mRNA localization in polarized intestinal epithelial cells

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An important feature of enteroctye maturation is the asymmetrical distribution of proteins and mRNA. This study was designed to determine the mechanisms responsible for mRNA asymmetry during enterocyte maturation and to provide a reliable in vitro model for investigations of this process.

MATERIALS AND METHODS

Vectors. Fusion reporter genes were synthesized to contain β-galactosidase (β-gal) and enhanced green fluorescence protein (GFP) linked upstream to the 3′-UTR of the mRNA of interest and were cloned into vector pCA13 (Microbix, Toronto, Ontario, Canada), under the control of the cytomegalovirus promoter (Pcav). A polyadenylation signal (PAS) was

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inserted downstream to ensure mRNA stability. Results were confirmed by vector sequencing (Tufts University Sequencing Core Facility, Department of Physiology, Tufts University School of Medicine, Boston, MA). Three expression vectors were constructed: for SI mRNA, Pcmv/β-gal/GFP/SI-3'UTR/PA/pcA13; for villin mRNA, Pcmv/β-gal/GFP/villin-3'UTR/PA/pcA13; and for the control, Pcmv/β-gal/GFP/PA/FPS/pcA13. β-Gal is a large protein that in short-term culture remains at the site to which its mRNA is targeted in fibroblasts (13). The linked GFP provided a fluorescent tag that could be analyzed by confocal microscopy. We further hypothesized that the addition of β-gal to GFP as a large fusion protein might enhance its capacity as a proxy marker for the position of its mRNA.

**Transient transfections.** Caco-2 cells were chosen due to their capacity to express a phenotype similar to differentiated absorptive enterocytes (8–10, 19, 23). Preconfluent cells were transfected by using electroporation (14) with 10 μg of expression plasmid (40 μg total DNA) and plated at 100% confluence on fibroblastic cell culture inserts (Becton-Dickinson, Bedford, MA) in DMEM supplemented with 10% FCS (GIBCO-BRL, Rockville, MD). Cells were maintained at 37°C under 5% CO2 with 100% humidity. Media were changed three times per week during the indicated culture periods.

At the indicated times after transfection, cells were washed twice with PBS and then fixed and permeabilized in a 40:40:20 solution of acetone/methanol/water for ~ 15 s. After cells were washed twice again with PBS, orientation within the cell was elucidated by staining nuclei with 2 μg/ml propidium iodide (Sigma, St. Louis, MO) and staining the microvillus membrane of the cells with either 0.33 μM rhodamine-conjugated or Texas red-conjugated phalloidin (Molecular Probes, Eugene, OR) for 1 h at room temperature. Cells were then rinsed with PBS, mounted on glass slides with SlowFade Light mounting medium (Molecular Probes), coverslipped, and sealed with nail polish.

**Stable transfections.** The SI, villin, and control inserts described above were cut out of the pcA13 vectors and cloned into the backbone vector pEGFP-N1 (containing a neomycin-resistance cassette; Clontech, Palo Alto, CA) in place of the GFP coding sequence at the H1 sites. Cloned inserts were verified by vector sequencing (Tufts University Sequencing Core Facility). Probe sequences were as follows:

- For villin, 5'-GCACAACTCTTCTGTGGCAAATGCCAGTTTT-3' and 5'-AAGGCATATTTAA-3';
- For the control, 5'-GGCTATCTTATTTTGTTTAATTATGCTT-3' and 5'-AAATACCATGCTTTTGCAGACAGACTGTCG-3'.

Monolayer images were scored on a blinded basis by two of the investigators for the presence of the GFP. Control monolayers were transfected with the transfected proxy construct and expressed as the percentage of specifically localized GFP in each region. Statistical analysis was performed by using a two-tailed ANOVA (GraphPad Instat Software, San Diego, CA), and significance was assigned as P < 0.05.

For detection of GFP in stable transfection experiments, horizontal fluorescent images were obtained every 0.5 or 1.0 μm on an Odyssey XL laser scanning confocal microscope (Noran), and the images were recorded with Noran Intervision 2D/3D Image Analysis software. Intracellular location of the GFP marker proteins, monolayers were selected at random when their apical and basal positions were clearly defined, a signal was present, and the control at the same intensity and contrast settings did not display a detectable signal (n = 85). Once the top and bottom positions of the monolayer were established, the monolayer was optically sectioned into 0.5-μm slices on an x-z plane by using the appropriate laser channel for each stain and merging all channels to create the final image. These z-section monolayer images were scored on a blinded basis by two of the investigators for the presence of the GFP. After the scoring was performed, results were correlated with the transfected proxy construct and expressed as the percentage of specifically localized GFP in each region. Statistical analysis was performed by using a two-tailed ANOVA (GraphPad Instat Software, San Diego, CA), and significance was assigned as P < 0.05.

For detection of GFP in stable transfection experiments, horizontal fluorescent images were obtained every 0.5 or 1.0 μm on an Odyssey XL laser scanning confocal microscope (Noran), and the images were recorded with Noran Intervision 2D/3D Image Analysis software. Intracellular location of the GFP was established by determining the apical and basal membranes of randomly selected single cells (n = 33) containing observed GFP and recording the 0.5-μm intervals at which the GFP was visualized. Control monolayers were imaged by using the same contrast and intensity settings to verify that no detectable signal was present. The data were expressed as the percentage of GFP localized in the intracellular compartment of interest. Statistical analysis was con-
ducted by using two-tailed ANOVA (GraphPad Instat Software), and significance was assigned at $P < 0.05$.

For in situ hybridization experiments, transfected construct mRNA was detected by confocal microscopy on the Bio-Rad Zeiss laser scanning confocal microscope as described above. Laser intensity and contrast levels were set by using the negative controls (as described above) to establish background. The image was kept if it met the following selection criteria: a monolayer of intact nuclei, visible microvillus membrane staining, and visible probe. The Bio-Rad .pic file format was converted to a .tiff format, and 47 individual cells that displayed a visible probe were digitally cut out of the image by using PaintShop Pro 7 (Jasc Software, Eden Prairie, MN) and pasted into an unlabeled collage. The intracellular position of probe within each cell in the collage was then assessed by two separate investigators on a blinded basis and scored as follows: 0 = no sorting; 1 = mostly apical; 2 = apical/perinuclear; 3 = mostly perinuclear; 4 = perinuclear/basal; 5 = mostly basal.

Statistical analysis. Statistical significance was determined by using a coded, nonparametric contingency table, with the null hypothesis assigned as equal distribution into each intracellular region (GraphPad Instat Software). Significance was defined as $P < 0.05$.

RESULTS

Morphology of monolayer. Under the conditions used in these experiments, we initially established that Caco-2 cells grown on plastic were not sufficiently or reproducibly polarized to allow assessment of mRNA sorting (data not shown). In contrast, cells grown on fibrillar-collagen inserts demonstrated a polarized appearance including a well-developed microvillus membrane at approximately day 3 postconfluency, as shown also by others (8–10, 19, 23). This morphology persisted at all subsequent time points studied (Fig. 1). When grown on other substrates, collagen I, fibronectin, or laminin, the Caco-2 cells demonstrated distinctly sparser microvilli and a shorter cell height, resulting in a squat, almost squamous appearance, compared with cells grown on the fibrillar-collagen matrix at the same time points. Accordingly, all subsequent experiments were conducted with the fibrillar-collagen-coated inserts.

Transient transfections. It had been shown previously that SI mRNA expression levels in Caco-2 cells peak at approximately day 10 postconfluency, plateau for ~5 days, and then decline thereafter (5, 23). Assuming that the subcellular machinery for sorting mRNAs would be operational at the time of maximum expression, we initially chose 10 days postconfluency as the time point for examining the sorting of SI and villin mRNAs. However, once the Caco-2 cells became differentiated, they proved entirely refractory to transfection by either electroporation or lipid techniques; therefore, it was necessary first to transfect preconfluent cells and then to culture them to the appropriate level of differentiation. By using this approach, optimal expression of GFP after transient transfection by either electroporation (10 μg) or Effectene (2 μg; Qiagen) occurred between 3 and 8 days postconfluence, with some fluorescence remaining after 14 days (data not shown). Because electroporation yielded a greater percentage of cells transfected, this method was chosen as the vehicle of choice for all further transient transfection experiments. Interestingly, in contrast to the ease of detection of transfected mRNA proxies, the abundance of native SI and villin mRNA per cell was lower than the detection limit by using in situ hybridization (data not shown).

Localization of the GFP proxy within the cell was determined by optical sectioning of the cells in 0.5-μm slices from the apical membrane through the basal surface and then scoring masked images to establish the intracellular location of the GFP. The data are shown in Fig. 2. Most of the SI proxy protein (GFP) appeared to sort to the apical portion of the cell in concordance with the in vivo expression ($n = 32$) (2). The villin proxy protein, on the other hand, appeared...
predominantly perinuclear and basal (n = 30). Due to variability in the intracellular localization of the GFP, there was no statistically significant difference in the intracellular location of any of the construct proteins. The control construct protein was found evenly distributed within the cells (n = 23).

**Stable transfections.** In an effort to control for the variation in location of GFP, and to attempt to increase expression, stably transfected Caco-2 cell lines were created for the SI and villin constructs. Cells were imaged as described above from days 3 through 7 postconfluence. No significant difference was observed in the transfected Caco-2 cells cultured on 3D collagen inserts for 3 days postconfluence. Images obtained by using cells at 3 days postconfluence (n = 33 cells) are shown in Fig. 3. These data demonstrate that the predominant sorting of the SI mRNA construct tended to be apical and perinuclear and that the predominant localization of the villin mRNA construct tended to be perinuclear and basal. However, these differences were not statistically significant. No significant differences were noted in GFP sorting when additional time points were compared.

**In situ hybridization.** Because of the variability in the intracellular localization of GFP seen in Figs. 2 and 3, it was possible that the GFP/β-gal fusion protein might not anchor at the final destination of the mRNA as reported by others (13). Accordingly, detection of transfected construct mRNA was accomplished by using transient transfection techniques followed by in situ hybridization to localize the proxy mRNAs within single cells on a reconstructed x-z axis.

In situ hybridizations were conducted by using fluor-conjugated oligonucleotides against the 3'-UTRs of the transiently transfected constructs at days 6–8 postconfluence. Initial experiments were performed with a hybridization temperature of 50°C, in accordance with the protocol established for in situ hybridization of adult intestinal sections in this laboratory (2, 15, 16). Hybridization in cell monolayers, however, proved difficult at this temperature, because the cells were too fragile and morphology was unacceptably compromised. Others (12) have reported successful hybridization of fibroblast cell cultures by using labeled DNA oligonucleotide probes at 37°C (see also http://singerlab.aecom.yu.edu/protocols). This temperature, however, did not provide a sufficient hybridization signal. Accordingly, an intermediate temperature of 42°C was adopted with reproducible results (Fig. 4).

Forty-seven well-oriented cells that displayed a discernable brush-border, basal membrane, and intact nucleus were scored for intracellular mRNA localization by two different investigators. The 47 cells were chosen from 19 different experiments. Analysis of those cells suitable for morphometry showed that 76% of the SI proxy mRNA localized to the top half of the cell (defined as apical or a combination of apical and perinuclear sorting), whereas 49% of the villin proxy mRNA localized to the basal half of the cell (defined as basal or a combination of basal and perinuclear sorting) (P < 0.001) as shown in Fig. 5. The significant localization of the transfected SI construct mRNA contrasts with the lack of significant localization of the GFP protein proxy as shown in Fig. 2.
DISCUSSION

This study demonstrates that Caco-2 cells in culture achieve and maintain sufficient polarity to allow visualization of intracellular events, including differential sorting of transfected mRNAs to specific intracellular locations. The value of this cell line as an in vitro model for native enterocytes has been well established (8–10, 19, 23). Furthermore, the model system described in this study displays distinct sorting patterns for SI and villin mRNA proxy constructs. This provides a means for investigating the control of mRNA sorting and its potential role in development and in the maintenance of intestinal epithelial cell polarity.

Investigations in other cell types have identified similarities in some of the mechanisms of mRNA sorting. Trans-acting, sequence-specific regions in the 3′-UTR of sorted mRNAs bind specific proteins that then complex with motor proteins to form a translocation particle. These particles move along the cytoskeletal apparatus until they reach their final intracellular destination, in which they anchor by an as yet incompletely understood process (17). The cytoskeletal components involved may be either microtubules, microfilaments, or both at alternate points in the sorting process (4). The differential sorting displayed in the current experiments by the SI and villin proxy constructs will facilitate a delineation of the mechanisms responsible for localization of these mRNAs in intestinal epithelial cells.

In the present experiments, the SI proxy mRNA localized apically within the cell, mimicking the pattern of SI mRNA localization seen in native human enterocytes (2, 15). The detection of the 3′-UTR sequence apically in the cells may indicate that the mRNA was anchored at its site of function. The apical localization of this mRNA presumably facilitates translation as well as the insertion of the mature SI enzyme into the apical membrane. It is important to note that the SI construct mRNA was more distinctly localized than that of the SI proxy protein GFP. This was most likely due to intracellular diffusion of the GFP synthesized during posttransfection culture of the Caco-2 cells. Accordingly, GFP cannot be used as a marker for SI localization in this model, unlike the findings of Kislauskis et al. (12) in fibroblasts in which β-gal could be used as a proxy marker for β-actin mRNA in short-term culture. Having established a reliable model system, we have now begun mutational analysis and mapping of the SI 3′-UTR to determine the specific sequences necessary for this localization.

In contrast to native enterocytes, in which villin mRNA is predominantly localized basally (15), in Caco-2 cells the villin proxy mRNA is localized less precisely. There are two possible explanations for this observation. First, there might be altered sorting mechanisms in Caco-2 cells in culture compared with enterocytes in vivo. Bacchi and Gown (1) found that, although villin protein was usually expressed apically in colonic adenocarcinomas, cytoplasmic and basolateral mislocalization was also identified. Because Caco-2 cells derive originally from colon carcinoma, this might contribute to a sorting pattern for the villin mRNA that is distinct from that seen in vivo in small intestine (15). Alternatively, a second possible explanation is that villin may be one of the rare genes whose sequences that govern sorting and/or anchoring of mRNA at the intracellular destination do not lie completely within the 3′-UTR (18). Experiments are currently underway to clone other portions of the mRNA into the villin proxy construct to determine whether this is the case.

An important component of the mRNA sorting system is the trans-acting mRNA binding protein(s) that mediate(s) attachment of the mRNA to the motor assembly (18). These proteins have been identified and cloned for several mRNA species with the use of electrophoretic mobility shift assays. Investigations in other genes have shown that the sequences that govern sorting are often multiparticle (13) and widely spaced within the 3′-UTR, implying that folding and secondary structure of the mRNA is critical in protein binding to these sequences. Further support for this theory comes from the proteins involved in sorting β-actin in fibroblasts and Veg1 mRNA in Xenopus oocytes (21, 22). These proteins display a significant homology to one another but bind to different primary sequences in their respective mRNAs. However, analysis of these mRNAs by computational folding programs predicts a similar stem-loop structure within each of the mRNAs at the sequences shown to be necessary for their localization (3). As a consequence of the present study, it will now be possible to establish which proteins and elements of the cytoskeleton (microtubules and/or microfilaments) are involved in the sorting mechanisms for these intestinal mRNAs and to elucidate the predicted protein intermediates (likely, the cytoskeletal motor proteins), which may also mediate the translocation of these mRNAs.

By extending the current findings, it may also be possible to identify the role of mRNA sorting in the establishment and maintenance of intestinal epithelial cell polarity during differentiation and in diseases that alter enterocyte shape, such as gluten-sensitive enteropathy. The model system we report here is a crucial first step in investigating this important process.

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