Clathrin in gastric acid secretory (parietal) cells: biochemical characterization and subcellular localization

CURTIS T. OKAMOTO,1 JOSEPH G. DUMAN,2 KAMALA TYAGARAJAN,2 KENT L. MCDONALD,2,4 YOUNG Y. JENG,1 JEANA McKINNEY,1 TRUDY M. FORTE,3 AND JOHN G. FORTE2

1Department of Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles 90089-9121; and 2Department of Molecular and Cell Biology, 3Lawrence Berkeley Laboratory, and 4Electron Microscope Lab, University of California, Berkeley, California 94720

Received 22 January 2000; accepted in final form 3 April 2000

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: C. T. Okamoto, Dept. of Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 1985 Zonal Ave., Los Angeles, CA 90089-9121 (E-mail: cokamoto@hsc.usc.edu).

The regulation of the trafficking of membrane transporters is becoming widely recognized as a basic mechanism by which cells, epithelial cells in particular, regulate solute transport (8). The gastric parietal (oxyntic) cell represents a model system in which to study the means by which solute transport can be regulated by the vesicular trafficking of a membrane transporter, the H-K-ATPase (13, 21). In the resting parietal cell, the gastric H-K-ATPase is sequestered in an intracellular system of tubulovesicular membranes. When cells are stimulated to secrete HCl, the tubulovesicular membranes fuse with the canalicular (apical) membrane, thus delivering the H-K-ATPase to the apical membrane. When the stimulus is removed, the H-K-ATPase is retrieved from the canalicular membrane, and the tubulovesicular compartment is re-established (22).

The tubulovesicular compartment of the parietal cell has recently been demonstrated to contain key components of the essential machinery to regulate the trafficking of the H-K-ATPase, such as the small GTPases rab11 (10) and rab25 (26), and proteins implicated in vesicular docking/fusion, such as syntaxin 3 and vesi
cle-associated membrane protein (VAMP) (10, 41). Some of these components, including rab11, show secretagogue-stimulated changes in subcellular membrane localization (11), and additional functional studies suggest that rab11 is a key regulator of H-K-ATPase trafficking (20). In addition, a tyrosine-containing motif in the cytoplasmic domain of the β-subunit of the heterodimeric H-K-ATPase has been implicated to serve as a sorting signal for the reinternalization of the H-K-ATPase from the apical membrane on cessation of acid secretion (17). The motif in the β-subunit is strikingly similar to the internalization signal in the transferrin receptor that allows the transferrin receptor to interact with clathrin adaptor protein-2 (AP-2) at the plasma membrane. Clathrin and an AP-1 clathrin adaptor have been recently identified on tubulovesicles (39). Moreover, the AP-1 adaptor and the H-K-ATPase appear to interact, as shown by their copurification from tubulovesicles solubilized with a nondenaturing detergent (39). Thus significant progress in recent
years has been made in cataloging components of the molecular machinery ostensibly involved in the regulation of H-K-ATPase trafficking.

In the present study the role of clathrin in the regulation of trafficking of the H-K-ATPase in gastric parietal cells was investigated by biochemical and morphological approaches. Clathrin from H-K-ATPase-rich membranes was further characterized immunologically and by mass spectrometry. Clathrin and adaptors formed baskets in vitro. Immunofluorescent labeling and electron-microscopic localization of clathrin in resting parietal cells suggest a role for clathrin in the regulation of membrane traffic between tubulovesicular membranes and the canalicular membrane. Immunofluorescent labeling of clathrin in secretagogue-stimulated parietal cells suggests that clathrin is not translocated to the apical membrane with the H-K-ATPase and may therefore play a direct role in H-K-ATPase trafficking at another stage of the secretory cycle.

MATERIALS AND METHODS

Materials. Anti-clathrin heavy chain monoclonal antibodies (MAbs) X-22 (36) and TD.1 (36); the anti-clathrin light chain MAbs X-16 (1), LCB.1 (1), and CON.1 (36); and anti-α-adaptin MAb AP.6 (15) were obtained from two sources: as kind gifts of Dr. Frances Brodsky (University of California, San Francisco) and from X-22, TD.1, CON.1, and AP.6 hybridoma cell culture supernatants. The hybridomas were purchased from the American Type Culture Collection. Anti-light chain MAbs X-16, 1:1,000 for anti-light chain MAb LCB.1, and 1:1,000 for anti-light chain MAb CON.1. Hybridoma cell culture supernatants containing TD.1 and CON.1 were also used neat for Western blotting. Goat anti-mouse-HRP secondary antibody was used at 1:20,000 dilution. Blocking of nitrocellulose was done in 1–5% nonfat milk or 0.2% BSA in Tris-buffered saline. HRP was detected by ECL, and the signal was visualized on Kodak Bio-Max X-ray film.

Hydroyxapatite chromatography of coat proteins from purified gastric microsomes. Clathrin coat proteins and other peripheral membrane proteins were stripped from purified gastric microsomes (starting with 3–30 mg of microsomal membrane proteins) essentially according to the protocol of Keen et al. (29). The stripped proteins were dialyzed overnight against three changes of a solution of 125 mM mannitol, 40 mM sucrose, 1 mM EDTA, and 5 mM PIPES buffer, pH 6.7 (MSEP). The dialysate was applied to a 5-ml hydroxyapatite column equilibrated with MSEP, and proteins were eluted by a stepwise gradient of 12 ml each of 10, 100, 200, and 400 mM sodium phosphate, pH 7.0. NaN₃ was added to the eluted fractions, and these fractions were stored at 4°C. For SDS gels and Western blots, proteins in 0.3- to 1.2-ml aliquots were precipitated with 10% TCA and resuspended in SDS gel buffer before electrophoresis.

In-gel digestion. A slightly modified in-gel digestion method from Rosenfeld et al. (44) was performed. Protein bands (~5 µg of protein) were minced, and the gel slices were destained with three washes of 50% acetonitrile-25 mM NH₄HCO₃ (~10 min each). The destained gel pieces were dried in a Speedvac (Savant, Farmingdale, NY) for 30 min and then rehydrated in 50 µl of 25 mM NH₄HCO₃ (pH 8.0) with 0.01 µg/µl trypsin. The slices were overlaid with 50 µl of 25 mM NH₄HCO₃ and incubated for 15 h at 37°C. Peptides were recovered by three extractions of the digestion mixture with 50% acetonitrile-5% trifluoroacetic acid. All supernatants were pooled, concentrated to 5 µl in a Speedvac, and brought back up to 25 µl in 50% acetonitrile-5% trifluoroacetic acid. The peptide mix was stored at −20°C until further analysis.

Matrix-assisted laser desorption delayed extraction reflection time of flight mass spectrometry of clathrin peptides. Aliquots (~5%) of unseparated tryptic digests were cocrystallized with α-cyano-4-hydroxycinnamic acid and analyzed using a matrix-assisted laser desorption delayed extraction reflection (MALDI) time of flight (TOF) mass spectrometry (MS) instrument (Perceptive Biosystems, Voyager Elite mass spectrometer, Framingham, MA) equipped with a nitrogen laser at the University of California, San Francisco, Mass Spectrometry Facility. Measurements were performed in a positive ionization mode. All MALDI spectra were externally calibrated using a standard peptide mixture. For postsource decay (PSD) spectra, tryptic peptides were fractionated by reverse-phase microbore HPLC. PSD spectra were acquired on a TOFSpec SE MALDI-TOF MS (Micromass, Manchester, UK) with a nitrogen laser and operated in the reflectron mode.

Database searches for protein identification. Experimentally determined masses were used for database interrogation with use of MS-Fit software (16, 43). PSD data interrogation was performed using MS-Tag. Both software programs were developed at the University of California, San Francisco, Mass Spectrometry Facility and are available on the World Wide Web at http://prospector.ucsf.edu. Protein searches were carried out in the National Center for Biotechnology Information protein database and the SwissProt database by using a protein molecular weight of 150–250.
kDa, a peptide mass tolerance of 0.5 Da, and a minimum match of 50% of peptides observed in the total digest.

**Immunogold staining protocol.** For immunogold labeling, LR White sections were picked up on 100-mesh nickel grids coated with Formvar film and carbon, incubated in blocking buffer (5% BSA, 0.1% fish gelatin, and 0.05% Tween 20 in PBS) for 30 min, and then incubated with primary antibodies diluted in blocking buffer for 1.5–2 h. MAb X-22 was used as undiluted cell culture supernatant; MAb 2G11 cell culture supernatant was diluted 1:5. The commercially procured MAb23 was diluted 1:100. Sections were rinsed in PBS-Tween, and then PBS and incubated for 1 h in secondary antibodies conjugated to 10-nm gold particles (goat anti-mouse IgG F(ab')2 (H + L); Ted Pella) diluted 1:20 in blocking buffer. Sections were washed as described above, fixed in 0.5% glutaraldehyde in PBS for 5 min, and rinsed in PBS and water. Sections were poststained in 2% uranyl acetate for 5 min and in lead citrate for 3 min. Identically treated samples were stained with secondary antibody only as controls and revealed no labeling pattern.

**Fractionation of sucrose density gradient-purified gastric microsomes on discontinuous glycerol velocity gradients.** The antibody partial purification of sucrose density gradient-purified gastric microsomes on discontinuous glycerol velocity gradients was adapted from Saleem et al. (45). Gastric sucrose microsomes (200 μg) sedimenting at the 32% barrier on sucrose density gradients were diluted to 0.4 mg/ml in MSEP. It was layered on top of a discontinuous glycerol gradient comprised of 0.5 ml each of 20, 40, and 80% glycerol, diluted in MSEP. The sample was centrifuged in an RPS5S-485 Sorvall swinging bucket rotor at 55,000 rpm for 30 min in a Sorvall RC M120EX minicentrifuge. The membranes sedimenting at each interface were collected, diluted to 1.0 ml with MSEP, and recentrifuged at 150,000 g for 40 min. The pellets were resuspended in SDS gel sample buffer and analyzed by SDS-PAGE and Western blot. For the membranes sedimenting at the 20 and 40% glycerol layers, the entire samples were loaded onto the gels; for membranes sedimenting at the 80% layer, one-eighth to one-fourth of the sample was loaded onto the gels.
mucosa was digested in MEM supplemented with HEPES buffer (MEM-HEPES) containing 0.125 mg/ml collagenase (Sigma Chemical) and 0.25 mg/ml BSA at 37°C for ~30 min. The reaction was stopped by threefold dilution of the digestion solution with MEM-HEPES. Because of their large size, relatively intact gastric glands settled out in 10–15 min, leaving individual cells suspended in the medium. The suspended cells were strained through nylon mesh and washed three times with MEM-HEPES. Cells were next incubated for 30 min in medium B (DMEM-F-12; GIBCO, Rockville, MD) supplemented with 20 nM HEPES, 0.2% BSA, 10 mM glucose, 8 nM EGF, 1× SITE (selenium, insulin, and transferrin) medium (Sigma Chemical), 1 mM glutamine, 100 U/ml penicillin-streptomycin, 400 μg/ml gentamicin sulfate, 25 μg/ml amphotericin B, 15 μg/ml genetin, and 20 μg/ml novobiocin, pH 7.4, to prevent yeast infection. Cells were then plated onto coverslips coated with Matrigel (Collaborative Biomedical, Franklin Lakes, NJ) and incubated at 37°C in culture medium A (medium B without amphotericin B).

For comparison, the relative amounts of clathrin in crude CCVs from hog brain and hog gastric microsomes were assessed in Coomassie blue-stained gels (Fig. 1C). Clathrin heavy chain, migrating as a 160-kDa protein band, is the major protein in the brain CCVs (lane 1). In contrast, H-K-ATPase is the major protein in gastric microsomes, whereas some ~160-kDa migrating protein bands are also visible (lane 2). The relative amounts of clathrin associated with purified gastric microsomes and brain CCVs were estimated from

![Figure 1](http://apcphys.org/)

Fig. 1. Immunodetection of clathrin heavy chain on purified gastric microsomes of parietal cells. A: Coomassie blue-stained SDS gel of sucrose density gradient-purified gastric microsomes from rabbit gastric mucosa (24 μg protein/lane). Position of the H-K-ATPase α-subunit (HKα) is indicated. Lane 1, microsomes purified at the 27% sucrose layer; lane 2, microsomes purified at the 32% layer. Apparent molecular masses are indicated, and the identity of the markers is as follows: myosin, β-galactosidase, phosphorylase B, BSA, ovalbumin, and carboxic anhydrase. B: Western blot of purified gastric microsomes (15 μg protein/lane) with anti-clathrin heavy chain monoclonal antibody (MAb) TD.1. Lane 1, 27% sucrose layer; lane 2, 32% sucrose layer. Immunoreactivity was detected by enhanced chemiluminescence (ECL) with a 2-min exposure to film. Position of prestained molecular mass markers (myosin and β-galactosidase) is shown. C: comparison of clathrin content of crude clathrin-coated vesicles (CCVs) from brain and purified gastric microsomes. Coomassie blue-stained SDS gel of crude CCVs from hog brain and purified gastric microsomes (10 μg protein/lane) is shown. Lane 1, crude CCVs from hog brain; lane 2, hog gastric microsomes purified at the 32% layer. D: Western blot with MAb TD.1. Lane 1, CCVs from hog brain (5 μg); lane 2, hog gastric microsomes purified at the 32% layer (5 μg). Signal detected by ECL with a 2-min exposure.
Western blots probed with MAb TD.1 (Fig. 1D). Brain CCVs (lane 1) contain ~20 times more clathrin than gastric microsomes (lane 2). One of the reasons that brain CCVs may possess a higher specific content of clathrin than gastric microsomes is that the preparation of brain CCVs includes an extraction step with 1% Triton X-100. This treatment would be expected to solubilize most membrane proteins and therefore deplete the cargo proteins for CCVs, but clathrin and associated proteins would remain and, therefore, be enriched, in pelletable complexes. When crude or purified gastric microsomes are subjected to the same extraction procedure, clathrin is recovered but not enriched in the Triton X-100-insoluble pellet, even though the H-K-ATPase is quantitatively extracted (data not shown). Thus there appear to be differences between clathrin on brain CCVs and purified gastric microsomes that confer greater stability of clathrin on brain CCVs to the Triton X-100 extraction procedure.

Purified hog gastric microsomal membrane fractions were also probed for clathrin light chains. The immunodetection of clathrin light chain A (LCa) and light chain B (LCb) in gastric microsomes and brain CCVs was performed with the MAbs X-16 (LCa specific) and LCB.1 (LCb specific) (1). LCa (Fig. 2A) is clearly present in gastric microsomes (lane 2). Although LCb is not detectable in gastric microsomes in the immunoblot shown in Fig. 2B (lane 2), it becomes detectable when higher amounts of microsomal protein are assayed. The light chains from brain appear to be of higher relative molecular weight on SDS gels than their counterparts in gastric microsomes, possibly because of the presence of neuron-specific inserts in LCa and LCb (9, 30).

By immunoblotting with MAb CON.1, which recognizes both light chains equally well (36), the ratio of LCa to LCb can be determined in a single sample. The immunoblot in Fig. 2C suggests that LCa is the predominant light chain associated with gastric microsomes (lanes 3 and 4), and these results are consistent with the results from the immunoblots by use of the isof orm-specific MAbs (Fig. 2, A and B). From densitometric analyses of these Western blots, the ratio of LCa to LCb is 3.1 ± 1.1 (SD, n = 11 blots from 8 different animals).

Fractionation of gastric microsomal clathrin by hydroxyapatite chromatography. Purified gastric microsomes were stripped of their clathrin coats (and other peripheral membrane proteins) by incubation with 0.5 M Tris·HCl and 2 mM EDTA. After dialysis in a low-ionic-strength buffer, the stripped proteins were applied to a hydroxyapatite column, and proteins were eluted by a stepwise sodium phosphate gradient. A major protein, eluted by the addition of 0.2 M sodium phosphate and clearly visible on Coomassie blue-stained gels (Fig. 3A, lane 4), was identified as clathrin heavy chain on the basis of its immunoreactivity with anti-clathrin heavy chain MAb 23 (Fig. 3B) and TD.1 (not shown). Clathrin light chains (with approximately the same ratio of LCa to LCb as in isolated membranes) were also detected in Western blots of the 0.2 M sodium phosphate eluate (Fig. 3C). From densitometric analyses of Coomassie blue-stained gels, clathrin heavy chain was determined to comprise 14 ± 3% (SD, n = 10 independent preparations) of the total protein eluted in this fraction. By comparison, in crude CCVs isolated here (Fig. 1C), clathrin comprises ~12% of the total protein. If we assume that the recovery of clathrin heavy chain by hydroxyapatite chromatography is 100%, we estimate that gastric microsomes contain ~17 μg of clathrin heavy chain per milligram of microsomal protein. If one assumes that the H-K-ATPase comprises minimally 50% of the purified gastric microsomal protein, a ratio of 50 copies of H-K-ATPase per copy of clathrin heavy chain can be calculated.

Finally, as reported previously, the majority of γ-adaptin (Fig. 3D) and a β-adaptin that is apparently immunologically distinct from the conventional β1- and β2-adaptins (not shown) also eluted into this fraction (38). This fraction was used to characterize gastric microsomal clathrin heavy chain by mass spectrometry.

Characterization of gastric microsomal clathrin by MS. The 160-kDa gel band immunoreactive with anti-clathrin heavy chain MAbs (Fig. 3, A and B, lane 4) was subjected to in-gel trypsinolysis, and the recovered peptide mixture was analyzed by MALDI-TOF-MS to yield a peptide mass fingerprint (Fig. 4). When a database search was performed with 17 input peptide masses, only conventional human, bovine, and rat clathrin heavy chain (clathrin heavy chain I) matched the input criteria given in MATERIALS AND METHODS.

Fig. 2. Identification of clathrin light chain isoforms on purified gastric microsomes by immunoblotting. A: Western blot of crude CCVs from hog brain (3 μg) and hog gastric microsomes, 32% layer (10 μg), with anti-light chain A MAb X-16. Signal was detected by ECL with a 2-min exposure. B: Western blot of crude CCVs from hog brain (3 μg) and hog gastric microsomes, 32% layer (10 μg), with anti-light chain B MAb LCB.1. Signal was detected by ECL with a 1-min exposure. C: Western blot of crude CCVs from hog brain (lanes 1 and 2) and hog gastric microsomes (lanes 3 and 4) with anti-light chain (common) MAb CON.1. Lane 1, 3 μg of CCVs; lane 2, 5 μg of CCVs; lane 3, 10 μg of 32% gastric microsomes; lane 4, 20 μg of 27% gastric microsomes. Positions of light chains A and B (LCa and LCb) are indicated. Signal was detected by ECL with a 15-s exposure.
These observed peptides are compared with the predicted masses from tryptic digestion of clathrin heavy chain I in Table 1, all agreeing within a mass accuracy of 0.2 Da.

Confirmation of the 160-kDa band as clathrin heavy chain I was obtained from sequence information based on PSD spectra. To obtain good PSD spectra, the total tryptic peptide mixture was fractionated by reverse-phase microbore HPLC before MALDI-MS. A representative PSD spectrum of peptide mass-to-charge ratio (m/z) 1,415.82 (also seen in total digest, Fig. 4) is shown in Fig. 5, which matches a single sequence, 1011IVLDNSVFSEHR1022, from clathrin heavy chain. Similar PSD sequence confirmation was obtained on peptides with m/z 1,126.6 (1398Val-Arg1406), 1,304.7 (355Asn-Arg366), 1,433.8 (469Ser-Arg481), 1,943.0 (1482Thr-Arg1498), and 1,971.2 (1227Leu-Arg1245) (data not shown). All peptides identified from PSD data are indicated by underline in Table 1. The peptide mass and sequence data thus verified gastric microsomal clathrin to be the conventional clathrin heavy chain (clathrin heavy chain I) isoform.

In vitro polymerization of gastric microsomal clathrin and adaptors. Proteins stripped from purified gastric microsomes with 0.5 M Tris buffer were dialyzed against a low-ionic-strength buffer in the absence or presence of Ca\(^{2+}\). The polymerized proteins were sedimented and compared with the nonpolymerized proteins. The overall polypeptide pattern of the sedimented proteins (Fig. 6A, lane 2) differs from that of the proteins remaining in the supernatant (Fig. 6A, lane 3). The prominent 160-kDa protein in the sedimentable material was confirmed to be clathrin heavy chain by immunoblot analysis (not shown). Also, as shown in the Western blot in Fig. 6B, γ-adaptin was quantitatively recovered in a sedimentable complex. However, the quantitative polymerization of clathrin and adaptors did not appear to depend on the presence of Ca\(^{2+}\) in the dialysis buffer. Of the total recovered clathrin, 69 ± 21% (SE, n = 5 independent experiments) was found in the pellet in the absence of Ca\(^{2+}\), whereas 59 ± 30% (SE, n = 11 independent experiments) was found in the pellet in the presence of Ca\(^{2+}\).
An aliquot of the hydroxyapatite fraction enriched in gastric microsomal clathrin and AP-1 adaptors (200 mM sodium phosphate fraction; Fig. 3, lane 4) was dialyzed in low-ionic-strength buffer to induce the polymerization of clathrin and adaptors. As observed above, the polypeptide pattern of the sedimenting proteins differed significantly from those proteins remaining in the supernatant (Fig. 6, lanes 2 and 3), suggesting specificity in the proteins incorporated into the polymerized complex.

Negative-stain electron microscopy of polymerized clathrin and AP-1 adaptors from gastric microsomes revealed basketlike structures very similar to those polymerized in vitro from clathrin and adaptors from brain (Fig. 7). One difference between the clathrin cages assembled in vitro from brain and gastric microsomal clathrin is that gastric microsomal clathrin baskets appear to be of slightly larger diameter: 120 nm compared with 80 nm for reassembled clathrin-AP-1 adaptor baskets from brain (40). Another difference is that gastric microsomal clathrin baskets appeared to be less regular in shape than those from brain. Although other proteins present in the sedimentable material may play a role in determining the size and shape of clathrin baskets formed in vitro from gastric microsomal clathrin and adaptors, the polymerized baskets appear to be more similar, rather than dissimilar, to baskets polymerized in vitro from clathrin isolated from other sources.

**Immunofluorescent labeling of clathrin in digitonin-permeabilized resting parietal cells.** With most of our previously used immunostaining protocols, the distri-
The presence of clathrin at the canalicular membrane suggests that this membrane domain is endocytotically active, even in resting parietal cells. Thus other major proteins that regulate endocytosis at the cell membrane should also be present. Subsequent staining demonstrated that canalicular membranes are also immunoreactive for dynamin (Fig. 8B’) and the AP-2 clathrin adaptor subunit α-adaptin (MAb AP.6; Fig. 8C’). This report is the first to identify clathrin and α-adaptin at the canalicular membrane of parietal cells and confirms the enrichment of dynamin at the canalicular membrane in parietal cells (11). Moreover, the expression of this immunoreactive form of dynamin appears to be significantly higher in parietal cells than in the nonparietal glandular cells. These results suggest that the budding of CCVs at the canalicular membrane is mediated by the AP-2 clathrin adaptor and a member of the dynamin family of GTPases.

Identification and localization of clathrin-coated pits and vesicles in parietal cells by thin-section electron microscopy of isolated rabbit gastric glands prepared by high-pressure rapid freezing and freeze substitution. Previous ultrastructural studies have not been able to demonstrate convincingly the presence of clathrin-coated membranes in parietal cells (7, 23, 28, 47). Yet, the biochemical and immunofluorescence data suggest that clathrin is a significant component of tubulovesicles and apical membranes. Thus we sought to reexamine the identification and localization of clathrin in parietal cells at the ultrastructural level. Isolated rabbit gastric glands were prepared for thin-section electron microscopy by high-pressure rapid freezing and freeze substitution. A low-magnification (×4,000) image of a resting, nonsecreting parietal cell is shown in Fig. 9A; this micrograph demonstrates the excellent morphological preservation of parietal cells by the high-pressure rapid-freezing technique. Prominent features observed previously in electron micrographs of resting cells that are also observed here are the numerous mitochondria, intracellular canaliculi (which are invaginations of the apical membrane), and, barely visible, the elaborate system of tubulovesicles in proximity to intracellular canaliculi. At higher magnification, coated membranes are clearly visible along the intracellular canaliculus, which also includes nicely preserved microvilli and their microfilaments (Fig. 9B). Coated pits are prominent at the canalicular membranes (Fig. 9C, arrows), and most of the coated pits appear at the base of canalicular microvilli. On average, these pits are 60–90 nm diameter. Coated membranous structures in the subapical cytoplasm (Fig. 9C, arrowhead) are occasionally visible. The ability to identify a significant number of coated pits at the canalicular membrane reinforces the conclusion that this membrane in resting parietal cells is an endocytotically active zone. Moreover, these coats possess a morphology that is highly reminiscent of conventional clathrin-coated membranes, suggesting that clathrin is involved in endocytic processes at the canalicular membrane.

Fig. 7. Electron micrographs of gastric microsomal clathrin and AP-1 clathrin adaptors assembled in vitro into baskets. Baskets were visualized by negative staining with 1% uranyl acetate. Clathrin and AP-1 clathrin adaptors were assembled by dialysis of the fraction from hydroxyapatite chromatography that is enriched in clathrin. The pelletable material was resuspended and processed for negative-stain electron microscopy. Scale bar, 100 nm.

Fig. 8A). The canaliculi within parietal cells are clearly identifiable as a network of tubular structures projecting from the gland lumen (Fig. 8, A–C). The lumen of the gland, including the apical membranes of parietal cells as well as all other cell types in the gastric gland, is also prominently stained with BODIPY FL-phallacidin (Fig. 8A). The canaliculi within parietal cells are clearly identifiable as a network of tubular structures projecting from the gland lumen (Fig. 8, A–C). The lumen of the gland, including the apical membranes of parietal cells as well as all other cell types in the gastric gland, is also prominently stained with BODIPY FL-phallacidin. Abundant intracellular staining for clathrin is evident in parietal cells, with a significant amount of staining associated with the regions peripheral to canalicular membranes; this staining pattern is consistent with clathrin immunoreactivity on tubulovesicular membranes. Interestingly, clathrin immunoreactivity is also readily visible at the apical canalicular membranes of parietal cells (Fig. 8A’, arrowheads), as indicated by its colocalization with F-actin staining of canaliculi (Fig. 8A).
Despite the excellent preservation of coated pits at the canalicular membrane, a distinctive clathrin-like coat is not observed on tubulovesicles, although proteinaceous material is occasionally visible on the cytoplasmic faces of tubulovesicular membranes. Thus the ultrastructural identification of clathrin on tubulovesicles could not be made by this protocol. On the other hand, an interesting morphological feature of these resting parietal cells is the presence of numerous subapical tubular and cup-shaped membranous structures (Fig. 9, B and C), ranging from 45 to 60 nm diameter and from 300 to 380 nm long. With respect to shape, these structures are reminiscent of the subapical endocytotic/transcytotic vesicles observed in Madin-Darby canine kidney (MDCK) cells (25), but the tubules in parietal cells appear to be somewhat larger.

Immunogold electron-microscopic localization of clathrin and H-K-ATPase in resting parietal cells. Isolated rabbit gastric glands were processed for immunogold localization of clathrin and H-K-ATPase within resting parietal cells. The immunogold localization of clathrin by use of anti-clathrin heavy chain MAb X-22 is shown in Fig. 10A in a region including and surrounding a canaliculus. Gold particles clearly decorate invaginated membranes or pits at the canalicular surface, usually at the bases of microvilli (arrowheads). These data thus confirm that the coated pits observed in Fig. 9, B and C, are comprised of clathrin. In Fig. 10A and in the more extensive subapical cytoplasmic view in Fig. 10B, densely staining, 80- to 100-nm-diameter vesicular profiles are also decorated with gold particles, as are the ends of the tubulovesicles (arrows). Thus, although a distinctive coat was not observed on these membranes by standard electron-microscopic staining protocols, clathrin can be localized to these sites by immunogold labeling. Similar results were obtained with another anti-clathrin heavy chain antibody, MAB 23 (not shown). This identification of clathrin at the ultrastructural level on tubulovesicles of parietal cells very likely corresponds to the clathrin identified biochemically on purified gastric microsomes.
To assess the degree to which the distributions of clathrin and H-K-ATPase overlap, the anti-H-K-ATPase β-subunit antibody MAb 2G11 was used for immunogold staining of H-K-ATPase (Fig. 11). As expected, anti-H-K-ATPase staining was observed predominantly in two places in resting parietal cells: along the central regions of intracellular tubular membrane profiles and along the microvillar membranes of

Fig. 9. Transmission electron micrographs of isolated rabbit gastric glands fixed by high-pressure rapid freezing. A: low-power magnification of a parietal cell in a gastric gland. Numerous electron-dense mitochondria (m) and intracellular canaliculi (c) are visible. A glancing section through a nucleus (n) is also seen. Tubulovesicles are barely visible at this magnification in the regions around the intracellular canaliculi. B: high-power magnification of a region surrounding an intracellular canaliculus with short microvilli projecting into the lumen of the canaliculus. Microfilaments are evident in the microvilli lining the lumen of the intracellular canaliculus. Several endocytic structures, some of which appear to possess a coat at the cytosolic face of the membranes, are visible. Mitochondria and numerous tubulovesicular structures are seen in the surrounding cytoplasm. C: higher-power view of a region surrounding an intracellular canaliculus. Several coated endocytic structures are seen invaginating from the canalicular surface (arrows) or as coated vesicles in the subapical cytoplasm (arrowhead). Cytoplasmic space also includes numerous tubulovesicles, several of which appear as indented saccules or C-shaped structures. Scale bars, 1 μm.
the canaliculus. The microvillar staining was more obvious along their lengths than at their bases. Despite the abundance of anti-H-K-ATPase staining, virtually no gold particles were associated with mitochondria or intracellular organelles other than tubulovesicles. However, anti-H-K-ATPase staining, although it appeared in the same general areas of clathrin staining (at the canalicular membrane and on tubulovesicles), did not appear to be concentrated at the same sites in which anti-clathrin immunolabeling was observed (in invaginations of the canalicular membrane and at the ends of tubulovesicles), although labeling for the H-K-ATPase could occasionally be found in pits at the canalicular membrane and in densely staining vesicles in the cytoplasm. Thus, overall, clathrin and the H-K-ATPase appear to be segregated to different regions or subsets of the apical canalicular membrane and tubulovesicles.

Subfractionation of gastric microsomes by glycerol gradient centrifugation. The immunogold labeling of clathrin in parietal cells suggests that at least two different types of intracellular membranes may possess a clathrin coat: the ends of tubulovesicles and the densely staining vesicles. Such membranes may cofractionate with “conventionally” purified H-K-ATPase-rich gastric microsomes. Thus we subjected purified gastric microsomes to additional fractionation on a discontinuous glycerol gradient. Figure 12 shows Coomassie blue-stained gels and Western blots of purified gastric microsomes subfractionated on a discontinuous
glycerol gradient. By this approach, a population of clathrin-rich and H-K-ATPase-poor membranes was identified at the 40% glycerol boundary, whereas the majority of H-K-ATPase was found in membranes sedimenting at the 80% glycerol boundary (Fig. 12A). It is clear from the immunoblots that clathrin is also present in the 80% glycerol fraction (Fig. 12B), and after correction for the total amount of protein, this fraction still contained the majority of the total microsomal clathrin. Interestingly, the 80% fraction is also enriched in the γ-adaptin subunit of the AP-1 clathrin adaptor (Fig. 12C). Thus the glycerol gradient can effect the separation of two types of clathrin-coated membranes, those poor in H-K-ATPase and those rich in H-K-ATPase, and may correspond to two (or more) intracellular populations of clathrin-coated membranes identified by immunogold electron microscopy.

**Immunofluorescent staining of clathrin, AP-1 clathrin adaptors, and H-K-ATPase in resting and stimulated primary cultures of rabbit parietal cells.** With the characterization of the steady-state localization of clathrin in resting cells, we sought to characterize the role of clathrin in the dynamic membrane trafficking processes occurring during functional resting-to-stimulated transition of parietal cells. Primary cultures of parietal cells have proven to be a good system with which to evaluate membrane recruitment and structural rearrangement associated with stimulation (2).

Within a few hours of being isolated and placed in culture, the apical canalicular membrane becomes sequestered into the parietal cell and now appears as a collection of vacuoles that are clearly identifiable by differential interference contrast microscopy or by labeling the membrane with probes for F-actin. Figure 13 shows cultured parietal cells in the resting state.
state stained variously for H-K-ATPase, clathrin (with MAb X-22), and F-actin. F-actin staining clearly demarcates the apical membrane vacuoles and the basolateral membrane surrounding the cell (Fig. 13, B and C). In resting cells, H-K-ATPase can be seen in a punctate distribution throughout the cytoplasm and, to some extent, within the apical membrane vacuoles (Fig. 13, A and B'). Staining with MAb X-22 indicates that distribution of clathrin is similar to that of H-K-ATPase, i.e., throughout the cytoplasm and within the apical membrane vacuoles (Fig. 13, A' and C').

Parietal cells respond vigorously to secretagogues, recruiting H-K-ATPase from the compartment of cytoplasmic tubulovesicles to the apical vacuoles and pumping HCl and water into those structures. As shown in Fig. 13, D–F, the apical vacuoles greatly expand as a consequence of 1) the fusion of tubulovesicles and 2) the osmotic force created by the pump. In this maximally stimulated configuration where the
vacuolar space occupies most of the viewable cell, staining for F-actin (Fig. 13, E and F), H-K-ATPase (Fig. 13, D and F'), and clathrin (Figs. 13, D' and E') appear to overlap largely at the periphery of the balloononed vacuolar structures. However, the shrinking of the residual cytoplasmic space makes it difficult to conclude anything regarding specific localization.

Treatment of parietal cells with proton pump inhibitors has been shown to produce a definitive pattern of stimulation-dependent structural changes. When stimulated parietal cells were treated with the pump inhibitor SCH-28080, H-K-ATPase was cleared from the cytoplasm and translocated to the apical membrane vacuoles, where it is colocalized with F-actin. The vacuoles become slightly enlarged, but there is ample remaining cytoplasmic space that has been cleared of H-K-ATPase (2, 20) (Fig. 13, H and H'). Thus membrane recruitment and trafficking of H-K-ATPase remained intact, even though ion transport was inhibited. On the other hand, staining with MAb X-22 reveals that although some clathrin is associated with the apical membrane vacuoles, much parietal cell clathrin remains distributed throughout the cytoplasm (Fig. 13, G and G'), sometimes even extending into the lamellipodia, thus demonstrating that a component of cytoplasmic clathrin can be segregated from H-K-ATPase on secretory activation.

Probing of parietal cell cultures with other anti-clathrin heavy chain (MAb 23; Fig. 14, A'–C') or light chain (MAb CON.1; Fig. 14, D'–F') antibodies or for the AP-1 clathrin adaptor (MAb 100/3; Fig. 14, G' and H') produced results identical to those for MAb X-22. In resting cells, clathrin was seen throughout the cytoplasm, as well as at the apical membrane vacuoles. When cells were stimulated in the presence of SCH-28080, the pattern of clathrin and AP-1 clathrin adaptor staining was not significantly different from that of resting cells, i.e., much of the signal for MAb 23, CON.1, and 100/3 remained distributed throughout the cytoplasm (Fig. 14, C', F', and H'). These data together suggest that clathrin and the AP-1 adaptor are not translocated en masse to the apical membrane on stimulation of the parietal cell and, therefore, do not appear to accompany the H-K-ATPase to the apical membrane in stimulated cells.

DISCUSSION

Biochemical characterization of gastric microsomal clathrin. Previously, clathrin and an AP-1 clathrin adaptor were identified on gastric microsomes from parietal cells and preliminarily characterized (39). In this study, gastric microsomal clathrin was characterized biochemically as a first step in the elucidation of its function in the regulation of membrane trafficking in the gastric parietal cell. Clathrin appears to constitute a significant fraction of the total peripheral membrane proteins of purified gastric microsomes. The enrichment of clathrin from gastric microsomes on hydroxyapatite columns reported here should serve as a convenient preliminary step in the purification of clathrin from gastric microsomes. Gastric mucus tissue may represent an easily obtainable source of clathrin from epithelial cells and should therefore facilitate the biochemical analysis of clathrin and associated proteins from a secretory epithelial cell.

Clathrin on gastric microsomes is apparently comprised of a conventional heavy chain and a light chain, with a predominance of LCa. The predominance of LCa on gastric microsomes, which are highly enriched in tubulovesicles, is an intriguing finding, given that tubulovesicles are a regulated secretory compartment. Previously, a predominance of LCb was demonstrated in cells that possess a regulated secretory pathway, such as those from brain and adrenal gland and in certain cultured cells, such as rat pheochromocytoma (PC-12) cells (1). On the other hand, LCa was found to predominate over LCb in cells that do not possess a regulated secretory pathway (cultured cells such as fibroblasts and MDCK cells) and cells in kidney. However, the present data suggest that perhaps the specific type of regulated secretory pathway may be important in dictating the ratio of LCa to LCb, rather than the presence of a regulated secretory pathway per se. Alternatively, the type of light chain may influence the size of CCVs or clathrin-coated tubules (9). For example, CCVs from brain are generally smaller than those from other tissues, which may be a reflection of not only a predominance of LCa, but also a result of both light chains containing neuronal tissue-specific inserts (9, 30). Consistent with this hypothesis, we observed that baskets polymerized from tubulovesicular clathrin and AP-1 adaptors are larger than those polymerized from brain clathrin and AP-1 adaptors (40).

Fig. 14. Immunofluorescent double labeling of F-actin and clathrin heavy chain, clathrin light chain, or γ-adaptin in cultured parietal cells under various secretory conditions. Primary parietal cell cultures were held in a resting state through the addition of 100 μM cimetidine (resting), maximally stimulated by the addition of 100 μM histamine and 30 μM IBMX (stimulated), or treated with the proton pump inhibitor SCH-28080 (5 μM) in addition to histamine plus IBMX (Stim + SCH-28080). After 25 min of incubation with the drugs, cells were fixed, permeabilized, and probed for F-actin (A, B, and C) and clathrin heavy chain by use of MAb 23 (A', B', and C'), for F-actin (D, E, and F) and clathrin light chain by use of CON.1 MAb (D', E', and F') or for F-actin (G and H) and γ-adaptin by use of MAb 100/3 (G' and H'). Morphological responses to stimulants plus inhibitor were the same as for Fig. 13; i.e., vacuoles expanded when stimulated and the enlarged swelling was prevented by the pump inhibitor. A large portion of clathrin, when probed for heavy chain (C') or light chain (F'), remained distributed throughout the cytoplasm in parietal cells stimulated in the presence of SCH-28080. Similarly, much of the signal for γ-adaptin (H') remained distributed throughout the cytoplasm in parietal cells stimulated in the presence of SCH-28080. In parallel tests, H-K-ATPase was found to be cleared from the cytoplasm and recruited to the apical membrane vacuoles when the cells were stimulated in the presence of SCH-28080 (not shown). Scale bars, 10 μm.
It is also known that clathrin light chains negatively affect polymerization of clathrin triskelions (55) and influence the stability of triskelions (27). The ratio of LCa to LCb may thus impart physiologically relevant properties to clathrin with respect to membrane trafficking in the parietal cell secretory cycle.

Despite the relative biochemical abundance of clathrin on purified gastric microsomes, shown here and in
previous work (39), a morphologically distinct clathrin coat has not been reported on tubulovesicles in any of the previous electron-microscopic analyses (7, 23, 28, 47). On the other hand, the clathrin baskets polymerized in vitro may differ markedly from those assembled onto membranes in vivo. For example, atypical clathrin-coated membranes have been reported in association with adhesion plaques (37) and at postsynaptic membranes (5). There is also evidence from yeast for a novel clathrin assembly complex (42). Moreover, the inability to detect clathrin on some membranes by standard electron-microscopic techniques may not be unique; only recently have clathrin-coated buds on endosomes been identified by immunoelectron microscopy in several different studies (18, 24, 34, 48). Thus the past inability to detect clathrin at the ultrastructural level on tubulovesicles may be a reflection of some fundamental properties of clathrin, such as its polymerized structure, that obfuscates its detection by standard electron-microscopic techniques.

Ultrastructural localization of clathrin on tubulovesicles. With the high-pressure rapid-freezing technique for preservation of gastric glands for immunoelectron microscopy, we have finally successfully identified clathrin on intracellular membranes resembling tubulovesicles in parietal cells. The amount of anticalclathrin labeling we observe on tubulovesicles at the ultrastructural level relative to the amount of H-K-ATPase immunoreactivity appears to be consistent with our biochemical estimation of the amount of clathrin relative to H-K-ATPase. An intriguing feature of the distribution of clathrin is its localization to specific sites on tubulovesicular membranes, i.e., at their ends. The implication of these findings is that clathrin may be involved in the formation of vesicles budding from tubulovesicles in resting parietal cells, with the canalicular membrane as a potential target membrane domain (see below). For example, clathrin on tubulovesicles may be involved in recycling of apical membrane components from tubulovesicles to the apical membrane, such as receptors for soluble N-ethylmaleimide-sensitive factor attachment protein (t-SNARES) like syntaxin 3 (41).

The other clathrin-coated intracellular membranes identified by immunogold labeling are the densely staining, 80- to 100-nm vesicles. The origin of these vesicles is unknown. They may represent endocytic vesicles, vesicles budded from tubulovesicles, or vesicles budded from another distinct subcellular membrane compartment, such as early endosomes. Alternatively, they may represent nascent buds from tubulovesicles viewed in cross section. They are not enriched in H-K-ATPase and may be the vesicles that can be subfractionated from purified gastric microsomes by glycerol gradients. Scaling up the glycerol gradient might allow for the further characterization of this subpopulation of clathrin-coated membranes.

Another unique morphological feature in parietal cells revealed by the high-pressure freezing technique is the appearance of tubulovesicles as cup-shaped tubules and flattened saccular membranes in the subapical cytoplasm. The cup-shaped tubules are morphologically similar to those observed in MDCK cells (25), although parietal cell tubules appear somewhat larger. In MDCK cells, these tubules were shown to be derived from an endocytic compartment and are thought to correspond to the “apical recycling compartment” in these cells (4, 6, 25, 51). Although these membranes in parietal cells are not precisely characterized, a large fraction of them appear to stain positively for H-K-ATPase and are likely to represent bona fide tubulovesicles. Also, the cup-shaped and saccular structures in parietal cells may be the equivalent of the apical recycling compartment or apical early endosomes of other epithelial cells.

Localization of clathrin and associated proteins at the canalicular membrane. In addition to clathrin, α-adaptin and a member of the dynamin family of large GTPases were also immunolocalized to canalicular membranes. Using the high-pressure rapid-freezing protocol and standard staining techniques or immunogold labeling, we have also been able to visualize coated pits and membranes at the canalicular surface that are morphologically very similar to conventional clathrin-coated pits. Taken together, these results provide the first evidence that the canalicular membrane of the resting parietal cell is endocytically active in a process involving clathrin, the AP-2 clathrin adaptor, and a dynamin. It would be of interest to identify the endocytic cargo in resting parietal cells to characterize further the role of clathrin in the physiology of resting cells; in this pathway, a possible candidate for endocytic cargo might be a v-SNARE, e.g., VAMP (10, 41). Interaction of v-SNARE with endocytic machinery has been shown in the case of synaptotagmin-AP-2 adaptor interactions at neuronal synapses (46, 56).

The detection of dynamin at the canalicular membrane confirms the findings of Calhoun et al. (11), who, using the same anti-dynamin MAb used in this study, reported the presence of dynamin on canalicular membranes in cultured parietal cells. The immunoreactivity of the canalicular membrane with the anti-dynamin MAb is intriguing, given the data presented suggesting that the expression of this immunoreactive form of dynamin in gastric glands appears to be almost exclusive to the parietal cell and enriched at the canalicular membrane. The anti-dynamin MAb was raised against a region in dynamin I (a COOH-terminal fragment spanning amino acids 698–851) that is a relatively conserved region among all the dynamins. Because dynamin I is thought to be neuron specific (49), it is likely that the anti-dynamin MAb is recognizing another member of the dynamin family (dynamin II or III). Inasmuch as many splice variants of dynamin I, II, and III have been identified (12), it will be of interest to identify at the molecular level the dynamin isoform expressed in parietal cells, particularly if this isoform is one that may be preferentially targeted to the apical membrane of all epithelial cells. Relative to this speculation, dynamin I heterologously expressed in MDCK cells is targeted to the apical membrane (3), whereas in pancreatic acinar cells, an endogenous dynamin II has
been immunolocalized to the apical membrane (50). Also, an endogenous dynamin in Caenorhabditis elegans is targeted to the apical membrane of its intestinal epithelial cells (31).

Clathrin and membrane trafficking in the parietal cell secretory cycle. Morphological data presented here suggest that the canalicular membrane of resting parietal cells is endocytically active, and this process is mediated by clathrin, the AP-2 clathrin adaptor, and dynamin. The endocytic cargo is likely to be destined for tubulovesicles, early endosomes, or some yet uncharacterized intracellular membrane compartment. To maintain the steady-state tubulovesicular or intracellular membrane surface area, membranes need to be recycled from these intracellular compartments to the apical membrane. This process also appears to be mediated by clathrin. Three models may account for the steady-state localization of clathrin on canalicular and intracellular membranes in resting parietal cells. The first model is one in which continuous endocytosis and recycling is occurring in resting cells, as occurs in most cells. Thus one would predict that the population of intracellular membranes would be comprised of a set of clathrin-coated early endosomal membranes and a distinct set of regulated secretory H-K-ATPase-rich tubulovesicular membranes that might not participate in the constitutive endocytic-recycling pathway and might not be clathrin coated. However, the clathrin-coated early endosomes may copurify with the H-K-ATPase-rich membranes on sucrose density gradients, thereby giving the impression at the biochemical level that clathrin resides on H-K-ATPase-rich membranes.

The second model is that constitutive membrane trafficking in resting cells may represent an extension of the “recovery” phase of the parietal cell after stimulation, in which the retrieval of membrane and H-K-ATPase from the apical membrane on the cessation of HCl secretion may be effected by a two-step process. One step is uptake of apical membrane followed by an extended phase of recovery involving more specific sorting of membrane proteins, such as SNARES or unretrieved H-K-ATPase, to their appropriate steady-state locations. These processes would be analogous to bulk flow membrane traffic and signal-mediated sorting, respectively (52). Both of these steps could be mediated by clathrin, with the second step requiring the action of clathrin and clathrin adaptors. A third model for clathrin in resting cells is that it performs two functions in two distinct populations of tubulovesicles. In one population of tubulovesicles, poor in H-K-ATPase, it could mediate the exchange of membrane and membrane protein with the apical membrane; this exchange may be required for some yet uncharacterized “housekeeping” function, analogous to trafficking through an early endosomal compartment, as described above. In another population of tubulovesicles, rich in H-K-ATPase, it could sequester fusogenic (i.e., v-SNARE-rich) domains to prevent premature fusion of tubulovesicles. In support of this dual hypothesis, fusogenic domains of Golgi membranes have been shown to be mechanically separable from other domains (19), and the AP-3 adaptor has been shown to interact with a synaptic vesicle v-SNARE to mediate the budding of synaptic vesicles from endosomes (18, 45). The identity of endocytotic and recycling cargo will be important to elucidate with respect to validation of these models, and these models are not necessarily mutually exclusive. Some approaches to evaluate the profiles of intracellular membranes of the parietal cell would be a more thorough fractionation and characterization of the membranes constituting a conventional preparation of purified gastric microsomes, the development of an in vitro budding assay, and colocalization of clathrin, H-K-ATPase, or other membrane markers at the immunoelectron-microscopic level.

Because the resting parietal cell appears to be endocytically active, on stimulation of the parietal cell, the volume of exocytosis must be stimulated such that it greatly exceeds that of endocytosis. In stimulated cells, clathrin is predominantly localized to the cytoplasm; thus clathrin does not appear to accompany the H-K-ATPase to the apical membrane on stimulation. Such an outcome might have been predicted if one considers that if tubulovesicles are the clathrin-coated membrane compartment, they must be uncoated before their fusion with the canalicular membrane. Thus, in this scenario, it would appear that clathrin’s main role in the parietal cell secretory cycle may be the retrieval of membrane and H-K-ATPase when HCl secretion ceases. Alternatively, clathrin may remain intracellular because of its association with membranes, such as early endosomes described above, that do not fuse with the apical membrane on stimulation. One challenge will be to develop a recycling model in which these hypotheses might be tested.

In summary, the morphological and biochemical data reported here suggest that the pattern of membrane trafficking and the proteins regulating this trafficking in parietal cells may be more complex than previously thought. However, these data have given us the ability to establish a framework for developing testable hypotheses to elucidate the function of clathrin in parietal cells and, by extension, in apical membrane trafficking in secretory epithelial cells. The tools are now available to launch a multidimensional approach to address this fundamental issue in epithelial cell biology with the parietal cell as a model system.

The authors thank the laboratory of Dr. Vincent Lee for rabbit stomachs and Drs. Frances Brodsky, Andy Wilde, and Shu-Hui Liu for generous gifts of antibodies and advice.

Mass spectra were obtained at the University of California, San Francisco, Mass Spectrometry Facility, which was supported by the Biomedical Research Technology Program of the National Center for Research Resources Grants RR-01614 and RR-08282. This work was supported by grants from the University of Southern California Gastrointestinal and Liver Diseases Center, the National American Heart Association, the Burroughs Wellcome Fund, the American Foundation for Pharmaceutical Education (C. T. Okamoto), and National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-51588 (C. T. Okamoto) and DK-10141 and DK-38972 (J. G. Forte).
REFERENCES


36. Okamoto CT, Karam SM, Jeng YY, Forte JG, and Goldenring JR. Identification of clathrin and clathrin adaptors on


