

# Membrane-Cytoskeleton Dynamics in Rat Parietal Cells: Mobilization of Actin and Spectrin upon Stimulation of Gastric Acid Secretion

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**Abstract.** The gastric parietal (oxyntic) cell is presented as a model for studying the dynamic assembly of the skeletal infrastructure of cell membranes. A monoclonal antibody directed to a 95-kD antigen of acid-secreting membranes of rat parietal cells was characterized as a tracer of the membrane movement occurring under physiological stimuli. The membrane rearrangement was followed by immunocytochemistry both at the light and electron microscopic level on semithin and thin frozen sections from resting and stimulated rat gastric mucosa. Double labeling experiments demonstrated that a specific and massive mobilization of actin, and to a lesser extent of spectrin (fodrin), was involved in this process. In the resting state, actin and spectrin were mostly localized beneath the membranes of all cells of the gastric gland, whereas the bulk of acid-secreting membranes appeared dif-

fusely distributed in the cytoplasmic space of parietal cells without any apparent connection with cytoskeletal proteins. In stimulated cells, both acid-secreting material and actin (or spectrin) extensively colocalized at the secretory apical surface of parietal cells, reflecting that acid-secreting membranes were now exposed at the lumen of the secretory canaliculus and that this insertion was stabilized by cortical proteins.

The data are compatible with a model depicting the membrane movement occurring in parietal cells as an apically oriented insertion of activated secretory membranes from an intracellular storage pool. The observed redistribution of actin and spectrin argues for a direct control by gastric acid secretagogues of the dynamic equilibrium existing between nonassembled (or preassembled) and assembled forms of cytoskeletal proteins.

**T**HE morphological changes displayed by gastric parietal (oxyntic) cells under hormonal stimulation were the first clue pointing to these cells as the site responsible for the secretion of gastric acid (11, 16, 18, 31). In the resting state, the cell cytoplasm is packed with a smooth tubulovesicle system that once was compared to endoplasmic reticulum (15). 60–80% of the protein content of these membranes consist of an inactivated form of the proton pump complex, the  $H^+-K^+$  ATPase (29, 30). By contrast, in the stimulated state, this membranous structure is reorganized into another fully active membrane entity: the secretory canaliculus, also called the intracellular canaliculus. This latter membrane system appears physically connected with the apical membrane and is fully invaginated with numerous microvilli at maximal stimulation of secretion (7, 11, 17). It is now considered that the membrane of the canaliculus is composed of the material stored as tubulovesicles in resting cells. However, no indisputable mechanism to explain this membrane movement has yet been demonstrated. Some have proposed that there exists a continuity between the resting

tubulovesicle system and the apical membrane (the osmotic extension hypothesis; reference 3). At present, however, most data support a second model (the fusion hypothesis; reference 6); in this model, the resting tubulovesicles are physically separated from the apical membrane (7, 9). Upon stimulation, these cytoplasmic membranes fuse with the apical membrane and generate the secretory canaliculus by successive insertion of membrane elements into a preexisting form of the canaliculus. Thus, in this latter proposal, the resting intracellular tubulovesicles represent a pool of stored inactive material which, upon stimulation, becomes inserted at the apical face concomitant with the activation of the enzyme system (the proton pump).

At the biochemical level, some important processes can now be understood thanks to the characterization of the nonelectrogenic gastric proton pump, the  $H^+-K^+$  ATPase (30). This enzyme was first detected as a 95–100-kD prominent component of purified porcine gastric tubulovesicles (29, 30) and was successively characterized in all other mammalian stomachs. The  $H^+-K^+$  ATPase was recently solubi-

lized in an active form (34), making studies possible after reconstitution of the enzyme into artificial proteoliposomes (26). The catalytic subunit of the rat enzyme has been cloned and sequenced (32). It displayed 62% amino acid sequence homology with the Na<sup>+</sup>-K<sup>+</sup> ATPase from sheep kidney. In addition to the gastric ATPase, a K<sup>+</sup>-Cl<sup>-</sup> cotransport was characterized in purified gastric vesicles. A low Cl<sup>-</sup> conductance and a valinomycin-dependent permeability to K<sup>+</sup> ions are specific of resting membranes. Stimulated gastric vesicles, on the contrary, possess a much higher Cl<sup>-</sup> conductance and a permeability to K<sup>+</sup> ions independent to valinomycin (14, 35, 39, 40).

Membranes associated with the gastric ATPase behave differentially in a centrifugal field, depending on whether or not they have been purified from resting or stimulated animals (13, 14, 39, 40). Resting membranes are much lighter than stimulated membranes and it is assumed that the former consist of tubulovesicles and the latter of secretory canaliculus and apical membranes. These physical differences might be explained if one proposes that stimulated membranes are heavier because they are decorated with a cytoskeletal cortex at their cytoplasmic face (2, 21, 23). On the other hand, intracellular resting tubulovesicles may be free of any cortical association. It has been shown that these tubules were not associated with electron dense material (10, 38). Additional data are consistent with the involvement of the parietal cell cytoskeleton in the membrane transformations occurring in these cells during the process of acid secretion. EGF and more recently transforming growth factor- $\alpha$  have been found to be potent inhibitors of acid secretion (10, 27). Ultrastructural studies showed that parietal cells were unable to shift from resting to stimulated state when treated first with the growth factor followed by a physiological stimulus. The authors proposed that the inhibitory effect of EGF was a consequence of one of its pleiotropic effects on the cell cytoskeleton (10). Another study showed that, *in vitro*, rat parietal cells were unable to accumulate the weak base aminopyrine (a method to assay acid secretion *in vitro*) when treated in the presence of cytochalasin D, whereas colchicine had no effect (28). Actin-based cytoskeleton appeared, therefore, to be involved in this process.

Gastric parietal cells represent a unique biological model to study apical membrane movements, from an intracellular storage pool, and back to it, modulated by physiological stimuli. This model should be of great help to understand how a membrane structure is inserted at a directed site in the plasma membrane. This dynamic process integrates necessarily other biological parameters, and particularly should be linked to a coherent control of the state of assembly of the cell cytoskeletal infrastructure. To gain valuable information on the parietal cell system, it is first essential to have access to specific markers and these have been searched for a long time (7). Here, we use a mAb specific to a 95-kD luminal component of acid-secreting membranes of rat gastric parietal cells as a specific membrane movement tracer allowing an easy observation of the phenomenon. Double labeling experiments performed on resting or stimulated gastric tissues illustrate that a massive mobilization of the actin-spectrin cytoskeleton (2, 21, 23) appears necessary to stabilize the insertion of the intracellular storage membranes to the apical face of the cell upon stimulation of secretion. The data, fully compatible with the membrane fusion hypothesis (6), indi-

cate that a nonassembled pool of cortical proteins should exist in resting cells. In this state, these proteins are unable to associate with intracellularly stored secreting membranes. Thus, physiological stimuli exert, in parietal cells, a control of the dynamic assembly of cytoskeletal structures.

## Materials and Methods

### Animals

Adult Wistar rats were fasted for one night, 1 h before being sacrificed, rats were either treated with the H<sub>2</sub> antagonist cimetidine (100 mg/kg) intraperitoneally (resting conditions), or given standard food or injected with carbachol (350  $\mu$ g/kg) subcutaneously (stimulated conditions).

### Membrane Fractionation

Rat gastric fundic cells were prepared according to Gespach et al. (8). The preparation of mucosal membranes, isolated from resting and stimulated animals, is described elsewhere (23a).

### Antibodies

The production and purification of mouse monoclonal antibody mAb 146.14 is fully described elsewhere (23a).

Rabbit antisera against cytoskeletal proteins (actin,  $\alpha$ -actinin, myosin, tropomyosin, filamin, vinculin, talin, tubulin, and calpactin I, the 36-kD substrate of oncogene kinase) were kindly provided by Dr. Keith Burrigge (University of North Carolina, Chapel Hill, NC). Rabbit anti-villin antibody was a gift from Dr. D. Louvard (Institut Pasteur, Paris, France). Affinity-purified bovine brain spectrin (fodrin) and anti-clathrin antibodies were prepared and used as previously described (5, 22). Fig. 1 shows the monospecificity of the anti-actin (4) and anti-spectrin antibodies on rat gastric mucosa cells. A single band of 43 kD cross-reacted with anti-actin antibody, whereas one band of spectrin-immunoreactive material was selectively detected using anti-brain spectrin antibody at an expected molecular mass of 240 kD. Although it is not apparent on this autoradiograph, it is assumed that the gastric spectrin is composed of an  $\alpha$  and  $\beta$  chain, similar to the fodrin molecule.

### SDS-PAGE and Immunoblot Analysis

SDS-PAGE was performed in slab gels according to Laemmli (19). 15% gels contained 0.08% bisacrylamide. For immunoblotting, the method of Towbin et al. (37) was modified as in reference 22 using <sup>125</sup>I-protein A (Amersham Corp.; 5  $\times$  10<sup>5</sup> cpm/ml for 2 h) followed by autoradiography.

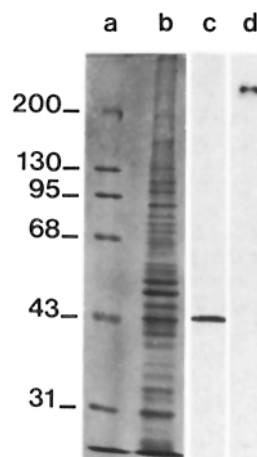
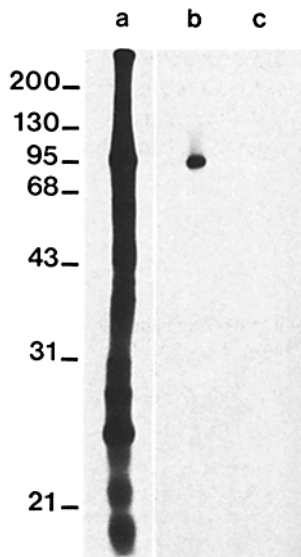


Figure 1. Characterization of immunoreactive antigens to anti-actin and anti-brain spectrin (fodrin) antibodies in rat gastric isolated mucosa cells. (Lanes a and b) Coomassie Brilliant Blue staining of molecular mass markers (a; indicated in kD on the left) and a total lysate from rat gastric isolated fundic mucosa cells (lane b). (Lanes c and d) Replica of lane b transferred on nitrocellulose incubated with anti-actin (lane c) or anti-brain spectrin (lane d) followed by radioiodinated protein A and autoradiography.



**Figure 2.** Immunoadsorption of 95-kD antigen on a mAb 146.14 affinity column.  $^{125}\text{I}$  radioiodinated, solubilized rat tubulovesicle membrane proteins (lane *a*) were incubated on a mAb 146.14-coupled Sepharose 4B column (lane *b*) or on a nonimmune affinity column (lane *c*). Affinity gels were extensively washed, eluted with sample gel buffer, analyzed on SDS-PAGE, and autoradiographed. The band eluted from the mAb 146.14 affinity gel (lane *b*) comigrated with the prominent 95-kD band ( $\text{H}^+\text{-K}^+$  ATPase) present in the membrane fraction (lane *a*).

### Iodination of Membrane Protein and Immunoadsorption Analysis

200  $\mu\text{l}$  of gastric membrane fraction (2–3 mg/ml) were iodinated by the chloramine T method (12) using 2 mCi  $^{125}\text{I}$  (New England Nuclear, Boston, MA) as described in reference 5. Iodinated membrane fractions were pooled and supplemented with a  $10\times$  solution of TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1%  $\text{NaN}_3$ ) containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 0.5 mM PMSF (complete TBS). After incubation for 15 min on ice, the membranes were spun for 1 h at 100,000  $g$  and the supernatant and the pellet were saved and analyzed on SDS-PAGE (essentially all proteins were found in the supernatant, not shown). Aliquots (1 ml) of solubilized iodinated membrane proteins were incubated 90 min on a rotating apparatus at  $4^\circ\text{C}$  with 100  $\mu\text{l}$  of a slurry of nonimmune mouse IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) coupled to Sepharose 4B (BrCN-activated Sepharose 4B; Pharmacia Fine Chemicals, Uppsala, Sweden) according to the manufacturer specifications (3.5 mg IgG/ml gel), then centrifuged for 2 min at 3,000  $g$  in a microfuge (Beckman Instruments, Inc., Palo Alto, CA), and the supernatant was saved. To one supernatant aliquot, 100  $\mu\text{l}$  of a slurry of nonimmune IgG-Sepharose 4B was added, and to a second one, 100  $\mu\text{l}$  of a slurry of purified mAb 146.14 coupled to Sepharose 4B (3.5 mg IgG/ml gel) was added. After a one-night incubation at  $4^\circ\text{C}$ , the gels were washed four times with 1 ml complete TBS followed by centrifugation at 3,000  $g$  for 2 min. After the final washing, the adsorbed material was eluted with 100  $\mu\text{l}$  Laemmli's sample. The recovered eluted material was then analyzed on SDS-PAGE followed by autoradiography at  $-80^\circ\text{C}$  on X-Omat AR film (Eastman Kodak Co., Rochester, NY) using an intensifying screen (2 d).

### Indirect Immunofluorescence Microscopy

For hybridoma screening, isolated rat gastric fundic cells were attached to polylysine-coated (0.1 mg/ml, 20 min at room temperature) 12-mm glass coverslips and then fixed, permeabilized (when necessary), and processed for indirect immunofluorescence microscopy as previously described (22). Frozen, semithin sections (0.5–0.8  $\mu\text{m}$ ) of rat gastric mucosa were prepared according to the method of Tokuyasu (36), using only paraformaldehyde as fixative (3% in PBS containing  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ , 1 h at room temperature). Frozen sections attached to glass slides were then processed for immunofluorescence microscopy. Antibodies were appropriately diluted in TBS containing 0.2% gelatin. Fluorophore-conjugated secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG) were from Nordic Immunological Laboratories. 10% rat serum was added to the second antibody solution. Cells and frozen sections were observed on a Leitz Dialux 20 microscope or a Zeiss photomicroscope III, both equipped with appropriate filters for fluorescein and rhodamine fluorescence and with a  $25\times/0.8$  or a  $63\times/1.4$  phase contrast oil-immersion lens.

### Ultrastructural Immunocytochemistry

Thin frozen sections were performed according to Tokuyasu (36). The rat gastric fundic mucosa was fixed with either 2% paraformaldehyde and 0.5% glutaraldehyde or with 3–8% paraformaldehyde in 100 mM potassium phosphate, pH 7.4, for 1 h at room temperature. Sections were labeled with an adequate dilution of purified mAb 146.14 and, when double labeling was performed, with rabbit anti-actin antiserum (1:100 dilution). After several washings, sections were then incubated with commercial (Janssen Pharmaceutica, Beerse, Belgium) secondary antibodies, goat anti-mouse IgG, and goat anti-rabbit IgG, respectively, coupled to 15- and 5-nm colloidal gold particles. The sections were then thoroughly rinsed and processed for electron microscopy.

### Results

#### *mAb 146.14 is a Marker of Acid-secreting Membrane Reorganization in Rat Parietal Cells*

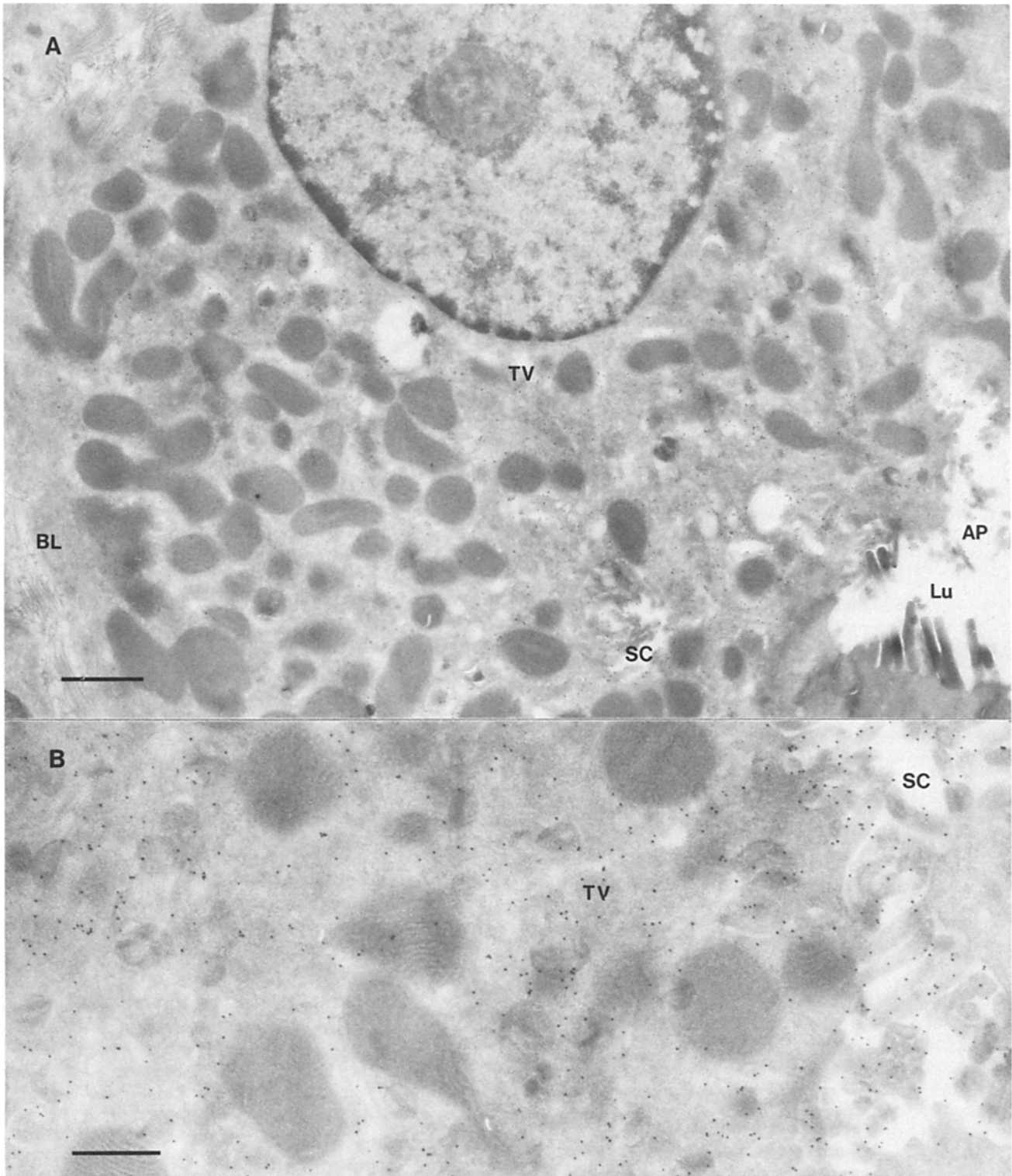
mAb 146.14 is a monoclonal antibody produced during the generation of mouse hybridoma against various antigens specific of the rat gastric mucosa. The antigen recognized by mAb 146.14 has been characterized elsewhere (23a). It consists of a 95-kD component (Fig. 2) only expressed in parietal cells (Figs. 3–6), predominantly present on an enriched acid-secreting membrane fraction purified from rat gastric tissue. The epitope is lumenally oriented (23a).

In the resting state, the 95-kD antigen was specifically concentrated in membranous areas related to acid secretion (Fig. 3, *A–B*). Labeling was only detected at the apical cell surface, at the luminal face of the membrane of the secretory canaliculus, as well as on intracellular tubulovesicular membranes. In contrast, no staining was observed in other parts of the cell such as the cell nucleus, the mitochondria, and the basolateral membrane.

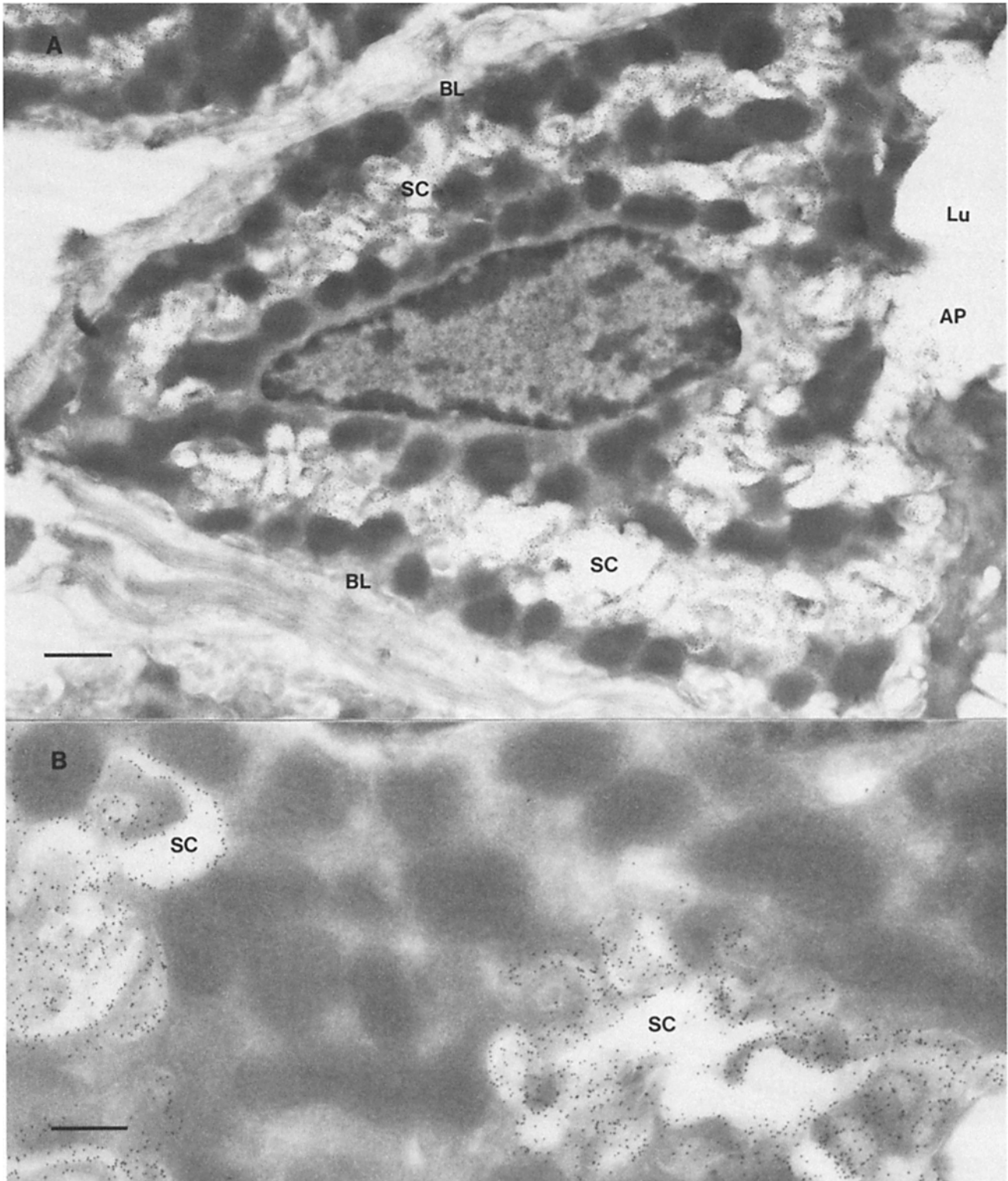
In the stimulated state, oxyntic cells underwent a remarkable change in morphology. The cell cytoplasm appeared free of membranous structures (tubulovesicles), which were now inserted at the extended apical cell surface forming the active secretory canaliculus. In this case, the labeling appeared uniquely distributed all over the newly formed apical plasma membrane (Fig. 4, *A–B*). No trace of residual label remained in the cell cytoplasm (Fig. 4 *B*). The microvilli of the secretory canaliculus were much more densely labeled than those of the preexisting structure in the resting state (compare Fig. 3 *B* with Fig. 4 *B*). In view of the results illustrated in Figs. 3 and 4, it clearly appeared that the 95-kD antigen was associated both with the inactive internal tubulovesicles in resting cells and with the active secreting membranes once inserted at the apical surface in stimulated cells.

#### *The Cytoskeletal Proteins Actin and Spectrin Are Mobilized during the Reorganization of Acid-secreting Membranes of Parietal Cells*

Double labeling experiments were performed on semi-thin frozen sections from resting and stimulated gastric tissues using mAb 146.14 to trace the acid-secreting membrane movement and different antibodies to various cytoskeletal proteins, to detect if any of these were associated with the membrane translocation. The cellular localization of two of these proteins, actin and spectrin, was particularly affected by this selective membrane movement. After stimulation, a massive actin reorganization was detected in parietal cells (Fig. 5), whereas a less dramatic but still noticeable redistribu-

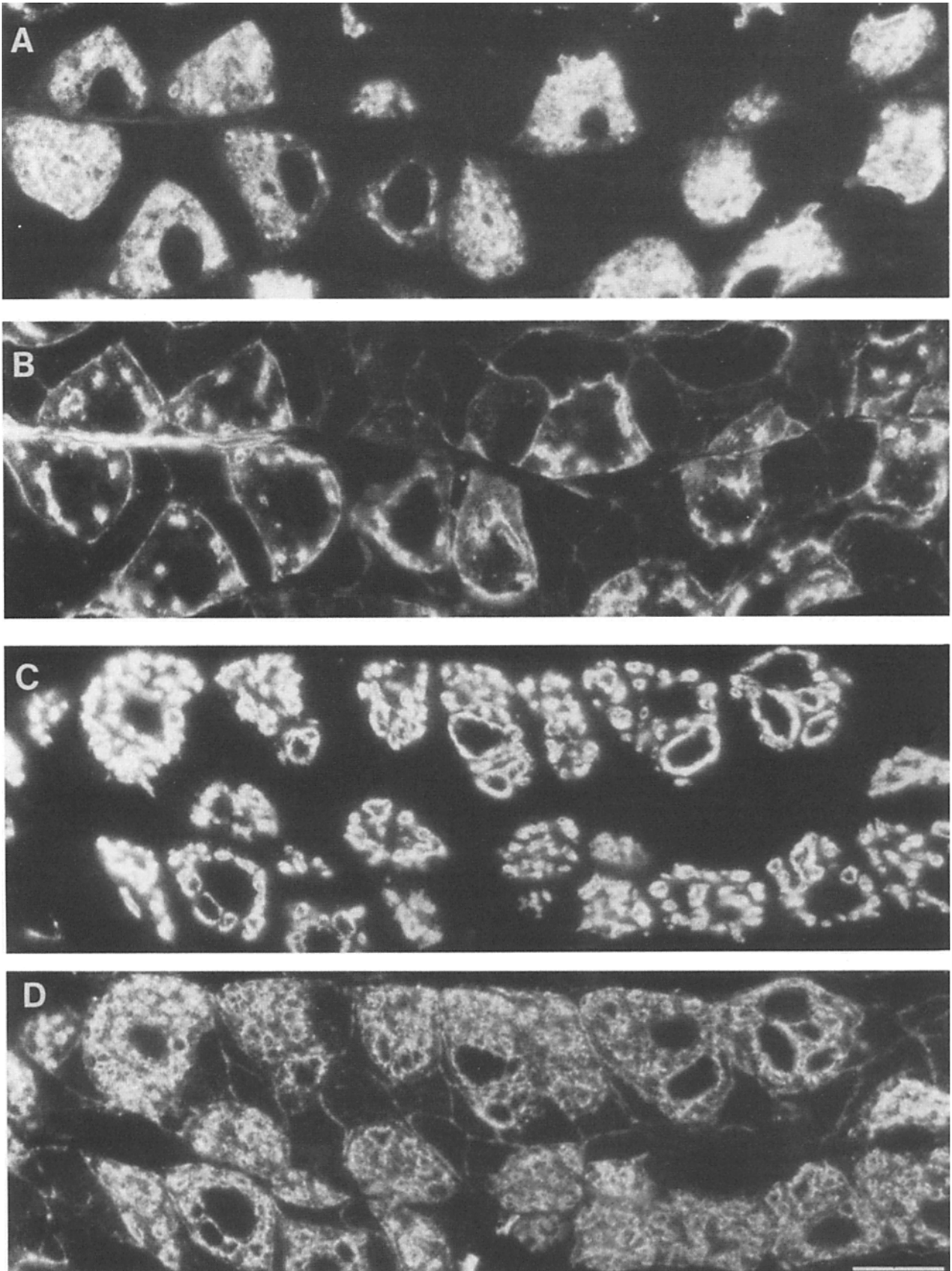


**Figure 3.** Ultrastructural characterization of 95-kD antigen in resting parietal cells. The 95-kD antigen was visualized by incubations of the section with mAb 146.14 followed by an appropriate second antibody coupled with 15-nm colloidal gold particles. *TV*, tubulovesicle membranes; *SC*, secretory canaliculus; *AP*, apical surface; *Lu*, lumen of the gastric gland; *BL*, basolateral membrane. (A) Low magnification of a half part of a parietal cell. Note the intense labeling of cytoplasmic structures (tubulovesicles), of secretory microvilli, and of the apical membrane of the parietal cell. By comparison, no label is present along the microvilli of the apical surface of a neighboring cell (bottom right of the micrograph). (B) Higher magnification of a part of another resting cell. Note the specific staining of cytoplasmic tubulovesicles and of microvilli of the preexisting secretory canaliculus. In comparison, mitochondria are unlabeled. Bars: (A) 1  $\mu$ m; (B) 500 nm.

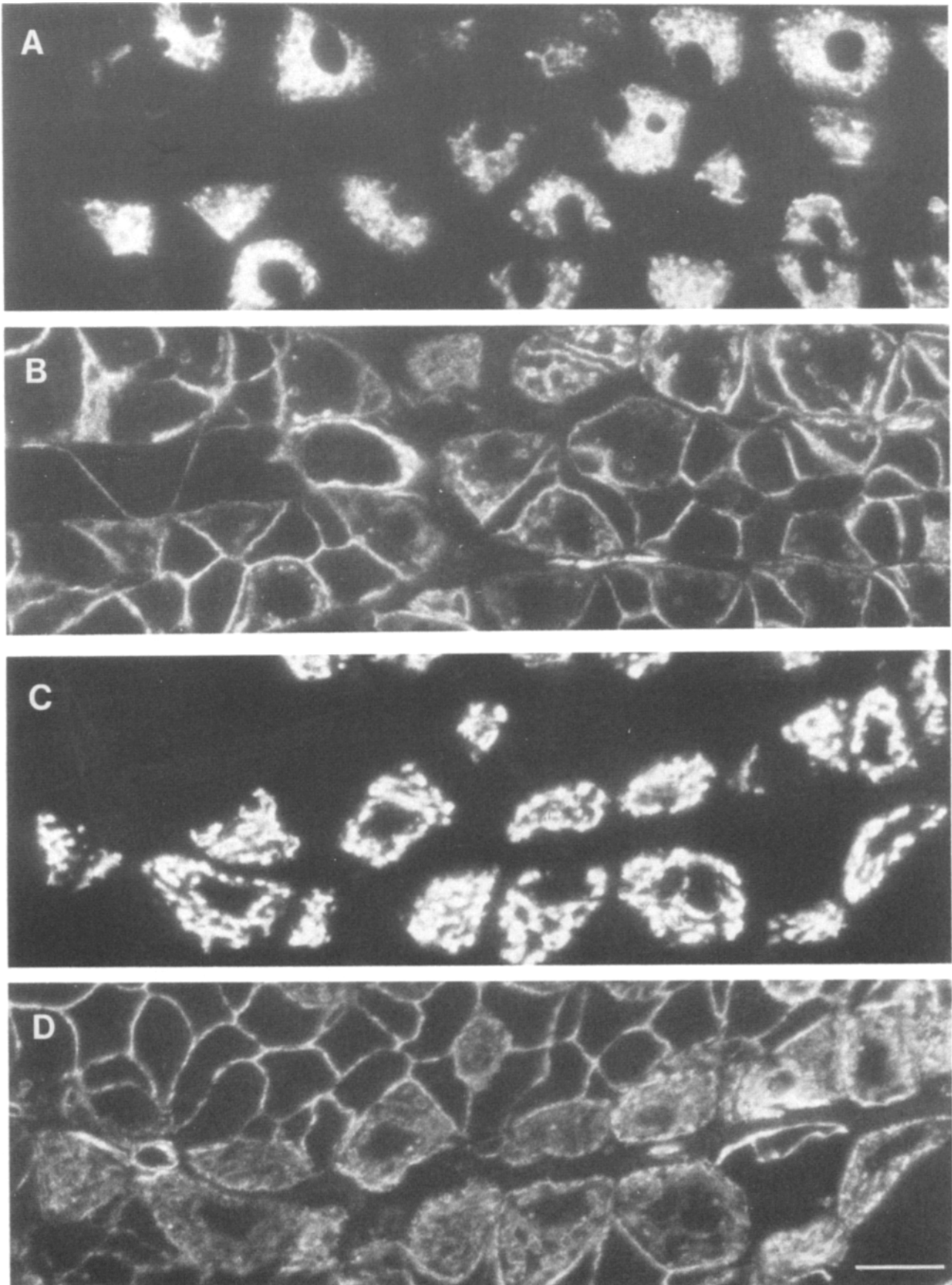


**Figure 4.** Ultrastructural characterization of 95-kD antigen in stimulated rat parietal cells. (A) The expanded secretory canaliculus (SC) appears physically associated with the apical membrane (AP), largely open to the lumen (Lu) of the gastric gland. All gold particles are decorating microvilli of secretory membranes. No labeling is detected in the cell cytoplasm, in the nucleus, and in the basolateral membrane. (B) Higher magnification of a part of another stimulated cell. Note the absence of intracytoplasmic labeling in contrast with the high density of gold particles associated with secretory membranes. Compare the density of labeling of the secretory canaliculus with that found in resting cell (see Fig. 3 B). Bars: (A) 1  $\mu$ m; (B) 500 nm.





**Figure 5.** Actin redistribution in rat parietal cells upon stimulation of acid secretion. Semithin, longitudinal frozen sections of fixed rat gastric fundic mucosa were double labeled with mAb 146.14 (*A* and *C*) and rabbit anti-actin antibody (*B* and *D*), followed by fluorescein-conjugated goat anti-mouse IgG (*A* and *C*) and rhodamine-conjugated goat anti-rabbit IgG (*B* and *D*) antibodies. (*A* and *B*) Identical frozen section from resting gastric mucosa selectively viewed with fluorescein (mAb 146.14, *A*) and rhodamine (anti-actin, *B*) optics. (*C* and *D*) Identical frozen section from stimulated gastric mucosa selectively viewed with fluorescein (*C*) and rhodamine (*D*) optics. Bar, 15  $\mu$ m.



**Figure 6.** Spectrin mobilization in rat parietal cells upon stimulation of acid secretion. Semithin, longitudinal frozen sections of fixed rat gastric fundic mucosa were double labeled with mAb 146.14 and affinity-purified anti-brain spectrin (fodrin) antibody followed by appropriately conjugated second antibodies as in Fig. 5. (*A* and *B*) Identical frozen section from resting gastric mucosa selectively viewed with fluorescein (mAb 146.14, *A*) and rhodamine (anti-spectrin, *B*) optics. (*C* and *D*) Identical frozen section from stimulated gastric mucosa selectively viewed with fluorescein (mAb 146.14, *C*) and rhodamine (anti-spectrin, *D*) optics. Bar, 15  $\mu\text{m}$ .

(Fig. 5), whereas a less dramatic but still noticeable redistribution of parietal cell spectrin (fodrin) was evident (Fig. 6).

In the resting state, actin and spectrin were localized in various amounts along the apical and basolateral membranes of all cells of the gastric gland. Actin was more represented in parietal cells than in nonparietal cells, whereas spectrin was equally distributed. In parietal cells, some patches of actin and spectrin fluorescence were scattered throughout the cells (Figs. 5 *B* and 6 *B*). These patches were always found colocalizing with similar mAb 146.14 fluorescence spots, each time one could well-distinguish the latter among the overall intracellular distribution of 95-kD antigen. However, the bulk of the mAb 146.14 fluorescence in resting cells was mostly diffuse and noncoincident with spectrin and actin labeling.

In stimulated tissues, a totally different fluorescence pattern was obtained (Fig. 5, *C* and *D* and Fig. 6, *C* and *D*). The fluorescent changes were specifically localized in parietal cells, whereas actin and spectrin distribution appeared unaffected in nonparietal cells. Within stimulated parietal cells, both mAb 146.14 and either actin or spectrin fluorescence appeared coincident. The fluorescence of actin and spectrin was somewhat blurred compared to the sharpness of the staining obtained with mAb 146.14. In contrast to the diffuse and mottled intracellular labeling observed in resting cells, mAb 146.14 fluorescence now appeared in more restricted areas, in agreement with the new insertion of apical secreting membranes.

Redistribution of other cytoskeletal proteins, such as tubulin and clathrin, was not observed (not shown). Other proteins ( $\alpha$ -actinin, myosin, tropomyosin, filamin, calpactin I, vimentin, villin, vinculin, and talin) were tested for their presence by use of antibodies known to cross react with their counterparts expressed in rat smooth muscle and rat fibroblasts or in rat intestinal epithelium but were absent or expressed at a too low level in parietal cells to draw any conclusion at this level of observation.

To clarify the redistribution of the cell cytoskeleton observed at the light level with respect to the acid-secreting membrane movement occurring in parietal cells, double labeling experiments were performed at the electron microscope level, using mAb 146.14 and anti-actin. Both antibodies were revealed using secondary antibodies coupled with 15- and 5-nm colloidal gold particles, respectively. In resting conditions (Fig. 7) intracellular inactive acid-secreting membranes (tubulovesicles) were labeled with mAb 146.14, whereas only a few 5-nm colloidal gold particles (actin) were scattered throughout this area of the cell. In the same cell, however, lumenally exposed membranes were organized as microvilli and double labeled with mAb 146.14 and anti-actin (Fig. 7 *B*). A similar double distribution of 95-kD antigen and actin was observed in the more elongated microvilli forming the secretory canaliculus of stimulated cells (Fig. 8 *A*). As shown, actin labeling was intense in the internal part of microvilli as well as in some intracytoplasmic submembranous structures beneath the microvilli. In Fig. 8 *B*, the labeling with anti-actin antibodies of a basolateral membrane of a parietal cell is presented as a control. In this area of the cell, depending on previous results obtained at the light level, labeling with anti-actin was expected (see Fig. 5), whereas no acid-secreting membrane was present.

A tentative quantification of the respective labeling of actin

and 95-kD antigen was performed in different parts of parietal cells related to acid-secretion on six different sections. Two areas, specifically chosen on a morphological basis, were scored in each resting cell; one, located intracellularly, corresponded to inactive tubulovesicles, the other to apical membranes or preexisting secretory canaliculus. In each case, the number of respective colloidal gold particles were recorded. In stimulated cells, the record was made at the level of the secretory canaliculus, the only place where double labeling was observed. In all cases, the labeling ratio, i.e., the number of 15-nm gold particles per number of 5-nm gold particles, was determined. As shown in Table I, in resting cells, the mean labeling ratio varied within a factor of  $\sim 14$  times between the internal tubulovesicle area (mean ratio 0.23) and the lumenally inserted secretory membranes (mean ratio 3.5). The ratio increased slightly (1.6 times) in stimulated canaliculus membranes (mean ratio 0.36) when compared to the same area of resting cells, reflecting that the density of 95-kD antigen was higher in active microvilli than in inactive ones.

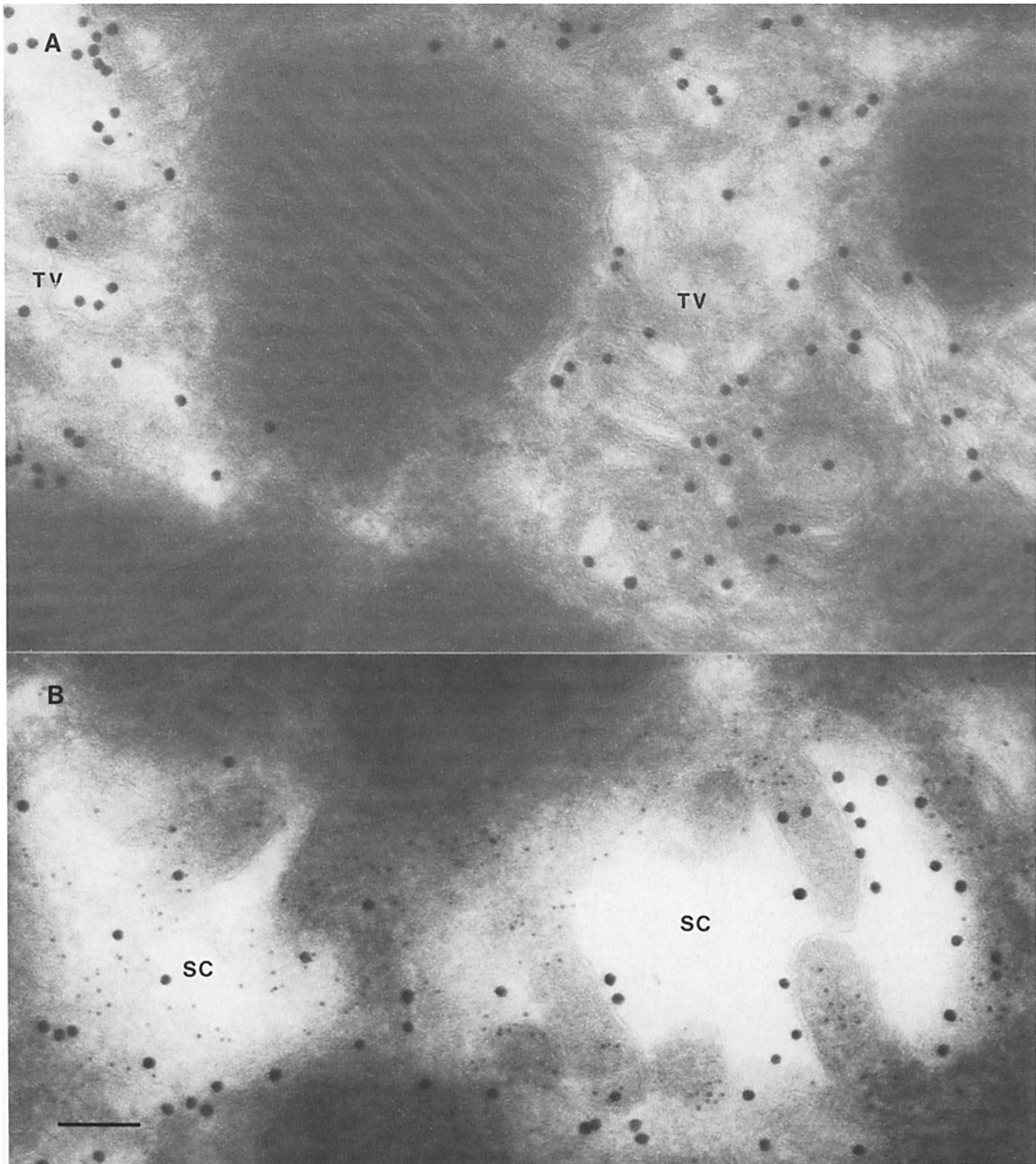
### *Membrane Translocation and Cytoskeletal Reorganization Are Rapid and Concomitant Events*

Fasted rats were subcutaneously injected with a single dose of carbachol and sacrificed at various intervals of time. The gastric fundic mucosa was fixed and processed for double immunostaining with mAb 146.14 and the F actin marker 7-nitrobenz-1,3-diazole-phalloidin (1), or rhodamine-phalloidine, thus preventing interference with any staining due to G actin material. Compared to resting conditions, a significant change in the pattern of mAb 146.14 fluorescence was detected as soon as 5 min after injection (results not shown). At this time, mAb 146.14 fluorescence still showed a diffuse and mottled distribution inside parietal cells, as in resting conditions, but new intense patches of fluorescence were also present. These structures always colocalized with F actin. This indicated that some internal membranes have already translocated to the apical cell surface and that actin reorganization occurred concomitant with this new insertion of membranes. At 10 min after injection and later on, all parietal cells were in a stimulated state, as evidenced by the specific pattern of mAb 146.14 fluorescence (results not shown). At these times, both mAb 146.14 and rhodamine-phalloidin fluorescence colocalized in parietal cells, as was previously found with anti-actin antibody (Fig. 5, *C* and *D*).

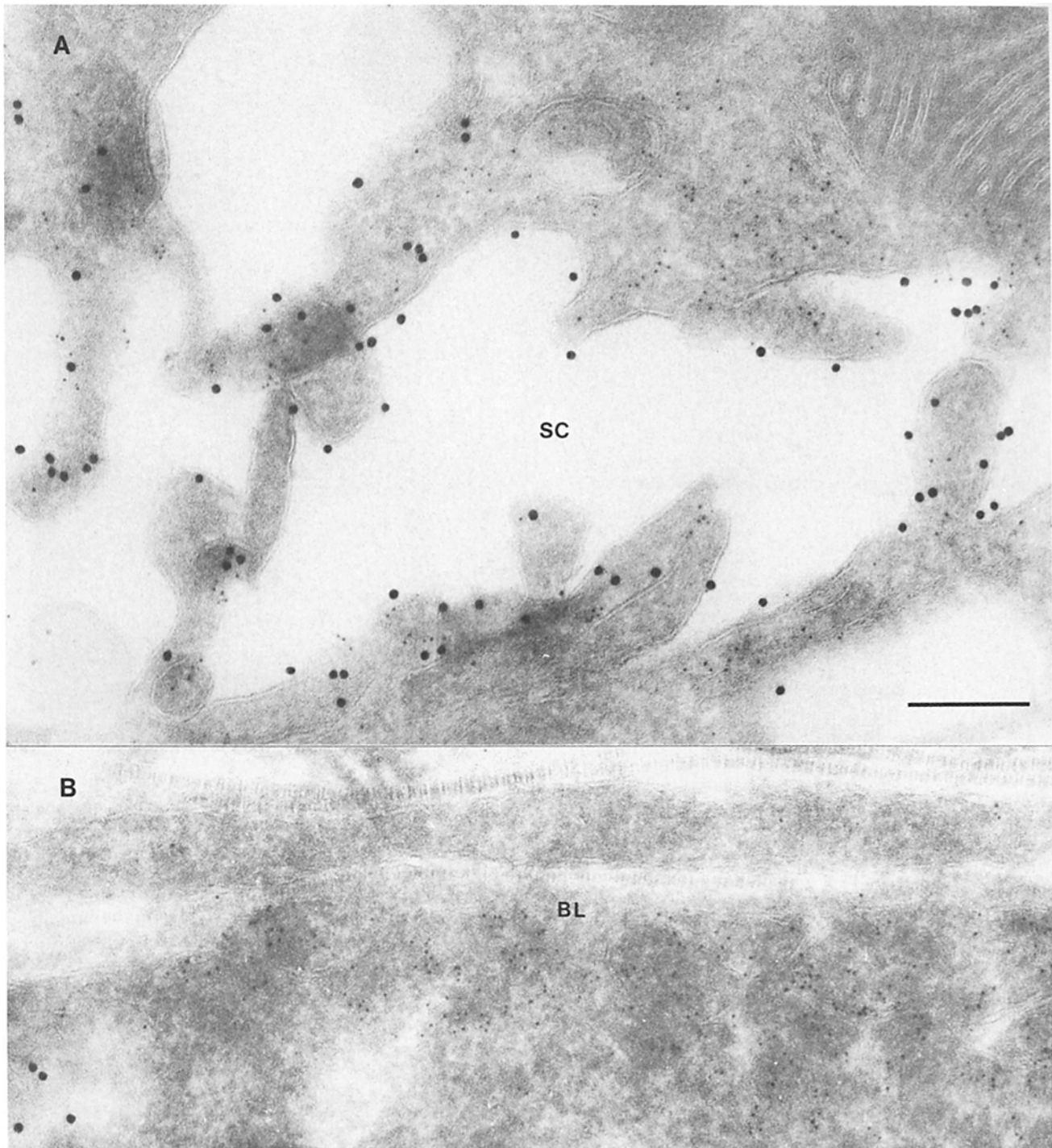
### *Discussion*

In this study, we have used a monoclonal antibody, mAb 146.14, characterized as a marker of acid-secreting membranes of rat gastric parietal cells to study the role of the cell cytoskeleton in the membrane translocation occurring upon stimulation of secretion. The antibody recognizes a 95-kD membrane protein, possibly part of the gastric proton pump (23a). The ultrastructural localization of the antigen (Figs. 3, 4, 7, and 8) clearly indicated that it was only expressed on membranous structures known to be related to gastric acid secretion. In resting cells, the labeling was associated with intracytoplasmic structures, composed of tubulovesicle membranes. Additionally, apical membranes and those of the secretory canaliculus (always present in reduced amount





**Figure 7.** Ultrastructural characterization of acid-secreting membranes and actin cytoskeleton in resting parietal cells. *A* and *B* are two different fields of the same cell. (*A*) Intracytoplasmic tubulovesicles (*TV*) are labeled with 15-nm gold particles (specific for 95-kD antigen labeled with mouse mAb 146.14) and appear free of any connection with actin microfilament. Only a few 5-nm gold particles (specific for actin labeled with rabbit anti-actin) are apparent in the field. (*B*) The secretory canaliculus (*SC*) contains acid-secreting membranes organized in small microvilli open to the lumen of the gland. They represent an extension of the apical cell surface. An intense colocalization of the two sizes of gold particles is apparent. 15-nm gold particles (95-kD antigen) are mostly aligned along the surface of the secretory membrane. 5-nm gold particles (actin) are more concentrated in the inner part of the microvilli (the morphology of microvilli on the left was less preserved than that on the right) indicating the presence of microfilaments in these structures. Bar, 100 nm.



**Figure 8.** Ultrastructural characterization of acid-secreting membranes and actin cytoskeleton in stimulated parietal cells. (A) A view of the extended secretory canaliculus (SC; apical surface) of a stimulated parietal cell identified by the presence of 95-kD antigen along the secretory membrane (15-nm gold particles). The presence of actin cytoskeleton is demonstrated by the specific labeling of microvilli with actin antibody and revealed by 5-nm gold particles. Note that actin labeling appears internal and is in some cases quite deep at the base of the microvilli (it is particularly apparent in the right of the upper part of the micrograph). (B) This view shows the specific and expected presence of actin cytoskeleton along the basolateral membrane of the parietal cell, while no 95-kD antigen is detected. Bar, 200 nm.

in resting cells) were also labeled. These latter structures were the only ones heavily labeled in stimulated cells, whereas intracellular staining has totally disappeared. Altogether, these data indicate that inactive internal membranes are composed in part of the 95-kD antigen and that these

membranes and the 95-kD antigen are both translocated and inserted at the apical surface upon stimulation of secretion.

The resolution offered by the semithin frozen section method at the light microscope level enabled us to trace this membrane reorganization with mAb 146.14. It has been

**Table I. Quantitative Comparison of the Respective Association of Actin Cytoskeleton with Acid-secreting Membranes in Different Areas of Resting and Stimulated Parietal Cells**

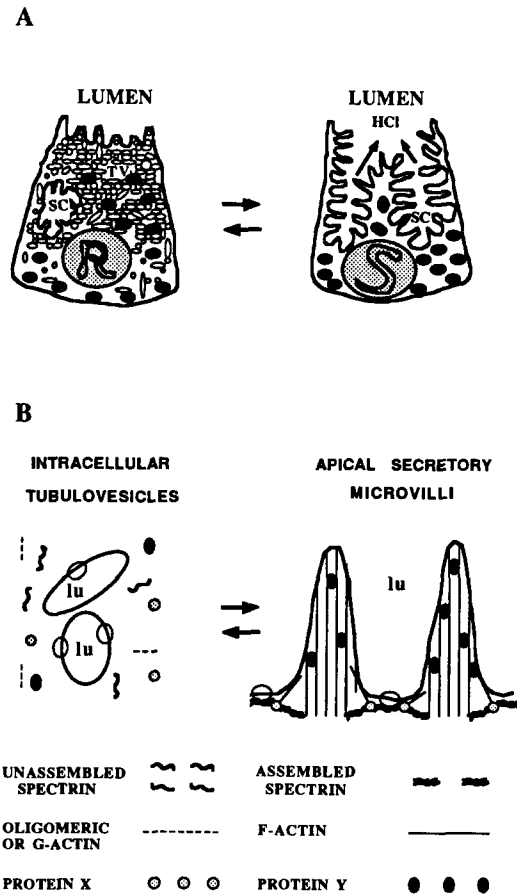
Section No.	Canaliculus			Tubulovesicles			B/A
	95 kD	Actin	Ratio A	95 kD	Actin	Ratio B	
<b>Resting</b>							
1	92	320	0.29	217	37	5.86	20.2
2	98	310	0.32	252	42	6.00	18.7
3	64	520	0.12	364	213	1.71	14.2
4	100	357	0.28	56	14	4.00	14.3
5	142	718	0.20	264	151	1.75	8.7
6	42	230	0.19	188	126	1.49	7.8
Mean	—	—	0.23	—	—	3.5	14.0
<b>Stimulated</b>							
7	156	519	0.30				
8	194	467	0.41				
9	335	721	0.46				
10	273	868	0.31				
11	262	637	0.41				
12	137	461	0.30				
Mean	—	—	0.36				

Quantitative comparison of the respective association of actin cytoskeleton with acid-secreting membranes in different areas of resting and stimulated parietal cells. Sections 1–6 were recorded on specimen from resting tissues, whereas sections 7–12 were from stimulated stomachs. In each resting cell, two areas were selected, secretory canaliculus and intracytoplasmic tubulovesicles, and scored. Values represent the number of 15-nm gold particles (95 kD) and the number of 5-nm gold particles (actin) recorded in each chosen area. B/A represents the value of ratio B/ratio A and reflects the relative degree of variation in the association of secretory membranes with actin cytoskeleton in the different cell compartments. The higher value of the mean ratio A in stimulated cells compared with that found in resting cells reflects that more 95-kD antigen was present on secretory canaliculus membranes of stimulated cells than in membranes of resting cells.

extensively used to follow any variation in the gross organization of cytoskeletal elements upon stimulation of parietal cells with a wide range of antibodies. Among the various cytoskeletal proteins assayed, an important mobilization of actin and spectrin molecules was specifically observed. Non-parietal cells were not affected by the cytoskeletal reorganization observed in parietal cells, thus suggesting that it was specifically linked to the biological activity of these cells. Fluorescent sections indicated that, upon stimulation, actin and spectrin colocalized in some extent with acid-secreting membranes, in addition to their well-characterized location at the contour of parietal and neighboring cells (Fig. 5, C and D and Fig. 6, C and D). This colocalization, in the stimulated state, reflected the fact that the intracellular storage pool of secreting membranes has been apically inserted, considerably expanding the surface of the canaliculus, which was decorated at the cytoplasmic face by cortical proteins in agreement with earlier observations by Vial and Garrido (38).

This interpretation was further supported by double labeling experiments at the electron microscope level. From a quantitative survey of different sections in various areas of parietal cells, it appeared that internal inactive tubulovesicles labeled with mAb 146.14 were devoid of any association with actin structures in resting cells (Fig. 7 and Table I). As soon as these membranes were inserted at the apical surface, they

became organized as microvilli which appeared more elongated in stimulated cells than in resting ones. Both types were associated with actin-supporting structures that presumably represent microfilaments running parallel to the long axis of the microvilli (Fig. 9). An intense labeling with anti-actin antibodies was also detected intracytoplasmically beneath the microvilli membranes in areas mostly devoid of 95-kD antigen. In some cases, this internal labeling was up to 300 nm thick at the microvilli base. It indicates that an actin meshwork may also exist beneath the microvilli. This is consistent with fluorescence data of actin distribution in stimulated cells appearing in partial coincidence with 95-kD



**Figure 9.** Tentative drawing of the structural organization of acid-secreting membranes in parietal cells. (A) Drawing of the morphological changes affecting parietal cells upon stimulation of secretion. R, resting cell; S, stimulated cell; TV, tubulovesicles; SC, secretory canaliculus. (B) Model of organization of acid-secreting membranes and actin-cytoskeleton in resting and stimulated conditions. The left drawing presents internal inactive tubulovesicle membranes bearing unfunctional cytoskeletal anchoring membrane protein(s). In this state, a dispersed pool of cytoskeletal protein may exist in the cell cytoplasm as a nonassembled (or preassembled) pool. On the right, the structural organization of active apical microvilli is presented. Actin microfilaments run parallel along the microvilli axis. At the base, an actin-spectrin-based meshwork, anchored on functional membrane protein(s), stabilizes the overall architecture. Proteins X and Y are additional cytoskeletal proteins likely to be present. They might respectively be similar to protein 4.1- (2, 23) and fimbrin- (25) like proteins. Lu, luminal face of the membrane.

antigen. The location of 95-kD antigen is strictly limited at the luminal surface of the membrane, whereas actin microfilaments are located both inside the corresponding microvilli and at their base.

The analysis of actin distribution, both at the light and electron microscope level, helps in the interpretation of the fluorescence data recorded with anti-brain spectrin (fodrin) antibody. The main spectrin fluorescence appeared along basolateral membranes of all cells. Upon stimulation, a significant amount of spectrin was also concentrated among acid-secretory structures of parietal cells. As for actin, the compared distribution of both spectrin and 95-kD antigen colocalized to some extent, but the spectrin fluorescence was more blurred as if it was present in less restricted areas than 95-kD antigen. The fluorescence signals indicated that much less spectrin was present compared to actin. The occurrence of spectrin in much lower amount with a fuzzy distribution argues for its presence as part of a meshwork in association with actin beneath apical secretory microvilli (see below). The exact nature of the spectrin cross reacting molecules involved is not known. By immunoblot analysis, a single species of 240 kD has been detected in the range expected for a fodrin-like component. Until the parietal protein is characterized, one cannot rule out that some nonconventional form of spectrin may exist in parietal cells.

The cytoskeletal reorganization observed in stimulated cells, so massive and specifically localized and so timely concomitant with the acid-secreting membrane movement, suggests a crucial role for these structural proteins in the dynamic process of membrane insertion at the apical cell surface. This is consistent with the inhibitory effects of cytochalasin D observed on the uptake of aminopyrine by stimulated isolated rat parietal cells (28) and of several growth factors (10, 27). Based on what is generally known about the inner structure of microvilli (25) and on the ultrastructural localization performed in this study, actin microfilaments presumably assemble as a driving and stabilizing force for the elongation of the numerous microvilli generated by the apical insertion of acid-secreting membrane elements. The weaker mobilization of spectrin certainly accounts for its organization as a meshwork beneath the membrane, at the base of the microvilli, and presumably for its absence from the inner microvilli skeleton as it occurs in the brush-border of the intestinal epithelium (25).

The morphological modifications occurring in these cells raise some questions related to our present knowledge of the assembly of the actin-spectrin cytoskeleton. Upon stimulation, actin might be mobilized from a soluble sequestered actin pool that might consist, for example, of a G actin-profilin complex that might be dissociated under second messenger action (20). Alternatively, a preexisting pool of oligomeric F actin may be presently dispersed throughout the cytoplasm in resting state. The qualitative differences in labeling observed between the staining performed with anti-actin antiserum (presumably staining G and F actin) and NBD-phalloidin or rhodamine-phalloidin (strictly reacting with F actin; not shown) do not allow a definite answer.

Since membrane insertion is rapid (within minutes), protein synthesis should not be involved in this process. Therefore, in the resting state, the existence of a cytoplasmic pool of nonassembled (or preassembled) spectrin molecules and of other additional accessory cytoskeletal elements may be predicted to provide enough material to stabilize the 10-fold

extension (7, 9) of the apical surface of stimulated parietal cells. Thus, the situation would be different to what has been described for avian erythrocytes in which nonorganized spectrin molecules are readily degraded in the cytoplasm (24, 41). Finally, the nature of and the dynamic status of the spectrin anchoring protein(s) and related molecules (2, 21, 23) associated with these membranes are of considerable interest regarding the assembly of cortical proteins. There is a real possibility (Fig. 9) that these anchoring proteins are still physically connected with resting tubulovesicles membranes, if all membrane components are translocated. This would imply that their conformation would be affected by some posttranslational modification, for instance phosphorylation, under the control of physiological stimuli. This would, in turn, explain why spectrin molecules would be unable to associate with them, preventing any cytoskeletal structures from assembling. Alternatively, the modulation of the state of assembly of these proteins might only involve a physiological control at the level of the spectrin molecules. Answers to most of these questions will be provided when all protein components of acid-secreting membranes as well as of peripheral proteins have been clearly identified.

The mobilization of actin and spectrin in stimulated parietal cells has several implications for the biology of gastric acid-secreting cells and raises also fundamental questions of a more general interest. Although one cannot rule out that a physical continuity between internal tubulovesicles and apical membrane may still exist in resting cells, our data are fully compatible with the fusion hypothesis mechanism of membrane insertion (6), since the reorganization of actin and spectrin molecules observed in stimulated cells is a good indication of the extent of membrane addition that occurred. The results strengthen existing evidence (17, 33) suggesting that the membrane movement involves all membrane components, as it is the case for the 95-kD antigen.

Moreover, the data show that parietal cells represent a model of rapid and polarized insertion of inactive secreting membranes from an intracellular storage pool to the apical plasma membrane. How this insertion is correctly directed to the apical surface remains totally unknown. It is interesting to note that, although rat gastric  $H^+-K^+$  ATPase shares 62% amino acid sequence homology with sheep kidney  $Na^+-K^+$  ATPase (32), the former is an apical membrane protein, whereas the latter is an epithelial basolateral membrane marker. If the  $H^+-K^+$  ATPase, the most prominent protein of gastric acid-secreting membranes (29, 30), is responsible for the correct apical insertion of these membranes, the insertion signal could reside in the  $NH_2$ -terminal hydrophilic domain, since it is the region that differs most extensively with  $Na^+-K^+$  ATPase sequence. Alternatively, this region may be involved in cation discrimination as proposed (32). Whatever the location and the nature of the insertion signal are, if one is present, the apical membrane insertion site should be regulated, presumably by some reversible posttranslational modifications; the secretory membranes are expressed either intracytoplasmically or at the apical cell surface depending on the state of cell stimulation. The molecular processes involved in the necessary internalization of these membranes once the cells return back to a resting state are equally ignored.

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## References

- Barak, L. S., R. R. Yocum, E. A. Nothnagel, and W. W. Webb. 1980. Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3-diazole-phalloidin. *Proc. Natl. Acad. Sci. USA.* 77: 980-984.
- Bennett, V. 1985. The membrane cytoskeleton of human erythrocytes and its implications for more complex cells. *Annu. Rev. Biochem.* 54:273-304.
- Berglinth, T., D. R. DiBona, S. Ito, and G. Sachs. 1980. Probes of parietal cell function. *Am. J. Physiol.* 238:G165-G176.
- Burridge, K. 1976. Changes in cellular glycoproteins after transformation: identification of specific glycoproteins and antigens in sodium dodecylsulfate gels. *Proc. Natl. Acad. Sci. USA.* 73:4457-4461.
- Burridge, K., T. Kelly, and P. Mangeat. 1982. Nonerythrocyte spectrins: actin-membrane attachment proteins occurring in many cell types. *J. Cell Biol.* 95:478-486.
- Forte, T. M., T. Machen, and J. G. Forte. 1977. Ultrastructural changes in oxyntic cells associated with secretory function: a membrane-recycling hypothesis. *Gastroenterology.* 73:941-955.
- Forte, J. G., J. A. Black, T. M. Forte, T. Machen, and J. M. Wolosin. 1981. Ultrastructural changes related to functional activity in gastric oxyntic cells. *Am. J. Physiol.* 241:G349-G358.
- Gespach, C., D. Bataille, C. Dupont, G. Rosselin, E. Wunsch, and E. Jaeger. 1980. Evidence for a cyclic AMP system highly sensitive to secretin in gastric glands isolated from the rat fundus and antrum. *Biochim. Biophys. Acta.* 630:433-441.
- Gilbert, A. J., and S. J. Hersey. 1982. Morphometric analysis of parietal cell membrane transformations in isolated gastric glands. *J. Membr. Biol.* 67:113-124.
- Gonzalez, A., J. Garrido, and J. D. Vial. 1981. Epidermal growth factor inhibits cytoskeleton-related changes in the surface of the parietal cells. *J. Cell Biol.* 88:108-114.
- Helander, H., and B. I. Hirschowitz. 1972. Quantitative ultrastructural studies on gastric parietal cells. *Gastroenterology.* 63:951-961.
- Hunter, W. M., and F. Greenwood. 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature (Lond.).* 194:495-496.
- Im, W. B., D. P. Blakeman, J. M. Fieldhouse, and E. Rabon. 1984. Effect of carbachol or histamine stimulation on rat gastric membranes enriched in (H<sup>+</sup>-K<sup>+</sup>)-ATPase. *Biochim. Biophys. Acta.* 772:167-175.
- Im, W. B., D. P. Blakeman, and J. P. Davis. 1985. Studies on K<sup>+</sup> permeability of rat gastric microsomes. *J. Biol. Chem.* 260:9452-9460.
- Ito, S. 1961. The endoplasmic reticulum of gastric parietal cells. *J. Biochem. Cytol.* 11:333-347.
- Ito, S. 1968. Anatomic structure of the gastric mucosa. In *Handbook of Physiology, Section 6, Alimentary Canal.* C. F. Code, editor. American Physiology Society, Bethesda, MD. 705-741.
- Ito, S. 1981. Functional gastric morphology. In *Physiology of the Gastrointestinal Tract.* L. R. Johnson, editor. Raven Press, New York. 517-550.
- Ito, S., and G. Schofield. 1974. Studies on the depletion and accumulation of microvilli and changes in the tubulovesicular compartment of mouse parietal cells in relation to gastric acid secretion. *J. Cell Biol.* 63:364-382.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680-685.
- Lassing, I., and U. Lindberg. 1985. Specific interaction between phosphatidylinositol 4,5-bisphosphate and prolactin. *Nature (Lond.).* 314:472-474.
- Mangeat, P. H. 1988. Interaction of biological membranes with the cytoskeletal framework of living cells. *Biol. Cell.* 64:261-282.
- Mangeat, P. H., and K. Burridge. 1984. Immunoprecipitation of nonerythrocyte spectrin within live cells following microinjection of specific antibodies: relation to cytoskeletal structures. *J. Cell Biol.* 98:1363-1377.
- Marchesi, V. T. 1985. Stabilizing infrastructure of cell membranes. *Annu. Rev. Cell Biol.* 1:531-561.
- Mercier, F., H. Reggio, G. Devilliers, D. Bataille, and P. Mangeat. 1989. A marker of acid-secreting membrane movement in rat gastric parietal cells. *Biol. Cell.* In press.
- Moon, R. T., and E. Lazarides. 1984. Biogenesis of the avian erythroid membrane skeleton: receptor-mediated assembly and stabilization of ankyrin (goblin) and spectrin. *J. Cell Biol.* 98:1899-1904.
- Mooseker, M. S. 1985. Organization, chemistry, and assembly of the cytoskeletal apparatus of the intestinal brush border. *Annu. Rev. Cell Biol.* 1:209-241.
- Rabon, E., R. D. Gunther, A. Soumarmon, S. Bassilian, M. Lewin, and G. Sachs. Solubilization and reconstitution of the gastric H,K-ATPase. 1985. *J. Biol. Chem.* 260:10200-10207.
- Rhodes, J. A., J. P. Tam, U. Finke, M. Saunders, T. Bernanke, W. Silen, and R. A. Murphy. 1986. Transforming growth factor  $\alpha$  inhibits secretion of gastric acid. *Proc. Natl. Acad. Sci. USA.* 83:3844-3846.
- Rosenfeld, G., E. McAllister, and W. J. Thompson. 1981. Cytochalasin inhibition of isolated rat gastric cell function. *J. Cell Physiol.* 109: 53-57.
- Saccomani, G., H. B. Stewart, D. Shaw, M. Lewin, and G. Sachs. 1977. Characterization of gastric mucosal membranes. IX. Fractionation and purification of K<sup>+</sup>-ATPase-containing vesicles by zonal centrifugation and free-flow electrophoresis technique. *Biochim. Biophys. Acta.* 465: 311-330.
- Sachs, G., H. H. Chang, E. Rabon, R. Schackman, M. Lewin, and G. Saccomani. 1976. A nonelectrogenic H<sup>+</sup> pump in plasma membranes of hog stomach. *J. Biol. Chem.* 251:7690-7698.
- Sedar, A. W., and M. H. F. Friedman. 1961. Correlation of fine structure of the gastric parietal cell (dog) with functional activity of the stomach. *J. Biophys. Biochem. Cytol.* 11:349-363.
- Shull, G. E., and J. B. Lingrel. 1986. Molecular cloning of the rat stomach (H<sup>+</sup> + K<sup>+</sup>)-ATPase. *J. Biol. Chem.* 261:16788-16791.
- Smolka, A., H. F. Helander, and G. Sachs. 1983. Monoclonal antibodies against H<sup>+</sup> + K<sup>+</sup> ATPase. *Am. J. Physiol.* 245:G589-G596.
- Soumarmon, A., F. Grelac, and M. J. M. Lewin. 1983. Solubilization of active (H<sup>+</sup> + K<sup>+</sup>)-ATPase from gastric membrane. *Biochim. Biophys. Acta.* 732:579-585.
- Takeguchi, N., and Y. Yamazaki. 1986. Disulfide cross-linking of H,K-ATPase opens Cl<sup>-</sup> conductance, triggering proton uptake in gastric vesicles. Studies with specific inhibitors. *J. Biol. Chem.* 261:2560-2566.
- Tokuyasu, T. K. 1973. A technique for ultracytometry of cell suspension and tissues. *J. Cell Biol.* 57:551-561.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedures and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Vial, J. D., and J. Garrido. 1976. Actin-like filaments and membrane rearrangement in oxyntic cells. *Proc. Natl. Acad. Sci. USA.* 73:4032-4036.
- Wolosin, J. M., and J. G. Forte. 1981. Changes in the membrane environment of the (K<sup>+</sup> + H<sup>+</sup>)-ATPase following stimulation of the gastric oxyntic cell. *J. Biol. Chem.* 256:3149-3152.
- Wolosin, J. M., and J. G. Forte. 1981. Functional differences between K<sup>+</sup>-ATPase rich membranes isolated from resting or stimulated rabbit fundic mucosa. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 125:208-212.
- Woods, C. M., and E. Lazarides. 1985. Degradation of unassembled  $\alpha$ - and  $\beta$ -spectrin by distinct intracellular pathways: regulation of spectrin topogenesis by  $\beta$ -spectrin degradation. *Cell.* 40:959-969.