochemistry of intestinal tissue of transgenic mice

Transmission electron micrographs of small-intestinal enterocytes, derived from transgenic mice expressing EGE' (upper left panel) and EGE'' (upper right panel), that have been labelled with EGE' antibody revealed by horseradish hydrogen peroxidase-conjugated secondary antibody. Scale bar = 2 μ m. EGE' was localized in small vesicles towards the basolateral aspects of the cells ($\downarrow$), with little evidence for microvilli (B) staining (upper left panel), while GPI-anchored EGE'' (upper right panel) was localized at the microvilli (B) tip (*), small vesicles ($\downarrow$) and multivesicular bodies ($\downarrow\downarrow$). The nucleus in both micrographs is identified by N. Confocal laser scanning micrographs of small-intestinal villi, from mice synthesizing EGE' (lower left panel) or EGE'' (lower right panel), stained with EGE' antibody. Immunoreactive EGE was revealed by FITC-conjugated secondary antibody (green) and alkaline phosphatase by a red histochemical substrate. Scale bar = 50 μ m. FITC fluorescence was largely cytoplasmic, interior to red alkaline phosphatase staining in EGE' mice (lower left panel), whereas FITC EGE' staining was largely exterior to the alkaline phosphatase staining in EGE'' mice, with significant intervillous luminal localization of EGE'' (lower right panel).

organs analysed (lung, heart, liver, spleen and stomach; results not shown). As with the EGE' transgenic lines, EGE'' was expressed predominantly in the jejunum and ileum (Figure 2). TEM of small-intestinal enterocytes derived from transgenic lines expressing EGE'', and labelled with anti-EGE' antibodies, revealed that a significant proportion of the endoglucanase was located on the apical microvilli (Figure 4, upper right panel), with no evidence for EGE'' at the basolateral surface of the intestinal cells. The enzyme was detected both in small intracellular vesicles and in large multivesicular bodies (Figure 4, upper right panel). In contrast, TEM of small-intestinal enterocytes synthesizing EGE' indicated that the endoglucanase was not targeted to the apical surface but was localized in small vesicles that accumulated towards the basolateral surface of the enterocytes (Figure 4, upper left panel). Examination of dual-labelled EGE''-expressing intestinal cells by confocal microscopy revealed that the endoglucanase co-localized with alkaline phosphatase to the apical surface. The EGE'' was exterior to the

alkaline phosphatase staining (Figure 4, lower right panel), consistent with the strong EGE'' staining at the microvilli tips observed by TEM (Figure 4, upper right panel). In addition, significant EGE'' staining was also observed in the small-intestinal lumen (Figure 4, lower right panel). In contrast, confocal microscopy of cells that synthesized non-GPI-anchored EGE' showed that the enzyme was cytoplasmic, with minimal co-localization with alkaline phosphatase at the apical surface (Figure 4, lower left panel), consistent with the weak EGE' staining of the brush-border membrane observed by TEM (Figure 4, upper left panel). The luminal location of a proportion of EGE'' was consistent with the structure of the hybrid protein; the GPI anchor was fused to the catalytic domain of EGE' via a proteinase-sensitive hydroxyamino acid-rich linker sequence [7,15]. Indeed, treatment of Caco-2 cells expressing EGE'' with trypsin rapidly released the polypeptide from the apical membrane [7]. To confirm the secretory route of the GPI-anchored endoglucanase, EGE'' antigen was quantified in the blood and

Figure 5 Western analysis of EGE expressed by transgenic mice

Western analysis was performed with anti-EGE' antiserum, as described by Hall et al. [21], on protein extracted from the small-intestinal epithelium (left panel) and luminal contents (right panel) of mice expressing EGE' (lane 1), and non-transgenic animals (lane 2). *Escherichia coli*-derived EGE' treated with small-intestinal proteinases is shown in lane 3 (right panel). The molecular masses (in kDa) of the immunoreactive polypeptides are shown.

small-intestinal lumen of these animals. The data, presented in Figure 3, showed that the ratio of blood/small-intestinal lumen EGE' was 0.17:1. This is the reverse of the ratio observed for the non-GPI-anchored endoglucanase. This value is an underestimate of the EGE' that has been sorted to the apical surface of the epithelial cells, as it does not take account of the enzyme that is still attached to the apical membrane. Western analysis of luminal proteins showed that the secreted form of EGE' had a molecular mass of 36 kDa, which was identical with non-glycosylated EGE' treated with small-intestinal proteinases (Figure 5). In contrast, intracellular EGE' had a molecular mass of 50 kDa (Figure 5), which is similar to the size of glycosylated EGE' expressed by several mammalian cell lines [6,18,20]. It is therefore likely that α -glycosidases in the small-intestinal lumen deglycosylate the secreted enzyme. Glycosylated and deglycosylated forms of the enzyme have equivalent endoglucanase activities and kinetic characteristics [20].

DISCUSSION

The primary objective of this study was to evaluate the influence of the Thy-1 GPI anchor on the secretory route of EGE. When EGE' was expressed in small-intestinal enterocytes of transgenic mice, more than 80% of the extracellular form of the enzyme was located in the bloodstream, suggesting that the endoglucanase was secreted through the basolateral membrane of the intestinal cells. It could be argued that any EGE' secreted into the gastrointestinal tract would be rapidly degraded by pancreatic and other luminal proteinases, and thus the relative absence of the enzyme in the small-intestinal lumen might not necessarily reflect a non-apical secretory route for the endoglucanase. However, EGE' is resistant to intestinal proteinases and remains fully active when secreted into the small-intestinal lumen [21]. Thus the results shown in Figure 3 indicate that most EGE' in small-intestinal enterocytes is exported through the basolateral membrane. As there is no evidence in this prokaryotic enzyme for the evolution of sorting signals other than the N-terminal

signal peptide [6], the secretory route of EGE' defines the default pathway of protein secretion from these cells *in vivo*. The results described above are comparable with previous studies *in vitro* that have defined the default pathway in Caco-2 cells, a culture model for differentiated small-intestinal enterocytes, which is predominantly (70%) through the basolateral membrane [10,22,23].

Recent results have suggested that the N-glycosylation of rat growth hormone targets the protein to the apical surface of MDCK cells [24]. EGE' is N-glycosylated in Caco-2 and MDCK cells *in vitro* [6,7], as well as in intestinal enterocytes *in vivo*. However, this glycosylation does not result in the apical targeting of this bacterial protein, suggesting that a role for N-glycans in apical protein sorting is not generic.

Results from confocal microscopy and TEM of intestinal tissue indicated that EGE' was associated with the apical membrane of small-intestinal enterocytes *in vivo*. In contrast, *in vitro* studies revealed a significant proportion of GPI-anchored EGE' at the basolateral surface of enterocyte-like Caco-2 cells, with subsequent transcytosis to the apical surface [7]. Results presented in this report provide no evidence for basolateral delivery of the GPI-anchored endoglucanase *in vivo*. The observation that EGE' was located only in the cytoplasm of intestinal cells confirmed that the GPI anchor targeted EGE' to the apical membrane of small-intestinal enterocytes *in vivo*.

TEM of intestinal enterocytes revealed that the bacterial protein was located in both small and large vesicles. GPI-anchored proteins have similarly been shown to accumulate in multivesicular bodies in a variety of non-polarized cells *in vitro*, including those of intestinal epithelial origin [25,26]. Field et al. [25] have shown that in CHO and COS cells, failure to cleave the C-terminal protein sequence that directs the addition of a GPI anchor prevents the proteins from entering the *cis*-Golgi complex, and they are then degraded in large non-lysosomal vesicles. It is therefore possible that in a proportion of EGE' molecules the C-terminal region, which signals the addition of the GPI anchor, is not cleaved and the endoglucanase, instead of entering the *cis*-Golgi complex, is degraded in large multivesicular bodies.

In view of the dominant role that the GPI anchor plays in directing EGE' to the apical membrane, it is probable that in small-intestinal enterocytes this sequence also determines the cellular location of several endogenous GPI-anchored proteins that associate with the apical membrane. However, in contrast with EGE', where a small proportion of the enzyme (less than 15%) is secreted through the basolateral membrane, endogenous GPI-anchored proteins are present at very low levels in the blood. This might be explained by the identification of apical sorting signals in the ectodomain of some GPI-anchored proteins, such as decay accelerating factor, Thy-1.2 and placental alkaline phosphatase [8–11], although the generality of this observation has yet to be demonstrated for GPI-anchored polypeptides derived from small-intestinal enterocytes.

To conclude, this report directly demonstrates that the GPI anchor functions as a dominant apical sorting signal in small-intestinal enterocytes *in vivo* and thus is likely to play a major role in targeting endogenous proteins to the apical surface of these cells. In addition, in view of the potential applications of generating transgenic mammals with the capacity to secrete exogenous proteins into the gastrointestinal tract [21], we have demonstrated the utility of the GPI anchor for directing heterologous polypeptides into the small-intestinal lumen.

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