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The *Dri 42* Gene, Whose Expression Is Up-regulated during Epithelial Differentiation, Encodes a Novel Endoplasmic Reticulum Resident Transmembrane Protein*

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A search for novel genes that are up-regulated during development and differentiation of the epithelial cells of the intestinal mucosa led us to the isolation of the *Dri 42* cDNA clone (Dri, differentially expressed in rat intestine). The nucleotide sequence of the full-length cDNA has shown that it encodes a 35.5-kDa protein with one consensus sequence for *N*-linked glycosylation and alternating hydrophilic and hydrophobic domains. To determine the intracellular localization of Dri 42 we have raised polyclonal antibodies in hens against a bacterially produced Dri 42-glutathione *S*-transferase fusion protein. Immunofluorescence detection with these antibodies has shown specific staining of the endoplasmic reticulum (ER) in the relatively undifferentiated fetal rat intestinal cell line FRIC B and in sections of rat small intestine. ER membrane localization of Dri 42 was confirmed by laser confocal microscopy of polarized Madin-Darby canine kidney cells overexpressing a Dri 42-chloramphenicol acetyltransferase (CAT) fusion protein by transfection. Pulse labeling experiments on transiently transfected cells demonstrated that the protein does not acquire Golgi modifications up to 4 h after synthesis, thus indicating that Dri 42 is an ER resident protein. The transmembrane disposition of Dri 42 was studied using *in vitro* insertion of Dri 42-CAT fusion proteins into microsomal membranes. The fusion proteins consisted of several different lengths of truncated Dri 42 and a reporter protein, CAT, that was linked in-frame after each hydrophobic segment. We found that hydrophobic segments H1, H3, and H5 had a signal/anchor function, and that membrane insertion of Dri 42 was achieved co-translationally by the action of a series of alternating insertion signals and halt transfer signals, resulting in the exposure of both termini of the protein to the cytosolic side. The functional implications of the structure and localization of Dri 42, whose primary sequence does not share significant homology to any previously described protein, are discussed.

The epithelial cells of the gastrointestinal mucosa undergo constant renewal in the adult small intestine and represent therefore a good model to study the mechanisms of epithelial differentiation (1). The three differentiated cell types that constitute the mature villus epithelium (absorptive enterocytes, goblet cells, and enteroendocrine cells) all arise from a progenitor stem cell, anchored in the crypt compartment, and acquire a differentiated phenotype during migration from crypt to villus tip, where they eventually detach from the underlying mesenchyme and shed into the lumen. Paneth cells are the only cell type of this epithelium which migrates in the opposite direction during differentiation, as they are exclusively located at the crypt base. Commitment to differentiation and loss of proliferation capacity occur at very early stages of the migration process along the crypt-villus axis, before the cells reach the crypt-villus junction. They are accompanied by increasing expression of specific proteins that are necessary to perform the absorptive functions of the mature epithelium. In the enterocytes, which represent the most abundant villus cell type, the major classes of proteins whose expression is turned on during differentiation are digestive enzymes, transport proteins, and structural components of the microvillar cytoskeleton.

Several studies have been conducted in recent years to identify the key factors involved in transcriptional activation of differentiation-specific intestinal genes. Among the transcription factors that have been shown to bind to intestinal gene promoters, some are expressed also in other tissues (members of the hepatic nuclear factor, GATA, retinoic acid receptor, and retinoid X receptor families) (2), while others appear to be intestine-specific (Cdx1 and Cdx2) (3, 4). Molecular analysis of the promoters of cloned intestinal genes has shown that each of them contains distinct combinations of binding sites for different transcription factors, and a clear picture has not emerged yet of the general mechanisms underlying the regulation of intestinal gene transcription (5). One aspect that can be further pursued to provide information on common mechanisms is the isolation of novel genes that are up-regulated during development and differentiation in the small intestine. In our laboratory, differentially expressed genes have been isolated in the past few years from rat intestinal epithelium by subtractive hybridization. Among the cDNA clones that we have identified with this approach, two novel genes have been previously re-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y07783.

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ported (6). Both genes, named *Dri 27*[1] and *Dri 42* (*Dri*, differentially expressed in rat intestine), share similar *in vivo* expression characteristics. In particular, the results of Northern hybridization experiments in developing rat intestine indicate a gradual increase in the steady-state levels of *Dri 42* mRNA between the last week of pregnancy and the second week after birth. During this period, the morphogenetic events that lead to formation of a functional gastrointestinal tract occur in parallel with the process of epithelial cell differentiation. Moreover, expression of the *Dri 42* gene is induced during developmental maturation also in other rat epithelia (liver, kidney, and lung). The geographical distribution of *Dri 42* mRNA in the adult small intestine is highest in the distal jejunum and ileum. *In situ* hybridization experiments on sections of adult rat small intestine have shown that transcription of *Dri 42* mRNA occurs along the entire villus epithelium, as well as in a cluster of positively hybridizing cells at the crypt base, presumably corresponding to the differentiated cell types that are present in this heterogeneous compartment. Therefore, *Dri 42* gene expression appears to be up-regulated not only during development, but also in adult animals, along both the horizontal (duodenum-to-ileum) and the vertical (crypt-to-villus) axes of the intestinal epithelium.

In this paper, we report the molecular analysis of the novel clone *Dri 42* and the intracellular localization and membrane topology of the encoded protein.

MATERIALS AND METHODS

Tissue Preparation and RNA Extraction—The portion of small intestine between the pylorus and the ileocecal valve was dissected from Sprague-Dawley rats, anesthetized by intraperitoneal injection of 20 mg/100 g body weight of Farmotal (Farmitalia-Carlo Erba, Milano, Italy). The dissected tissue was quickly rinsed in cold Hank's buffered saline (Flow Laboratories, Irvine, United Kingdom) and immediately frozen in liquid nitrogen. Total RNA was extracted from frozen pulverized tissues as described by Chirgwin *et al.* (7). Poly(A) RNA was purified by affinity chromatography on oligo(dT)-cellulose, type 3 (Collaborative Research Inc., Bedford, MA).

cDNA Library—The rat intestinal cDNA library used in this study was synthesized as described previously (6), by oligo(dT)-priming of 5 μg of poly(A)-RNA extracted from dissected villi of 21-day old rats. Double-stranded cDNA fragments were linked to adaptors (8) containing *Eco*RI, *Sph*I, and *Bam*HI restriction sites. Adapted cDNA fragments were size fractionated by chromatography on Sephacryl S-1000 (Pharmacia Biotech, Uppsala, Sweeden), and cloned into the *Eco*RI site of the phage vector λZAPII (Stratagene, La Jolla, CA). The library was screened by standard procedures, using radioactively labeled cDNA fragments as hybridization probes. Subcloning of cDNA fragments from positively hybridizing phage plaques was performed by *in vivo* excision of insert-containing pBluescript SK− from plaque-purified, recombinant λ phages, according to the instruction by the manufacturer.

DNA Sequence Determination—The DNA sequence was determined on double stranded templates using the Sanger's method of dideoxy chain termination, as modified by the use of Sequenase (U. S. Biochemical Corp., Cleveland, OH) and ^{35}S-labeled dATP (9).

DNA Constructs—Fusion constructs for *in vitro* translation and for Dri 42 protein bacterial expression were obtained from fragments generated by polymerase chain reaction, using Dri 42-specific primers with unique restriction sites at their 5′ ends. Pwo I DNA polymerase (Boehringer Mannheim Italia, Milan, Italy) was used for amplification as recommended by the manufacturer. Polymerase chain reaction primers had 20 nucleotides of identity with the template, 6 nucleotides encoding restriction sites, and 6 C/G residues at their 5′ end to facilitate restriction digestion. All constructs were tested by sequencing of the fusion junctions. The pGEM-3Z-CAT vector used for the Dri 42-CAT fusion constructs (p42CAT 1–5) was obtained as described in Ref. 10. The p42CAT 1–5 constructs were obtained by subcloning *Dri 42* cDNA fragments between position 410 (the *Dri 42* ATG codon) and positions 623, 750, 1093, 1140, or 1345 (within the *Dri 42* open reading frame) into *Hin*dIII-*Sal*I digested pGEM-3Z-CAT. The pGEM 42 construct was obtained by subcloning the *Sma*I-*Sac*I fragment between positions 385 and 1204 of clone *Dri 42a* into pGEM-3Z (Promega Co., Madison, WI). The pGEX2T-Dri 42 construct, used for expression of the protein in *Escherichia coli*, was obtained by subcloning the *Dri 42* cDNA fragment between positions 568 and 1205 into *Bam*HI-*Sma*I digested pGEX-2T (Pharmacia Biotech). The pcDNA-3 42-CAT construct used for transfection of MDCK cells was obtained by subcloning the *Hin*dIII-*Bam*HI fragment, containing the entire Dri 42 coding region fused to the CAT gene, from p42CAT-5 into *Hin*dIII-*Bam*HI digested pcDNA-3 (Invitrogen BV, Leek, The Netherlands).

In Vitro Transcription and Translation—*In vitro* transcription of linearized templates was carried out using SP6 RNA polymerase and reagents from the Promega *in vitro* transcription system. The pGEM-42 vector was linearized using *Fsp*I while all the p42CAT fusion constructs were linearized using *Sma*I. *In vitro* translation was carried out using a rabbit reticulocyte lysate system (Promega Co.) in the presence of ^{35}S-labeled methionine (1000 Ci/mmol, DuPont NEN, Milan, Italy). The reaction mixture was incubated for 90 min in the presence or absence of canine pancreas microsomal membranes (Promega Co.). Processing for SDS-PAGE of translation products obtained from *Dri 42* templates (Fig. 2), as well as Endo H treatment of products translated in the presence of microsomes were carried out as described in Ref. 10. p42CAT 1–5 translation products (Fig. 3*B*) were immunoprecipitated before SDS-PAGE as described by Anderson and Blobel (11), using unconjugated rabbit antibody to chloramphenicol acetyltransferase (anti-CAT, 5′-3′ Prime Inc., Boulder, CO). For post-translational assay (Fig. 4*B*), *in vitro* translation of p42CAT-4 was carried out with the addition of microsomal membranes to the reaction mixture 60 min after initiation of translation, together with 1 mM puromycin to prevent further translation. Samples were then diluted three times with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, incubated in the presence of 3 mM tetracaine for 10 min at room temperature, and then digested with 50 μg/ml trypsin and chymotrypsin for 30 min at room temperature. The reaction was stopped by addition of protease inhibitors (10 μg/ml Trasylol, 3 mM phenylmethylsulfonyl fluoride, and 150 μg/ml soybean trypsin inhibitor) and incubated on ice for 10 min.

Production of Antibodies—The *Dri 42* cDNA fragment encoding amino acids 54–265 was subcloned in the bacterial expression plasmid pGEX-2T (Pharmacia Biotech), in-frame with the glutathione *S*-transferase coding region. The resulting 51-kDa fusion protein was produced in *E. coli* cells at a maximum level after 2 h of isopropyl-1-thio-β-D-galactopyranoside induction, followed by rapid degradation. Due to the presence of 11 cysteine residues in the primary sequence, the GST-Dri 42 fusion protein was found to precipitate in the inclusion bodies (12). To raise polyclonal antibodies against Dri 42, 100 μg of inclusion bodies, prepared as described in Ref. 13, were suspended in 1 × phosphate-buffered saline and injected into the breast muscle of 20-week-old hens (14). Four booster injections of the same suspension were given at 1, 2, 3, and 4 weeks after the first injection. At the end of the immunization period, chicken IgY were purified from egg yolks by polyethylene glycol precipitation (15). The antibody solution was further cleaned by affinity chromatography on Sepharose 4B-immobilized *E. coli* protein extracts (13).

Cell Cultures—FRIC B lines (fetal rat intestinal cells) were grown as described previously (16). MDCK cells (17) were grown as described in Ref. 18.

Immunofluorescence—For immunocytochemical localization the cells were fixed in 2% paraformaldehyde in PBS+ (1 × phosphate-buffered saline containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$) for 30 min at room temperature and treated by conventional immunofluorescent techniques. For intracellular labeling, cells were permeabilized with 0.05% saponin in PBS+. For tissue immunofluorescence, portions of proximal duodenum, jejunum, and distal ileum from 21-day-old rats were dissected and longitudinal sections were prepared as described in Ref. 19. The following antibodies were used: primary antibodies: chicken polyclonal anti-Dri 42 (this paper); rabbit polyclonal anti-CAT (5′-3′ Prime Inc.); rabbit polyclonal anti-calnexin C (StressGen Biotechnologies Co., Victoria, British Columbia, Canada). Secondary antibodies used were tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-chicken IgG (Jackson Labs, West Grove, PA) and TRITC-labeled anti-rabbit IgG (Cappel, Organon Teknika Co., Durham, NC).

Transfection of MDCK Cells—MDCK cells were transfected either with calcium phosphate, as described in Ref. 20, or with Lipofectamine (Life Technologies, Inc. Italia, Milan, Italy) as described by the manu-

[1] The abbreviations used are: Dri, differentially expressed in rat intestine; CAT, chloramphenicol acetyltransferase; Cdx, caudal-type homeobox; ER, endoplasmic reticulum; FRIC, fetal rat intestinal cells; GST, glutathione *S*-transferase; MDCK, Madine-Darby canine kidney; PAGE, polyacrylamide gel electrophoresis; TRITC, tetramethylrhodamine isothiocyanate.

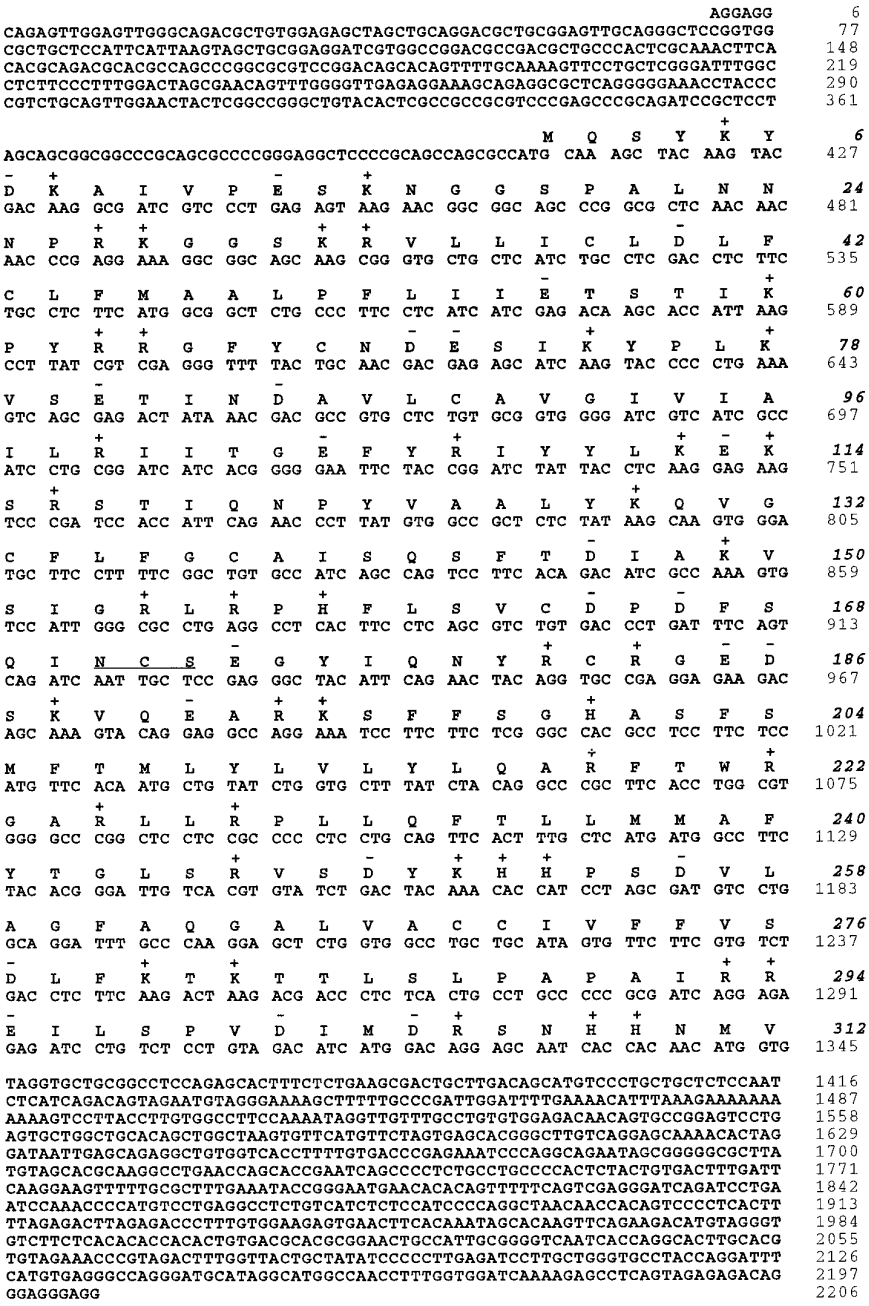

FIG. 1. **Nucleotide sequence and the deduced amino acid sequence of *Dri 42* cDNA.** The complete cDNA sequence was obtained from the two overlapping clones *Dri 42a* (nucleotides 1–1453) and *Dri 42b* (nucleotides 332-2206). + and − superscripts indicate charged amino acids; the consensus *N*-linked glycosylation site (*NCS*) is *underlined*. The hydrophobic segments (H1-H6) are located between residues 34–58 (H1), 82–103 (H2), 130–145 (H3), 201–217 (H4), 230–245 (H5), and 257–276 (H6). *Plain* numbers represent nucleotide sequence, *boldface* refers to amino acid sequence. The sequence has been submitted to the EMBL, GenBank, and DDBJ Nucleotide Sequence data bases, where it is listed under the accession number Y07783.

facturer. Metabolic labeling of transfected cells was carried out 48 h after transfection, essentially as described in Ref. 21. Immunofluorescent cells were viewed with a Leitz Diaplan microscope and with a Sarastro 2000 confocal microscope. Computer aided reconstruction of confocal images (Sylicon Graphics) was performed using the Image Space software (Molecular Dynamics).

RESULTS

Isolation and Molecular Analysis of Full-length Dri 42 cDNA Clones—The *Dri 42* cDNA fragment originally isolated by subtractive hybridization was 300 base pairs long and did not contain an open reading frame (6). To isolate a full-length clone we constructed a rat intestinal cDNA library, enriched in epithelial-specific transcripts, using poly(A)$^+$ RNA extracted from dissected intestinal villi (see "Materials and Methods"). The 300-base pair fragment was used as hybridization probe to isolate larger size clones from this library. Screening of 2×10^5 recombinant phage plaques resulted in the isolation of two *Dri 42* clones (*42a* and *42b*), containing inserts of approximately 1.5 and 1.9 kilobases, respectively. Determination of the nucleotide sequence of the two clones showed that they overlapped in the central portion, containing a complete open reading frame, but differed in the lengths of the 5′- and 3′-untranslated regions, as indicated in the legend to Fig. 1. The 3′ end of clone *42a* lies within a stretch of 11 adenine residues at position 1481 in the 3′-untranslated region. This internal poly(A) stretch probably functioned as annealing site for the oligo(dT)$_{18}$ that was used to prime reverse transcriptase during first strand cDNA synthesis. Both clones were sequenced over their entire length and in the region encompassing the open reading frame the DNA sequence was determined on both strands. The complete nucleotide sequence of the *Dri 42* cDNA and the deduced primary sequence of the encoded protein are shown in Fig. 1. The Dri 42 primary sequence consists of 312-amino acid residues (corresponding to a molecular mass of 35.5 kDa) and contains a consensus *N*-linked glycosylation site at position 171. The hydropathy analysis of this sequence (Fig. 3*A*), based on Kyte and Doolittle algorithms (22), revealed that Dri 42 contains six

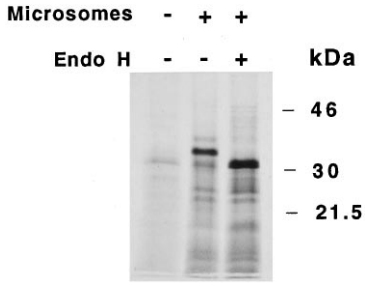

FIG. 2. **Dri 42 is a glycoprotein.** Transcripts obtained *in vitro* from the pGEM42 construct were translated in a rabbit reticulocyte lysate in the presence or absence of canine pancreas microsomal membranes. The products carried out in the presence of microsomes were divided into two aliquots, one of which was treated with Endo H. Both aliquots, as well as the translation products obtained in the absence of microsomes, were analyzed in SDS-PAGE.

hydrophobic segments (H1-H6), which are located between residues 34–58 (H1), 82–103 (H2), 130–145 (H3), 201–217 (H4), 230–245 (H5), and 257–276 (H6). This structure is indicative of a polytopic membrane protein without a cleavable signal sequence.

Dri 42 Is a Glycoprotein—Since the primary structure of Dri 42 contained a consensus sequence for *N*-linked glycosylation, we examined whether the site was indeed glycosylated, using *in vitro* translation of construct pGEM-42 (see "Materials and Methods") in the presence of dog pancreas microsomes. The primary translation products of the mRNA transcribed from this plasmid contained a major band of about 32 kDa (Fig. 2, *lane 1*), which is the expected size of the peptide encoded by this construct. When the mRNA was translated in the presence of dog pancreas microsomes, an additional band with a molecular mass of 35 kDa was obtained (*lane 2*). This band shifted to 32 kDa by Endo H digestion (*lane 3*), which corresponds to the size of the primary translation product, indicating that membrane insertion of Dri 42 was initiated by an internal insertion signal sequence, and that the downstream peptide containing the consensus sequence for *N*-linked glycosylation was translocated through the microsomal membranes into the lumen. As three hydrophobic segments (H1-H3) are present before the consensus *N*-linked glycosylation site, we can predict four different possibilities for glycosylation to occur, depending on which of the three hydrophobic segments has topogenic function. 1) If H2 and H3 lack topogenic function, insertion of Dri 42 is initiated by H1 and the downstream peptide is translocated through the membrane into the lumen. 2) If H1 and H3 lack topogenic function, the insertion and translocation processes are initiated by H2. 3) If H1 and H2 lack topogenic function, insertion of Dri 42 and translocation of the downstream peptide are initiated by H3. 4) If all three hydrophobic segments have topogenic function, insertion of Dri 42 is initiated by H1 and the downstream translocation is halted by H2. A second cycle of insertion is initiated by H3 and the downstream peptide is translocated into the lumen. Whichever is the case, the results clearly indicate that Dri 42 is a glycoprotein.

Dri 42 Is a Polytopic Membrane Protein—The observations derived from hydropathy analysis (Fig. 3*A*) and from the results of *in vitro* translation of Dri 42 (Fig. 2) strongly suggested that Dri 42 was a polytopic membrane protein. To examine the membrane topology of Dri 42, we constructed fusion proteins consisting of five truncated amino-terminal segments of Dri 42, linked in-frame at amino acid residues 72 (p42CAT-1), 114 (p42CAT-2), 228 (p42CAT-3), 244 (p42CAT-4), and 312 (p42CAT-5) to CAT, a reporter protein that contains a consensus sequence for *N*-linked glycosylation (23). In these fusion proteins the CAT reporter was connected after each of the Dri

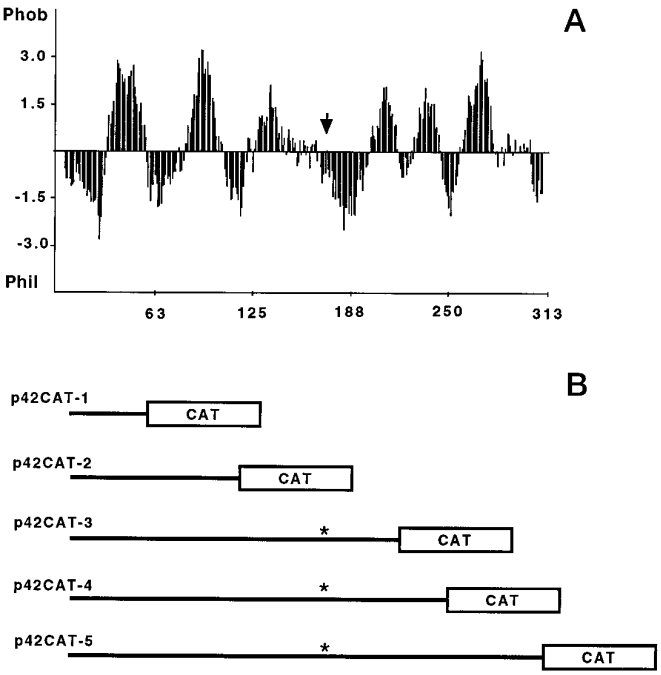

FIG. 3. **Schematic view of the Dri 42-CAT fusion constructs used for *in vitro* translation.** *A,* hydropaticity profile of the Dri 42 protein predicted by the Kyte and Doolittle algorithms (22). *Phob* and *Phil* indicate hydrophobicity and hydrophilicity, respectively. *Numbers* in the bottom refer to amino acid residues from the amino terminus. An *arrow* indicates the position of the consensus *N*-linked glycosylation site. *B,* Dri 42-CAT fusion constructs: a *horizontal line* before each *rectangle* indicates the Dri 42 coding region present in the construct, upstream of the CAT. Position of the left corner of each rectangle corresponds to the ligation site between the truncated Dri 42 and CAT. An *asterisk* indicates the position of the consensus *N*-linked glycosylation site.

42 hydrophobic segments, with the exception of segment H3 that occurs immediately upstream of the endogenous *N*-linked glycosylation site, so that the topogenic properties of hydrophobic segments can be examined sequentially in their native context. Signal/anchor and halt transfer sequences are identified by characterization of the glycosylated products after *in vitro* translation, as the COOH-terminal CAT moiety can be glycosylated only if the hydrophobic segment immediately preceeding the *N*-linked glycosylation site leads it to face the inner portion of microsomes. Therefore, the occurrence of *N*-linked glycosylation is used as a marker for luminal disposition of the COOH-terminal flanking region.

The primary translation products of p42CAT-1, -2, -3, -4, and -5 transcripts (referred to from now on as CAT 1–5) show a major band with molecular mass of approximately 35, 39, 51, 53, and 60 kDa, respectively (Fig. 4*A, lanes 1, 4, 7, 10,* and *13*). When the transcripts were translated in the presence of dog pancreas microsomes, an additional band was obtained with CAT-1, -3, -4, and -5 (*lanes 2, 8, 11,* and *14*), but not with CAT-2 (*lane 5*). The molecular mass of the additional band was larger than the respective primary translation product by approximately 3 kDa (for CAT-1, -3, and -5) or 6 kDa (for CAT-4). Endo H treatment shifted their molecular mass to those of their primary translation products (*lanes 3, 9, 12*, and *15*). As the Dri 42 fragment shorter than 170 amino acid residues does not contain any consensus *N*-linked glycosylation site, the increase in molecular mass of CAT-1 was due to glycosylation of the CAT moiety. Therefore, CAT-1 translocation was initiated by segment H1, which functioned as a signal/anchor, and its downstream peptide was translocated into the microsomal lumen, where the reporter protein was glycosylated (Fig. 5*A*). On

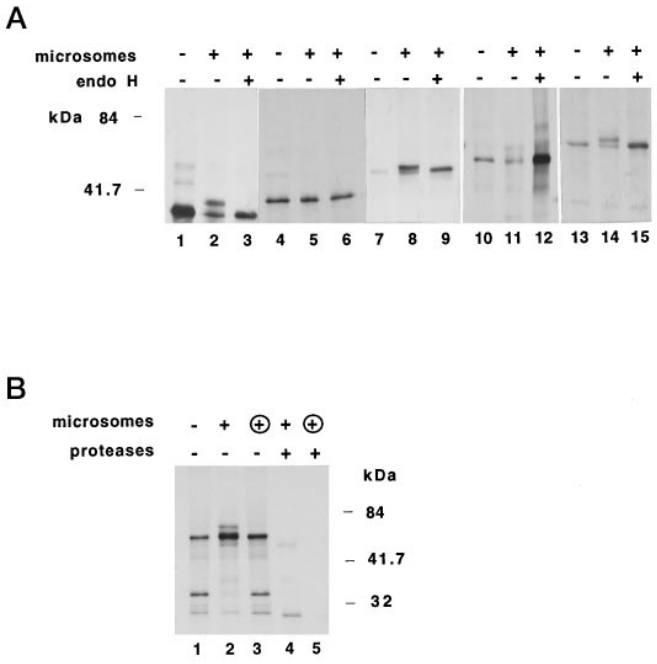

FIG. 4. **Co-translational insertion of Dri 42 into microsomal membranes by the action of alternating signal/anchor and halt transfer signals.** *A,* transcripts obtained from the constructs in Fig. 3 were translated *in vitro* in the absence (*lanes 1, 4, 7, 10,* and *13*) or presence (*lanes 2, 5, 8, 11,* and *14*) of microsomal membranes, and the translation products were immunoprecipitated with anti-CAT antibody. One aliquot of the immunoprecipitates from translation mixtures containing microsomal membranes was treated with Endo H (*lanes 3, 6, 9, 12,* and *15*). *Lanes 1–3*, p42CAT-1; *lanes 4–6*, p42CAT-2; *lanes 7–9*, p42CAT-3; *lanes 10–12*, p42CAT-4; *lanes 13–15*, p42CAT-5. *B*, transcripts obtained from the p42CAT-4 construct were translated in the absence or presence of pancreas microsomes. The products obtained in the absence of microsomes were divided into three aliquots. One aliquot was used as control for post-translation incubation (*lane 1*), the remaining two were further incubated in the presence of microsomal membranes and puromycin (*lane 3*). One aliquot (*lane 5*) was then digested with proteases. Protease digestion was also carried out on one aliquot of the translation products obtained in the presence of microsomal membranes (*lane 4*). All samples were subjected to immunoprecipitation, followed by SDS-PAGE fractionation.

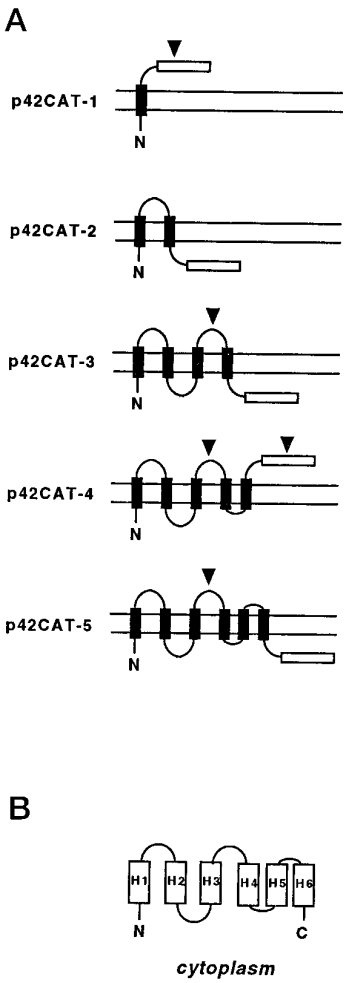

FIG. 5. **Membrane topology of Dri 42.** *A*, schematic drawing of the orientation of the Dri 42-CAT fusion proteins shown in Fig. 4*A*. *Closed rectangles* represent membrane spanning regions. *Open rectangles* represent the reporter protein CAT. *Arrows* indicate the positions where consensus *N*-linked glycosylation sites were glycosylated. The luminal and cytoplasmic sides of microsomes are above and under the two parallel lines, respectively. *N* represents the amino terminus. *B,* predicted membrane topology of Dri 42. *Open rectangles* labeled H1-H6 represent the disposition of the six hydrophobic segments within microsomal membranes. *N* and *C* indicate the amino and carboxyl termini, respectively.

the contrary, CAT-2 was not glycosylated, although it contains segment H1, indicating that segment H2 halted translocation of its downstream peptide. Therefore, the reporter protein remained unglycosylated in the cytosol. These results indicate that glycosylation of the consensus *N*-linked glycosylation site in Dri 42, shown in Fig. 2, occurred by the fourth process of those previously hypothesized: H3 has a signal/anchor function and initiates the second cycle of translocation of Dri 42. As the truncated portions of Dri 42 longer than 175 amino acid residues (CAT-3, -4, and -5) contained the endogenous *N*-linked glycosylation site, the 3-kDa increase in molecular mass that occurs in CAT-3 and CAT-5 was due to single glycosylation at this site, while the 6-kDa increase observed in CAT-4 resulted from double glycosylation of both Dri 42 and reporter protein (Fig. 4*A, lane 11*). Therefore, the results in *lanes 7–15* indicated that the second cycle of CAT-3 translocation was initiated by segment H3, and that segment H4 acts as a second halt signal. In CAT-4, the third cycle of translocation was initiated by segment H5 and its downstream peptide was translocated into the microsomal lumen, where the reporter protein was glycosylated. Thus two *N*-linked glycosylations occur in this product. However, in CAT-5 the third cycle of CAT translocation was terminated by segment H6, functioning as third halt signal, and its downstream reporter protein remained unglycosylated in the cytosol. It should be stressed here that the results shown in *lanes 8, 11,* and *14* display only one glycosylated form (with a molecular mass higher by either 3 or 6 kDa than that of their respective non-glycosylated forms), indicating that all Dri 42 peptides whose translocation was initiated by H3 were then halted by H4. Similarly, translocation of all the downstream portions of the halted peptides was initiated again by H5 and then halted by H6. Thus, each of the hydrophobic segments in the Dri 42 sequence showed strong topogenic function. These results on the transmembrane disposition of Dri 42-CAT fusion proteins are schematically summarized in Fig. 5*A*, and the resulting model for transmembrane disposition of this polytopic protein, which includes the results shown in Fig. 2, is depicted in Fig. 5*B*.

The results of *in vitro* translation of p42CAT fusion constructs strongly suggested that Dri 42 was co-translationally inserted into microsomal membranes. To confirm the phenomenon, the transcript of p42CAT-4 was translated in a reticulocyte lysate system for 60 min, dog pancreas microsomes were added at the end of translation and the mixture was incubated for additional 30 min in the presence of translational inhibitors (Fig. 4*B, lane 3*). As a control, the same transcript was trans-

lated in the presence of microsomes from the beginning of the incubation period (*lane 2*). One aliquot of both samples was then subjected to protease digestion before immunoprecipitation (*lanes 4* and *5*). Since the reporter protein in CAT-4 is translocated into the microsomal lumen, if the translocation process occurs exclusively by a co-translational mechanism, it should not be glycosylated nor protected by digestion with proteases when microsomal membranes are added post-translationally. The results in Fig. 4*B*, demonstrate that this is indeed the case: the translation products carried out in the presence of microsomes (*lane 2*) displayed the additional glycosylated band. When these products were digested by proteases before immunoprecipitation with anti-CAT antibody, the two major bands disappeared, yielding a fragment with a molecular mass of approximately 27 kDa which corresponds to the molecular mass of the CAT moiety linked to the Dri 42 hydrophobic segment H5 (*lane 4*). The fragment has a similar molecular mass than the faster migrating band present in *lanes 1* and *3*, which was not detected after proteolysis (*lane 5*). Therefore, the band in *lane 4* was considered to represent truly a membrane protected fragment. The translation products incubated with post-translationally added microsomes contained a major band with the same molecular mass as the primary translation products (*lanes 1* and *3*), which was completely digested by proteases into fragments that did not immunoprecipitate with anti-CAT antibody (*lane 5*). Overall, these results show that only addition of microsomal membranes during the translation reaction protects the carboxyl-terminal CAT moiety from protease attack, thus demonstrating co-translational membrane insertion of Dri 42.

Dri 42 Is Localized in the Endoplasmic Reticulum—Having determined that Dri 42 is a transmembrane protein, its intracellular localization was examined immunochemically and biochemically. For the first approach, polyclonal antibodies were raised in hens against a fusion protein consisting of amino acids 54–265, linked in-frame to bacterial glutathione *S*-transferase, which was encoded by plasmid pGEX2T-Dri 42 (described under "Materials and Methods"). Purified anti-Dri 42 antibodies recognized a specific band by Western blotting of *E. coli* protein extracts, corresponding to the GST-Dri 42 fusion protein (results not shown). These antibodies were used for immunochemical localization of Dri 42 in tissue culture cells and tissue sections. The results of immunofluorescent staining with the anti-Dri 42 antibody on cryostat sections of adult rat small intestine are shown in Fig. 6, *A* and *B*. Fluorescent staining was detected predominantly in the intracellular portion of both villus (*panel A*) and crypt (*panel B*) epithelial cells. No staining could be detected on the basolateral domain of the plasma membrane, although the apical brush border showed a diffuse positive signal. Since the mucous layer that protects the brush-border membrane of the enterocytes from chemical attacks may cause artifacts due to nonspecific adhesion of the antibodies, we chose to examine the localization of Dri 42 in the fetal rat intestinal cell line FRIC B (16). The results, reported in Fig. 6*C*, showed that the anti-Dri 42 antibody exclusively stained intracellular structures, which appeared as complicated fine networks that were more concentrated in the perinuclear region and diverged to the peripheral region. This pattern closely resembles a typical staining image of ER networks obtained by using antibodies against ER marker proteins, such as calnexin (24).

To confirm the above results in a polarized epithelial cell line, MDCK cells that had been transiently transfected with a Dri 42-CAT fusion protein (construct pcDNA3–42CAT, described under "Materials and Methods") were examined by confocal laser scanning microscopy, using a specific anti-CAT

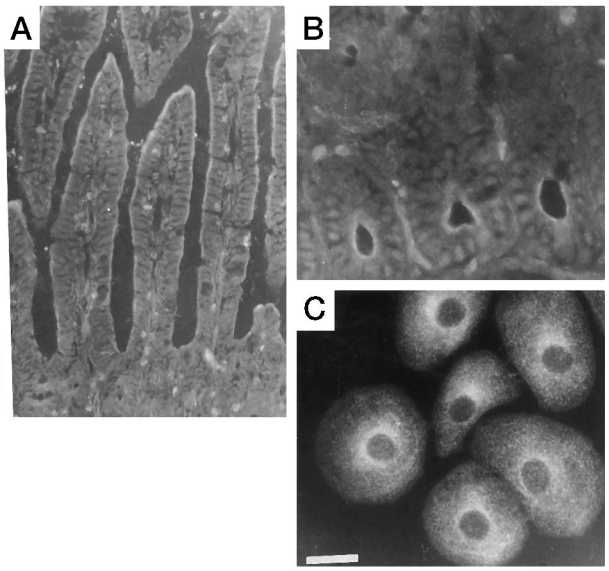

FIG. 6. **Immunofluorescent detection of Dri 42 in intestinal tissue and cultured cells.** *A,* transverse section of jejunum from a 21-day-old rat. *B*, higher magnitude view of the crypt region. *C,* permeabilized FRIC B cells. All samples were stained with anti-Dri 42 primary antibodies and rhodaminated anti-chicken IgG secondary antibodies. *Scale bars*, 55 μm (*A*), 35 μm (*B*), 20 μm (*C*).

antibody. Polarized intestinal cell lines (Caco 2) could not be used for this experiment because they differentiate after 2 weeks in culture, and therefore cannot be transiently transfected in the differentiated state. On the other hand, MDCK cells, derived from kidney epithelium, are a widely used *in vitro* model for the study of differentiated traits, since they acquire a polarized phenotype as they reach confluence (17). The results of computer-aided reconstruction of 50 confocal images, acquired at 0.2-μm intervals (Fig. 7*A*) showed intense staining of an intracellular compartment which was very similar to the ER networks visualized by a control anti-calnexin antibody on the same cells (Fig. 7*C*). Furthermore, a transverse section shows no staining of the plasma membrane with the anti-Dri 42 antibody (Fig. 7*B*), but rather a distribution of fluorescent staining above and around the nucleus. Again, this distribution corresponds to the localization of the ER in a polarized epithelial cell, as shown by staining with the anti-calnexin antibody (Fig. 7*D*).

ER localization of Dri 42 was also examined biochemically, taking advantage of the intrinsic nature of *N*-linked glycoproteins, which are sensitive to Endo H digestion as long as they remain in the ER, while they become resistant once they are transferred to the Golgi apparatus where their *N*-linked sugar moieties are further modified (25). MDCK cells that had been transiently transfected with pcDNA3–42CAT were metabolically labeled with [^{35}S]methionine/cysteine for 10 min, followed by 0, 1, 2, and 4 h chase in the presence of an excess amount of unlabeled methionine/cysteine. In this fusion protein the entire coding region of Dri 42 is present (see "Materials and Methods"), but only the endogenous Dri 42 *N*-linked glycosylation site, not that in the reporter protein, is glycosylated *in vitro* (Fig. 4*A, lane 14*). The CAT moiety was used only as the antigenic site for immunoprecipitation with anti-CAT antibodies, since the anti-Dri 42 antibodies could not immunoprecipitate satisfactorily. Immunoprecipitated proteins from cell lysates were split into two aliquots, one of which was digested by Endo H, while the other was left as control. The results in Fig. 8 demonstrated that, even after 4 h of chase, Dri 42 remained sensitive to Endo H treatment. Immunoprecipitates from the culture medium of transfected cells were also analyzed in the

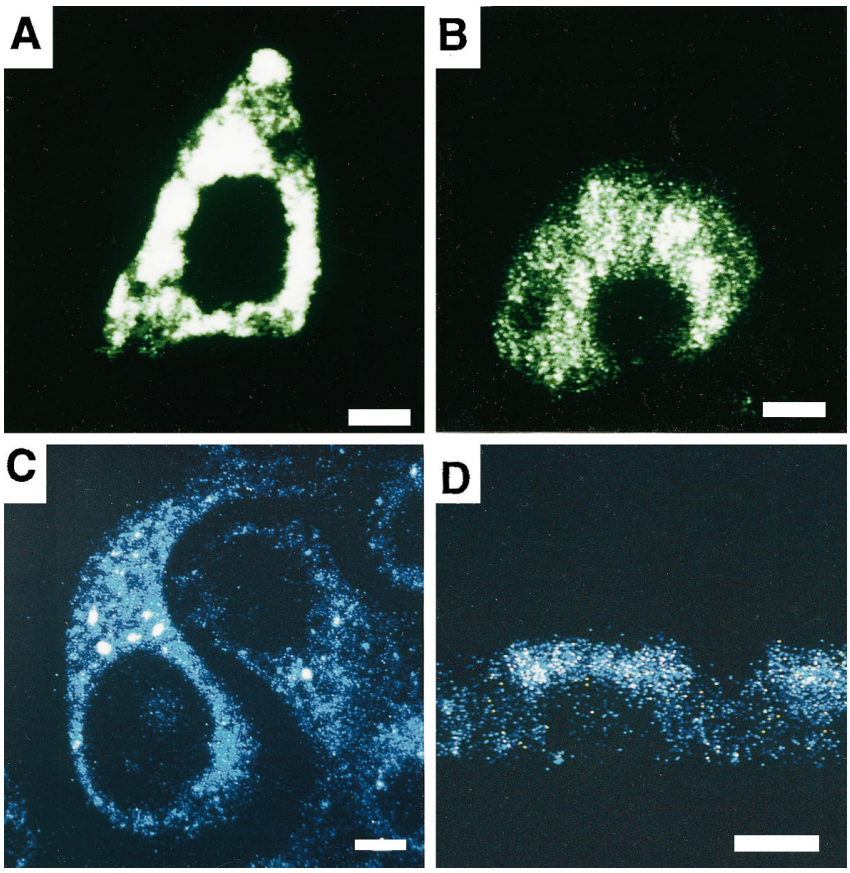

FIG. 7. **Immunolocalization of Dri 42 in polarized epithelial cells by confocal laser scanning microscopy.** MDCK cells, transiently transfected with pcDNA-3/42-CAT were stained 48 h after transfection with either anti-CAT (*A* and *B*) or with anti-calnexin antibodies (*C* and *D*), followed by fluorescein-conjugated anti-rabbit IgG. *A*, three-dimensional reconstruction of 50 confocal images, acquired at 0.2-μm intervals. *B*, transverse section across the ER region and the nucleus. Laser scanning was acquired on a plane orthogonal to that shown in *A*, tangential to one side of the cell nucleus. *C*, three-dimensional reconstruction of 12 confocal images, acquired at 0.1-μm intervals. *D*, transverse section of the cell in *panel C*. Increasing fluorescence intensity from green to white (*A* and *B*) and from blue to white (*C* and *D*). *Scale bars*, 5 μm.

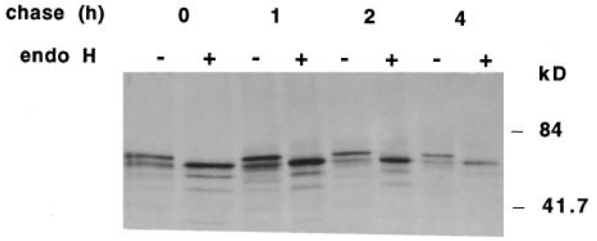

FIG. 8. **The *N*-linked sugars of Dri 42 do not acquire Golgi modifications.** 48 h after transfection with pcDNA-3/42-CAT, MDCK cells were pulse-labeled with [^{35}S]methionine for 10 min and chased in the presence of unlabeled methionine for 0, 1, 2, and 4 h. At the end of the chase period the cells were lysed. Immunoprecipitates with anti-CAT antibody from each cell lysate were divided into two aliquots, one of which was treated with Endo H. All samples were analyzed by SDS-PAGE.

same way and confirmed that no secretion of Dri 42 occurred during the chase period (results not shown). These results strongly support the immunocytochemical observation that Dri 42 is an ER resident protein. In addition, as an increase in the molecular mass of 3 kDa due to a single *N*-linked glycosylation was observed, this result confirmed that membrane disposition of hydrophobic segment H6, observed in the *in vitro* translation of p42CAT-5 (Fig. 4*A, lanes 13–15*) was the same also in intact cells.

DISCUSSION

In this paper we have presented the following findings: (*a*) the primary structure of the novel protein Dri 42, deduced from the nucleotide sequence of the full-length cDNA, consists of 312 amino acid residues and one consensus *N*-linked glycosylation site at position 171; (*b*) the *N*-linked glycosylation site was confirmed to be glycosylated by *in vitro* translation of the Dri 42 transcript in a reticulocyte cell-free system, in the presence of dog pancreas microsomes and followed by Endo H treatment; (*c*) Dri 42 is a polytopic membrane protein, whose membrane insertion is co-translationally initiated by the first hydrophobic segment (H1), while the following hydrophobic segments (H2-H6) are inserted by the action of alternating halt transfer and insertion signals, resulting in the cytosolic location of both NH_2 and COOH termini; (*d*) immunofluorescence microscopy, and a biochemical method that utilizes sensitivity of *N*-linked glycoproteins to Endo H treatment, have shown that Dri 42 is exclusively localized in the ER membrane.

Topogenic Properties of Hydrophobic Segments in Dri 42—In vitro membrane insertion experiments (Fig. 8) have shown that all six hydrophobic segments of Dri 42 function either as signal/anchor (H1, H3, and H5) or as halt transfer signals (H2, H4, and H6). As previously demonstrated with membrane insertion of Na,K-ATPase α subunit (10), the results of such *in vitro* assays reflect the *in vivo* membrane topology, since these properties have been determined in the native context. Further support in this direction comes from the results obtained with the fusion protein 42 CAT-5, which showed the same membrane disposition determined *in vitro* when expressed in transiently transfected MDCK cells (see Fig. 4*A, lanes 13–15*, and Fig. 8). All six Dri 42 hydrophobic segments detected by hydropathy analysis had topogenic function, although some of them had very low average hydrophobicity indices. The average hydrophobicity index, calculated according to Kyte and Doolittle (22), is generally considered to be a primary concern for the topogenicity determinant. These values for Dri 42 hydrophobic segments were 2.11 (Hl), 2.25 (H2), 0.85 (H3), 1.29 (H4), 1.49 (H5), and 2.22 (H6). Although the values for H3, H4, and H5 were relatively low, these segments definitely functioned as either signal/anchor or halt transfer sequences, indicating that there must be another determinant within the hydrophobic segment. These results raised the interesting question of which are the major determinant(s) for the topoge-

nicity of such segments in Dri 42. Several investigators have recently reported that in a polytopic membrane protein not all hydrophobic segments detected by hydropathy analysis have topogenic function. Examples of such proteins are: CHIP 28 water channel protein (26), human protein P-glycoprotein (27), H,K-ATPase α subunit (28), and Na,K-ATPase α subunit (29). Among the hydrophobic segments of these proteins with average hydrophobicity indices lower than 2.0, some did have topogenic function. Further analysis of these segments led to an interesting observation: when the largest average index of 12 consecutive amino acid residues within a hydrophobic segment (defined as a core hydrophobicity index) is larger than 2, the segment shows topogenic function even if the average hydrophobicity index is lower than 2. The core hydrophobicity indices of Dri 42 hydrophobic segments H3, H4, and H5 were calculated to be 1.65 (H3), 1.43 (H4), and 1.83 (H5). These values are still too low to support their topogenic function. Therefore, there must be strong factors other than hydrophobicity involved in topogenic determination. Hartmann *et al.* (30) and von Heijne (31) reported that the distribution of charged amino acids of both NH_2- and COOH-terminal flanking regions is an important factor for the topogenic determinant. Hartmann *et al.* (30) found a striking correlation between membrane topology of the first signal/anchor sequence and the charge difference of the 15 COOH-terminal and NH_2-terminal flanking amino acids. The difference was calculated using the formula: $\delta = C - N$, where δ represents the charge difference, while C and N represent the sum of the assigned values of charged amino acid residues present in the COOH-terminal and NH_2-terminal 15 flanking residues, respectively. lf δ is larger than 0, the COOH terminus of the hydrophobic segment is found on the luminal side, while if the value is negative, the NH_2 terminus of the segment is found on the cytosolic side. Using their formula, we obtained the following values for the charge difference of Dri 42 hydrophobic segments: −3 (H1), +3 (H2), −0.5 (H3), +2.5 (H4), −3 (H5), and +0.5 (H6). Therefore, in our case the calculated orientation of each hydrophobic segment into the lipid bilayer perfectly matches the orientation determined by *in vitro* membrane insertion experiments (Fig. 8*A*). For this reason we conclude that the topogenic properties of the six hydrophobic segments in Dri 42 is determined by both hydrophobicity and charge difference between NH_2-terminal and COOH-terminal 15 flanking residues. Dri 42 appears to represent an exceptional case, because the charge effect on the orientation of hydrophobic segments in a polytopic membrane protein is applicable in most cases only to the first one that contains a signal/anchor sequence (28–30).

A Novel Mechanism May be Involved in the ER Retention of Dri 42—Biochemical and immunofluorescence studies clearly demonstrated ER localization of Dri 42 (Figs. 4–6). The primary sequence, however, does not contain any of the COOH-terminal consensus sequences for ER retention of membrane proteins, such as *X*KK*XX*, K*X*K*XX*, and *XX*KK*X* (25). However, Dri 42 contains two lysine residues at the extreme NH_2 terminus, within the sequence MQSYKYDK. Jackson *et al.* (25) have demonstrated that COOH-terminally located double lysine residues function as ER retention signal in HeLa cells. It remains to be established whether two amino-terminal lysines can also function as ER retention signal. If this case proves to be true for Dri 42, an alternative interpretation of ER retention signals could be provided, as two lysines within 8 residues from either terminus could play an important role in the ER retention of membrane proteins. Alternatively, if the double lysine signal functions only at the carboxyl terminus, the most likely interpretation of our results is that Dri 42 remains in the ER membrane by a new mechanism.

Functional Implications of the Dri 42 Unique Sequence—We have reported in this paper the molecular and biochemical analysis of the novel protein Dri 42, whose expression was previously shown to increase during epithelial differentiation in rat small intestine as well as in kidney, liver, and lung (6). *Dri 42* steady-state mRNA levels were found to increase also during *in vitro* differentiation of human adenocarcinoma cells Caco 2, while no difference was observed in the mouse myogenic C2 cell line, before and after myotube differentiation (data not shown). Therefore, although the *Dri 42* gene is expressed ubiquitously, differentiation-dependent regulation of its expression appears to be specific for epithelial tissues. This observation led us to analyze further the Dri 42 gene product and its possible role in differentiating epithelia. Isolation and sequence analysis of a full-length cDNA clone encoding rat Dri 42 allowed to deduce the primary sequence of this protein, which does not share relevant sequence homology with previously identified proteins of known function. The only significant homology that was found in the EMBL and GenBank sequence data bases is with a recently isolated protein of yet unknown function (HIC-53), which is induced transcriptionally by hydrogen peroxide treatment of *ras*-transformed mouse osteoblasts (32). Although the cloned portion of HIC-53 is incomplete at the amino terminus, the two proteins share 48% homology over a stretch of 211 amino acid residues (33–244 in the Dri 42 sequence), encompassing the first five hydrophobic segments of Dri 42. We have determined that the Dri 42 protein contains six transmembrane regions and both the amino and carboxyl termini on the cytoplasmic side of the membrane. Such structure, together with the molecular mass and the membrane topology of Dri 42, is similar to that of aquaporins, the recently described water channel proteins of the plasma membrane (reviewed in Ref. 33). On the basis of sequence homology, Dri 42 and HIC 53 may therefore represent a novel class of channel proteins. Further support in this direction comes from the features that are conserved between these two proteins. They both contain a repeating motif $(R\text{-}X\text{-}X)_n$ (between residues 218–230 in the Dri 42 sequence), which has been implicated in the conformational change that accompanies voltage gating in ion channel proteins, and a potential leucine zipper immediately downstream of this motif (residues 278, 285, and 292) (Ref. 34, and references therein). We do not have any evidence that Dri 42 can interact with itself or with other proteins, but it is tempting to speculate that it might be a subunit of an ER-specific channel. Outside of the apparent conservation of these motifs, the overall sequence homology with ion channel proteins is not very significant, but it is of interest to note that all channel proteins cloned so far have been localized in the plasma membrane. Chloride channels have been described in the ER, which may be involved in facilitating calcium uptake into the organelle or to associate with protein-conducting channels (Ref. 35, and references therein), but no molecular data are yet available for sequence comparison.

Further studies on the role of Dri 42 in intestinal epithelial differentiation, its putative function as a channel protein, and the mechanism of its ER retention are currently in progress in our laboratory.

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The *Dri 42* Gene, Whose Expression Is Up-regulated during Epithelial Differentiation, Encodes a Novel Endoplasmic Reticulum Resident Transmembrane Protein

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