A Strategy for Isolation of cDNAs Encoding Proteins Affecting Human Intestinal Epithelial Cell Growth and Differentiation: Characterization of a Novel Gut-specific *N*-myristoylated Annexin

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Abstract. The human intestinal epithelium is rapidly and perpetually renewed as the descendants of multipotent stem cells located in crypts undergo proliferation, differentiation, and eventual exfoliation during a very well organized migration along the crypt to villus axis. The mechanisms that establish and maintain this balance between proliferation and differentiation are largely unknown. We have utilized HT-29 cells, derived from a human colon adenocarcinoma, as a model system for identifying gene products that may regulate these processes. Proliferating HT-29 cells cultured in the absence of glucose (e.g., using inosine as the carbon source) have some of the characteristics of undifferentiated but committed crypt epithelial cells while postconfluent cells cultured in the absence of glucose resemble terminally differentiated enterocytes or goblet cells. A cDNA library, constructed from exponentially growing HT-29 cells maintained in inosine-containing media, was sequentially screened with a series of probes depleted of sequences encoding housekeeping functions and enriched for intestine-specific sequences that are expressed in proliferating committed, but not differentiated, epithelial cells. Of 100,000 recombinant phage surveyed, one was found whose cDNA was derived from an apparently gut-specific mRNA. It encodes a 316 residue, 35,463-D protein that is a new member of the annexin/lipocortin family. Other family members have been implicated in regulation of cellular growth and in signal transduction pathways. RNA blot and in situ hybridization studies indicate that the gene encoding this new annexin exhibits region-specific expression along both axes of the human gut: (*a*) highest levels of mRNA are present in the jejunum with marked and progressive reductions occurring distally; (*b*) its mRNA appears in crypt-associated epithelial cells and increases in concentration as they exit the crypt. Villus-associated epithelial cells continue to transcribe this gene during their differentiation/translocation up the villus. Immunocytochemical studies reveal that the intestine-specific annexin (ISA) is associated with the plasma membrane of undifferentiated, proliferating crypt epithelial cells as well as differentiated villus enterocytes. In polarized enterocytes, the highest concentrations of ISA are found at the apical compared to basolateral membrane. In vitro studies using an octapeptide derived from residues 2–9 of the primary translation product of ISA mRNA and purified myristoyl-CoA:protein *N*-myristoyltransferase suggested that it is *N*-myristoylated. In vivo labeling studies confirmed that myristate is covalently attached to ISA via a hydroxylamine resistant amide linkage. The restricted cellular expression and acylation of ISA distinguish it from other known annexins. The paradigm used to identify and characterize this gene product from HT-29 cells should be generally applicable for defining other sequences that may regulate proliferation and differentiation in the human gut epithelium.

THE intestinal epithelium is composed of four principal terminally differentiated cell types (reviewed in Neutra et al., 1983): (*a*) absorptive cells or enterocytes: (*b*) mucus-producing goblet cells; (*c*) a complex population of enteroendocrine cells; and (*d*) Paneth cells that synthesize lysozyme plus defensins (Ouellette et al., 1989) and have been postulated to form a component of the physical/biochemical barrier that prevents bacterial translocation across the gut. This epithelium undergoes continuous and rapid renewal. Studies using normal (Winton et al., 1988, 1989; Winton and Ponder, 1990; Griffiths et al., 1988), chimeric (Schmidt et al., 1985*b*, 1988, 1990; Ponder et al., 1985) and transgenic mice (Sweetser et al., 1988*a*,*b*; Cohn et al., 1991; Roth et al., 1991*b*) suggest that a single, active multipotent stem cell, located near the base of each intestinal crypt, sustains this renewal-giving rise to descendants that undergo amplification (Potten and Loeffler, 1990), commitment, and differentiation into each of the four cell types (Cheng and Leblond, 1974; Ponder et al., 1985). Proliferation is restricted to the mid-crypt region where ~150 of the 250 cells

in each (mouse) small intestinal crypt pass through the cell cycle every 12 h (Potten and Loeffler, 1990). Gut epithelial cell differentiation occurs during a remarkably well-organized, bipolar migration (Schmidt et al., 1985*a*,*b*). Enterocytes, goblet cells, and enteroendocrine cells differentiate as they migrate in vertical, coherent bands from the crypt upward to an "extrusion" zone located at or near the villus tip. Migration/differentiation/exfoliation is completed in ~3 d in the mouse and in ~5 d in humans (reviewed in Potten and Loeffler, 1990). In contrast, Paneth cells differentiate during a downward migration to the crypt base where they have a residence time of ~4 wk in humans.

The intestinal epithelium thus is able to establish and maintain a precise balance between proliferation and differentiation. It represents an attractive model system for studying these processes since they are rapid and perpetual, involve a monoclonal population of cells (i.e., derived from a single, ultimate stem cell; Winton et al., 1988, 1989; Winton and Ponder, 1990; Roth et al., 1991*b*), and occur in an easily defined anatomic space.

The intestine is also able to establish and maintain spatial differentiation along its duodenal-to-colonic axis. For example, the number of goblet cells and the distribution of enteroendocrine cell subpopulations vary as a function of location along the cephalocaudal axis of the gut (Neutra, 1983; Roth et al., 1990). Studies with intestinal isografts suggest that the gut's stem cell population may be programmed to retain spatial memory before maturation of the crypt-to-villus axis (Rubin et al., 1991). Thus, gut stem cells may be characterized by their seemingly enormous proliferative potential (insuring both self-renewal and renewal of the epithelium) as well as their ability to retain positional information.

Such a spatially well-organized system invites a number of questions. First, what factors regulate proliferation and differentiation? Second, to what extent are the processes of proliferation and differentiation mutually exclusive? For example, would the differentiation program be affected if the zone of proliferation is expanded beyond the crypt onto the villus or if the rate of migration is accelerated or retarded? Third, is geographic differentiation along duodenal-to-colonic axis related to, or accompanied by, subtle differences in proliferation/migration? Transgenic and chimeric mice have been very useful for examining lineage relationships among the gut's epithelial cell populations and for mapping *cis*-acting elements that regulate the cellular and geographic patterns of transcription of several genes in fetal, neonatal, and aging animals (Ponder et al., 1985; Sweetser et al., 1988*a*,*b*; Hauft et al., 1989; Roth et al., 1990, 1991*a*,*b*; Roth and Gordon, 1990; Rubin et al., 1991; Hansbrough et al., 1991). However, a cell culture model that could mimic, at least to some degree, the processes of proliferation, commitment, and gut epithelial cell differentiation would facilitate an analysis of the first two questions.

HT-29 is a cell line derived from a human colon adenocarcinoma (Fogh and Trempe, 1975). These cells proliferate rapidly when cultured in the presence of glucose. Upon reaching confluence, most cells continue to divide and form a multi-layer. Only a small proportion of cells have an enterocyte-like phenotype (Pinto et al., 1982; Lesuffleur et al., 1990). Pinto et al. (1982) noted that if HT-29 cells were cultured for several passages in the absence of glucose (e.g., using galactose as the carbon source), they would stop dividing upon reaching confluence, form a true monolayer, and "differentiate"—acquiring many of the features of enterocytes including morphologic polarization, an apical brush border with brush border specific hydrolases, and tight junctions. Louvard and co-workers (Huet et al., 1987) reported that confluent monolayers of differentiated HT-29 cells exhibited two phenotypes: 10% of cells resembled the mucus-secreting goblet cell while ~90% were enterocyte-like. Since all of the cells were derived from a single clone, they concluded that HT-29 cells are multipotent. Together these results suggest that (*a*) HT-29 cells which have been cultured in the presence of glucose may resemble multipotent intestinal stem cells; (*b*) proliferating HT-29 cells that have been cultured in the absence of glucose may resemble pre-differentiated crypt cell populations that are committed to differentiate into either enterocytes or goblet cells; and (*c*) cells that have been cultured in the absence of glucose and have been maintained in a confluent state resemble terminally differentiated enterocytes or goblet cells. These observations also suggest that the HT-29 cell line could be exploited as a model system for identifying potential molecular mechanisms which regulate epithelial cell proliferation/commitment/differentiation along the crypt-to-villus axis of the (human) gut. In this report, we describe a strategy for surveying a cDNA library, derived from proliferating HT-29 cells grown in the absence of glucose, to identify potential mediators of these processes. This survey has yielded a cDNA encoding a novel gut-specific annexin.

Materials and Methods

Cells and Culture Conditions

HT-29 cells were obtained from Dr. Alain Zweibaum (INSERM U178, Villejuif, France) and cultured in Dulbecco's modified minimal essential medium (D-MEM) containing 10% dialyzed FBS, plus glucose (final concentration = 25 mM) or inosine (2.5 mM). Cells were grown in an atmosphere of 10% CO2/90% air at 100% humidity. HT-29/ino[1] cells were cultured in the presence of inosine and absence of glucose for at least four passages (15–20 cell doublings) before use (Wice et al., 1985). "HT-29 glc" cells were always exposed to glucose as their exclusive carbon source. HT-29/ino and HT-29/glc cells were initially plated at densities of 2×10^6 and 1×10^6 cells/25 cm^2, respectively. "Log phase" cells were harvested 3-4 d after plating while "postconfluent" cells were recovered 18–22 d after plating.

CaCo-2, SW480, and HeLa (CCL2 and CCL2.2) cells were obtained from the ATCC (Rockville, MD). CaCo-2 cells were cultured in D-MEM/25 mM glucose/20% nondialyzed FBS/1× nonessential amino acids (Gibco Laboratories, Grand Island, NY). SW480 cells were cultured in D-MEM exactly as described for HT-29 cells. HeLa cells were grown in MEM-α supplemented with 10% dialyzed FBS (clone CCL2.2) or glucose-free MEM/Earle's salts (CCL2.2) containing 10% dialyzed FBS and 1 mM inosine (Wice et al., 1981). "Log phase" and "postconfluent" CaCo-2, SW480, and HeLa cells were defined in exactly the same way as described above for HT-29 cells.

Procurement of Human Tissues

Full thickness segments of gut, harvested along the duodenal-to-colonic axis, were obtained from adult organ donors through the National Disease Research Interchange (Philadelphia, PA). Additional tissue samples were obtained as surgical specimens. All tissues were snap-frozen in liquid nitro-

1. *Abbreviations used in this paper*: HT29/ino, HT-29 cells grown in the absence of glucose and the presence of 2.5 mM inosine for at least four passages; HT-29/glc, HT-29 cells grown exclusively in the presence of 25 mM glucose; ISA, intestine-specific annexin (Gut-2); NMT, myristoylCoA: protein *N*-myristoyltransferase; L-FABP liver fatty acid binding protein.

gen and stored at −140°C. Tissues were procured using guidelines and protocols approved by our University's Human Studies Committee.

Isolation of RNA

Total cellular RNA was extracted from cultured cells and from human tissues using guanidinium thiocyanate and further purified by centrifugation through cesium chloride gradients (Chirgwin et al., 1979). RNA integrity was established after electrophoresis through methylmercury agarose or formaldehyde agarose gels (Thomas, 1980). PolyA$^+$ RNA was prepared from total cellular RNA by oligo-dT cellulose (Type 7, LKB-Pharmacia, Uppsala, Sweden) chromatography. Samples of total cellular RNA isolated from the entire small intestine of 12.5- and 15-wk human fetuses were kindly provided by Dr. Nicholas O. Davidson (University of Chicago).

Synthesis and Screening of a cDNA Library Prepared from Proliferating HT-29/ino Cells

A cDNA library of 6.7×10^6 independent recombinant phage was produced by directional cloning of double stranded cDNA into the XbaI/EcoRI site of Lambda GEM-2 (Promega Biotec, Madison, WI). The strategy for screening this library with subtracted cDNA probes was as follows. PolyA$^+$ RNA, prepared from HT-29/ino cells harvested during log phase, was reverse transcribed in the presence of [^{32}P]dCTP with an XbaI-oligodT primer-adaptor and avian myeloblastosis virus (AMV) reverse transcriptase (Davis et al., 1984). This cDNA contains sequences complementary to HT-29-specific mRNAs as well as to "housekeeping" mRNAs. An unknown fraction of the former mRNAs should be intestine-specific. To reduce the number of cDNAs encoding "housekeeping" functions, the radiolabeled probe (0.1 μg, specific activity = 1,300 cpm/pg) was incubated with 1.2 μg of biotinylated polyA$^+$ RNA (Sive and St. John, 1988) prepared from proliferating HeLa cells cultured in the presence of glucose. This epithelial cell line was derived from a human cervical adenocarcinoma (Gey et al., 1952; Jones et al., 1971) and should contain mRNAs that could hybridize with "housekeeping", but not with HT-29-specific, sequences present in the HT-29/ino/log cDNA preparation. Hybridization was allowed to proceed at 65°C for 40 h in a 5 μl reaction mixture containing *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (50 mM, pH 7.6), SDS (0.2%), EDTA (2 mM), and NaCl (0.5 M). Unhybridized, single-stranded, [^{32}P]labeled cDNAs were separated from [^{32}P]cDNA:biotinylated mRNA hybrids using avidin and the phase partitioning method described in Sive and St. John (1988). A second round of subtraction, identical in all respects to the first, was then performed. The final preparation of single stranded [^{32}P]labeled cDNA typically represented ~10% of the starting material. (N.B. control experiments using either radiolabeled biotinylated mRNA alone, or labeled cDNA alone, indicated that the recovery of counts after the phase partition protocol is >95%). This fraction, enriched for HT-29 "specific" sequences, was termed the "plus" probe.

Since the "plus" probe undoubtedly contained "housekeeping" sequences that were not removed by the subtraction procedure, we also screened the library with a "minus" probe designed to identify phage containing "housekeeping" cDNAs. This "minus" probe was generated by synthesizing [^{32}P]labeled cDNA from polyA$^+$ RNA prepared from log phase HeLa/glc cells and hybridizing it (0.1 μg, specific activity = 1,300 cpm/pg) with biotinylated polyA$^+$ RNA (1.2 μg) prepared from proliferating HT-29/ino cells. Hybridization conditions were identical to those described above. After avidin/phase separation and a second cycle of subtraction, ~10% of the input counts were recovered as single-stranded cDNA. This fraction should contain cDNAs that are specific to HeLa cells as well as housekeeping sequences which were not removed by the subtraction protocol. Because our HT-29/ino library cannot contain any HeLa specific cDNAs, any recombinant phage that hybridize with the "minus" probe should have cDNA inserts derived from "housekeeping" mRNAs.

100,000 recombinant phage from our proliferating HT-29/ino cDNA library were screened as follows. Duplicate filters were prepared (Sambrook et al., 1989) from each Petri dish containing 25,000 plaques. All eight filters were incubated in a solution containing formamide (50%), NaCl (0.75 M), sodium phosphate (50 mM, pH 7.4), EDTA (5 mM), 1.5× Denhardt's solution (1× Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), dextran sulfate (10%), SDS (0.1%) sheared, denatured salmon sperm DNA (0.1 mg/ml), and the [^{32}P]labeled "minus" probe (3×10^5 cpm/ml). After a 40-h incubation at 42°C, filters were washed (final stringency = 0.1× SSC at 65°C) and subjected to autoradiography (typically filters were exposed for 7 d to Kodak XAR film at −80°C using one intensifying screen). As noted above, phage in the HT-29/ino library that reacted with this "minus" probe should contain "housekeeping" cDNAs. Annealed probe was removed from the filters by incubating them for 4 h at 65°C in the hybridization solution described above (minus dextran sulfate). The filters were then hybridized with the [^{32}P]labeled plus probe (note that both plus and minus probes had equivalent specific activities). Plaques that were plus probe positive and minus probe positive were considered to have cDNAs encoding "housekeeping" proteins. Their dual reactivity was presumed to arise from the fact that the subtraction employed during preparation of the "plus" probe failed to remove all housekeeping sequences. After sequential screening with the "minus" followed by the "plus" probes, 15 plaques were identified that reacted on duplicate filters with the "plus" probe but not the "minus" probe. 11 plaques remained positive after secondary and tertiary rounds of screening.

Phage DNA was prepared from each of the 11 purified plaques (Sambrook et al., 1989). Their cDNA inserts were excised by digestion with XbaI and EcoRI and purified by agarose gel electrophoresis and glass bead capture (Geneclean, Bio 101, LaJolla, CA). Each insert was labeled with [^{32}P] using random hexanucleotide primers (Feinberg and Vogelstein, 1983, 1984), denatured, and subsequently used to probe Southern blots containing all 11 purified XbaI/EcoRI fragments. The results of this survey indicated that the 11 inserts were derived from four unique cDNAs (data not shown).

DNA Sequencing

XbaI/EcoRI fragments were subcloned into pBluescript/KS (Stratagene, LaJolla, CA). Nested deletions were generated from each end of the insert using Exonuclease III, Mung Bean nuclease, and the protocol supplied in Strategene's Exo/Mung kit. The sequenase II system (United States Biochemical, Cleveland, OH) which is based upon the dideoxynucleotide chain termination method was employed for nucleotide sequencing: M13 "universal", "reverse" M13, T3, or T7 primers were utilized together with denatured, double-stranded DNA templates. Oligodeoxynucleotide primers were produced using phosphoramite chemistry and an Applied Biosystems Model 380B Synthesizer to complete the sequencing of regions that were not encompassed by the nested deletions. Both strands were completely sequenced.

Northern Blot Analysis

Total cellular RNA (5–10 μg) containing bound ethidium bromide was fractionated by denaturing formaldehyde-agarose gel electrophoresis, and transferred to nitrocellulose filters (Thomas, 1980). Gels, as well as the nitrocellulose filters containing bound RNA, were examined under short wave UV light to verify that all lanes contained similar amounts of intact RNA and that the efficiency of transfer of RNA was similar for all samples. Blots were probed with purified XbaI/EcoRI fragments labeled with [^{32}P] using the random primer method or with [^{32}P]cRNAs prepared by in vitro run off transcription of linearized recombinant pBluescript plasmids according to protocols supplied by Promega Biotec (Madison, WI). Hybridization with cRNA probes was conducted at 60°C in a solution containing formamide (50%), SSC (5×), sodium phosphate (50 mM, pH 7.4), SDS (0.1%), Denhardt's solution (2.5×), and sheared, denatured salmon sperm DNA (0.2 mg/ml). Blots were subsequently washed (final stringency = 0.1× SSC at 70–75°C). Each blot contained internal standards of purified in vitro transcribed mRNA (0.3–1000pg) to allow quantitation of the absolute levels of a given mRNA species in preparations of total cellular RNA. Blots were scanned using a Betagen Image Analyzer (Betagen, Waltham, MA).

In Situ Hybridizations

These analyses were performed on cryostat sections of human jejunum and colon based on the method described in Ausubel et al. (1989). Pieces of frozen tissue were embedded in OCT and 10-μm sections were collected on slides that had been coated twice with gelatin. Sections were fixed for 20 min at room temperature in a fresh solution of 4% paraformaldehyde (prepared in PBS) and then dehydrated in graded alcohols. Samples were acetylated with acetic anhydride and dehydrated once more. Hybridization solution (formamide [50%], dextran sulfate [10%], Denhardt's solution [1×], EDTA [1 mM], Tris [10 mM, pH 8.0], NaCl [0.3 M], DTT [10 mM], yeast tRNA [0.5 mg/ml], and [^{35}S]labeled cRNA [5×10^6 cpm/ml, prepared as described below]) was added to the tissue sections (80 μl/slide). A coverslip was placed over the slide, its edges were sealed with DPX Mountant (BDH Ltd., Poole, England), and hybridization was allowed to proceed for 12–16 h at 55°C. At the conclusion of this incubation, the cover-

slip was removed, the section was washed four times in 4× SSC at room temperature (5 min/wash), and then treated for 30 min at 37°C with RNase A (20 μg/ml of buffer containing NaCl (0.5 M), Tris (10 mM, pH 8.0), and EDTA (1 mM)). After a 30-min wash in 0.1× SSC at 55°C, the sections were dehydrated with graded alcohols, and the slides were dipped in Kodak NTB2 emulsion. Slides were exposed at 4°C, then developed, fixed, stained with hematoxylin, and counterstained with Eosin Y.

Labeled cRNA and mRNA probes for these in situ hybridization studies were generated in the presence of [^{35}S]UTP using linearized, recombinant, pBluescript/KS DNA, and T3 or T7 RNA polymerase. The specific activity of the purified in vitro transcribed RNAs was 2,000 cpm/pg. Their unique specificities were established by Northern blot hybridization using cRNA and mRNAs produced in an identical fashion except [^{32}P]dCTP was substituted for [^{35}S]UTP in the reaction mixture.

Generation of Antipeptide Antibodies

An 18 amino acid peptide (GNRHAKASSPQGFDVDRC-NH_2) was synthesized using the Merrifield method with an Applied Biosystems Model 430 Synthesizer as described in Towler et al. (1987). This peptide spans residues 2–18 of the primary translation product of Gut-2 cDNA (see below) and contains an additional COOH-terminal cysteine. 4 mg of the purified octadecapeptide was coupled to 2 mg of Keyhole Limpet hemocyocyanin via this cysteine using 3-maleimidobenzyoyl-*N*-hydroxy-succinimidester (Ausubel et al., 1989). After separation from unbound peptide, the conjugate was emulsified with Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO) and used to immunize two female New Zealand white rabbits. A total initial dose of 300 μg conjugate was administered at five intradermal sites. 4 wk later, and at 2-wk intervals thereafter, rabbits were boosted with 150–300 μg antigen in Freund's incomplete adjuvant. Each animal produced antibodies that recognize the free peptide as determined by ELISA.

Western Blot Analyses

Cells or tissues were homogenized with a Tissue Mizer (Tekmar, Cincinnati, OH) in 2× Laemmli loading buffer (Laemmli, 1970) containing the following protease inhibitors: E-64 (1 μg/ml), aprotinin (5 μg/ml), leupeptin (5 μg/ml), PMSF (0.5 mM), phosphoramidon (50 μg/ml), pepstatin (1 μg/ml), and EDTA (5 mM). After boiling, the proteins were separated by electrophoresis through 10% polyacrylamide gels containing 0.1% SDS and then transferred onto nitrocellulose membranes. The resulting Western blots were incubated with preimmune or immune rabbit sera in Blotto (5% [wt/vol] non-fat dry milk, antifoam A [0.03%], Tween-20 [0.04%], sodium azide [0.1%], NaCl [0.15 M], and Tris [10 mM, pH 7.5]) for 1–2 h at room temperature. Blots were washed in Blotto (four washes of 10 min each at room temperature). Antigen–antibody complexes were detected by incubating the washed blots with [^{125}I]Protein A (1 μCi/ml Blotto; specific activity = 2–10 μCi/μg) for 30 min at room temperature followed by four washes with Blotto (10 min/wash; each wash performed at room temperature).

Immunocytochemical Studies

Frozen samples of human jejunum or colon were embedded in OCT and 10-μm cryostat sections were prepared. Sections were fixed for 10 min in 100% methanol at 0°C, air dried, and stored at −20°C until use. Sections were rehydrated in PBS and incubated for 30 min at room temperature in blocking buffer (BSA [1%], powdered skim milk [0.2%], and Triton X-100 [0.3%] prepared in PBS). Primary antibodies were diluted in blocking buffer and incubated with sections for 60 min at room temperature. Sections were subsequently washed with PBS (four washes of 5 min each) and then incubated for 60 min at room temperature with secondary antibodies (obtained from Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 in blocking buffer containing 5% normal goat sera. Sections were washed with PBS and mounted in PBS/glycerol (1:1 vol/vol).

Immunocytochemical analyses of cultured cells were conducted in a similar fashion except that cells were cultured on glass coverslips and rinsed with PBS before being processed as described above.

The following primary antibodies were employed: mouse monoclonal HSI4/34 (antihuman sucrase-isomaltase, antibody HSSI9 in Beaulieu et al., 1989, final dilution = 1:200); mouse monoclonal WE9 (antigoblet cell mucin; Phillips et al., 1988; 1:10 dilution of hybridoma medium); mouse mAb HBB 3/755/42 which recognizes dipeptidyl-peptidase IV (Hauri et al., 1985; 1:200); plus the rabbit polyclonal anti-GNRHAKASSPQGFDVDRC sera (1:1,000).

In Vitro MyristoylCoA:Protein N-Myristoyltransferase Assay

To determine whether the primary translation product of Gut-2/ISA mRNA (see below) is a substrate for myristoylCoA:protein *N*-myristoyltransferase (NMT), an octapeptide representing its residues 2-9 (GNRHAKAS-NH2) was synthesized and added to a coupled in vitro assay system developed by Towler and Glaser (1986) and described in several of our earlier publications (Rudnick et al., 1990, 1991; Duronio et al., 1990*b*). Briefly, the assay measures the transfer of myristate from myristoylCoA to the amino-terminal Gly residue of peptide acceptors. It consists of two steps. The first involves enzymatic generation of radiolabeled myristoylCoA using [^{3}H]-myristate and *Pseudomonas* acylCoA synthetase. The second step involves addition of purified NMT (Rudnick et al., 1990) together with an octapeptide containing residues 2–9 of the candidate sequence. The enzymatically generated acylpeptide is purified from the reaction mixture by reverse phase HPLC and detected by an in-line scintillation counter (Kishore et al., 1991).

Metabolic Labeling Studies

Monolayers of proliferating HT-29/ino cells were washed twice with PBS and then incubated with glucose-free DMEM containing 2.5 mM inosine, 10% dialyzed and delipidated FBS (Cham and Knowles, 1976), and (*a*) [9,10-^{3}H]myristic acid or [9,10-^{3}H]palmitic acid (39 Ci/mmole; 200 μCi/ml) (New England Nuclear, Boston, MA) or (*b*) L-[^{35}S]methionine (1,000 Ci/mmol; 100 μCi/ml). At the end of the 4-h labeling period, monolayers were washed twice in PBS. Cells were then lysed in PBS containing sodium deoxycholate (1%), Triton X-100 (1%), SDS (0.1%), E-64 (1 μg/ml), EDTA (5 mM), leupeptin (5 μg/ml), aprotinin (5 μg/ml), PMSF (0.5 mM), phosphoramidon (50 μg/ml), and pepstatin A (1 μg/ml). Cellular DNA was sheared by passing the lysate through a 26-g syringe needle. Insoluble material was removed by centrifugation for 2 min at 12,000 *g*. The resulting supernatant was subsequently incubated for 12–16 h at 4°C with antipeptide serum (1 μl serum/125 μg HT-29/ino cell protein). Protein A–Sepharose (Pharmacia, Uppsala, Sweden) was added and the incubation continued for 1–2 h at room temperature. The beads were then harvested by centrifugation, washed five times with the same lysis buffer described above except the SDS concentration was 1% and NaCl was added to a final concentration of 0.65 M. After a final wash with PBS, the bound antigens were eluted by reduction and heat denaturation in 2× Laemmli sample loading buffer and then separated by electrophoresis through 10% polyacrylamide gel containing SDS (0.1%) (Laemmli, 1970). Gels were processed as described in Heuckeroth and Gordon (1989) and Johnson et al. (1990).

Results and Discussion

HT-29 Cells as a Model System for Identifying Gene Products that Affect Intestinal Epithelial Cell Growth and Differentiation

Logarithmically growing HT-29 cells, maintained in media containing inosine as a carbon source, represent a useful starting point for identifying sequences that may modulate the balance between proliferation and differentiation of epithelial cells along the crypt-to-villus axis of the human gut. Selection of these cells for construction of a cDNA library was based on the following observations and suppositions. First, exponentially growing HT-29/ino cells offer the opportunity for obtaining gene products that may regulate proliferation of undifferentiated intestinal epithelial cells in the crypt. Second, it appears that genes expressed during exponential growth in inosine are sufficient to "induce" the terminal differentiation program, i.e., if HT-29 cells are cultured in the absence of glucose (e.g. in inosine) for several passages without ever being allowed to reach confluence, and glucose is then added when confluency is achieved, they will still differentiate into both enterocyte-like and goblet cell-like phenotypes (B. Wice and G. Trugnan, unpublished observations). In contrast, >95% of cells that have been cul-

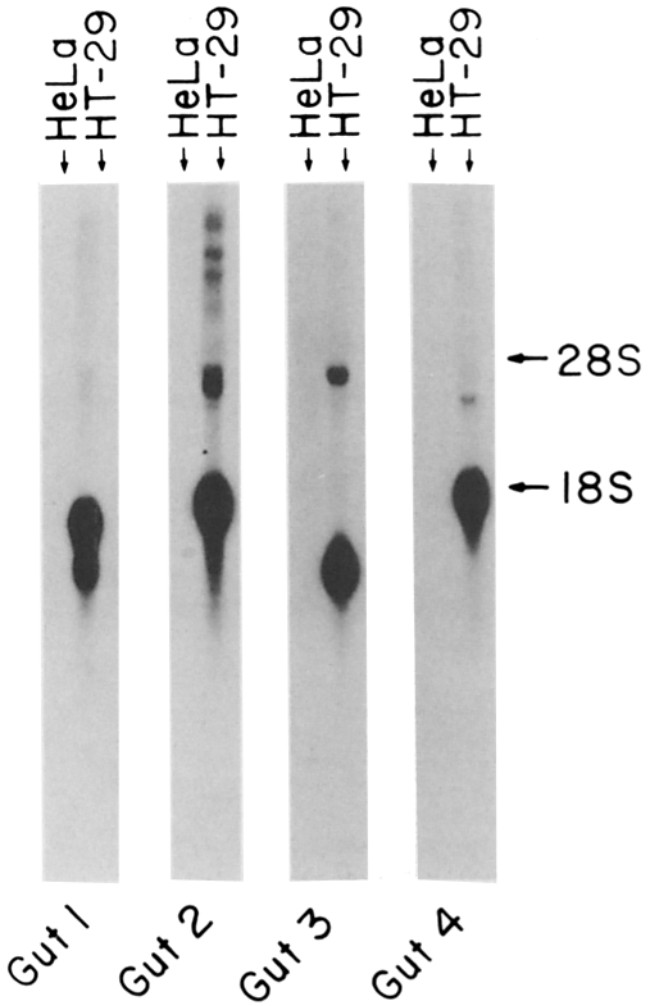

Figure 1. Northern blot analysis of RNA prepared from proliferating HT-29/ino and HeLa/glc cells using cDNAs recovered from the library screening. [^{32}P]labeled cDNA inserts from each of four independent recombinant phage DNA were used to probe blots of total cellular RNA (10 μg/lane) prepared from the indicated cell lines. The positions of migration of 28S and 18S rRNA are indicated. Note that Gut-1 cDNA reacts with two different abundant mRNAs, whereas Gut-2, Gut-3, and Gut-4 cDNA each hybridize to a single dominant transcript. These latter cDNAs do react with larger and less abundant transcripts present in the total cellular RNA samples. They presumably represent partially processed nuclear transcripts which are present in HT-29/ino but not HeLa cells. Note that the exposure time for this autoradiograph was 4 d allowing us to emphasize the degree of confidence that underlies the conclusion that Gut-1, Gut-2, Gut-3, and Gut-4 mRNAs are not present HeLa cells.

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   1  CGTTGCTGTCGGTAAACTTTGCCTGTAGGAGGACTGATCTCTTAATGAAATACAGAAAAA
  61  CCATCTCAGAAAAAGGAAAATGGGCAATCGTCATGCTAAAGCGAGCAGTCCTCAGGGTTT
                      M  G  N  R  H  A  K  A  S  S  P  Q  G  F    14
 121  TGATGTGGATCGAGATGCCAAAAAGCTGAACAAAGCCTGCAAAGGAATGGGGACCAATGA
       D  V  D  R  D  A  K  K  L  N  K  A  C  K  G  M  G  T  N  E    34
 181  AGCAGCCATCATTGAAATCTTATCGGGCAGGACATCAGATGAGAGGCAACAAATCAAGCA
       A  A  I  I  E  I  L  S  G  R  T  S  D  E  R  Q  Q  I  K  Q    54
 241  AAAGTACAAGGCAACGTACGGCAAGGAGCTGGAGGAAGTACTCAAGAGTGAGCTGAGTGG
       K  Y  K  A  T  Y  G  K  E  L  E  E  V  L  K  S  E  L  S  G    74
 301  AAACTTCGAGAAGACAGCGTTGGCCCTTCTGGACCGTCCCAGCGAGTACGCCGCCCGGCA
       N  F  E  K  T  A  L  A  L  L  D  R  P  S  E  Y  A  A  R  Q    94
 361  GCTGCAGAAGGCTATGAAGGGTCTGGGCACAGATGAGTCCGTCCTCATTGAGTTCCTGTG
       L  Q  K  A  M  K  G  L  G  T  D  E  S  V  L  I  E  F  L  C   114
 421  CACGAGGACCAATAAGGAAATCATCGCCATTAAAGAGGCCTACCAAAGGCTATTTGATAG
       T  R  T  N  K  E  I  I  A  I  K  E  A  Y  Q  R  L  F  D  R   134
 481  GAGCCTCGAATCAGATGTCAAAGGTGATACAAGTGGAAACCTAAAAAAAATCCTGGTGTC
       S  L  E  S  D  V  K  G  D  T  S  G  N  L  K  K  I  L  V  S   154
 541  TCTGCTGCAGGCTAATCGCAATGAAGGAGATGACGTGGACAAAGATCTAGCTGGTCAGGA
       L  L  Q  A  N  R  N  E  G  D  D  V  D  K  D  L  A  G  Q  D   174
 601  TGCCAAAGATCTGTATGATGCAGGGGAAGGCCGCTGGGGCACTGATGAGCTTGCGTTCAA
       A  K  D  L  Y  D  A  G  E  G  R  W  G  T  D  E  L  A  F  N   194
 661  TGAAGTCCTGGCCAAGAGGAGCTACAAGCAGTTACGAGCCACCTTTCAAGCCTATCAAAT
       E  V  L  A  K  R  S  Y  K  Q  L  R  A  T  F  Q  A  Y  Q  I   214
 721  TCTCATTGGCAAAGACATAGAAGAAGCCATTGAAGAAGAAACATCAGGCGACTTGCAGAA
       L  I  G  K  D  I  E  E  A  I  E  E  E  T  S  G  D  L  Q  K   234
 781  GGCCTATTTAACTCTCGTGAGATGTGCCCAGGATTGTGAGGACTATTTTGCTGAACGTCT
       A  Y  L  T  L  V  R  C  A  Q  D  C  E  D  Y  F  A  E  R  L   254
 841  GTACAAGTCGATGAAGGGTGCGGGGACCGATGAGGAGACGTTGATTCGCATAGTCGTGAC
       Y  K  S  M  K  G  A  G  T  D  E  E  T  L  I  R  I  V  V  T   274
 901  CAGGGCCGAGGTGGACCTTCAGGGGATCAAAGCAAAGTTCCAAGAGAAGTATCAGAAGTC
       R  A  E  V  D  L  Q  G  I  K  A  K  F  Q  E  K  Y  Q  K  S   294
 961  TCTCTCTGACATGGTTCGCTCAGATACCTCCGGGGACTTCCGGAAACTGCTAGTAGCCCT
       L  S  D  M  V  R  S  D  T  S  G  D  F  R  K  L  L  V  A  L   314
1021  CTTGCACTGAGCCAAGCCAGGGCAATAGGAACACAGGGTGGAACCACCTTTGTCAAGAGC
       L  H
1081  ACATTCCAAATCAAACTTGCAAATGAGACTCCCGCACGAAAACCCTTAAGAGTCCCGGAT
1141  TACTTTCTTGGCAGCTTAAGTGGCGCAGCCAGGCCAAGCTGTGTAAGTTAAGGGCAGTAA
1201  CGTTAAGATGCGTGGGCAGGGCACCTTGAACTCTGGCTTAGCAAGCATCTAGGCTGCCTC
1261  TTCACTTTCTTTTAGCATGGTAACTGGATGTTTTCTAAACACTAATGAAATCAGCAGTTG
1321  ATGAAAAAACTATGCATTTGTAATGGCACATTTAGAAGGATATGCATCACACAAGTAAGG
1381  TACAGGAAAGACAAAATTAAACAATTTATTAATTTTCCTTCTGTGTGTTCAATTTGAAAG
1441  CCTCATTGTTAATTAAAGTTGTGGATTATGCCTCT(A)32
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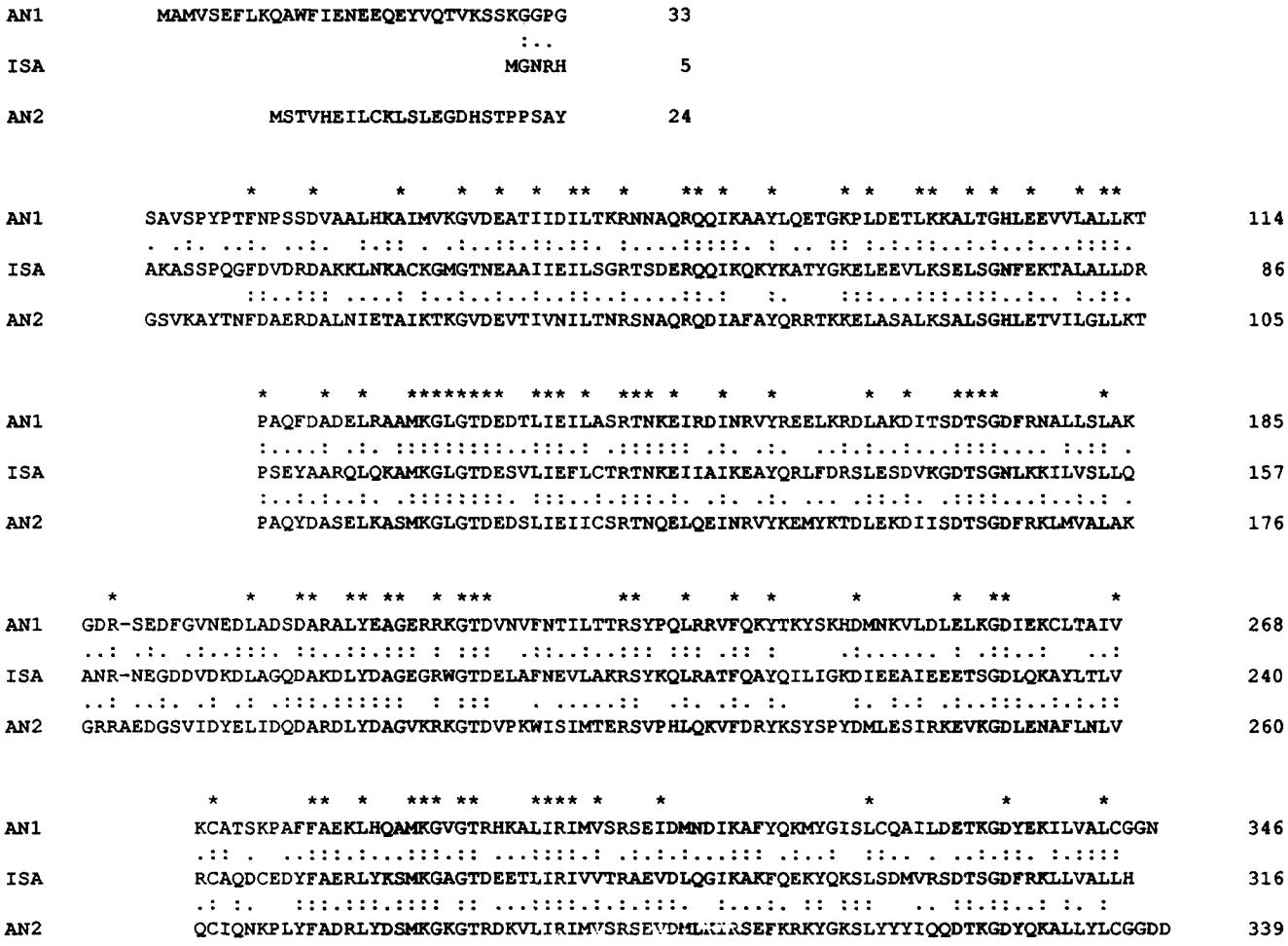
Figure 2. Nucleotide sequence of ISA (Gut-2) cDNA and its deduced primary translation product.

tured in the presence of glucose during logarithmic growth fail to acquire the ability to express a differentiation program. Third, if the differentiated state represents the product of continued active regulation rather than the result of a series of irreversible binary decisions (Blau and Baltimore, 1991), nonproliferating, postconfluent cells should express genes that regulate differentiation. However, devising a strategy for identifying these cDNAs in a library constructed from postconfluent HT-29/ino cells appeared to us to be more difficult than one constructed from proliferating, undifferentiated (but committed) HT-29/ino cells since we anticipated a large number of sequences in the former to be markers, rather than regulators, of the differentiated state.

The protocol used for screening an exponentially growing, HT-29/ino cDNA library for intestine-specific sequences that may regulate epithelial cell growth and/or differentiation is discussed in Materials and Methods. Four unique cDNAs were identified from the 100,000 recombinant phage surveyed. These cDNAs were subsequently incubated with Northern blots of total cellular RNA prepared from proliferating HT-29/ino and proliferating HeLa/glc cells. As expected from the experimental design of the library screen, each of the labeled cDNAs only hybridized to transcripts present in HT-29 cells (Fig. 1). To determine whether any or all of these cDNAs were relatively specific for the intestinal epithelium, an additional set of Northern blots was constructed containing total cellular RNA from adult human small intestine, stomach, liver, kidney, heart, and placenta. All four cDNAs reacted with unique mRNAs present in small intestine. Each of these four mRNAs appeared to be identical in size to those detected in proliferating HT-29/ino cellular RNA (data not shown). The mRNA tentatively named Gut-2 was the only one whose accumulation was limited to the small bowel: Gut-1 mRNA is also present in liver, Gut-3 mRNA is present in liver and stomach, while

A



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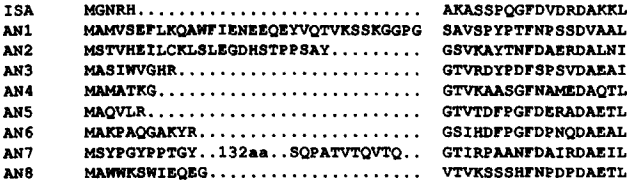


Figure 3. Alignment of human annexins. (*A*) FASTP alignment (Pearson and Lipman, 1988) of ISA with human annexins (AN) I and II. The reader is referred to Pepinsky et al. (1988) for the results of multiple sequence alignments between other members of the annexin family; : and . indicate identical amino acids and conservative substitutions, respectively, are present at this position in ISA and ANI or ANII. An asterisk indicates that the same amino acid is present at comparable positions of ISA, ANI, and ANII. (*B*) Alignment of the amino-terminal domains of the human annexins. The dots represent gaps inserted between their divergent amino-terminal domains and beginning of their conserved first repeating domain.

Gut-4 accumulates in stomach, kidney, and placenta (data not shown). We selected Gut-2 for further characterization given the fact that this limited survey suggested intestinal-specificity.

Gut-2 cDNA Specifies a Protein That Is a New Member of the Annexin family

The complete nucleotide sequence of Gut-2 cDNA is shown in Fig. 2. The size of the cDNA (1,507 bases) is in close agreement with the estimated length of its mRNA (~1,500–1,600 bases; Fig. 1). A favorable consensus sequence for initiation of translation surrounds the first ATG at nucleotides 80–83: an adenine is positioned three bases upstream from the Met codon while a guanosine is located four bases downstream (Kozak, 1987). A rare, but efficient polyadenylation signal (ATTAAA; Wickens, 1990) spans nucleotides 1,452–1,457. The deduced sequence of the primary translation product of Gut-2 mRNA contains 316 amino acids and has a calculated mass of 35,463 D.

A search of protein and nucleic acid databases was conducted. A survey of versions 27.0 and 67.0 of the EMBL and GenBank DNA databases, respectively, with FASTA (Pearson and Lipman, 1988) yielded 43 annexin (lipocortin) cDNA entries, representing eight unique human annexins,

that are related to Gut-2. Each of these cDNAs shares 50–60% identity with Gut-2 cDNA over a span of 900–1,000 nucleotides. This level of identity is limited to their protein coding regions.

FASTP alignments (Pearson and Lipman, 1988) revealed that Gut-2 has 44% identity with human annexin I over a 313 amino acid span and 44% identity with human annexin II over a 302 residue region (Fig. 3 *A*). Based on this degree of identity, we concluded that Gut-2 represents a new member of this protein family and named it intestine-specific annexin (ISA).

The annexins were originally described as steroid-inducible proteins that inhibit phospholipase A_2 (PLA_2) activity in vitro (Flower et al., 1984). The annexins/lipocortins were originally named based on their proposed functions. Different groups have assigned different names to the same protein, resulting in a complex and confusing nomenclature. The reader is referred to Pepinsky et al. (1988), Geisow et al. (1990), and Crumpton and Dedman (1990) for tables listing the various names for annexins/lipocortins. We have used the term annexin as suggested by Crompton and Dedman (1990). Pepinsky and co-workers were able to purify a number of annexins based upon their calcium-dependent inhibition of PLA_2 and isolate the corresponding cDNAs (Huang et al., 1986; Wallner et al., 1986; Pepinsky et al., 1986, 1988). While the precise physiological functions of these proteins have not been established, a number of observations suggest that they may play important roles in the regulation of cellular growth and in signal transduction pathways. For example, annexin I (p35) is the major physiological substrate for the EGF receptor/tyrosine kinase in vivo (Sawyer and Cohen, 1985; De et al., 1986). Annexin II (p36; Saris et al., 1986; Huang et al., 1986), is the dominant phosphorylated protein in Rous sarcoma virus transformed fibroblasts, leading to the suggestion that it is a substrate for the $p60^{v\text{-}src}$ tyrosine kinase (Erikson and Erikson, 1980). Monomeric annexin II and phosphoglycerate kinase have also been implicated (Jindal and Vishwanotha, 1990; Jindal et al., 1991) as the two cofactors in the "primer recognition protein complex" that interacts with DNA polymerase α, the enzyme responsible for synthesizing the lagging strand of DNA during DNA replication. Annexin III is identical to inositol 1:2 cyclic phosphate 2-phosphohydrolase (Ross et al., 1990) and probably controls the intracellular levels of all inositol cyclic phosphates. These cyclic phosphates are thought to be important for the sustained, or delayed, action of effectors in signal transduction pathways since they remain in stimulated cells longer than the noncyclic forms. Overexpression of annexin III in 3T3 cells results in a reduction in their density at confluency (Ross et al., 1991). Annexin VII can act as a voltage-dependent calcium channel (see Pollard and Rojas, 1988; Burns et al., 1989). In addition to proposed regulatory roles in growth and signal transduction, a large number of annexins have also been implicated in exocytosis (Zaks and Creutz, 1990*a*,*b*; Creutz et al., 1987; Ali et al., 1989; Drust and Creutz, 1988; Creutz, 1981; Hong et al., 1981; Creutz et al., 1978).

Previous alignments of annexins have revealed a considerable degree of overall sequence similarity (~50%; see Pepinsky et al., 1988). Moreover, each of the annexins has been highly conserved over the course of mammalian evolution: the human and rat homologues of annexins I, III, and V share 89, 85, and 89% identity, respectively (Pepinsky et al., 1988), while human and mouse annexin II are ~98% identical (Saris et al., 1986). A domain of ~70 amino acids is repeated four times in the 35–40-kD annexins and eight times in the 68-kD annexins. These domains are believed to be important for calcium and phospholipid binding (reviewed in Crompton et al., 1988). Calcium, in turn, is required for the association of annexins with membranes and/or cytoskeletal proteins. Unlike their highly conserved repetitive domains, the amino-terminal regions of each annexin have diverged considerably, raising the possibility that this domain of 5–154 residues is important for the unique function of each annexin. For example, the unique tyrosine phosphorylation sites for annexins I and II are located in this domain. ISA lacks a comparably positioned amino-terminal Tyr. (While phosphorylation can affect the calcium and phospholipid binding properties of some annexins [Schlaepfer and Haigler, 1987; Powell and Glenney, 1987], the precise physiologic relevance of their phosphorylation is generally unclear.)

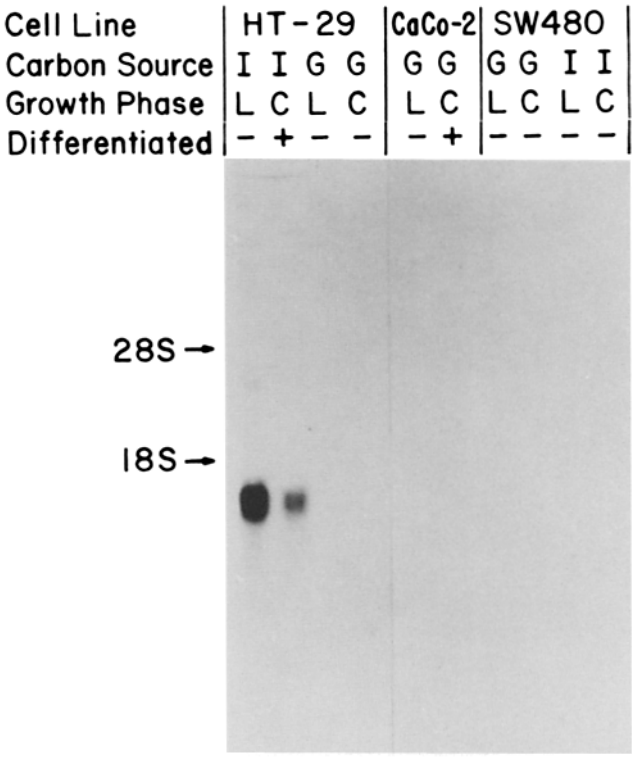


Figure 4. Expression of ISA mRNA in a variety of cell lines derived from human colonic adenocarcinomas as a function of their growth and differentiation. Northern blots were prepared from formaldehyde agarose gels containing 10 μg of each sample of total cellular RNA and probed with a full-length [^{32}P]labeled Gut-2 cDNA. Exposure time for the autoradiograph of the washed blot = 4 d. Carbon source: *I*, inosine; *G*, glucose. Growth phase: *L*, logarithmically growing cells; *C*, cells maintained in a confluent state for 10–14 d. Differentiation was based on the presence of immunoreactive sucrase-isomaltase, aminopeptidase N, and/or dipeptidylpeptidase IV.

Expression of ISA in Human Intestinal Epithelial-Derived Cell Lines

Annexin I may play a role in the differentiation programs of

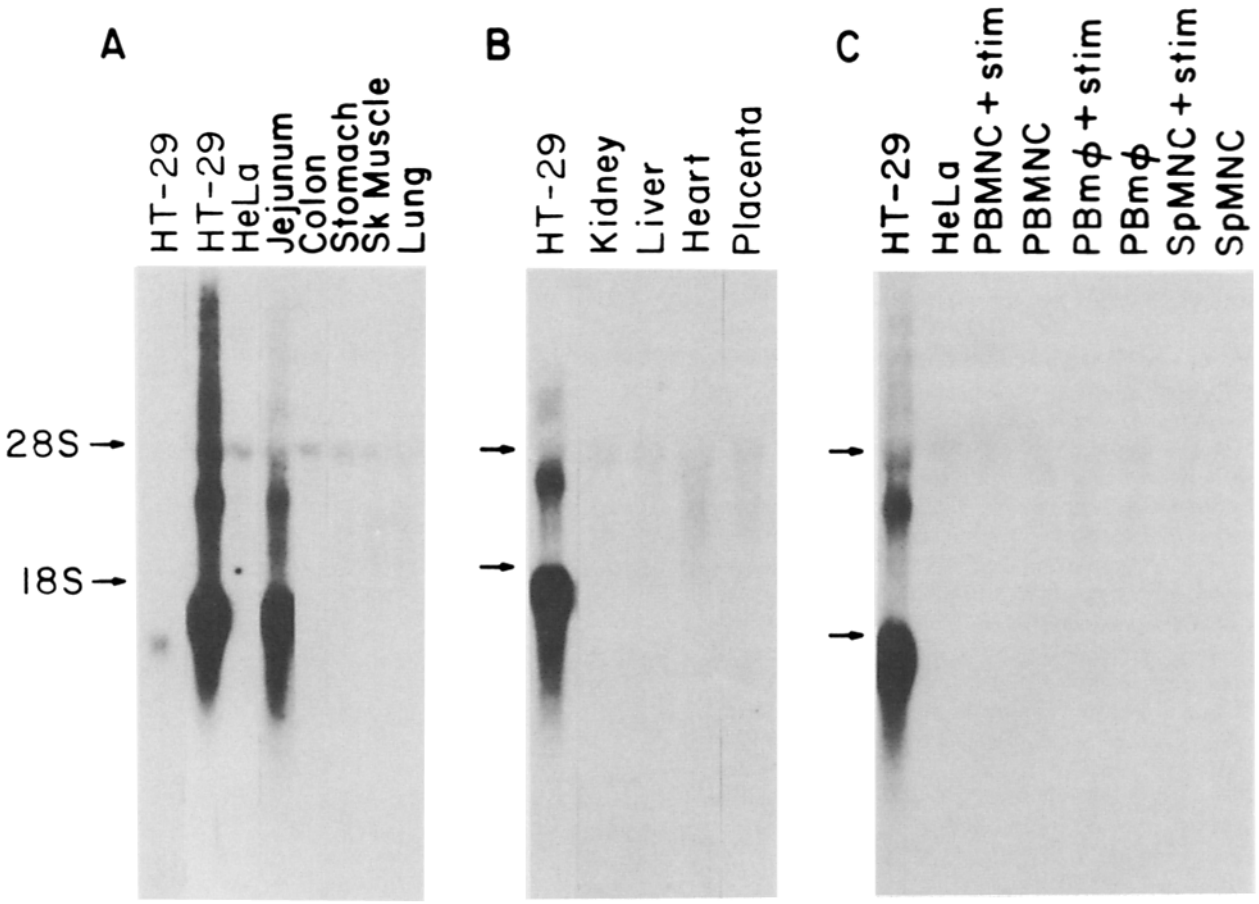


Figure 5. Surveys of ISA mRNA levels in adult human tissue RNAs and in cellular components of the immune system. Northern blots containing 5 μg of total cellular RNA (*A* and *B* and the HT-29/ino sample in *C*) or 10 μg of total cellular RNA (all the other samples in *C*) were probed with a full-length [^{32}P]labeled ISA cRNA probe as described in Materials and Methods. The exposure time of the autoradiographs = 6 d except for the left hand lane of panel *A* containing log phase HT-29/ino RNA which shows the same gel but exposed for only 3 h. *PBMNC*, peripheral blood mononuclear cells; *PBmφ*, peripheral blood macrophages; *SpMNC*, splenic mononuclear cells; + *stimulated*, cells treated with mitogen for 24 h before they were harvested. The faint band observed in all samples is due to hybridization of the labeled ISA cRNA probe with 28S rRNA despite the high stringency wash conditions employed.

some cultured cell lines (William et al., 1988; Violette et al., 1990). Therefore, we next defined the pattern of ISA expression in various human gut-derived cell lines to determine if it is regulated as a function of their growth and/or differentiation. Fig. 4 shows that ISA mRNA is readily detected in proliferating HT-29/ino cells (i.e., cells committed to differentiate). Postconfluent HT-29/ino cells that are expressing their differentiation program exhibit a modest (less than twofold) reduction in the concentration of ISA mRNA relative to that observed in exponentially growing cells. HT-29/glc cells do not contain detectable levels of this mRNA regardless of the growth phase sampled (Fig. 4). Since very few (<5%) HT-29/glc cells differentiate when they become confluent (Pinto et al., 1982; Lesuffleur et al., 1990), it appears that accumulation of ISA mRNA is restricted to committed or differentiated HT-29 cells.

CaCo-2 (Fogh et al., 1977) and SW480 (Leibowitz et al., 1976) are two other cell lines derived from human colon adenocarcinomas. CaCo-2 cells will differentiate spontaneously in culture after they become confluent, even in the presence of glucose (Pinto et al., 1983). Unlike HT-29 cells, they will only express an enterocyte-like phenotype. SW480 cells do not differentiate in culture regardless of the growth conditions employed (Chantret et al., 1988). ISA mRNA was not detectable in proliferating or postconfluent CaCo-2 cells (Fig. 4). In addition, it was not detectable in proliferating HeLa cells or in growing or postconfluent SW480 cells whether cultured in glucose or inosine containing media (Fig. 4 and data not shown). These observations allow us to conclude that accumulation of ISA mRNA is not simply a cellular response to being cultured in the absence of glucose. The data are compatible with the notion that ISA could play a role in regulating the growth and/or differentiation of some, but not all, intestinal epithelial cell lines.

The Northern blot shown in Fig. 4 was subsequently reprobed with an oligonucleotide (5′-CCAGGCCTGCTTGAGGAATTCTGATACCATTGCCAT-3′) complementary to the region of human annexin I mRNA encoding residues 1–18 of its primary translation product (Wallner et al., 1986). Annexin I mRNA was detected in HT-29, CaCo-2, and SW480 cells, although its steady-state levels varied several fold as a function of cell type, growth phase, and culture conditions (data not shown). Annexin I mRNA was also detected in HeLa/glc and in a human hepatocyte-like cell line (HepG2).

Thus, annexin I gene expression is not restricted to intestinal epithelial cell lines nor can its expression be correlated with their ability to differentiate. The presence of annexin I mRNA in a wide variety of cell lines and tissues (Pepinsky et al., 1986; Huang et al., 1986) is typical for annexins (Gould et al., 1984; Greenberg et al., 1984; Shadle et al., 1985; De et al., 1986; Pepinsky et al., 1986; Kaetzel et al., 1989). ISA appears to represent an exception to this "rule".

ISA mRNA Is Not Detectable in a Variety of extra-intestinal Adult Human Tissues

Although our initial survey of six human tissue RNAs (stomach, small intestine, kidney, liver, heart, placenta) indicated that ISA mRNA was limited to the intestine (Fig. 5), we extended these studies to other cellular populations to further assess its distribution in adult humans. Quantitative RNA blot hybridization analysis indicated that the steady-state level of ISA mRNA in exponentially growing HT-29/ino cells is ~15 pg/μg total cellular RNA. RNA prepared from skeletal muscle, lung, fibroblasts, peripheral blood macrophages, peripheral blood, or splenic mononuclear cells also failed to react with ISA cDNA (Fig. 5, *A–C*). These mononuclear cells express high levels of annexin I (Fava et al., 1989 and data not shown), but do not contain detectable ISA mRNA even if RNA is prepared 24 h after mitogenic stimulation with phytohemagglutinin plus phorbolmyristic acid (mononuclear cells) or with *S. aureus* Cowan I (macrophages) (Fig. 5 *C*).

Expression of ISA Appears to Be Temporally and Spatially Regulated in the Human Intestinal Epithelium

To further define ISA gene expression in the human intestine, we analyzed a series of samples of duodenum, jejunum, ileum, and colon from each of three organ donors aged 32, 36, and 72 yr. Total cellular RNA was isolated from two separate portions of each full thickness intestinal segment from each donor. Fig. 6 shows that the steady-state levels of ISA mRNA varies dramatically along the duodenal-to-colonic axis. Highest levels are encountered in the jejunum (~4 pg/μg total cellular RNA, see Fig. 5). Levels in the duodenum and ileum are two- to fourfold lower while those in the distal colon are ~90 fold lower. Remarkably, there was little variation in the absolute levels of ISA mRNA within each comparable segment harvested from each of the three organ donors (Fig. 6, *A* and *B*).

The liver fatty acid binding protein (L-FABP) gene is expressed in hepatocytes and enterocytes. It has been used as a model for mapping *cis*-acting elements that regulate cellular and geographic differentiation of the gut epithelium in the mouse (Sweetser et al., 1988*a*; Roth et al., 1990, 1991*b*; Roth and Gordon, 1990; Cohn et al., 1991; Rubin et al., 1991). Control experiments employing a human L-FABP cDNA (Lowe et al., 1985) revealed that the distribution of its mRNA along the duodenal-to-colonic axis was distinct from that of ISA mRNA, varying less than twofold between segments (Fig. 6, *A* and *B*). This pattern of accumulation of L-FABP mRNA is similar to the results of previous immunocytochemical surveys of its protein product in different regions of the human intestinal tract (Suzuki et al., 1988).

We were able to examine two samples of human fetal total

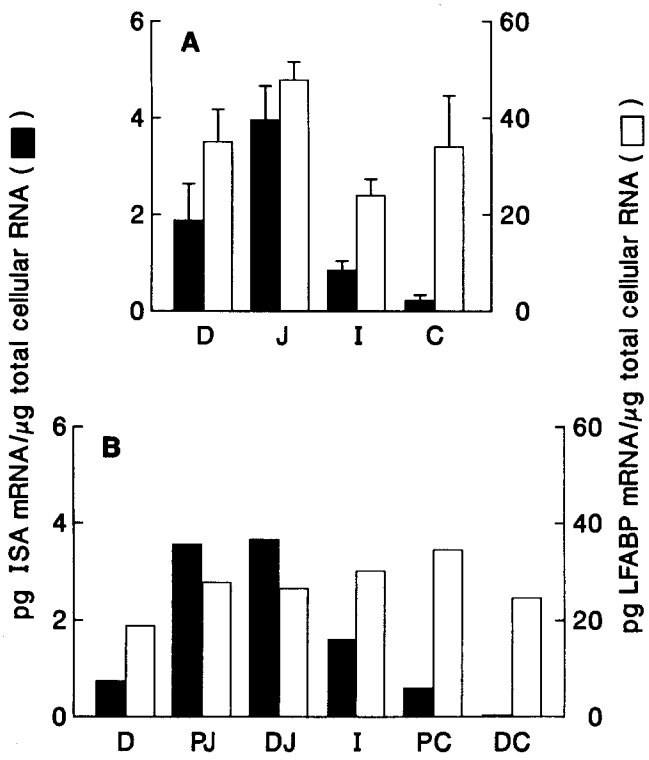
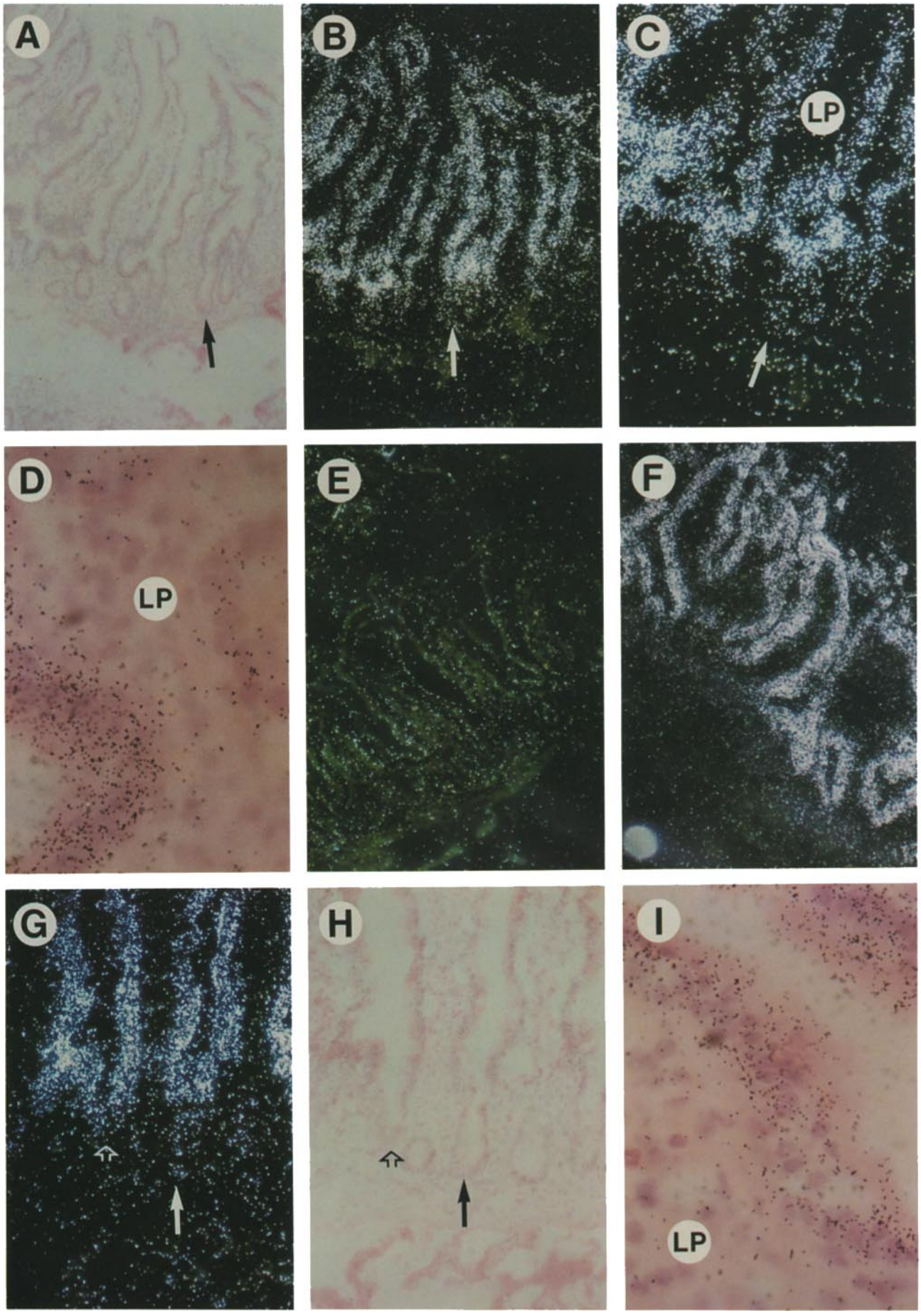


Figure 6. Distribution of ISA and L-FABP mRNAs along the duodenal to colonic axis of the adult human intestine. *A* represents the results of surveying two RNA samples prepared from each segment of two organ donors' intestines. Note that samples from the entire length of intestine from each of these donors was screened by quantitative RNA blot hybridization analysis as described in Materials and Methods. *B* shows the results of an analysis of a third organ donor whose duodenal-to-colonic axis was more extensively dissected. Single total cellular RNA samples were prepared from each of the indicated segments. *D*, duodenum; *PJ*, proximal jejunum; *J*, mid jejunum; *DJ*, distal jejunum; *I*, ileum; *PC*, proximal colon; *C*, mid colon; and *DC*, distal colon.

small intestine RNA. The level of ISA mRNA in 12.5-wk fetal gut was ~10-fold higher than in adult jejunum while its concentration in a sample of 15-wk intestine was similar to that in the adult (data not shown). It is intriguing to note that the relatively "elevated" level of ISA mRNA in 12.5-wk fetal gut occurs just at the completion of cytodifferentiation of its epithelium (this period spans the eighth through twelfth weeks of gestation; Dahlqvist and Lindberg, 1966; Moxey and Trier, 1978, 1979; Auricchio et al., 1981). While these observations are compatible with the notion that ISA is involved in intestinal epithelial cell growth and differentiation, it is important to exercise caution in interpreting the data since the range in ISA mRNA levels between individuals at each developmental stage could not be assessed. However, note that the steady-state levels of ISA mRNA in comparable regions of gut harvested from several adult individuals are quite similar (Fig. 6).

A series of in situ hybridization experiments was performed to determine if the ISA gene is transcribed in gut epithelial cells and, if so, whether its mRNA demonstrates variations in steady-state levels as cells differentiate during their translocation along the crypt-to-villus axis. A [^{35}S]-labeled cRNA probe complementary to nucleotides 1–156 of ISA mRNA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(Fig. 2) was used for these studies because of its lack of sequence identity with other human annexin mRNAs. Control Northern blot hybridizations employing this cRNA (labeled with ^{32}P) gave results that were identical to those shown in Figs. 4 and 5.

In the adult jejunum, epithelial cells located at the crypt/villus junction possess the highest levels of ISA mRNA with much lower intracellular concentrations noted in epithelial cells situated in the lower and mid thirds of the crypt (Fig. 7, *A–D*). Only modest variations in ISA mRNA levels were noted as cells migrate from the base of the villus to the apical extrusion zone. ISA mRNA was not detectable in the nonepithelial cellular populations of the lamina propria, in smooth muscle myocytes, or in components of the enteric nervous system (Fig. 7, *A–D*). No signal was observed in control jejunal sections incubated with an in vitro transcribed, radiolabeled mRNA probe derived from nucleotides 1-156 of ISA cDNA (Fig. 7 *E*). The pattern of ISA mRNA accumulation differs from that of L-FABP mRNA which is present in villus-associated enterocytes but not in proliferating, undifferentiated crypt cellular populations (Fig. 7, *F–I*).

The colon lacks villi. Studies in chimeric mice indicate that differentiating colonic epithelial cells migrate up from the crypt to a surface cuff of epithelium that surrounds the crypt orifice (Schmidt et al., 1985*a*,*b*). This hexagonal shaped cuff represents the colonic homologue of the small intestinal villus. Surveys of sections obtained from adult human colon indicated that ISA mRNA is present at very low levels in epithelial cells situated in the lower portions of the crypt. It is not detectable in the differentiated colonocytes located in the surface cuff (data not shown). These data are consistent with the results of our Northern blot studies (Fig. 6). They contrast with colonic expression of L-FABP mRNA which first becomes detectable in mid-crypt–associated epithelial cells and is expressed at high levels as cells differentiate during their migration to the surface epithelial cuff (data not shown).

Immunologic Studies of ISA Accumulation/Targeting in Cultured Human Intestinal Cell Lines

Polyclonal rabbit antibodies were raised against residues 2–18 of ISA to further examine this protein's cellular origins and intracellular destination. Its amino-terminal domain was selected for generation of antibodies because of the lack of sequence similarity with other annexins (see Fig. 3 *B* and below). Western blot studies using antipeptide sera prepared from two different rabbits revealed two immunoreactive polypeptides with calculated masses of 36 and 42 kD in denatured, reduced protein extracts of proliferating/undifferentiated and postconfluent/differentiated HT-29/ino cells (Fig. 8 *A*). Similar-size proteins were detected in Western blots of jejunal, duodenal and colonic homogenates (Fig. 8 *B*). Both antisera revealed that the relative steady-state concentrations of the two immunoreactive polypeptides in these intestinal segments roughly parallel the steady-state levels of ISA mRNA (compare Fig. 8 *B* with Fig. 6, *A* and *B*). As in the case of ISA mRNA, the polypeptides were not detectable (with either antisera) in homogenates prepared from growing or confluent HeLa/glc cells, human placenta, spleen, liver, or lung (Fig. 8, *A* and *B*). Moreover, these bands were not observed when duplicate blots were probed with preimmune serum (data not shown) or when the immune sera were preadsorbed with peptide (e.g., Fig. 8 *C*). It is important to note that lung, liver, spleen, and placenta contain abundant quantities of various annexins (Gould et al., 1984; Greenberg et al., 1984; Shadle et al., 1985; De et al., 1986; Pepinsky et al., 1988; Kaetzel et al., 1989). Together these results suggest that our antisera recognize ISA and are specific for this annexin. The calculated size of the smaller of the two reactive species was identical to that predicted for the primary translation product of ISA mRNA. The structural relationship between this species and the larger immunoreactive polypeptide remains to be determined, although they share a common amino terminal modification (see below).

The anti-ISA sera was subsequently incubated with methanol-fixed cultured cells. Fig. 9 (*A* and *B*) shows ISA is present in most proliferating HT-29/ino cells. Staining was not observed with preimmune serum or if the antiserum was preincubated with the octadecapeptide (data not shown). A striking finding was the high concentration of immunoreactive protein at or near the plasma membrane.

Growing or confluent HeLa/glc cells, which contain no

Figure 7. In situ hybridization studies of ISA and L-FABP mRNA accumulation along the crypt-to-villus axis of the adult human jejunum. Two [^{35}S]labeled cRNA probes were used for these studies: (*a*) a 156 nucleotide ISA fragment (spanning nucleotides 1–156 in Fig. 2) obtained by runoff transcription of a recombinant, EcoRI digested pBluescript/KS plasmid with T7 RNA polymerase; (*b*) a 418 nucleotide human L-FABP fragment obtained after BamHI digestion of pBluescript/KS containing a 418-bp PstI DNA derived from pHF658 (Lowe et al., 1985) and in vitro runoff transcription with T3 RNA polymerase. (*A*) A section of human jejunum that had been incubated with the ISA cRNA probe, stained with hematoxylin and eosin, and viewed under bright field illumination (100×); (*B*) the same section as shown in A but viewed under darkfield to "enhance" the signal from exposed silver grains. The arrows in *A* and *B* point to the base of a crypt. Exposure time = 14 d; (*C*) higher power view of B illustrating low level accumulation of ISA mRNA in crypt associated epithelial cells (*arrow*). The lamina propria (*LP*) contains no detectable signal over background (200×); (*D*) higher power bright field view of *A* showing villus-associated epithelial cells containing ISA mRNA (1,000×). Again, cellular populations in the lamina propria do not appear to express this annexin; (*E*) section taken from the same jejunal segment but probed with a control [^{35}S]labeled ISA mRNA (rather than cRNA) probe of identical specific activity and length. No signal above background is seen in this darkfield view (exposure time = 14 d, 100×); (*F*) darkfield view of a section of human jejunum after incubation with a human L-FABP cRNA probe. Note that accumulation of this mRNA is confined to villus-associated enterocytes. Exposure time = 6 d (100×); (*G*) higher power view of section shown in *F* demonstrating that the L-FABP is transcriptionally activated as cells traverse the crypt-villus junction (indicated by the *open arrows*, 200×); (*H*) bright field view of *G*. The closed arrow in this panel and in *G* point to the base of the crypt; (*I*) higher power view of jejunal villus-associated epithelium shown in H (1,000×). Note the absence of signal in the lamina propria (*LP*). Control experiments using a [^{35}S]labeled L-FABP mRNA probe of equal specific activity failed to produce any signal above background in adjacent jejunal sections (data not shown).

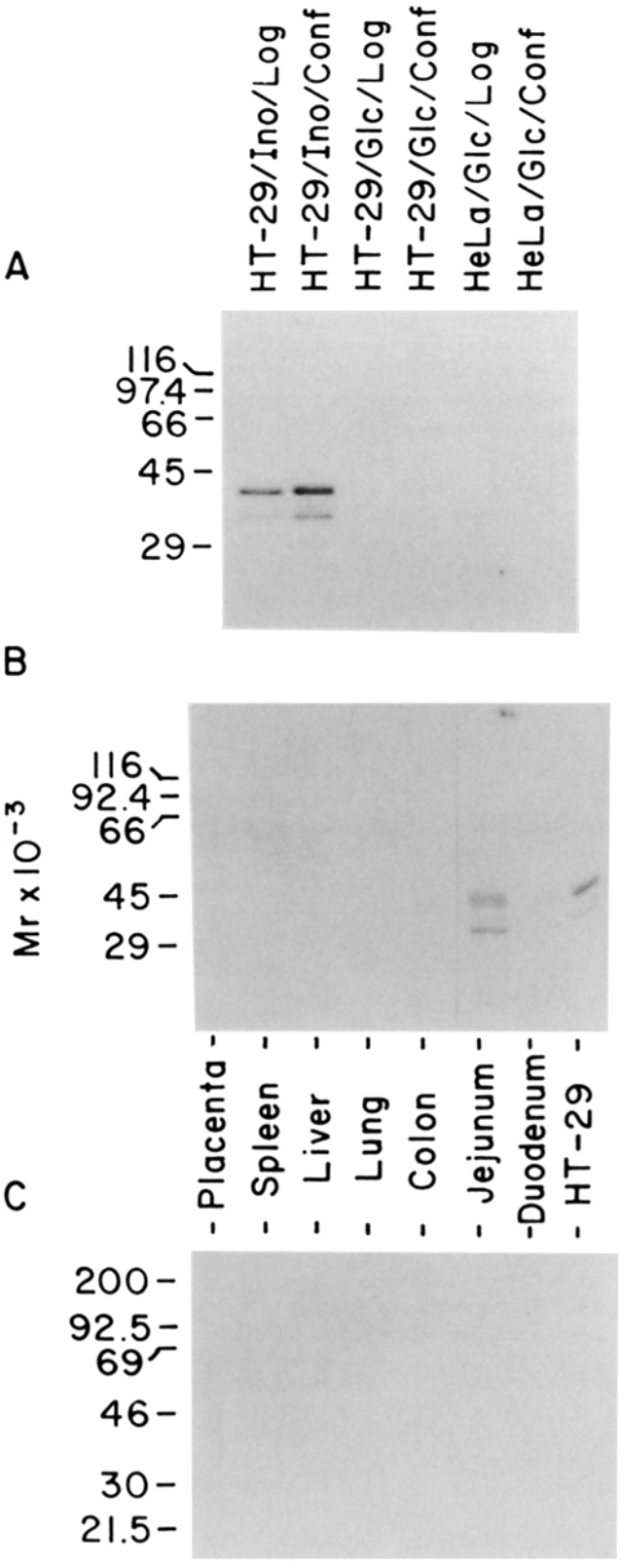

Figure 8. Western blot surveys of HT-9, HeLa, and adult human tissue homogenates for the presence of ISA. (*A*) Analysis of total cell lysates (10 μg protein/lane). Samples were reduced, denatured, fractionated by electrophoresis through 10% polyacrylamide gels containing SDS (0.1%), and transferred to nitrocellulose before being probed with a rabbit polyclonal antisera directed against residues 2–18 of the primary translation product of ISA mRNA. The antibody was diluted 1:10,000. Antigen–antibody complexes were visualized with [^{125}I]protein A. The washed blot was subjected to autoradiography for 3 d. Note that faint 36- and 42-kD bands were observed in HT-29/Glc cell lysates. Proliferating HT-29/ino cells contain slightly higher steady state levels of ISA mRNA than postconfluent cells (Fig. 4). The data shown in this panel indicate that the levels of ISA are comparable in the two cellular populations, raising the possibility that either translational control mechanisms contribute to the regulation of ISA expression or that the stability of this protein changes after HT-29/ino cells become confluent. (*B*) Western blot of proteins contained in human tissue homogenates. Each lane contains 20 μg of protein. The blot was probed with the same antisera as used in *A* but now diluted 1:2,000. Exposure time of the autoradiograph = 16 h. No immunoreactive protein was detected in placenta, spleen, liver, and lung even after a 3-d exposure. (*C*) Duplicate of the blot shown in *B* but incubated with antisera that had been preadsorbed with the octadecapeptide. The dilution of the sera was identical to that employed in *B*. Exposure time = 16 h. N. B. no signal was seen after a 3-d exposure (data not shown).

detectable levels of ISA mRNA (Figs. 4 and 5) or protein (Fig. 8) are not stained with the antisera. Less than 2% of HT-29/glc cells were ISA-positive. The few cells that were positive exhibited a pattern of peripheral membrane staining similar to HT-29/ino cells (data not shown). This is consistent with the observation that very few HT-29/glc cells differentiate in vitro and with the results obtained from Northern (Fig. 4) and Western (Fig. 8 *A*) blot studies.

Immunocytochemical Studies of ISA Expression in Adult Human Jejunum and Colon

Fig. 10 *A* demonstrates that the apical brush border of polarized, villus-associated, jejunal enterocytes is intensely stained by the antipeptide serum. The basolateral membrane is less prominently stained. Faint cytoplasmic staining can also be appreciated. Epithelial cells located in the crypt also contain ISA (Fig. 10 *B*), consistent with the distribution of its mRNA along the crypt-to-villus axis (Fig. 7). Even in these undifferentiated cells, ISA appears to be sorted to the plasma membrane with higher concentrations present at the apical surface. No staining was noted in cellular populations that reside in the lamina propria. Control experiments using preimmune sera (Fig. 10 *C*) or peptide-adsorbed immune sera (Fig. 10 *D*) establish the specificity of the reaction observed between our antibodies and ISA.

The pattern of ISA expression in the jejunum differs from that observed for sucrase-isomaltase (Fig. 10 *F*) or aminopeptidase N (data not shown). In differentiated enterocytes, these brush border hydrolases are confined to the apical membrane; no basolateral staining can be appreciated. Reduced levels of immunoreactive protein are detectable at the apical region of mid and upper crypt epithelial cell populations. The pattern of ISA accumulation also differs from (*a*) the distribution of mucus in differentiated jejunal goblet cells (Fig. 10 *E*) and (*b*) the intracellular distribution of annexin II and IV in enterocytes (annexin II is restricted to the terminal web region of the cell's apical border while annexin IV is only associated with the basolateral membrane [Gould et al., 1984; Greenberg et al., 1984; Massey et al., 1991]).

ISA is associated with the plasma membrane of epithelial

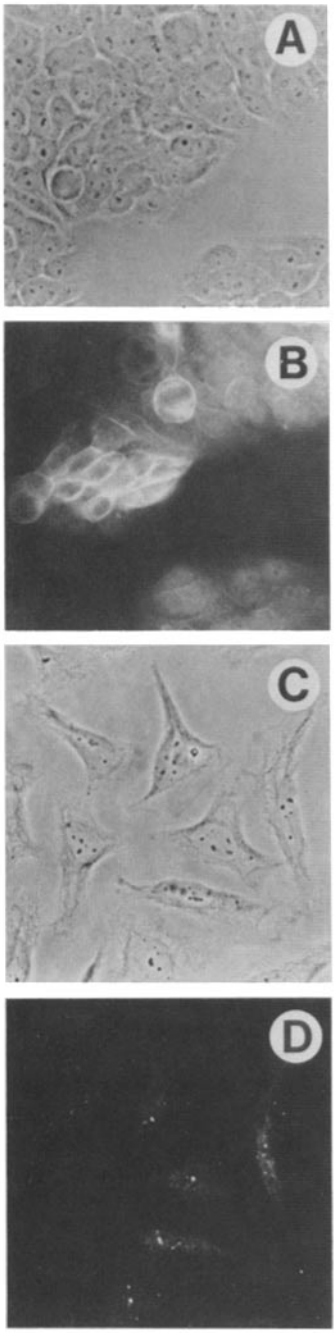

Figure 9. Immunocytochemical studies of HT-29/ino and HeLa/glc cells using an antipeptide antibody against the amino-terminal domain of ISA. HT-29/ino (*A* and *B*) or HeLa/glc (*C* and *D*) were plated on glass coverslips. 3 d later, cells were washed with PBS, fixed in MeOH, and incubated with the rabbit polyclonal antioctadecapeptide serum followed by FITC-conjugated goat antirabbit sera. (*A*) Phase-contrast view of HT-29/ino cells; (*B*) fluorescence microscopy of the same field as that shown in *A*. Note the intense staining seen at the periphery of these cells (500×). (*C*) HeLa/glc cells seen under phase; (*D*) fluorescence microscopy of the same cells as shown in *C* (500×).

cells located in colonic crypts (Fig. 10 *G*). In contrast to jejunal villus-associated enterocytes, ISA is not expressed in terminally differentiated colonocytes (Fig. 10 *G*). As in the small bowel, the lamina propria does not contain immunoreactive ISA.

ISA Is a N-Myristoylprotein

ISA contains a Gly at position 2. None of the other known annexins begins with Met-Gly (Fig. 3 *B*). Inspection of residues 2–9 of ISA suggested that it may be a substrate for myristoylCoA:protein *N*-myristoyltransferase (NMT; see Towler et al., 1988*b* for a summary of the peptide substrate specificity of this acyltransferase). This enzyme catalyzes the cotranslational attachment of myristate, a rare 14 carbon saturated fatty acid, to the amino-terminal Gly residues of a number of cellular and viral proteins (reviewed in Towler et al., 1988*b*; James and Olson, 1990; Gordon et al., 1991). These proteins have diverse functions and diverse intracellular destinations. They include serine/threonine and tyrosine kinases, protein phosphatases, as well as components of transmembrane signal transducing pathways, e.g., α subunits of heterotrimeric G proteins (Mumby et al., 1990; Linder et al., 1991). *N*-myristoylation can have a profound effect on targeting: Gly2→Ala mutagenesis of $p60^{v\text{-}src}$ blocks *N*-myristoylation, prevents its stable association with a plasma membrane–associated 32-kD receptor (Resh, 1989; Resh and Ling 1990), and blocks its ability to transform cells even though tyrosine kinase activity is not affected (Cross et al., 1984; Kamps et al., 1985; Buss et al., 1986). If the protein encoded by ISA mRNA were subjected to this form of acylation, it would be unique among annexins. Annexins I, II, and V are substrates for another amino-terminal processing enzyme-*N*-acetyltransferase (Biemann and Scoble, 1987; Glenney et al., 1986; Funakoski et al., 1987; also see Weber et al., 1987 for evidence that other annexins may have acetylated amino termini).

We used an in vitro NMT assay containing a myristoyl-CoA generating system, purified *S. cerevisiae* NMT, and an octapeptide representing residues 2–9 of ISA to determine whether this protein was likely to be a substrate for the acyltransferase. This in vitro system has been used to establish that the mammalian and yeast enzymes share a remarkable degree of similarity in their acylCoA and peptide substrate specificities (Towler et al., 1988*a*). The yeast NMT was used for these studies because large quantities of pure enzyme have been obtained from *E. coli* strains containing suitably constructed expression vector (Duronio et al., 1990*a*,*b*, 1991; Rudnick et al., 1990). As shown in Table I, the octapeptide GNRHAKAS-NH_2 has kinetic properties that are comparable to those of octapeptides representing residues 2–9 of other known mammalian *N*-myristoylproteins.

Metabolic labeling studies provided additional evidence that ISA is *N*-myristoylated. Incubation of HT-29/ino cells with [^{35}S]methionine and subsequent immunoprecipitation of cellular lysates with antipeptide sera resulted in recovery of two principal [^{35}S]polypeptides. The relative intensities of the labeled 36- and 42-kD bands (Fig. 11, lane *5*) were similar to the intensities of the 36- and 42-kD polypeptides noted in the Western blot shown in Fig. 8. The immunoprecipitated proteins are not abundant—examination of total cellular, [^{35}S]methionine-labeled proteins separated by single dimension SDS-PAGE failed to reveal prominent 36- or 42-kD species at the conclusion of the 4-h labeling period (Fig. 11, compare lanes *1–3* with lane *5*). The proteins were not detectable in HT-29/glc or HeLa cell lysates (Fig. 11, lanes *4* and *6*).

When HT-29/ino cells were incubated with [^{3}H]myristate for 4 h, 36- and 42-kD labeled polypeptides could also be recovered by the antipeptide sera (Fig. 11, lane *8*). Proteins of comparable size were not detected by the antibodies in HeLa/glc or HT-29/glc cell lysates prepared after a 4-h labeling with [^{3}H]myristate (Fig. 11, lanes *7* and *9*). In addition, a 4-h incubation of HT-29/ino cells with [^{3}H]palmitate failed to produce labeling of immunoreactive 36- or 42-kD

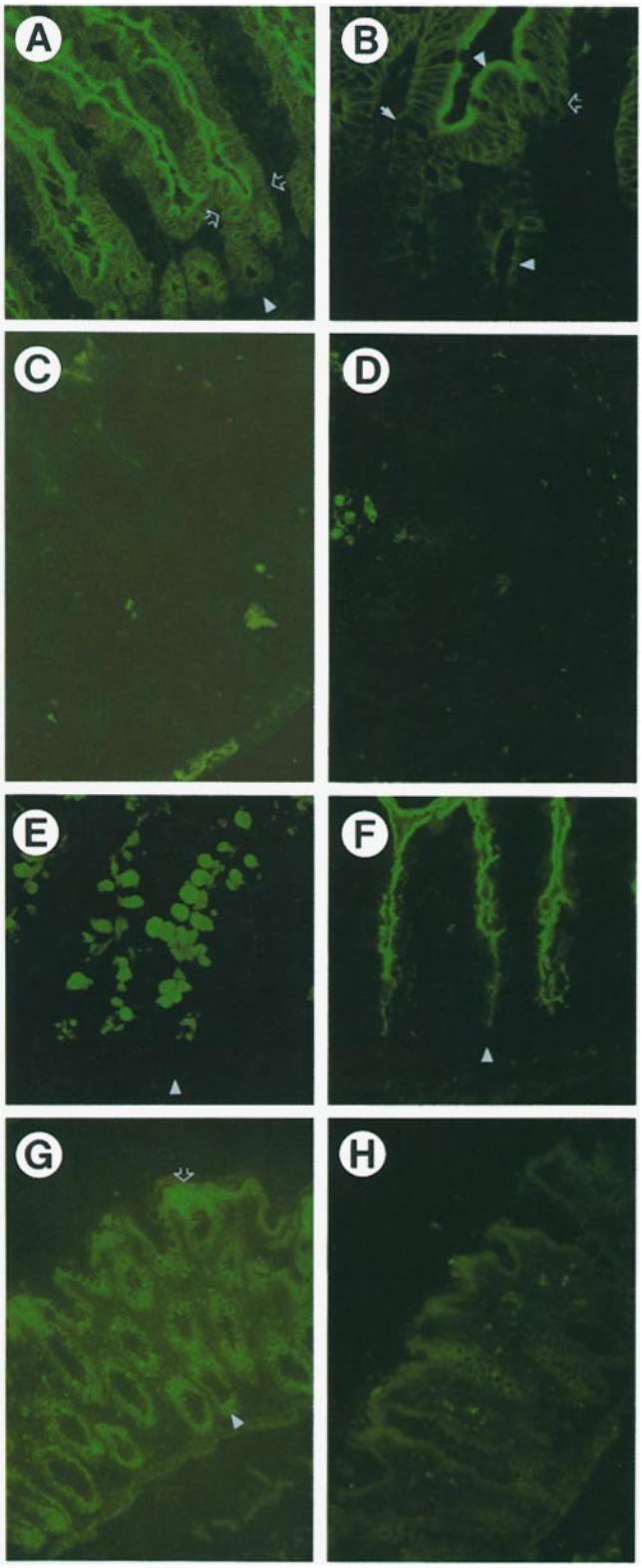

Figure 10. Immunocytochemical localization of ISA in human jejunal and colonic epithelial cells. (*A*) Section of adult jejunum incubated with the rabbit polyclonal antipeptide sera. Immunoreactive ISA was visualized using FTIC-conjugated goat antirabbit sera. The open arrows point to the crypt-villus junction while the closed arrowhead indicates the base of the crypt. The luminal surface of villus enterocytes stains intensely for ISA. Fainter staining of crypt epithelial cell membranes can be appreciated (200×). (*B*) Higher power view of *A* showing ISA associated with the apical membrane of villus-associated enterocytes (*closed arrowhead* at the top of *B*). Basolateral membrane staining is less intense. The crypt epithelial cells contain immunoreactive ISA but at lower levels than villus enterocytes and with less prominent polarization to the apical membrane (*arrowhead* at the bottom of the photo, 400×). The goblet cell denoted by the closed arrow does not appear to contain detectable levels of immunoreactive protein. (*C*) Jejunal section incubated with anti-ISA sera that had been pre-adsorbed with the peptide (400×). (*D*) Jejunal section incubated with preimmune sera (400×). (*E*) Section of jejunum from the same individual as shown in *A–D* incubated with a mouse monoclonal antibody directed against human mucins. Antigen-antibody complexes were visualized using FTIC-conjugated goat anti-mouse IgG antibodies. Mucus synthesized in villus goblet cells is readily visualized. The crypt base is denoted by the closed arrowhead (200×). (*F*) Jejunal section stained with a mouse monoclonal antibody directed against sucrase-isomaltase. This brush border hydrolase is present at high levels at the apical membrane of villus-associated enterocytes and at lower levels at the apical side of crypt epithelial cells (200×). (*G*) Section of colon incubated with the anti-ISA sera. Epithelial cells in colonic crypts contain immunoreactive protein. The intracellular distribution of ISA is less polarized than observed in jejunal enterocytes. The open arrow points to the surface epithelial cuff that surrounds a crypt orifice. Staining of these cells appears to be nonspecific since they are stained with preimmune sera (*H*, 100×).

Table I. In Vitro NMT Assay Using Octapeptides Derived from Residues 2–9 of ISA and Several Known N-myristoylproteins

Peptide sequence	K_m	V_m
	μM	%
GNRHAKAS (human intestine-specific annexin)	240	4
GNAAAARR (catalytic subunit of bovine cAMP-dependent protein kinase A)	40	100*
GSSKSKPK ($p60^{v\text{-}src}$)	40	43‡
GARASVLS (HIV-1 $Pr55^{gag}$)	2	34‡
GNEASYPL (calcineurin b)	2,300	55‡

* V_m data have been expressed as a percentage of that observed with the PK-A octapeptide (6.5×10^4 pmol/min/mg NMT.
‡ Taken from Towler et al. (1988*b*).
Conditions employed for the coupled in vitro NMT assay are described elsewhere (Rudnick et al., 1990). Peptide K_m was determined in the presence of 0.23 μM myristoylCoA using *S. cerevisiae* NMT. Experiments were performed three times, each time in duplicate. Averaged values are shown.

proteins (Fig. 11, lane *11*) even though the specific activity of this radiolabeled C16:0 fatty acid was identical to that of [^{3}H]myristate.

To verify that the [^{3}H]myristate had been covalently attached to ISA via an amide linkage, SDS-polyacrylamide gels containing labeled cellular proteins were treated with 1 M hydroxylamine (pH 10) or 1 M Tris, pH 10, before fluorography. Hydroxylamine, but not Tris, will hydrolyze thioester and oxyester bonds but not amide linkages. The intensities of the tritiated 36- and 42-kD polypeptides were identical in gels treated with hydroxylamine (Fig. 11, lane *8*) or Tris

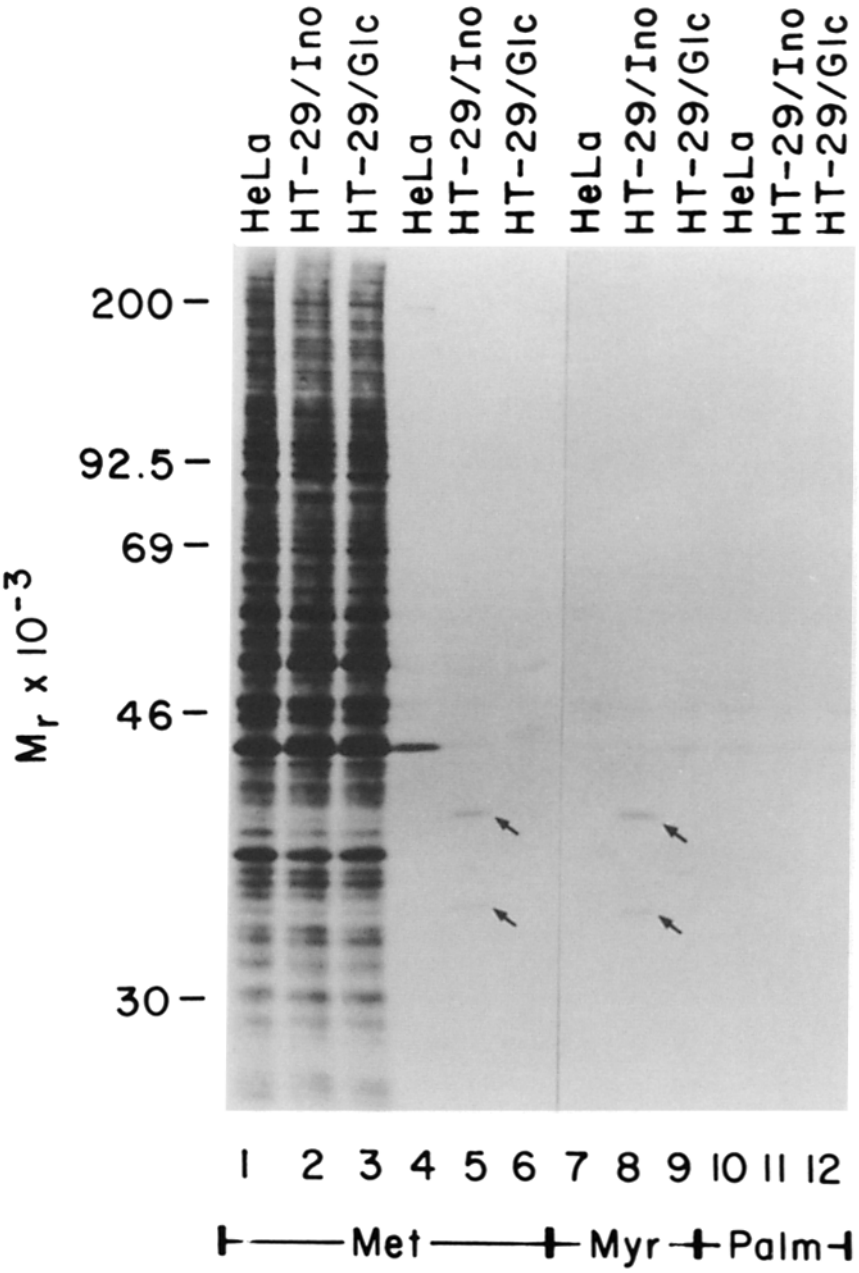

Figure 11. Metabolic labeling of proliferating HT-29/ino cells with [^{3}H]myristate, [^{3}H]palmitate, or [^{35}S]methionine. Cell lines were labeled with [^{35}S]methionine (lanes *1–6*), [^{3}H]myristate (lanes *7–9*), or [^{3}H]palmitate (lanes *10–12*) as described in Materials and Methods. Whole cell lysates (lanes *1–3*) or proteins recovered by immunoprecipitation with anti-ISA sera (lanes *4–12*) were reduced, denatured, and separated by SDS-PAGE. Duplicate gels were fixed and then treated with either 1 M hydroxylamine (pH 10) or 1 M Tris (pH 10) for 6 h at room temperature. The hydroxylamine-treated gel is shown. The following amounts of cellular protein were used to generate the data shown: lanes *1–3*, 1.7 μg; lanes *4–6*, 133 μg (before immunoprecipitation); lanes *7–12*, 267 μg (before immunoprecipitation). Lanes *1–6* and *7–14* were exposed for 14 h and 17 days, respectively. Note that (*a*) the intensity of [^{3}H]palmitate labeled bands in whole cell lysates was markedly reduced after treatment with hydroxylamine compared to Tris (data not shown; see text) and (*b*) that in the Tris-treated gel, no radiolabeled proteins were detected after immunoprecipitation of [^{3}H]-palmitate-labeled HT-29/ino cell lysates (data not shown).

(data not shown). In contrast, control experiments demonstrated total cellular HT-29/ino cellular proteins labeled with [^{3}H]palmitate all had hydroxylamine-sensitive linkages (data not shown).

Taken together, these results suggest that ISA is a substrate for human as well as *S. cerevisiae* myristoylCoA:protein *N*-myristoyltransferase. The fact that both the 36- and 42-kD polypeptides are *N*-myristoylated with comparable efficiencies indicates that their amino-terminal sequences are either very similar or identical (as defined by their interactions with NMT). The dependence of ISA on its myristoyl moiety for targeting to the enterocyte's basolateral and apical membranes can be assessed by mutagenesis of its Gly^2 codon and/or by metabolic labeling studies of HT-29/ino cells with myristic acid analogues containing oxygen, or sulfur, for methylene substitutions that produce marked reductions in hydrophobicity yet still allow the analogue CoA to remain substrates for NMT (Heuckeroth et al., 1988; Heuckeroth and Gordon, 1989; Bryant et al., 1991; Kishore et al., 1991).

In summary, ISA appears to be distinguished from other annexins by its extraordinarily limited cellular distribution and by its modification by myristoylCoA:protein *N*-myristoyltransferase. ISA's precise role, if any, in regulating intestinal epithelial cell proliferation and/or differentiation remains to be established. However, its patterns of expression (*a*) in cultured intestinal cell lines under various culture conditions and (*b*) along the crypt-to-villus axis of human intestine are consistent with such a proposed role. An analysis of its patterns of accumulation under conditions where the intestinal epithelium has lost its balance of proliferation and differentiation (e.g., during colorectal tumorigenesis) may offer an opportunity for further assessing its biological function as may transfection of HT-29, CaCo-2, and SW480 cells cultured under various conditions. Finally, the method used

to obtain ISA from our HT-29 cDNA library should be generally useful in identifying other sequences that may modulate the delicate balance between proliferation and differentiation which is maintained in the dynamic, perpetually renewing, human gut epithelium.

We thank David Rudnick for his assistance with the NMT assay; Daniel Podolsky, Andrea Quaroni, and Hans-Peter Hauri for mAbs; Steven Adams for peptide synthesis; Hazel Sive for suggestions concerning the subtraction hybridization protocols; Andrew Lassar for encouragement concerning the feasibility of the cloning strategy; Deborah Rubin for advice about in situ hybridizations; and Kevin Roth for helpful directions on immunocytochemical methods.

This work was supported by National Institutes of Health grant DK30292.

Received for publication 15 July 1991 and in revised form 19 September 1991.

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