

# (Na<sup>+</sup> + K<sup>+</sup>)-ATPase and Plasma Membrane Polarity of Intestinal Epithelial Cells: Presence of a Brush Border Antigen in the Distal Large Intestine that Is Immunologically Related to $\beta$ Subunit

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**Abstract.** The previously produced monoclonal antibody IEC 1/48 against cultured rat intestinal crypt cells (Quaroni, A., and K. J. Isselbacher. 1981. *J. Natl. Cancer Inst.* 67:1353-1362) was extensively characterized and found to be directed against the  $\beta$  subunit of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase as assessed by immunological and enzymatic criteria. Under nondenaturing conditions the antibody precipitated the  $\alpha$ - $\beta$  enzyme complex (98,000 and 48,000 *M.*). This probe, together with the monoclonal antibody C 62.4 against the  $\alpha$  subunit (Kashgarian, M., D. Biemesderfer, M. Caplan, and B. Forbush. 1985. *Kidney Int.* 28:899-913), was used to localize (Na<sup>+</sup> + K<sup>+</sup>)-ATPase in epithelial cells along the rat intestinal tract by immunofluorescence and immunoelectron microscopy. Both antibodies exclusively labeled the basolateral

membrane of small intestine and proximal colon epithelial cells. However, in the distal colon, IEC 1/48, but not C 62.4, also labeled the brush border membrane. The cross-reacting  $\beta$ -subunit-like antigen on the apical cell pole was tightly associated with isolated brush borders but was apparently devoid of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity. Subcellular fractionation of colonocytes in conjunction with limited proteolysis and surface radioiodination of intestinal segments suggested that the cross-reacting antigen in the brush border may be very similar to the  $\beta$  subunit. The results support the notion that in the small intestine and proximal colon the enzyme subunits are exclusively targeted to the basolateral membrane while in the distal colon nonassembled  $\beta$  subunit or a  $\beta$ -subunit-like protein is also transported to the apical cell pole.

THE (Na<sup>+</sup> + K<sup>+</sup>)-ATPase is a heterodimeric surface membrane protein complex that is composed of a catalytic 100-kD  $\alpha$  subunit and a 50-kD  $\beta$  subunit of unknown function (for reviews see Cantley, 1981; Jørgensen, 1982, 1986; Rossier, 1984; Glynn, 1985). This enzyme catalyzes the transmembrane exchange of Na<sup>+</sup> ions for K<sup>+</sup> ions, a process requiring ATP. Biochemical studies and the amino acid sequence deduced from complementary DNA suggest that the catalytic  $\alpha$  subunit spans the bilayer several times (Shull et al., 1985; Kawakami et al., 1985; Ovchinnikov et al., 1986, 1988; Chehab, 1987). A binding site for cardiac glycosides, such as ouabain, is located on the extracytoplasmic side while both the ATP-binding site and the phosphorylation site are located on the cytoplasmic side of the  $\alpha$  subunit. Contrary to the  $\alpha$  subunit which appears to lack covalently bound carbohydrates, the  $\beta$  subunit is a glycosylated transmembrane protein whose principal mass protrudes into the intercellular space (Girardet et al., 1983; Noguchi et al., 1986; Shull et al., 1986; Ovchinnikov et al., 1986; Brown et al., 1987).

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The two enzyme subunits are synthesized independently from separate mRNA species (Geering et al., 1985). Some studies have suggested that the  $\alpha$  subunit might be synthesized on free polysomes before its posttranslational assembly with the lipid bilayer, a process that may be mediated by the cotranslationally inserted  $\beta$  subunit (Sabatini et al., 1982; Hiatt et al., 1984). However, most authors now agree that both the  $\beta$  and  $\alpha$  subunits are membrane associated during their synthesis (Geering et al., 1985) and that subunit assembly occurs very rapidly (Fambrough and Bayne, 1983; Tamkun and Fambrough, 1986).

There is little doubt that in polarized epithelial cells functionally active (Na<sup>+</sup> + K<sup>+</sup>)-ATPase must be localized in the basolateral membrane domain of the cell surface under physiological conditions that are characterized by a luminal-to-serosal transport of Na<sup>+</sup> (Schultz, 1981; Ernst and Schreiber, 1981). However, immunolocalization studies with polyclonal or even monoclonal antibodies have led to conflicting results. While a majority of studies confirmed the principal basolateral localization of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase (Fambrough and Bayne, 1983; Kashgarian et al., 1985; Gerard et al., 1985; Gorvel et al., 1983; Yamamoto et al., 1984; Almers and Stirling, 1984), some authors have found opposite localiza-

tions of  $\alpha$ -subunit antigenic determinants: i.e., in the apical canalicular surface of liver cells (Schenk and Leffert, 1983; Takemura et al., 1984) or in association both with the basolateral and apical cell surface membrane in mammalian (Kyte, 1976a,b) or toad kidney cells (Kraehenbuhl, J. P., C. Bonnard, K. Geering, M. Girardet, and B. C. Rossier, unpublished observation) as well as in rat parotid glands (Conteas et al., 1986). Some authors speculated that the apical immunoreactivity may be due to the sodium channel-sharing antigenic sites with  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$   $\alpha$  subunit (Kraehenbuhl, J. P., C. Bonnard, K. Geering, M. Girardet, and B. C. Rossier, unpublished observation; Bonnard et al., 1984).

Clearly, studies on subunit assembly and subcellular localizations critically depend on the specificity of the antibodies used. In the present work we have extensively characterized a monoclonal antibody (IEC 1/48) against rat intestinal crypt cells (Quaroni and Isselbacher, 1981) which was found to be directed against the  $\beta$  subunit of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . This probe together with a previously characterized  $\alpha$ -subunit-specific monoclonal antibody (Kashgarian et al., 1985) was used to study the expression of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  along the intestinal tract by light and immunoelectron microscopy. The results suggest that enzymatically active  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , as probed by the anti- $\alpha$  monoclonal antibody, is exclusively confined to the basolateral aspect of the plasma membrane in both small and large intestinal epithelial cells while an unexpected cross-reactivity with distal colon brush borders was observed with the anti- $\beta$  monoclonal antibody.

## Materials and Methods

### Antibodies

The following mouse monoclonal antibodies were used: IEC 1/48 against cultured crypt cells of rat small intestine (Quaroni and Isselbacher, 1981); C 62.4 against  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$   $\alpha$  subunit of dog kidney (Kashgarian et al., 1985); and BB4/33/1 against rat intestinal aminopeptidase N (Quaroni and Isselbacher, 1985). In most experiments the antibodies were used in the ascites form. Antibody C 62.4 was partially purified by ammonium sulfate precipitation. In some instances the antibodies were further purified by DE-52 chromatography and coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to the information provided by the manufacturer. Antisera to the dog kidney  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  recognizing either both the  $\alpha$  and the  $\beta$  subunit or exclusively the  $\beta$  subunit were kindly provided by Dr. Daniel Louvard (Institut Pasteur, Paris, France) (Louvard, 1980). A rat monoclonal antibody against mouse  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$   $\beta$  subunit was kindly provided by Dr. C. Goridis (Centre d'Immunology, INSERM, Marseille, France) (Gorvel et al. 1983).

### Metabolic Labeling of Intestinal Cells by [ $^{35}\text{S}$ ]Methionine or [ $^3\text{H}$ ]Fucose

These experiments were either carried out *in vivo* or *in organ culture*. For the *in vivo* experiments 200–220-g Sprague-Dawley rats were fasted overnight and anesthetized with Nembutal (50 mg/kg body weight; Abbott Laboratories, Zug, Switzerland). The abdomen was opened and a ligature was placed in the proximal jejunum. 15 cm distal to the ligature the small intestine was closed with a small stainless steel metal clamp. At the proximal end of this 15-cm-long segment 500  $\mu\text{Ci}$  [ $^3\text{H}$ ]fucose (500  $\mu\text{Ci}$  in 1 ml prewarmed PBS) was injected into the lumen of the gut. The abdomen was closed with clamps and reopened after 3 h. The body temperature of the operated animals was maintained by exposure to infrared lamps. At the end of the experiments the intestinal segment was excised, flushed through with 50 ml ice-cold 0.9% NaCl containing 0.23 mM PMSF, placed on a glass

plate on ice, and longitudinally opened. The mucosa was scraped with a microscope slide. The scraped mucosa was processed to yield the "total membrane fraction" as described below.

Short-time organ culture was performed according to Browning and Tier (1969) using RPMI-1640 medium instead of Trowell T8 medium (Hauri et al., 1975). 1–4-mm<sup>2</sup> mucosal explants were cultured on stainless steel grids at 37°C in an atmosphere of 95% O<sub>2</sub>, 5% CO<sub>2</sub> for 3 h in the presence (or absence) of 20  $\mu\text{g}/\text{ml}$  tunicamycin, in methionine-free medium containing tunicamycin for 30 min, and in methionine-free medium containing 50  $\mu\text{Ci}/\text{ml}$  methionine and tunicamycin for 2.5 h. At the end of the labeling period the explants were washed several times in PBS, homogenized in 100 mM sodium phosphate, pH 8, containing 1% (wt/vol) Triton X-100 and 0.23 mM PMSF (designated "solubilization buffer"), and processed for immunoprecipitation.

### Subcellular Fractionation

To obtain a total membrane fraction, scraped mucosa of a 15-cm-long intestinal segment was homogenized in 5 ml of 60 mM mannitol, 2 mM Tris-HCl, 1 mM EGTA, pH 7.1, in the presence of 0.23 mM PMSF in a glass-TEFLON potter for 2 min with 20 up and down strokes. The homogenate was diluted to 30 ml with the same buffer and centrifuged at 2,800  $g_{av}$  for 15 min. The supernatants were centrifuged at 105,000  $g_{av}$  for 60 min, and the resulting pellet was designated "total membrane fraction."

Enterocytes were isolated at 0°C (Weiser, 1973; Bonkovsky et al., 1985) for the preparation of basolateral membranes (Weiser et al., 1978; Hagenbuch et al., 1985).

Colonic brush border membranes were purified from isolated colonocytes of proximal (i.e., the proximal half) or distal (i.e., the distal half) rat colon according to a recently developed method (Stieger et al., 1986). In brief, the cells were isolated in an EDTA-containing buffer and mildly homogenized so that their apical brush borders remained intact. The brush border caps were then recovered by Percoll density gradient centrifugation and treated with 1 M Tris-HCl at pH 8.2, which led to vesiculation of the brush border membrane. Subsequently, the brush border vesicles were isolated on discontinuous sucrose gradients. This method gives a 20-fold enrichment of brush border membranes vs. homogenate (Gorr et al., 1988).

### Immunoprecipitation

Membrane fractions or cell homogenates were solubilized in solubilization buffer (detergent-to-protein ratio >3) for 45–60 min on ice. The solubilized sample was then centrifuged at 105,000  $g_{av}$  for 60 min, and the resulting supernatant was used for direct immunoprecipitation by a previously described protein A-Sepharose method (Hauri et al., 1985). Before immunoprecipitation the samples were preabsorbed to protein A-Sepharose without antibody for at least 120 min at 4°C and the supernatant fraction was subsequently transferred to the immunobeads. Alternatively, an indirect immunoprecipitation technique was used in which the monoclonal antibody was bound to protein A-Sepharose via a rabbit anti-mouse immunoglobulin antiserum. In some experiments Triton X-100 was replaced by 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. For the immunoprecipitation of enzymatically active  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  the isolated basolateral membranes were solubilized for 15 min with the detergent dodecyloctaethyleneglycol (C<sub>12</sub>E<sub>8</sub>) (detergent-to-protein ratio = 1.5) in 1 mM triethanolamine, pH 7.6, containing 250 mM sucrose, 10 mM KCl or NaCl, 2 mM DTT, and 40  $\mu\text{g}/\text{ml}$  PMSF at room temperature. The nonsoluble particles were removed by a 10-min spin in an airfuge (Beckman Instruments, Inc., Fullerton, CA) at full speed in the cold room. The resulting supernatant was incubated with IEC 1/48 adsorbed to protein A-Sepharose beads, pH 8, for 90 min at 4°C. After two wash steps with 1 ml of 100 mM sodium phosphate, pH 8, containing C<sub>12</sub>E<sub>8</sub> and three wash steps with phosphate buffer lacking the detergent, the bead-associated K<sup>+</sup>-stimulated *p*-nitrophenyl-phosphatase activity was measured using buffers I, II, or III as described by Stieger et al. (1986).

### SDS-PAGE

One-dimensional PAGE in the presence of SDS was carried out according to Laemmli (1970). Mercaptoethanol was replaced by DTT and the samples were not boiled to avoid aggregation of the  $\alpha$  subunit but instead were incubated in a heating block at 60°C for 30 min before loading onto the gel. For the assessment of the subunit specificity of antibody IEC 1/48, the [ $^{125}\text{I}$ ]-labeled samples were treated with Laemmli's sample buffer at 20°C for

exactly 10 min. In that case, SDS-PAGE was run in the cold room at 4°C overnight. Individual gel lanes were cut out and stored at -20°C until the positions of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase  $\alpha$  and  $\beta$  subunits were established by autoradiography of lanes run in parallel. The frozen gel lanes were cut into 0.5-mm-thick slices with a gel slicer. Slices containing the  $\alpha$  or the  $\beta$  subunit were pooled, and the proteins were eluted by shaking in 1 ml of 100 mM Na phosphate buffer, pH 8, containing 1% (wt/vol) Triton X-100 and 0.23 mM PMSF overnight at 4°C. Eluted proteins were subjected to a second round of immunoprecipitation with either IEC 1/48 or C 62.4.

Samples for two-dimensional slab gel electrophoresis were prepared as described by Ames et al. (1976), and electrophoresis was performed as described by O'Farrell (1975): the second dimension was SDS-slab gel electrophoresis using 7-12% linear gradient acrylamide gels. After fixation, the gels were processed for fluorography using EN<sup>3</sup>HANCE (New England Nuclear, Boston, MA).

### Radioiodination

Triton X-100-solubilized basolateral or brush border membranes were labeled with carrier-free Na<sup>125</sup>I (Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland) using the glucoseoxidase-lactoperoxidase method as described previously (Hauri et al., 1985). The iodinated <sup>125</sup>I-protein fraction was used immediately for immunoprecipitation experiments or was frozen in aliquots in the presence of 0.1% BSA as cryoprotectant at -20°C and used within 3 wk.

Intact proximal or distal large intestinal segments were surface labeled with <sup>125</sup>I-sulfo-SHPP<sup>1</sup> according to Thompson et al. (1987). The large intestine was flushed through with 200 ml PBS containing 0.5 mM DTT. <sup>125</sup>I-sulfo-SHPP, prepared with 1 mCi Na<sup>125</sup>I, was filled into the intestinal segment in 500  $\mu$ l PBS, and the reaction was allowed to proceed at room temperature. After 30 min, the segment was flushed through with 60 ml ice-cold PBS-lysine, split open longitudinally, and briefly washed in a beaker containing PBS-lysine. The mucosa was lightly scraped with a microscope slide and homogenized in 1 ml of 100 mM sodium phosphate, pH 8, containing 2% Triton X-100, 0.1% sodium azide, 40  $\mu$ g PMSF, 10  $\mu$ g aprotinin, 5  $\mu$ g leupeptin, 17.5  $\mu$ g benzamidin, 1  $\mu$ g antipain, 1  $\mu$ g pepstatin, and 2 mM *O*-phenanthroline. After 60 min on ice, the sample was spun at 100,000 g for 60 min and the resulting supernatant was subjected to immunoprecipitation.

### Glycosidase Treatment

For the digestion with endo- $\beta$ -*N*-acetylglucosaminidase F (endo F; Boehringer Mannheim Biochemicals, Indianapolis, IN), the washed immunoprecipitates were boiled in 50  $\mu$ l of 0.1 M sodium phosphate, pH 6.1, containing 50 mM EDTA, 1% NP-40, 0.1% SDS, 1% 2-mercaptoethanol, and the above protease inhibitor cocktail. 1  $\mu$ l (490 U/ml) endo F was added together with a fresh aliquot of the protease inhibitor cocktail and the samples were incubated for 24 h at 37°C. In some instances the reaction was extended for up to 48 h after addition of a fresh aliquot of endo F at 24 h. The reaction was stopped by boiling in Laemmli's sample buffer.

### Peptide Mapping

The  $\beta$  subunit of immunisolated (Na<sup>+</sup> + K<sup>+</sup>)-ATPase was cut out of an unstained 7.5% SDS-polyacrylamide gel, digested with *Staphylococcus aureus* protease (V8), papain, or porcine elastase (Cleveland et al., 1977) as follows. The gel slices were soaked in 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, and 1 mM EDTA for 30 min, placed into the sample wells of a 15% SDS-polyacrylamide gel, and overlaid with sample buffer containing the appropriate amount of protease. When the bromophenol blue approached the bottom of the stacking gel, the current was turned off for 30 min to allow for proteolysis.

### Immunoblotting

The method of Towbin et al. (1979) was used to detect electrophoretically transferred proteins on nitrocellulose strips. The immunoreaction was performed essentially as described (Hauri and Bucher, 1986) using either

peroxidase-linked second antibody or <sup>125</sup>I-protein A to visualize the bound immunoantibody.

### Cryosectioning and Immunofluorescence Technique

Rats fasted overnight were killed by cervical dislocation, and their small or large intestines were rapidly flushed through with fixation buffer (2% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M Pipes-NaOH, pH 7, containing 5% [wt/vol] sucrose). The tissue was placed into fresh fixative and cut with a scalpel into 3  $\times$  2-mm fragments. After an overall fixation time of 60 min at room temperature, free aldehyde groups were quenched with glycine-containing 0.1 M Pipes buffer, pH 7 (3 drops of 0.5 M glycine/10 ml buffer), three times for 10 min. The tissue fragments were then infused with 2.3 M sucrose in 0.1 M Pipes, pH 7, placed onto the specimen holder, and rapidly frozen in liquid nitrogen. 0.5-1- $\mu$ m-thick sections were cut with a microtome (Ultracut E; Reichert Scientific Instruments, Buffalo, NY) with cyroattachment according to Tokuyasu (1978), transferred to gelatine-coated microscope slides, and immunolabeled with the monoclonal antibodies to (Na<sup>+</sup> + K<sup>+</sup>)-ATPase (ascites fluid diluted 1:75 in PBS) for 2 h. After four wash steps with PBS during 30 min, the binding of the antibodies was visualized by a rhodamine-conjugated rabbit anti-mouse IgG (Nordic Immunological Laboratories, Tilburg, Netherlands).

### Immunoelectron Microscopy

Sprague-Dawley rats were anesthetized with Inactin (10 mg/100 g body weight). The abdominal aorta was catheterized retrograde and clamped at the diaphragm. The abdominal organs were initially perfused with mammalian Ringer's solution and then fixed using the periodate lysine paraformaldehyde fixative of McLean and Nakane (1974) as modified (Kashgarian et al., 1985). Segments of small bowel and proximal and distal colon were removed after  $\sim$ 5 min of perfusion, the lumen was flushed with Ringer's solution and fixative, and 2-mm cross sections were postfixed for 6 h in the fixative. The preembedding labeling procedure used previously in the kidney was used for labeling of bowel segments with C62.4 and IEC 1/48 as primary antibodies (Kashgarian et al., 1985).

### Other Methods

Protein was determined with the protein assay kit (Bio-Rad Laboratories, Richmond, CA) using standard I.

## Results

### Monoclonal Antibody IEC 1/48 Is Directed against (Na<sup>+</sup> + K<sup>+</sup>)-ATPase

In a previous study the monoclonal antibody IEC 1/48 was found to bind specifically to rat small intestinal crypt cells in culture (Quaroni and Isselbacher, 1981). To visualize the antigen recognized by this antibody in the cultured intestinal cells, IEC-6 cells were metabolically labeled with [<sup>3</sup>H]proline and [<sup>3</sup>H]lysine, solubilized with Triton X-100, and incubated with the IEC 1/48 antibody bound to Sepharose 4B (Quaroni and Isselbacher, 1985). The affinity-purified antigen was analyzed by two-dimensional slab gel electrophoresis, which revealed two polypeptides of approximate molecular masses of 100 and 50 kD, respectively (Fig. 1). The 50-kD antigen appeared as closely spaced multiple spots, most likely due to charge heterogeneity. Since charge heterogeneity often is an expression of glycosylation (Marshall and Hokin, 1979) we suspected that the smaller protein might be a glycoprotein.

The immunofluorescence technique was used to localize the antigen defined by the IEC 1/48 antibody on cryosections of rat small intestine. Immunolabeling was exclusively confined to the basolateral aspect of the epithelial cells (Fig. 2 a) while the brush border of the enterocytes was not la-

1. *Abbreviations used in this paper:* endo F, endo- $\beta$ -*N*-acetylglucosaminidase F; SHPP, succinimidyl(hydroxyphenyl)propionate.



**Figure 1.** Identification of the IEC 1/48 antigen produced by cultured intestinal crypt cells by two-dimensional slab gel electrophoresis. IEC-6 cells were metabolically labeled by addition of 0.5 mCi/ml [ $^3\text{H}$ ]lysine and [ $^3\text{H}$ ]proline to lysine- and proline-free Dulbecco's minimal essential medium supplemented with dialyzed FCS for 24 h. Triton X-100-solubilized membrane proteins were incubated with IEC 1/48 antibody-Sepharose 4B beads, and the affinity-purified antigen was analyzed by two-dimensional gel electrophoresis followed by fluorographic detection of the labeled polypeptides.

beled. This latter domain was easily visualized by means of a monoclonal antibody against brush border aminopeptidase N (Fig. 2 c). Thus, the IEC 1/48 antigen is expressed in a polarized manner in small intestinal epithelial cells.

Fig. 3 demonstrates that the IEC 1/48 antibody precipitates two proteins of apparent 98,000 and 48,000  $M_r$  from detergent-solubilized basolateral membranes of rat small intestinal epithelial cells (lane 1). This pattern is very similar to the results obtained with cultured crypt cells (Fig. 1). Since Coomassie blue staining did not allow us to clearly visualize proteins in the 50,000- $M_r$  region because of comigration of the heavy chain of the antibody, detergent-solubilized basolateral membranes were radioiodinated before immunoprecipitation. This experiment showed that indeed only two major proteins were immunoprecipitable by the IEC 1/48 antibody (Fig. 3, lane 2). Furthermore, only the 48,000- $M_r$  protein but not the 98,000- $M_r$  protein incorporated [ $^3\text{H}$ ]fucose, indicating that the former protein was a glycoprotein of the complex type (Fig. 3, lane 3).

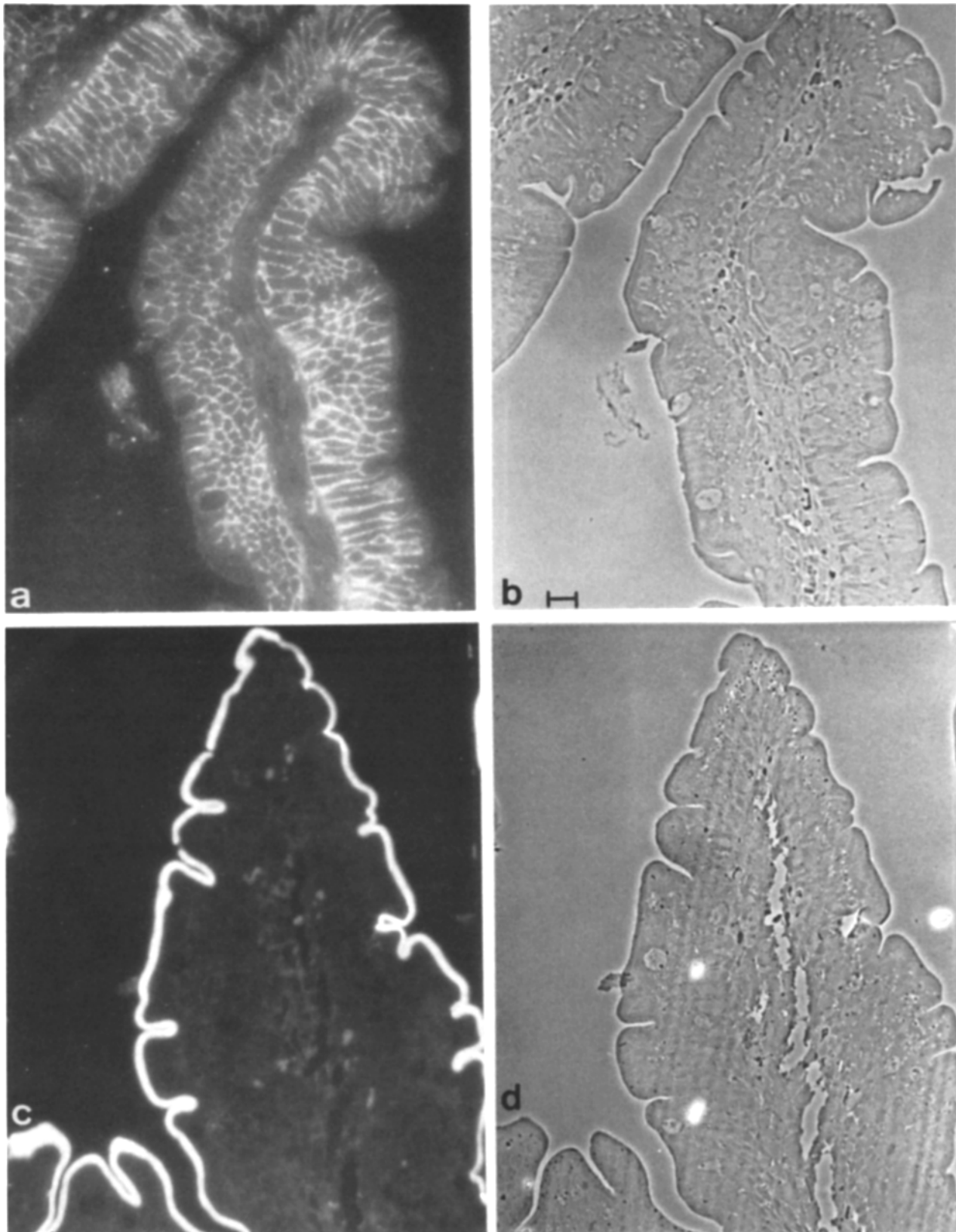
The features of the IEC 1/48 antigen are reminiscent of those of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . Immunoblotting experiments with polyclonal antibodies against dog kidney  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  confirmed this impression. The anti- $(\alpha + \beta)$  subunit polyclonal antibody was found to react with both the larger and the smaller protein of the IEC 1/48 antigen (Fig. 3, lanes 5 and 6). The  $\beta$ -subunit-specific polyclonal antibody only reacted with the smaller but not the larger protein of the IEC 1/48 antigen (Fig. 3, lanes 7 and 8). Since these polyclonal antibodies do not recognize any additional antigens on blots prepared with total basolateral membranes (not shown) we concluded that the IEC 1/48 antigen was most probably  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ .

Direct evidence for the IEC 1/48 antigen being  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  was obtained from immunoprecipitation experiments using  $\text{C}_{12}\text{E}_8$ -solubilized small intestinal basolateral membranes. The nonionic detergent  $\text{C}_{12}\text{E}_8$  allows the solubilization of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  in a partially active form (Esmann et al., 1979). We therefore tested the IEC 1/48 antibody to see if it could precipitate enzymatically active  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . This experiment revealed that 21% of the

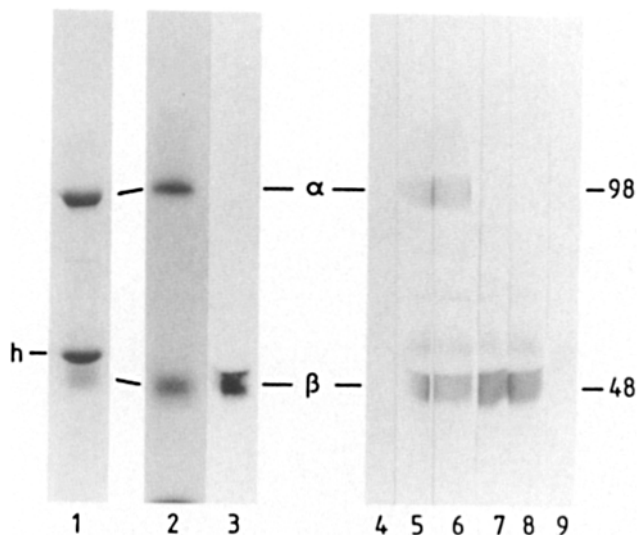
solubilized ouabain-sensitive  $\text{K}^+$ - $p$ -nitrophenyl-phosphatase activity (i.e., the dephosphorylation step of the  $[\text{Na}^+ + \text{K}^+]\text{-ATPase}$  reaction) was immunoprecipitable with the monoclonal antibody. Control experiments with nonimmune antibodies did not precipitate any measurable  $\text{K}^+$ - $p$ -nitrophenyl-phosphatase activity under these conditions, and all the non-ouabain-sensitive  $\text{K}^+$ - $p$ -nitrophenyl-phosphatase activity remained in the supernatant. Although enzyme recovery was not quantitative, probably due to the instability of the enzyme during prolonged incubations with  $\text{C}_{12}\text{E}_8$  (Esmann et al., 1979), the results clearly show that the IEC 1/48 antibody can specifically precipitate  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity.

The antibody did not react with its antigen on immunoblots which complicated the establishment of subunit specificity. We therefore eluted the  $\beta$  subunit from SDS-polyacrylamide gels run under relatively mild conditions (see Material and Methods). Aliquots of the eluted subunit were then immunoprecipitated with the IEC 1/48 antibody. Antibody C 62.4 was used as a control. Fig. 4 shows that the IEC 1/48 antibody preferentially precipitated the  $\beta$  subunit (lane 3) while the C 62.4 antibody, as expected, preferentially precipitated the  $\alpha$  subunit (lane 2) of the enzyme. IEC 1/48 appeared to also precipitate some  $\alpha$  subunit. However, this is considered background since it is not more than the amount of  $\beta$  subunit precipitated by the  $\alpha$ -subunit-specific C 62.4. The results strongly suggest that the IEC 1/48 antibody is directed against the  $\beta$  subunit of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ .

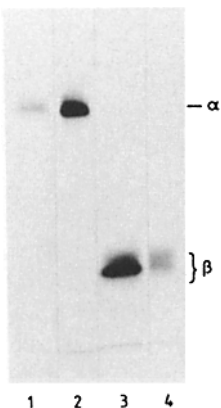
In light of the unexpected labeling patterns obtained with the distal colon (see below) it was important to establish that the IEC 1/48 antibody was directed against a protein and not a carbohydrate epitope. For this reason intestinal mucosal specimens were metabolically labeled in organ culture with [ $^{35}\text{S}$ ]methionine in the presence of tunicamycin to inhibit  $N$ -glycosylation. This experiment was performed with the small intestines of suckling animals since the incorporation of [ $^{35}\text{S}$ ]methionine was much higher than in adult tissue. The  $\beta$  subunit is known to carry three  $N$ -linked oligosaccharide side chains (Tamkun and Fambrough, 1986). Fig. 5 shows that in the presence of tunicamycin, a 35-kD protein



**Figure 2.** Immunofluorescence staining of rat jejunal mucosa. The mucosa was fixed with 2% formaldehyde and 0.1% glutaraldehyde before infusion and freezing in 2.3 M sucrose. 1- $\mu$ m cryosections were incubated with monoclonal antibody IEC 1/48 (a) or with monoclonal antibody BB4/33/1 against aminopeptidase N (c) followed by rhodamine-conjugated rabbit anti-mouse IgG. b and d are corresponding phase-contrast pictures. Bar, 10  $\mu$ m.

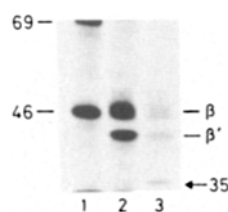


**Figure 3.** Identification of IEC 1/48 antigen by immunoprecipitation and immunoblotting. (Lane 1) Basolateral membranes of rat small intestine were detergent solubilized and precipitated with monoclonal antibody IEC 1/48 adsorbed to protein A-Sepharose at pH 8. The immunoprecipitate was separated on a 7.5% polyacrylamide gel in the presence of SDS. The gel was stained with Coomassie blue. (Lane 2) Detergent-solubilized basolateral membranes were radiiodinated by the lactoperoxidase-glucoseoxidase method and immunoprecipitated with the IEC 1/48 antibody. The immunoprecipitate was separated by SDS-PAGE and followed by autoradiography of the dried gel. (Lane 3) Rats were metabolically labeled with 500  $\mu$ Ci [ $^3$ H]fucose before the isolation of the IEC 1/48 antigen from purified basolateral membranes (fluorogram). (Lanes 4-9) Immunoblotting with polyclonal antibodies. The IEC 1/48 antigen was immunisolated by means of the monoclonal antibody. The immunoisolate was separated by SDS-PAGE; electrophoretically transferred to nitrocellulose; and immunolabeled with a rabbit anti-dog kidney  $\alpha$  and  $\beta$  ( $\text{Na}^+ + \text{K}^+$ )-ATPase antibody (lanes 5 and 6, two different basolateral membrane preparations), a rabbit anti-dog kidney  $\beta$  ( $\text{Na}^+ + \text{K}^+$ )-ATPase antibody (lanes 7 and 8), or nonimmune rabbit IgG (lanes 4 and 9, controls). Bound antibodies were detected by goat anti-rabbit peroxidase.  $\alpha$  and  $\beta$ , subunits of ( $\text{Na}^+ + \text{K}^+$ )-ATPase; *h*, heavy chain of IEC 1/48 antibody. Molecular masses of  $\alpha$  and  $\beta$  subunits are indicated in kilodaltons (7.5% SDS-gel).



**Figure 4.** Subunit specificity of monoclonal antibodies against ( $\text{Na}^+ + \text{K}^+$ )-ATPase. Detergent-solubilized basolateral membranes of rat small intestinal enterocytes were radiolabeled with  $\text{Na}^{125}\text{I}$  and separated by SDS-PAGE at 4°C. Slices containing either the  $\alpha$  or the  $\beta$  subunit of ( $\text{Na}^+ + \text{K}^+$ )-ATPase were cut out of the frozen gel and separately eluted with a Triton X-100-containing buffer at 4°C. Equal aliquots of the eluted radioactive proteins were then immunoprecipitated with either the IEC 1/48 antibody (lanes 1 and 3) or with the C 62.4 antibody (lanes 2 and 4). The immunoprecipitates were rerun on an SDS-polyacrylamide gel at room temperature and radioactive proteins were visualized by autoradiography. Note that antibody IEC 1/48 and C 62.4 preferentially precipitate the  $\beta$  and  $\alpha$  subunits, respectively, of ( $\text{Na}^+ + \text{K}^+$ )-ATPase. Similar results were obtained when the first gel was run with immunoprecipitated ( $\text{Na}^+ + \text{K}^+$ )-ATPase instead of with basolateral membranes (7.5% SDS-gel).

acrylamide gel at room temperature and radioactive proteins were visualized by autoradiography. Note that antibody IEC 1/48 and C 62.4 preferentially precipitate the  $\beta$  and  $\alpha$  subunits, respectively, of ( $\text{Na}^+ + \text{K}^+$ )-ATPase. Similar results were obtained when the first gel was run with immunoprecipitated ( $\text{Na}^+ + \text{K}^+$ )-ATPase instead of with basolateral membranes (7.5% SDS-gel).



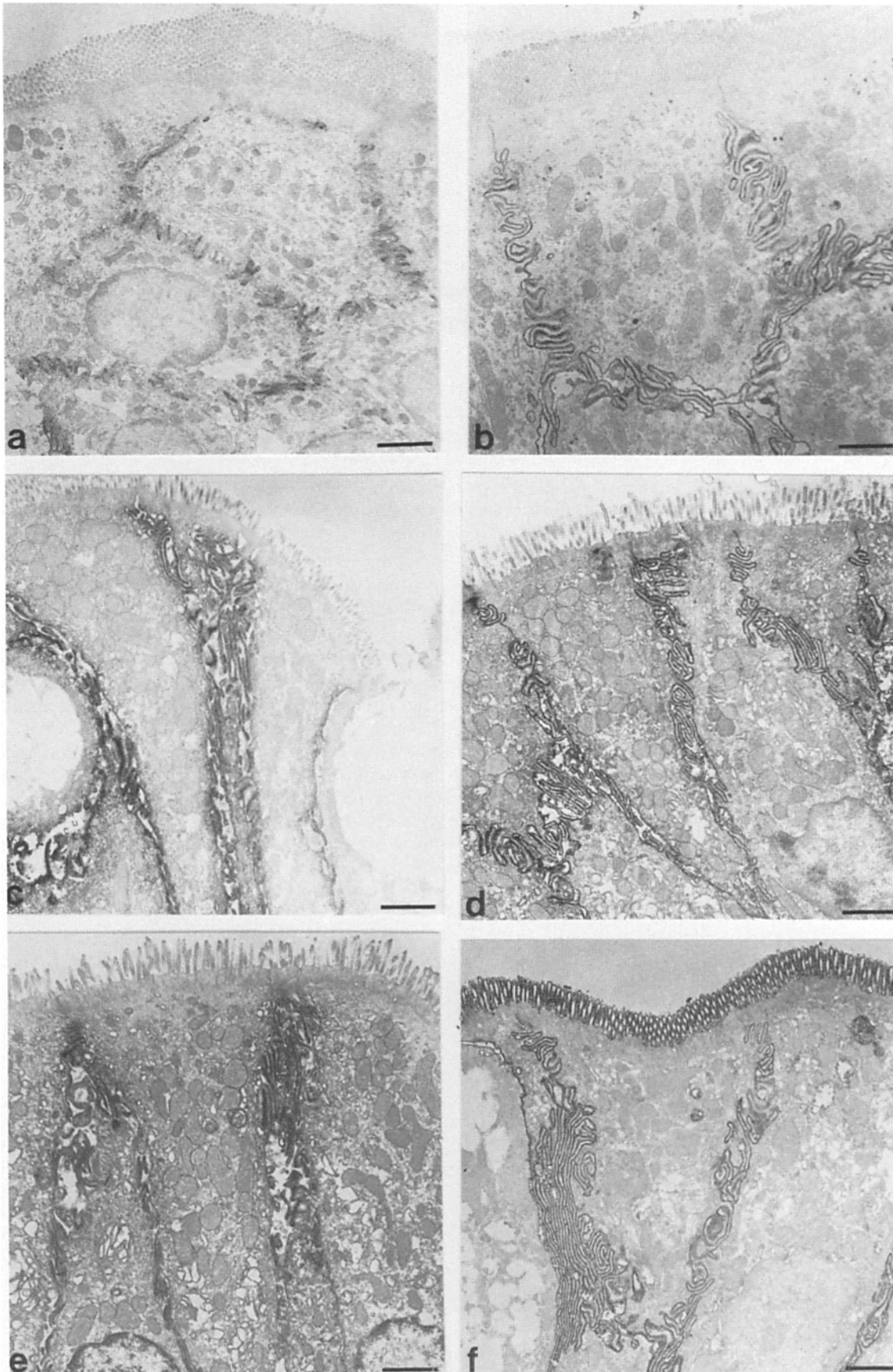
**Figure 5.** The IEC 1/48 antibody recognizes nonglycosylated  $\beta$  subunit after tunicamycin treatment. Small intestinal explants of a 12-d-old suckling rat were metabolically labeled with [ $^{35}$ S]methionine for 30 min in the presence (lane 3) or absence (lane 2) of 20  $\mu$ g/ml tunicamycin (see Material and Methods). Lane 1 indicates relative molecular mass markers in kilodaltons.  $\beta$ , complex-glycosylated  $\beta$  subunit of ( $\text{Na}^+ + \text{K}^+$ )-ATPase;  $\beta'$ , high-mannose form of the  $\beta$  subunit. Arrow indicates the position of the 35-kD nonglycosylated  $\beta$  subunit. Note that the block by tunicamycin is incomplete despite the high concentration used and that this inhibitor leads to the known overall reduction in methionine incorporation.

is immunoprecipitable with IEC 1/48 (in addition to some high-mannose and complex-glycosylated protein). This is most likely the nonglycosylated  $\beta$  subunit since its relative molecular mass agrees well with the calculated mass of the protein moiety deduced from its primary structure (Noguchi et al., 1986). The result suggests therefore that IEC 1/48 is directed against the protein backbone and not against carbohydrate.

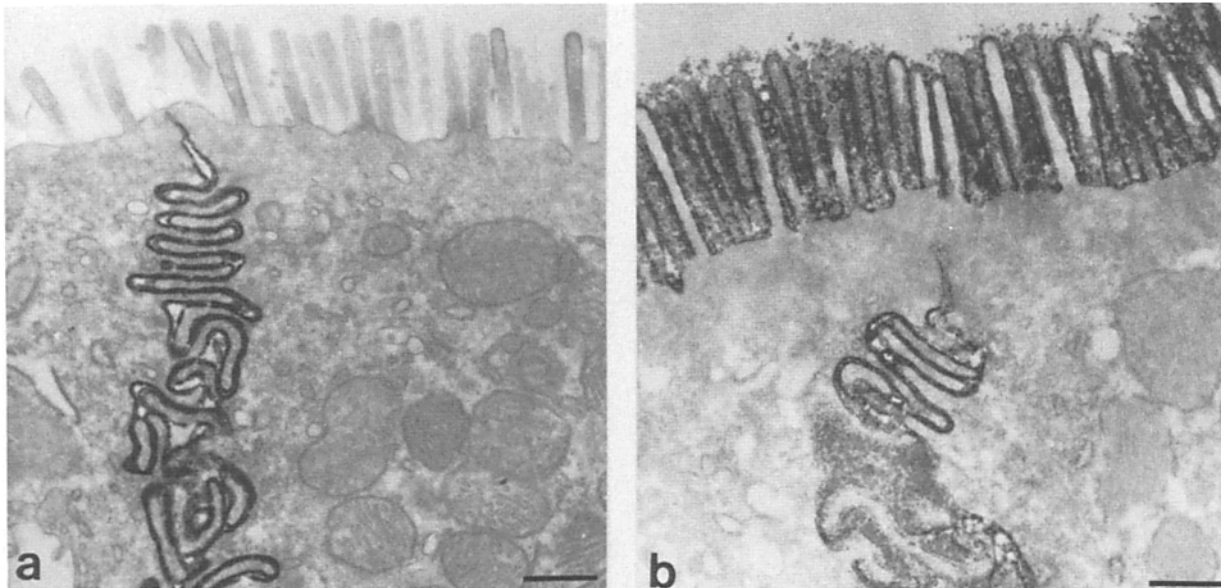
#### Localization of ( $\text{Na}^+ + \text{K}^+$ )-ATPase along the Intestine by Fluorescence Light and Immunoelectron Microscopy

Immunofluorescence microscopy showed a basolateral labeling pattern with C 62.4 in all parts of the small and large intestine (not shown). A basolateral pattern was also observed with IEC 1/48 in small and proximal large intestinal colonocytes. Surprisingly, in the distal half of the colon, however, this antibody also labeled the apical cell pole. To precisely localize the apical immunoreactivity, we performed immunoelectron microscopy. First, it was found that in rat kidney epithelial cells the IEC 1/48 antibody exhibited an exclusively basolateral immunoreactivity which was identical to that obtained with the C 62.4 antibody (Kashgarian et al., 1985). In the small intestine and the proximal colon, both antibodies exclusively labeled the basolateral membrane of epithelial cells (Fig. 6, *a-d*), while in the distal colon immunolabeling with IEC 1/48 was found associated both with the basolateral and the brush border membrane (Fig. 6 *f*). Furthermore, an immunoreaction was also observed in lysosomal structures. As with immunofluorescence, the immunolabeling of C 62.4 at the ultrastructural level was strictly confined to the basolateral membrane in epithelial cells of the distal colon (Fig. 6 *e*). At higher magnification (Fig. 7) it is apparent that the reaction product at the brush border due to IEC 1/48 is intimately associated with the membrane and, therefore, cannot be explained by peripheral adsorption to the mucosa of antigens derived from the luminal content. In the region of tight junctions no reaction product was seen, suggesting that this membrane domain may possess no or only minimal amounts of ( $\text{Na}^+ + \text{K}^+$ )-ATPase. A notable difference between the two antibodies relates to the pattern of the reaction product. C 62.4 gave a somewhat blurred deposit along the basolateral membrane which is due to the fact that the antibody is directed against a cytoplasmic domain of the enzyme leading to a diffusion of the reaction product into the cytoplasm before embedding (Kashgarian et al., 1985).





**Figure 6.** Immunolabeling of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  in the gut by immunoelectron microscopy. Small intestine (ileum, *a*), proximal colon (*c*), and distal colon (*e*) were labeled with monoclonal antibody C62.4 (anti- $\alpha$  subunit). Labeling is confined to the cytoplasmic aspect of the basolateral membranes of all intestinal segments. The pattern of labeling with IEC 1/48 (anti- $\beta$  subunit) of the small intestine (*b*) and proximal colon (*d*) is identical to that seen with C 62.4. The reaction product is localized to the external aspect of the basolateral plasma membrane and appears more distinct than that seen with C 62.4. In the distal colon (*f*) IEC 1/48 labels the external aspect of both apical and basolateral membranes in contrast to the pattern seen with C 62.4 in the same segment (*e*). Bars: (*a* and *d*)  $0.9 \mu\text{m}$ ; (*b*)  $0.8 \mu\text{m}$ ; (*c*, *e* and *f*)  $1.4 \mu\text{m}$ .



**Figure 7.** Immunolabeling of the distal colon with C 62.4 (a) and IEC 1/48 (b). While C 62.4 exclusively labels the cytoplasmic aspect of the basolateral membrane (a), IEC 1/48 labels the external aspect of both the apical microvilli and the basolateral membrane. The region of the tight junction is excluded in both instances. Bars, 0.4  $\mu\text{m}$ .

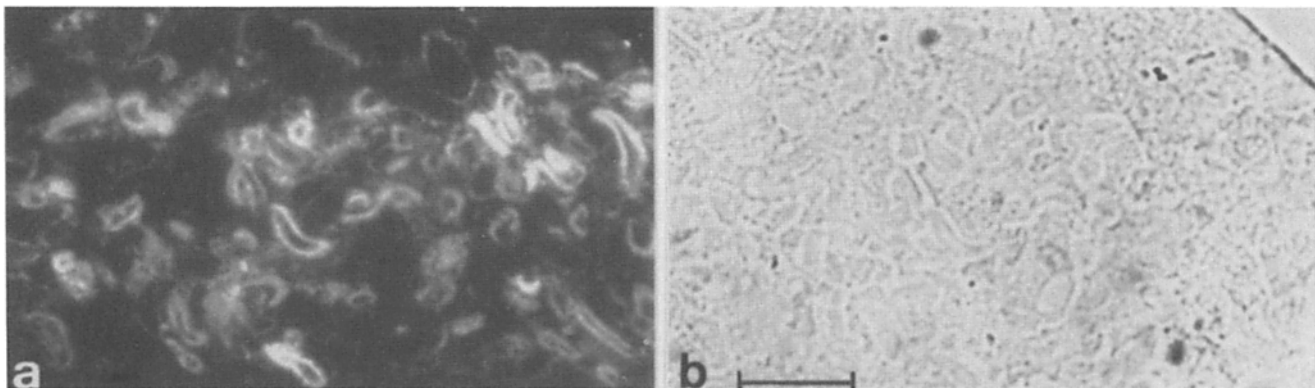
In contrast, with IEC 1/48 the reaction product was more defined. This may indicate that IEC 1/48 recognizes the extracytoplasmic side of the bilayer.

***Characterization of the Brush Border Antigen that Cross Reacts with the IEC 1/48 Antibody in the Distal Colon***

The cross-reacting brush border antigen remained associated with intact colonocytes during isolation (not shown) as well as with isolated brush borders after subcellular fractionation as assessed by immunofluorescence (Fig. 8). We take this as additional evidence for an intimate association of the antigen with the brush border. To identify the cross-reacting antigen we used the subcellular fractionation technique. A recently established procedure allows one to isolate intact brush border caps from colonocytes and, in a subsequent step, to further purify the brush border membrane in vesicular form (Stieger et al., 1985; Gorr et al., 1988). When the brush bor-

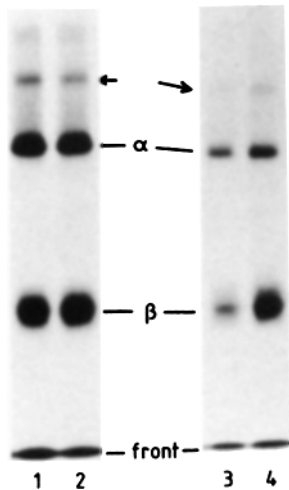
der caps or vesicles of distal colon were solubilized, radiolabeled with  $\text{Na}^{125}\text{I}$ , and immunoprecipitated with the IEC 1/48 antibody, no additional antigens were precipitated to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ -related proteins that were also present in the proximal colon (Fig. 9). Since the brush border does not contain  $\alpha$  subunit, its presence in this fraction reflects cross-contamination by basolateral membranes. However, a notable difference between the proximal and the distal samples concerned the ratio of radioactivity measured in the two subunits (Table I). The  $\beta$ -to- $\alpha$  ratio was significantly higher in the distal as compared with the proximal colon. With the purified brush border membrane fractions this difference was even more pronounced. These findings indicated that the cross-reacting antigen might comigrate on gels with basolateral  $\beta$  subunits.

To study the structural relationship between the cross-reacting antigen and  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$   $\beta$  subunit, limited proteolysis was carried out as described by Cleveland et al.



**Figure 8.** Immunolabeling of intact brush border caps with antibody IEC 1/48. (a) Isolated brush border caps (Stieger et al., 1986) were washed once with PBS and fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in PBS. The fixed and washed pellet was included in 10% gelatine, infused with 2.3 M sucrose in 2% paraformaldehyde-PBS, and processed for immunofluorescence microscopy as described in the legend to Fig. 2. (b) Corresponding phase-contrast micrograph. Bar, 10  $\mu\text{m}$ .





**Figure 9.** Immunoprecipitation of detergent-solubilized and radioiodinated brush border caps (lanes 1 and 3) or brush border membranes fractions (lanes 2 and 4) from proximal (lanes 1 and 2) or distal (lanes 3 and 4) colonocytes with monoclonal antibody IEC 1/48. The fractions were isolated according to Stieger et al. (1986).  $\alpha$  and  $\beta$ , positions of the subunits of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . Arrow indicates the position of an additional band that displayed an  $\alpha$ -subunit pattern in one-dimensional peptide mapping and, hence, might originate from aggregation of  $\alpha$ -subunit-derived proteolytic fragments. However, this band is not a dimer composed of  $\alpha$  and  $\beta$  subunits as was the case in Tamkun and Fambrough (1986).

(1977). Using *Staphylococcus aureus* protease V8, the peptide maps, obtained with the  $\beta$ -subunit area cut out from SDS-gels, were identical for proximal and distal colon samples (Fig. 10, cf. lanes 2 and 3). The patterns generated with elastase or papain were virtually identical for the two regions of the colon (Fig. 10). However, the two major proteolytic fragments of  $\beta$  subunit showed a higher electrophoretic mobility in the small as compared with the large intestine. Already the intact  $\beta$  subunit was found to be smaller in the small intestine (Fig. 11). Endo F digestions were performed to determine whether or not this molecular mass difference was due to the carbohydrate moiety as has been described for another glycoprotein that is present in the two intestinal segments (Gorr et al., 1988). Fig. 12 shows that this is indeed so. Treatment of immunisolated radioiodinated  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  with endo F lead to the appearance of two bands, the smaller of which displayed identical mobility in the two intestinal segments and had the same apparent relative molecular mass as the nonglycosylated  $\beta$  subunit after tunicamycin treatment. The larger band most probably is a partially glycosylated species that is endo F resistant. Indeed, it was not possible to digest this protein with higher glycosidase concentrations or extended incubation times (Fig. 12 b). Incomplete sensitivity to endo F has also been reported for the  $\beta$  subunits of *Torpedo californica* ( $\text{Na}^+ +$

$\text{K}^+)\text{-ATPase}$  (Noguchi et al., 1987). We conclude that the relative molecular mass differences of the  $\beta$  subunits are due to glycosylation. Furthermore, it is notable that, after endo F digestion, the colon sample did not exhibit additional protein bands which could account for the brush border fluorescence in the distal colon.

Finally an attempt was made to radiolabel the brush border in the intact large intestine by using the membrane-impermeable reagent  $^{125}\text{I}$ -sulfo-SHPP and to immunoprecipitate the labeled  $\beta$ -subunit-like brush border antigen with the IEC 1/48 antibody. With this approach much more  $\beta$  subunit was precipitable from the distal than from the proximal colon (Fig. 13). In both samples some  $\alpha$  subunit was also present, indicating a slight leakiness of the label to the basolateral membrane. However, the  $\beta$ -to- $\alpha$  ratio as measured by densitometric scanning was ten times higher for the distal than for the proximal sample. Collectively, these biochemical data support the notion of an apical  $\beta$ -subunit-like antigen in the distal colon that is not associated with  $\alpha$  subunit.

## Discussion

The major surprising result of the present study relates to the identification of an apical antigen in distal colonocytes that is related to the  $\beta$  subunit of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . In view of the controversies in the literature concerning localization and subunit assembly of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , it was important to carefully establish the specificity and the properties of the antibodies.

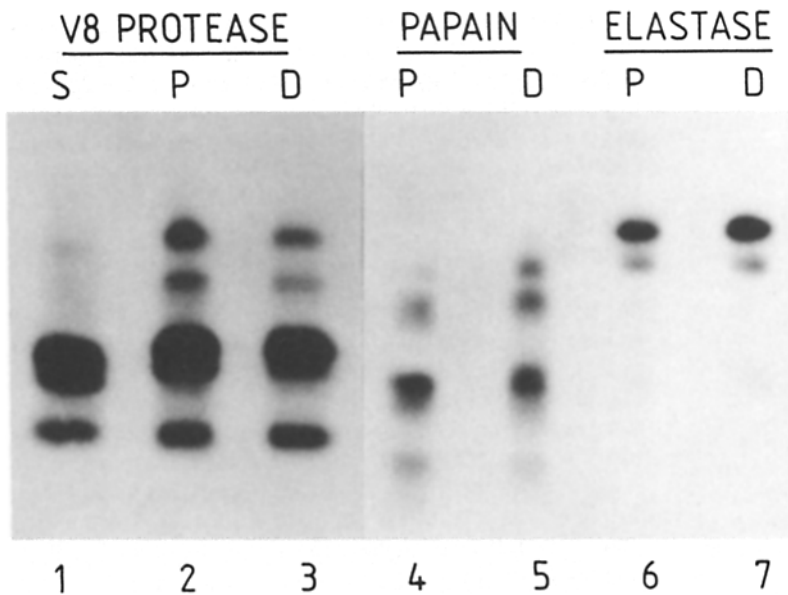
### Properties of Monoclonal Antibodies against $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$

The IEC 1/48 monoclonal antibody was originally prepared against cultured crypt cells of rat origin (Quaroni and Isselbacher, 1981). The following lines of evidence obtained in the course of this study now strongly suggest that this antibody is directed against  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . First, it binds to the basolateral membrane of small intestinal enterocytes, the site of functionally active  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . Second, it specifically immunoprecipitates two proteins from detergent-solubilized basolateral membranes of isolated enterocytes with apparent 98,000 and 48,000  $M_r$ s that are characteristic for the subunits of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . Third, the smaller protein is a glycoprotein since it incorporates [ $^3\text{H}$ ]-fucose and undergoes a mobility change during pulse-chase experiments that is characteristic for  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$   $\beta$  subunit carrying N-linked carbohydrates (Tamkun and Fam-

**Table I.** Radioactivity Associated with the Subunits of Immunoprecipitated  $^{125}\text{I}$ -Labeled  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}^*$

	Brush border caps			Brush border membranes		
	$\alpha$	$\beta$	$\beta$ -to- $\alpha$ ratio	$\alpha$	$\beta$	$\beta$ -to- $\alpha$ ratio
	cpm	cpm		cpm	cpm	
Proximal colon	1085 $\pm$ 125	1140 $\pm$ 106	1.1	1424 $\pm$ 135	1629 $\pm$ 116	1.1
Distal colon	414 $\pm$ 19	601 $\pm$ 54	1.5	729 $\pm$ 38	1215 $\pm$ 36	1.7

\* Brush border caps or membranes were isolated, detergent solubilized, and iodinated by the lactoperoxidase-glucoseoxidase method to equal specific radioactivity.  $5 \times 10^6$  cpm of each fraction were immunoprecipitated with the IEC 1/48 antibody and the immunoprecipitates were separated by SDS-PAGE. The positions of the subunits were identified by autoradiography. Subsequently, the individual subunits were excised from the dried gel and the radioactivity was determined by gamma counting. Given are the means  $\pm$  1 SD of samples run in triplicate.



**Figure 10.** Peptide mapping of the  $\beta$  subunit of the IEC 1/48 antigen isolated from small intestine (S), proximal colon (P), and distal colon (D). Small intestinal basolateral membranes or brush border membrane fractions of proximal or distal colonocytes were detergent solubilized, radioiodinated by the lactoperoxidase method, and immunoprecipitated with antibody IEC 1/48. The immunoprecipitates were separated by SDS-PAGE, and the area of the  $\beta$  subunit was cut out of the gel and processed for limited proteolysis (Cleveland et al., 1977). Shown is an autoradiogram of a 15% SDS-polyacrylamide gel exhibiting the peptide patterns obtained with *Staphylococcus aureus* V8 protease (0.5  $\mu\text{g/ml}$ ), papain (20  $\mu\text{g/ml}$ ), or elastase (250  $\mu\text{g/ml}$ ).

brough, 1986). The larger protein neither incorporates fucose nor changes its mobility on gels during biosynthesis, suggesting that it is not (or only minimally) glycosylated as has been shown for  $(\text{Na}^+ + \text{K}^+)\text{-ATPase } \alpha$  subunit (Jørgensen, 1982). Fourth, the immunisolated antigen reacted on Western blots with polyclonal antibodies against dog kidney  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . And, finally, immunocomplexes isolated with the IEC 1/48 antibody showed residual  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity.

The IEC 1/48 antibody specifically bound to the smaller subunit eluted from SDS-PAGE and, hence, was directed against the  $\beta$  subunit. Furthermore, experiments with tunicamycin showed that the antibody was directed against the protein rather than the carbohydrate moiety of the glycoprotein.

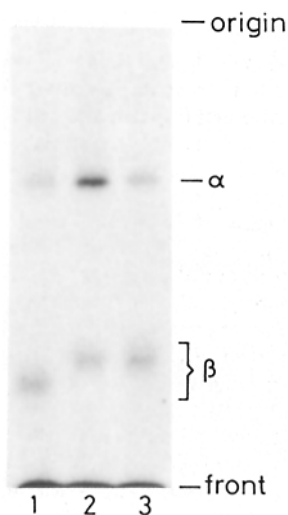
The characteristics and specificity of antibody C 62.4 have been described in a previous publication (Kashgarian et al., 1985). The antibody was found to specifically recognize  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ ; inhibit ouabain binding in the presence of  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Mg}^{2+}$ ; and bind to an epitope of the

cytoplasmic domain of the enzyme. C 62.4 specifically immunoprecipitated a 96,000-kD protein that was identified as  $(\text{Na}^+ + \text{K}^+)\text{-ATPase } \alpha$  subunit. Apparently the binding of the antibody to the  $\alpha$  subunit leads to a dissociation of the enzyme complex (Kashgarian et al., 1985; Caplan et al., 1986a). This was confirmed now by our studies with intestinal  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  which show that only minimal amounts of  $\beta$  subunit remain associated with the  $\alpha$  subunit in the immunoprecipitates. Although the antibody binds to a cytoplasmically exposed segment of the  $\alpha$  subunit, the ouabain binding in the extracytoplasmic part is also affected (Kashgarian et al., 1985). This suggests a gross conformational change by the antibody which may lead to the observed loss of the  $\beta$  subunit. C 62.4 can bind to the  $\alpha$  subunit of intestinal  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  that was first immunisolated with antibody IEC 1/48 as an enzyme complex. This shows that C 62.4 initially binds to assembled  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  and not (exclusively) to free  $\alpha$  subunit.

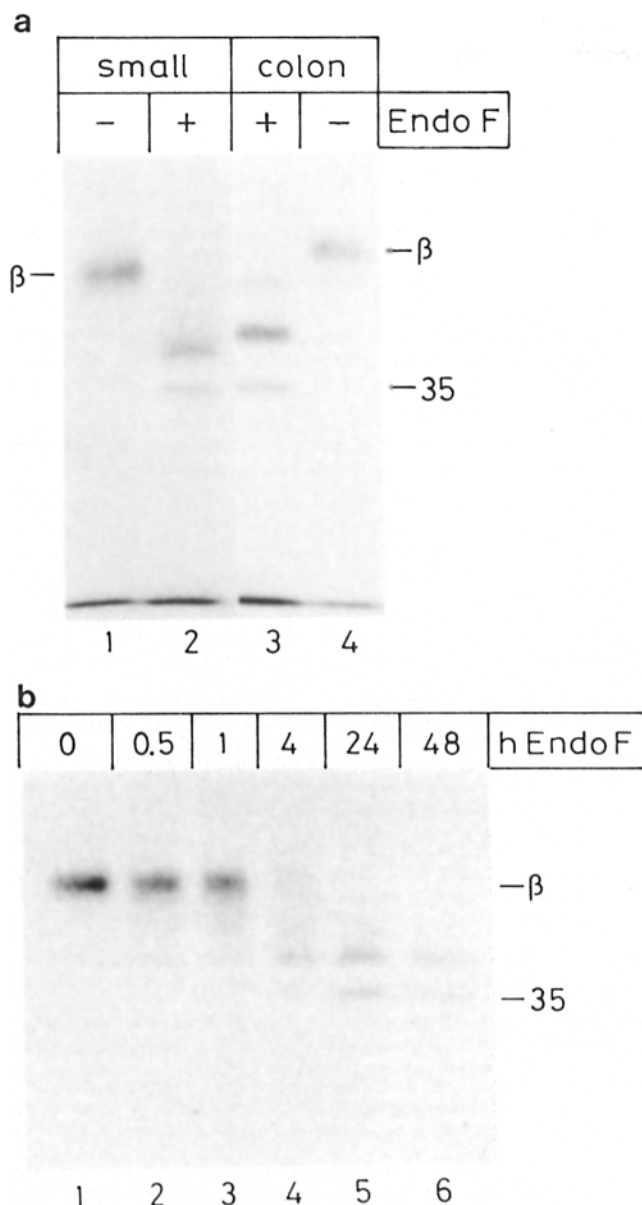
#### **Immunolocalization of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ in Intestinal Epithelial Cells**

Localization of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  by the immunofluorescence technique using intestinal cryosections suggests a decrease of immunoreactive enzyme level in the basolateral membrane in a proximal-to-distal direction. This parallels  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enzyme activities measured in homogenates of isolated epithelial cells from the different parts of the gut (our unpublished results). Both at the light and electron microscope level, immunoreactive  $(\text{Na}^+ + \text{K}^+)\text{-ATPase } \alpha$  and  $\beta$  subunits were exclusively restricted to the basolateral membranes of small intestinal and proximal large intestinal epithelial cells. The only intracellular organelle exhibiting an occasional positive reaction was the Golgi apparatus. This labeling pattern is in agreement with the notion of a direct delivery pathway of newly synthesized  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  to the basolateral plasma membrane (Tamkun and Fambrough, 1986; Caplan et al., 1986b).

An unexpected labeling pattern of the anti- $\beta$  antibody was observed with tissue sections of distal colon. This antibody



**Figure 11.** Comparison of the  $\beta$  subunits in different segments of the rat small intestine (autoradiogram). Total membrane fractions of small intestinal enterocytes (lane 1), proximal colonocytes (lane 2), and distal colonocytes (lane 3) were detergent solubilized, radioiodinated, and immunoprecipitated with the IEC 1/48 antibody against the  $\beta$  subunit. The immunoprecipitates were separated by SDS-PAGE.  $\alpha$ ,  $\alpha$  subunit;  $\beta$ ,  $\beta$  subunit.



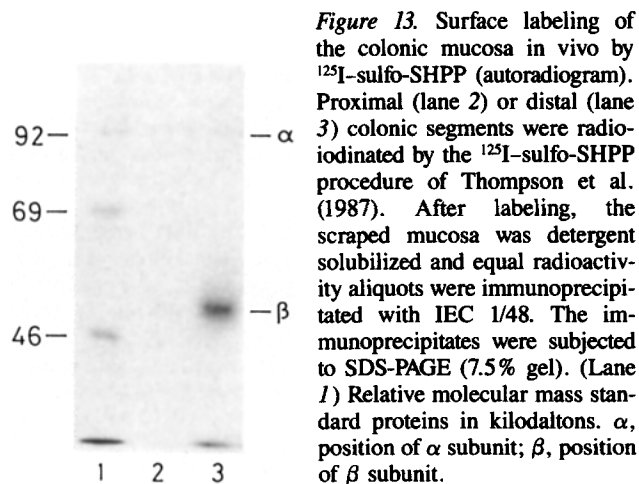
**Figure 12.** Digestion of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$   $\beta$  subunit with endo F (autoradiogram). (a) Small intestinal basolateral membranes (*small*) and distal colon brush border membranes (*colon*) were detergent solubilized, radioiodinated, and immunoprecipitated with the IEC 1/48 antibody. The immunoprecipitates were either digested with endo F (+) for 24 h or mock treated (-) before separation by SDS-PAGE. (b) Immunoisolated  $\beta$  subunit of the small intestine was digested with endo F in a time-dependent manner. To the 48-h endo F sample a fresh aliquot of endo F was added after 24 h.  $\beta$ , position of the complex-glycosylated  $\beta$  subunit; 35, relative molecular mass in kilodaltons of the smallest peptide obtained after endo F treatment.

bound to the brush border membranes and to apically localized vesicular structures, probably lysosomes, in addition to the basolateral membrane. Since the anti- $\alpha$  antibody exclusively decorated the basolateral membrane, the apical staining is unlikely to represent active  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . Furthermore, the results suggest that the labeled vesicles, at least for  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ -related antigens, may be part of the apical but not basolateral endocytotic pathway. A number

of studies have reported apical staining with antibodies against  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  in epithelial cells (Schenk and Leffert, 1983; Takemura et al., 1984; Kyte, 1976a,b; Conteas et al., 1986; Bonnard et al., 1984; Kraehenbuhl, J. P., C. Bonnard, K. Geering, M. Girardet, and B. C. Rossier, unpublished data). Kashgarian et al. (1985) have proposed that these results may be due to the presence of a highly antigenic contaminating protein copurifying with the  $\alpha$  subunit of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . It is important to note that the apical immunolabeling observed in this study is restricted to epithelial cells of distal colon and is neither found in kidney nor in liver epithelial cells; hence it is different from that of the previous studies.

### Characterization of the $\beta$ -Subunit-related Antigen in Brush Borders of Distal Colon

A number of trivial possibilities that may explain the unusual cross-reactivity with the brush border membrane could be excluded. First, the cross-reactivity is not due to a carbohydrate epitope common to the  $\beta$  subunit and a nonrelated brush border protein since the antibody can recognize the bona fide nonglycosylated as well as the high-mannose glycosylated forms with high specificity. Furthermore, the antigen does not survive immunoblotting, a procedure which rarely affects immunoreactivity of a carbohydrate epitope. Second, since most of our experiments were performed with  $^{125}\text{I}$ -labeled membranes, the antigen might have escaped detection provided it lacks tyrosine residues. However, no additional antigen was detectable when the membranes were either labeled chemically with  $[^{14}\text{C}]$ formaldehyde (Dottavio-Martin and Ravel, 1978; not shown) or  $^{125}\text{I}$ -sulfo-SHPP or metabolically with  $[^{35}\text{S}]$ methionine. Third, the antigen might have been lost into the Triton X-100-insoluble pellet before immunoprecipitation either by virtue of its association with the cytoskeleton, as with the intestinal 140-kD protein (Coudrier et al., 1983), or by its attachment to the lipid bilayer via phosphatidylinositol as with alkaline phosphatase, for instance (Low and Zilversmit, 1980). However, this appears unlikely since the cited examples show that these associations are not an all-or-nothing reaction; i.e., a substantial part of the 140-kD protein or alkaline phosphatase appears in the Triton-soluble fraction. And fourth, the unexpected cross-reactivity might be an odd feature of this particular



**Figure 13.** Surface labeling of the colonic mucosa in vivo by  $^{125}\text{I}$ -sulfo-SHPP (autoradiogram). Proximal (lane 2) or distal (lane 3) colonic segments were radioiodinated by the  $^{125}\text{I}$ -sulfo-SHPP procedure of Thompson et al. (1987). After labeling, the scraped mucosa was detergent solubilized and equal radioactivity aliquots were immunoprecipitated with IEC 1/48. The immunoprecipitates were subjected to SDS-PAGE (7.5% gel). (Lane 1) Relative molecular mass standard proteins in kilodaltons.  $\alpha$ , position of  $\alpha$  subunit;  $\beta$ , position of  $\beta$  subunit.

monoclonal antibody. Unfortunately, the polyclonal antibodies at our disposition were not specific enough in immunofluorescence experiments to confirm the apical labeling in the distal colon. However, when a rat monoclonal antibody against the  $\beta$  subunit of mouse ( $\text{Na}^+ + \text{K}^+$ )-ATPase was used (Gorvel et al., 1983) to localize this enzyme in the mouse colon by immunofluorescence on cryosections an identical pattern was found: basolateral labeling in the proximal colonocytes and both basolateral and apical labeling in distal colonocytes (Hauri, H.-P., and U. Eilers, unpublished observations). Again, no additional protein was immunoprecipitable from mouse distal colonocytes with this monoclonal antibody.

The antigen might be similar or identical to ( $\text{Na}^+ + \text{K}^+$ )-ATPase  $\beta$  subunit and therefore comigrate on gels with basolateral-derived  $\beta$  subunit. Evidence in support of this possibility came from experiments in which the radioactivity associated with the electrophoretically separated subunits was quantitated. The  $\beta$ -to- $\alpha$  ratio was higher in the distal than in the proximal brush border fractions. Moreover, in the distal but not in the proximal colon the  $\beta$ -to- $\alpha$  ratio increased with increasing purity of the brush border membrane fraction. Providing that the basolateral-derived  $\alpha$  chain itself and its association with the  $\beta$  chain are equally stable in both intestinal segments, this result suggests that the cross-reacting antigen indeed comigrated with basolateral  $\beta$  subunit. One-dimensional peptide mapping of radiolabeled protein in the  $\beta$ -chain region of the gel did not reveal any significant differences between samples of proximal and distal colon when V8 protease, elastase, or papain were used nor was an additional protein detectable after digestion of the colonic immunoprecipitate with endo F. Finally, radioiodination of the brush border in intact intestinal segments lead to the isolation of an electrophoretically indistinguishable  $\beta$ -subunit-like protein in distal, but not proximal, colon. Overall, the results are consistent with a model in which  $\beta$  subunit or a  $\beta$ -subunit-like protein is expressed in the brush border membrane of the distal colon.

What might be the function of such a  $\beta$ -subunit-like protein in distal colon brush borders? Two principal functions have been assigned to the distal colon: i.e.,  $\text{K}^+$  reabsorption that is mediated by an ouabain-insensitive  $\text{K}^+$ -ATPase (Gustin and Goodman, 1981, 1982) and  $\text{Na}^+$  uptake through an amiloride-sensitive sodium channel (Schultz, 1984). It remains to be elucidated if the  $\beta$ -subunit-like protein in distal colonocytes is in any way related to these functions.

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