

Identification of a 200-kD, Brefeldin-sensitive Protein on Golgi Membranes

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Abstract. A mAb AD7, raised against canine liver Golgi membranes, recognizes a novel, 200-kD protein (p200) which is found in a wide variety of cultured cell lines. Immunofluorescence staining of cultured cells with the AD7 antibody produced intense staining of p200 in the juxtannuclear Golgi complex and more diffuse staining of p200 in the cytoplasm. The p200 protein in the Golgi complex was colocalized with other Golgi proteins, including mannosidase II and β -COP, a coatomer protein. Localization of p200 by immunoperoxidase staining at the electron microscopic level revealed concentrations of p200 at the dilated rims of Golgi cisternae. Biochemical studies showed that p200 is a peripheral membrane protein which partitions to the aqueous phase of Triton X-114 solutions and is phosphorylated. The p200 protein is located on

the cytoplasmic face of membranes, since it was accessible to trypsin digestion in microsomal preparations, and is recovered in approximately equal amounts in membrane pellets and in the cytosol of homogenized cells. Immunofluorescence staining of normal rat kidney cells exposed to the toxin brefeldin A (BFA), showed that there was very rapid redistribution of p200, which was dissociated from Golgi membranes in the presence of this drug. The effect of BFA was reversible, since upon removal of the toxin, AD7 rapidly reassociated with the Golgi complex. In the BFA-resistant cell line PtK₁, BFA failed to cause redistribution of p200 from Golgi membranes. Taken together, these results indicate that the p200 Golgi membrane-associated protein has many properties in common with the coatomer protein, β -COP.

THE Golgi complex is a dynamic membranous network which serves as an assembly line and as a clearing house for the glycosylation, processing, transport, and sorting of newly synthesized and recycled proteins (13, 32). The individual Golgi stacks are comprised of a sequential array of cisternae and the functionally and structurally distinct *trans*-Golgi network (18). The intracellular transport of proteins into, out of, and between these Golgi subcompartments requires the movement of vesicles between membranes (30, 32, 36).

The *in vitro* reconstitution of vesicle transport between Golgi membranes has led to the identification of a number of membrane-associated and cytosolic proteins that are involved in Golgi membrane trafficking (28, 31, 36). For instance, the NEM sensitive factor (NSF) protein on Golgi membranes (4, 25, 47) and the soluble α , β , and γ soluble NSF attachment proteins (SNAPs) (8, 45) have essential roles in vesicle fusion. Membrane-associated coat proteins on the vesicles themselves are important for vesicle budding (11).

At least two major classes of vesicles associated with Golgi transport are distinguished by unique sets of coat proteins. Clathrin-coated vesicles are well known for transport in the endocytic pathway and for transport to and from the *trans*-Golgi network (reviewed in 6). The coats of these vesi-

cles consist of the heavy and light chains of clathrin in addition to adaptor complexes comprised of the 100-kD, α , β , and γ adaptins (19, 20, 33, 34, 35). More recently, another class of nonclathrin coated vesicles has been described (26, 31). These vesicles involved in transport within the Golgi complex and possibly between the rough ER and Golgi complex, have a transiently attached coat which contains an adaptin-like complex of α , β , γ , and δ coatomer proteins (COPS)¹ (11, 12, 39, 44).

Membrane-associated proteins on transport vesicles and Golgi membranes include members of GTP-binding protein families (G proteins). A role for G proteins and GTP hydrolysis in the process of vesicle trafficking was initially established through studies on yeast secretion mutants (37, 38). In mammalian cells, the rab 6 protein is found on Golgi membranes (17) and a number of other monomeric G proteins such as arf, rab 1, and rab 2 also appear to be involved in Golgi trafficking (2). We have recently shown that a heterotrimeric G protein, $G_{\alpha-3}$, is also a resident protein on Golgi cisternae and is able to regulate secretory trafficking (41). The precise functions of many of these Golgi mem-

1. *Abbreviations used in this paper:* BFA, brefeldin A; COP, coatomer protein; G proteins, GTP-binding protein families; NRK, normal rat kidney.

brane proteins, coat proteins, and regulatory G proteins are not yet known. It is already clear, however, that the processes of vesicle budding and membrane fusion involve intricate complexes of Golgi-associated proteins that must operate in a regulated fashion to allow the passage of itinerant proteins while maintaining the structure of the Golgi stack.

The fungal metabolite, Brefeldin A (BFA), has recently been shown to be a powerful tool with which to study the actions of Golgi proteins. Upon exposure of cells to BFA, there is a dramatic redistribution of Golgi membranes and proteins in the secretory pathway. BFA induces the fusion of some of the Golgi subcompartments with the rough ER by extension of retrograde tubular processes from the cis-Golgi network to the ER to form a unique BFA compartment (15, 22–24). BFA effectively stops the secretion and delivery of newly synthesized cellular proteins (16, 29) and viral proteins in infected cells (43, 46), sequestering them in the BFA compartment, often in a partially processed form. One of the initial effects of BFA is to prevent the association of the β -COP protein with Golgi membranes, an event which precedes the other, more dramatic morphological changes in the Golgi complex (9, 10, 12, 21, 31). Although the molecular target of BFA action is not currently known, the use of this drug now provides a means with which to manipulate Golgi proteins, enabling further investigation of their structure and function.

To fully understand vesicle trafficking in the Golgi, it is necessary to define all of the proteins involved in vesicle formation and fusion. To this end, we have raised a panel of mAbs to Golgi membrane proteins. One of these antibodies, AD7, showed strong immunofluorescence staining of the Golgi region. We find that the AD7 antibody recognizes a 200-kD peripheral membrane phosphoprotein, p200, in a variety of cells. The p200 protein exists in membrane-attached and soluble forms and is BFA sensitive, thus exhibiting behavior consistent with that of a Golgi-associated coat protein.

Materials and Methods

Cell Culture

Normal rat kidney (NRK) cells were maintained in DME supplemented with 10% FBS and PtK₁ cells were cultured in Eagle's medium (EMEM) with 10% FBS. NRK and PtK₁ cells were maintained in flasks, passaged every 4 d and grown on glass coverslips for immunofluorescence staining. MDCK cells (MDCK II) were grown in EMEM with Earle's salts containing 5% FBS and 10 mM HEPES, pH 7.3, as previously described (27).

Preparation of mAbs to Golgi Proteins

Golgi membranes were prepared from canine liver essentially as described by Fleischer (14). In brief, 50–100 g fresh liver was minced, suspended and homogenized in 3 vol of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, containing 1 mM PMSF. The crude homogenate was filtered through cheesecloth, adjusted to 1.5 M sucrose and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was overlaid with 6 ml each of 1.3 and 1.2 M sucrose (buffered as above), 7 ml of 1.1 M sucrose and 6 ml of 0.95 M sucrose, and then with 0.6 M sucrose to fill the tube. After centrifugation at 122,000 g for 1 h, Golgi membranes migrating at the 0.6–0.95 or 0.95–1.1 M interfaces were collected and assayed for galactosyltransferase activity.

Approximately 100 μ g of the 0.6–0.95 M interface membranes were extracted and phase partitioned with Triton X-114 (5) (see below). The detergent phase was emulsified in complete Freund's adjuvant and injected intraperitoneally into mice, which were later boosted twice with the same antigen. Spleen cells from the immunized Robertsonian (8.12) 5BnR mice

were fused to FOX NY myelomas using standard procedures (42) and hybridomas were selected at AAT medium. Culture supernatants were screened by immunofluorescence staining as described by Burke et al. (7) on MDCK cells. Cells with supernatants giving juxtannuclear staining, suggestive of Golgi localization, were cloned through three rounds of selection in soft agar and then grown in DMEM with 10% FBS. Spent medium from the AD7 clone was stored in the presence of sodium azide at 4°C and used as the source of antibody for all of the experiments described. Based on its reactivity with γ chain-specific secondary antibodies, and its reactivity with protein A, the AD7 mAb is an IgG, although subtype specificity has not yet been determined.

Other Antibodies

The mAb 53FC3 specifically recognizes the 135-kD mannosidase II Golgi enzyme and has been previously characterized (7). The rabbit anti-Golgi serum used for immunofluorescence staining, recognizes mannosidase II in addition to three other polypeptides and gives specific staining of the Golgi complex, as described previously (7). The M3A5 mAb to the 110-kD β -COP protein (12) was kindly provided by Dr. Thomas Kreis (European Molecular Biology Laboratory, Heidelberg, Germany). The H28E23 mAb to the influenza hemagglutinin (H1 subtype) protein (HA) was obtained from Dr. J. Yewdell (National Institutes of Health) and Dr. W. Gerhard (Wistar Institute, Philadelphia, PA).

FITC-conjugated, TRITC-conjugated, alkaline phosphatase-conjugated, and biotinylated second antibodies were obtained from Vector Laboratories (Burlingame, CA) and Tago Immunologicals (Burlingame, CA).

Immunocytochemistry

Immunofluorescence localization of Golgi complex-associated proteins was carried out on NRK and PtK₁ cells fixed in 3 or 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Nonspecific staining was blocked by washing cells with PBS containing either 0.5% BSA or 0.2% fish skin gelatin. Cells on coverslips were incubated with the appropriate primary antibody for 2 h, followed by washing and incubation for 1–2 h in FITC- or TRITC-labeled second antibodies. After washing in PBS, coverslips were mounted in *N*-propyl-gallate in glycerol and viewed on a microscope fitted for epifluorescence (microphot FXA; Nikon Inc., Melville, NY). Some cells were pretreated with BFA (Epicentre Technologies, Madison, WI) before fixation. BFA was stored frozen as a stock of 1 mg/ml in ethanol and added to the medium at a final concentration of 5 μ g/ml.

PtK₁ cells grown on 35-mm² plastic dishes were used for immunoperoxidase staining with the AD7 antibody. Cells were fixed in paraformaldehyde-lysine-periodate containing 0.01% glutaraldehyde for 4 h at 20°C, permeabilized with 0.005% saponin and incubated overnight at 4°C in primary antibody diluted in PBS containing 0.5% ovalbumin. Cells were then incubated with biotinylated goat anti-mouse IgG and avidin HRP. The reaction with DAB substrate, postfixation, and embedding for EM were carried out as previously described (40). Thin sections were cut and viewed on an electron microscope (model 400; Phillips Electronic Instruments Co., Mahwah, NJ).

Metabolic Labeling and Immunoprecipitation

Cells grown in 35-mm² petri dishes were metabolically labeled by overnight incubation in medium containing 50 μ Ci/ml of either [³⁵S]methionine (1,000 Ci/mmol, Amersham Corp., Arlington Heights, IL) or [³⁵S]-*trans*-label (1,000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) in methionine-reduced medium (final concentration 1/10th normal methionine), made from DMEM containing 5% FBS (dialysed against PBS) and 10% normal DMEM. For labeling with ³²P_{O₄, cells were first washed with phosphate-free DMEM and then incubated for 4 h in phosphate-free DMEM containing 5% FBS (dialysed against HEPES-buffered saline) and 1 mCi/ml ³²P_{O₄ (1,000 mCi/mmol, New England Nuclear, Boston, MA).}}

After labeling, cells were washed in PBS and lysed in 500 μ l of a buffer containing 50 mM triethanolamine, pH 7.4, 100 mM NaCl, 0.4% SDS, 1 mM DTT, 1 mM PMSF, and 1/1,000 CLAP (10 mg/ml in DMSO of each of chymostatin, leupeptin, antipain, pepstatin) and Triton X-100 was added to a final concentration of 0.2%. The lysate was centrifuged to remove insoluble material and incubated with 500 μ l of AD7 culture supernatant and 20 μ l of a 50% suspension of protein A-Sepharose in PBS. The mixture was rotated overnight at 4°C and then the protein A-Sepharose beads were collected by centrifugation, and washed 5 times in the same buffer containing 0.1% SDS and 0.5% Triton X-100, followed by 2 washes in 50 mM Tris,

pH 7.4. Proteins were released from the beads by boiling in SDS-PAGE sample buffer.

After immunoprecipitation of ^{32}P -labeled cells, the protein A-Sepharose pellets were washed in 50 mM Tris, pH 8.5, 0.1 mM EDTA and resuspended in 50 μl of this buffer. Samples were then incubated for 1 h at 37°C in the presence or absence of 1 U/ μl calf intestinal alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, IN). The Sepharose pellets were collected by centrifugation and resuspended in SDS-PAGE sample buffer.

Preparation of Microsomal Membranes

Confluent dishes of cells were labeled with [^{35}S]methionine, or were used unlabeled. Scraped cells were homogenized in ice-cold 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, buffer containing protease inhibitors. Nuclei and unbroken cells were removed by centrifugation at 1,000 g for 5 min at 4°C. The postnuclear supernatant was then centrifuged at 100,000 g for 1 h at 4°C to separate microsomal membranes (pellet) from cytosol (supernatant). The p200 protein was recovered from the pellet and supernatant by immunoprecipitation or detected in these fractions by Western blotting using the AD7 antibody.

Triton X-114 Extraction

Cells grown on 35-mm dishes were metabolically labeled overnight with ^{35}S -*trans*-label and then extracted on ice in 1 ml of a buffer containing 50 mM triethanolamine, pH 7.4, 100 mM NaCl, 0.5% Triton X-114, 1 mM DTT, 1 mM PMSF, and 1/1,000 CLAP. After removing insoluble material by centrifugation for 15 min at 4°C in a microfuge, the supernatant was warmed to 30°C for 3 min to effect phase separation of the Triton X-114 (5). Detergent and aqueous phases were collected by centrifugation at 3,000 rpm for 3 min at 20°C. Each phase was then used for immunoprecipitation with the AD7 antibody.

Accessibility of the AD7 Antigen to Trypsin

Confluent monolayers of MDCK cells in 6-cm dishes were labeled overnight with [^{35}S]methionine as described above. After labeling, cells were washed in Earle's MEM with 0.2% BSA and infected with 20 plaque-forming u/cell of influenza virus (A/PR8/34; H1 subtype) for 3 h at 37°C. Infected cells were pulse labeled for a further 20 min with [^{35}S]methionine, washed with ice-cold PBS containing 1 mM CaCl_2 , 0.5 mM MgCl_2 , and homogenized. Postnuclear supernatants were then digested for 15 min at 0°C with 50 $\mu\text{g}/\text{ml}$ trypsin-TPCK. As a control, one sample was brought to a concentration of 1% Triton X-100, 0.1% SDS, 0.15 M NaCl, 20 mM Tris-HCl, pH 8.6, before addition of the trypsin. Proteolysis was stopped by addition of 10 $\mu\text{g}/\text{ml}$ aprotinin and 100 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor. Samples were then used for immunoprecipitation with AD7 and H28E23 antibodies, using the methods described above.

Western Blotting

Microsomal membranes (100,000 g pellet) and cytosol fractions (100,000 g supernatant) were boiled in SDS-PAGE sample buffer and samples matched for protein concentration were loaded on SDS-PAGE gels. After electrophoresis, proteins were transferred to Immobilon membrane (Millipore Corp., Bedford, MA) and the membranes were then stained with Coomassie blue to ensure that all lanes contained equivalent amounts of transferred proteins. Destained membranes were incubated in blotting buffer (5% non-fat dry milk in 0.15 M NaCl, 1% Triton X-100, 20 mM Tris, pH 7.4) followed by incubation in specific antibody diluted in blotting buffer. After washing in blotting buffer, membranes were incubated with second antibody. Bound antibodies were detected by the enhanced chemiluminescence Western blotting detection system (Amersham Corp.) or by alkaline-phosphatase conjugates using NBT/BCIP as a substrate.

SDS-PAGE

Immunoprecipitated proteins, membrane and cytosol fractions, and cell extracts were boiled in SDS-PAGE sample buffer (1% SDS, 30 mM Tris, pH 6.8, 5% 2-mercaptoethanol, 12% glycerol) and electrophoresed using standard conditions on 8–10% Laemmli SDS-polyacrylamide gels. Bands were detected by fluorography using EN 3 HANCE (New England Nuclear) and dried gels were exposed to X-Omat AR film (Eastman Kodak Co., Rochester, MA).

Results

The AD7 Antibody Recognizes a 200-kD Peripheral Membrane Protein (p200)

Microsomal membrane proteins (100,000 g pellet) were prepared from cultured cell lines and screened by Western blotting using the AD7 mAb raised against dog liver Golgi membranes. The antibody specifically recognized a 200-kD protein (p200) in microsomes from NRK cells (Fig. 1 A, lane 1) and other cell lines tested. The molecular weight of this protein was judged to be \sim 200,000 because it migrated beside, or slightly above, the myosin molecular weight marker

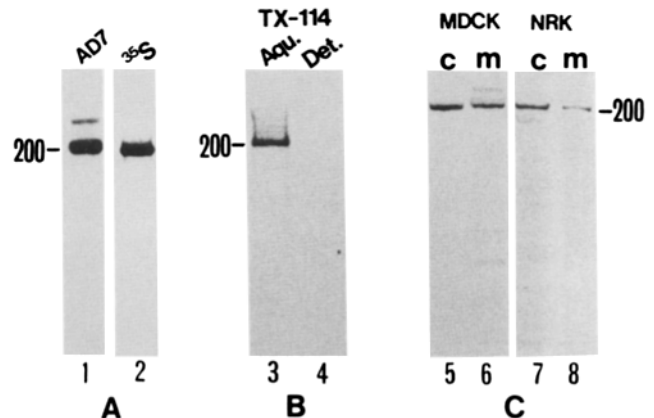


Figure 1. (A) The AD7 antibody recognizes a 200-kD protein. Western blotting and immunoprecipitation were used to identify the protein recognized by the AD7 antibody. (Lane 1) Western blotting of microsomal membranes of NRK cells with the AD7 antibody, bound antibody was detected by chemiluminescence. The antibody specifically recognizes a 200-kD protein; in some experiments a lighter band with a slightly slower mobility was also weakly recognized. (Lane 2) NRK cells were metabolically labeled overnight with [^{35}S]methionine and detergent extracts of the cells were used for immunoprecipitation with the AD7 antibody. The AD7 antibody specifically immunoprecipitates ^{35}S -labeled p200 from these cells. (B) p200 is a peripheral membrane protein. NRK cells were metabolically labeled with [^{35}S]methionine overnight and then extracted and partitioned in Triton X-114, as described in the text. The aqueous and detergent phases were collected and used for immunoprecipitation with the AD7 antibody. Immunoprecipitates were then analyzed by SDS-PAGE and fluorography. The p200 was immunoprecipitated only from the aqueous phase (lane 3) and not from the detergent phase (lane 4). (C) p200 is present in both the cytosol and on membranes in epithelial cells. (Lanes 5 and 6) MDCK cells were metabolically labeled overnight with [^{35}S]methionine, the postnuclear supernatant was centrifuged at 100,000 g and the supernatant (cytosol, c) and pellet (membranes, m) were used for immunoprecipitation with the AD7 antibody. ^{35}S -labeled p200 was present in both the cytosol (lane 5) and the membrane pellet (lane 6). (Lanes 7 and 8) NRK cells were homogenized and similarly fractionated. The high speed supernatant and pellet were run on SDS-polyacrylamide gels and transferred to Immobilon. Western blotting of these fractions was carried out with the AD7 antibody followed by alkaline phosphatase detection. The p200 protein was also found in both the cytosol (lane 7) and in the membrane pellet (lane 8) of NRK cells. In both the MDCK and NRK cells, slightly more of the p200 was recovered from the cytosol (lanes 5 and 7) than from the membrane pellets (lanes 6 and 8).

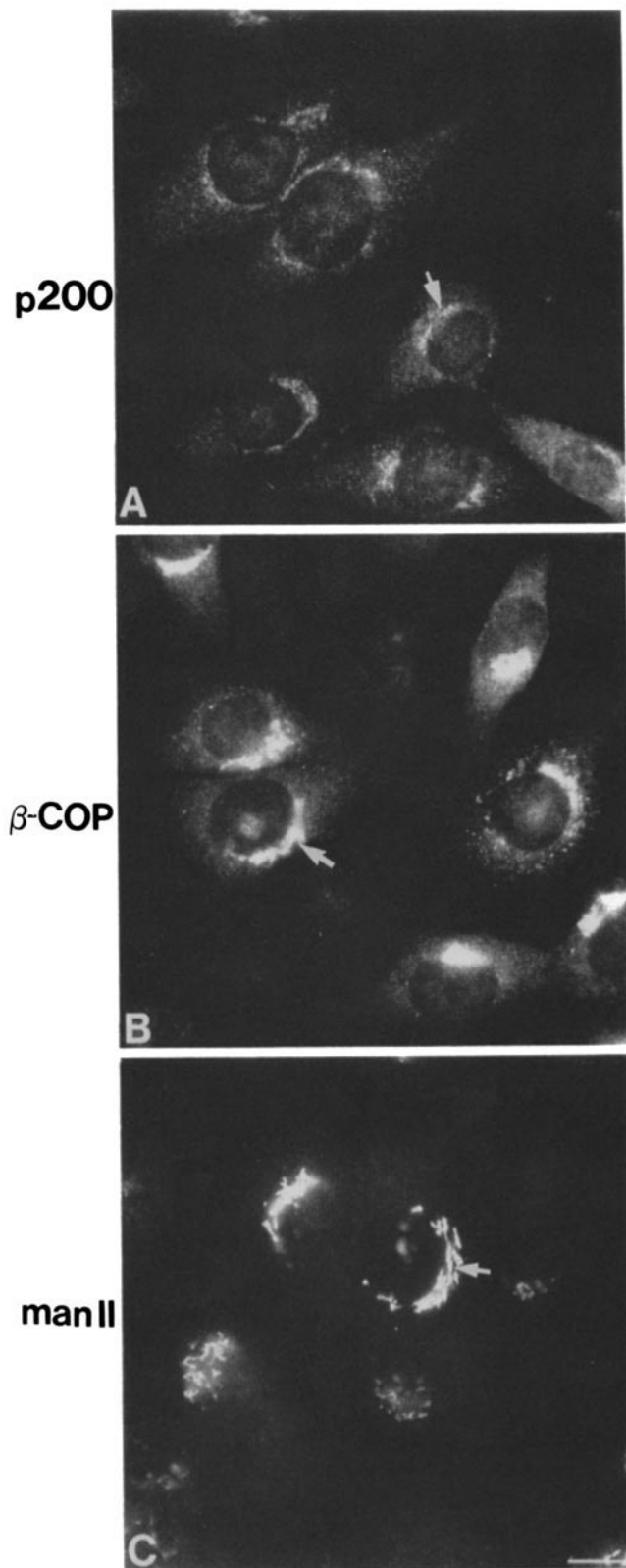


Figure 2. Immunofluorescence staining of NRK cells. NRK cells were fixed and permeabilized as described in the text and stained by immunofluorescence with the AD7 antibody to the p200, or with antibodies to β -COP and mannosidase II. (A) NRK cells stained with the AD7 antibody showed intense perinuclear staining in a Golgi-like distribution (arrow), and there was also less intense, finely punctate staining throughout the cytoplasm of these cells. (B)

(200 kD) on SDS-PAGE gels. In addition to this 200-kD protein band seen on Western blots, the antibody occasionally reacted weakly with a slightly slower migrating band (Fig. 1 A, lane 1) which may be related to the 200-kD protein. The p200 protein was also immunoprecipitated by the AD7 antibody from detergent extracts of NRK cells that had been metabolically labeled with [35 S]methionine (Fig. 1 A, lane 2).

The p200 protein was found in both membrane-associated and soluble forms in all of the cell lines tested (Fig. 1 C). In MDCK cells labeled overnight with [35 S]methionine, p200 was immunoprecipitated with the AD7 antibody from both the supernatant (100,000 g) and membrane pellet (100,000 g) fractions (Fig. 1 C, lanes 5 and 6). The p200 protein was also found by Western blotting in the supernatant and membrane pellet fractions from NRK cells (Fig. 1 C, lanes 7 and 8). In immunoprecipitates from both the supernatants and membrane pellets the 200-kD protein was the only protein recognized by the antibody. Typically, p200 was more abundant in the supernatants than in the membrane pellets, although the actual proportions varied between cell types.

Extracts of [35 S]methionine-labeled NRK cells were phase partitioned with Triton X-114 as described above. The p200 protein was immunoprecipitated and recovered entirely from the aqueous phase (Fig. 1 B, lane 3). The aqueous phase presumably contains soluble p200 and p200 which is associated with membranes as a peripheral membrane protein. The lack of any p200 in the detergent phase suggests that there is no hydrophobic, membrane-intercalated p200 in these cells.

The p200 Protein Is Localized in the Golgi Complex

The AD7 mAb was used for immunofluorescence staining in a variety of cell lines to localize the p200 protein. The typical staining pattern obtained with this antibody is demonstrated in NRK cells (Fig. 2 A) and in PtK₁ cells (see Fig. 10 A). In both cases, there was intense staining in a perinuclear crescent (Fig. 2 A) and fainter, finely punctate staining of the cytoplasm. This perinuclear staining was roughly coincident with the patterns obtained with antibodies to the Golgi-associated proteins, β -COP (Fig. 2 B) (12) and mannosidase II (Fig. 2 C) (3, 7). The AD7 staining of the p200 was similar to the staining of the β -COP protein, in that both had a vesicular appearance over the Golgi region and more diffuse staining throughout the cytoplasm. The staining of the p200 and β -COP proteins in the Golgi region was distinct from the staining of mannosidase II in the cisternae, which had a more linear and less punctate appearance.

To confirm that p200 is associated with the Golgi complex, NRK cells were double labeled with AD7 and a rabbit Golgi antibody. The rabbit anti-Golgi serum, which has been

A staining pattern similar to A, was seen with the antibody to β -COP, the Golgi vesicle coat protein, which was also localized in the perinuclear Golgi region (arrow) and in the cytoplasm. (C) NRK cells stained with an antibody to mannosidase II show perinuclear staining of the Golgi stacks (arrow). The Golgi staining of the mannosidase was more linear than Golgi staining of the p200 or the β -COP which both gave more punctate or vesicular staining over the Golgi area. Bar, 10 μ m.

used in our laboratory as a Golgi marker, gave perinuclear staining in NRK cells which colocalized precisely with the staining of the resident Golgi enzyme mannosidase II (Fig. 3, *A* and *B*). This polyclonal rabbit anti-Golgi serum and monoclonal AD7 antibody were then used together for colocalization of proteins in the same cells. The perinuclear staining of AD7 precisely matched the localization of the rabbit-anti-Golgi staining (Fig. 3, *D* and *E*) although the AD7 antibody gave additional cytoplasmic staining, as described above. This confirmed that the p200 protein is associated with the perinuclear Golgi complex.

The ultrastructural localization of p200 was examined by immunoperoxidase staining and EM in PtK₁ cells (Fig. 4, *A* and *B*). DAB reaction product was deposited heavily over the ends of the cisternae of Golgi stacks and appeared to originate from vesicle-like structures close to the Golgi complex and from the dilated rims of the Golgi cisternae. There was

no reaction product in the lumens of Golgi cisternae or on the cytoplasmic face of the central portions of the stacked cisternae. However, some diffuse reaction product was evident in the cytoplasm of these cells, reflecting the large amount of soluble p200 (Fig. 1 *B*). Organelles other than the Golgi complex were not stained by the AD7 antibody. The staining of Golgi membranes and cytoplasm seen in cells reacted with the AD7 antibody was specific since there was no comparable deposition of reaction product in the absence of antibody in control cells (Fig. 4 *C*).

The p200 Protein Is Accessible to Trypsin in Cell Homogenates

The apparent cytoplasmic disposition of p200 was confirmed biochemically by proteolysis of cell fractions. MDCK cells were labeled overnight with [³⁵S]methionine and infected

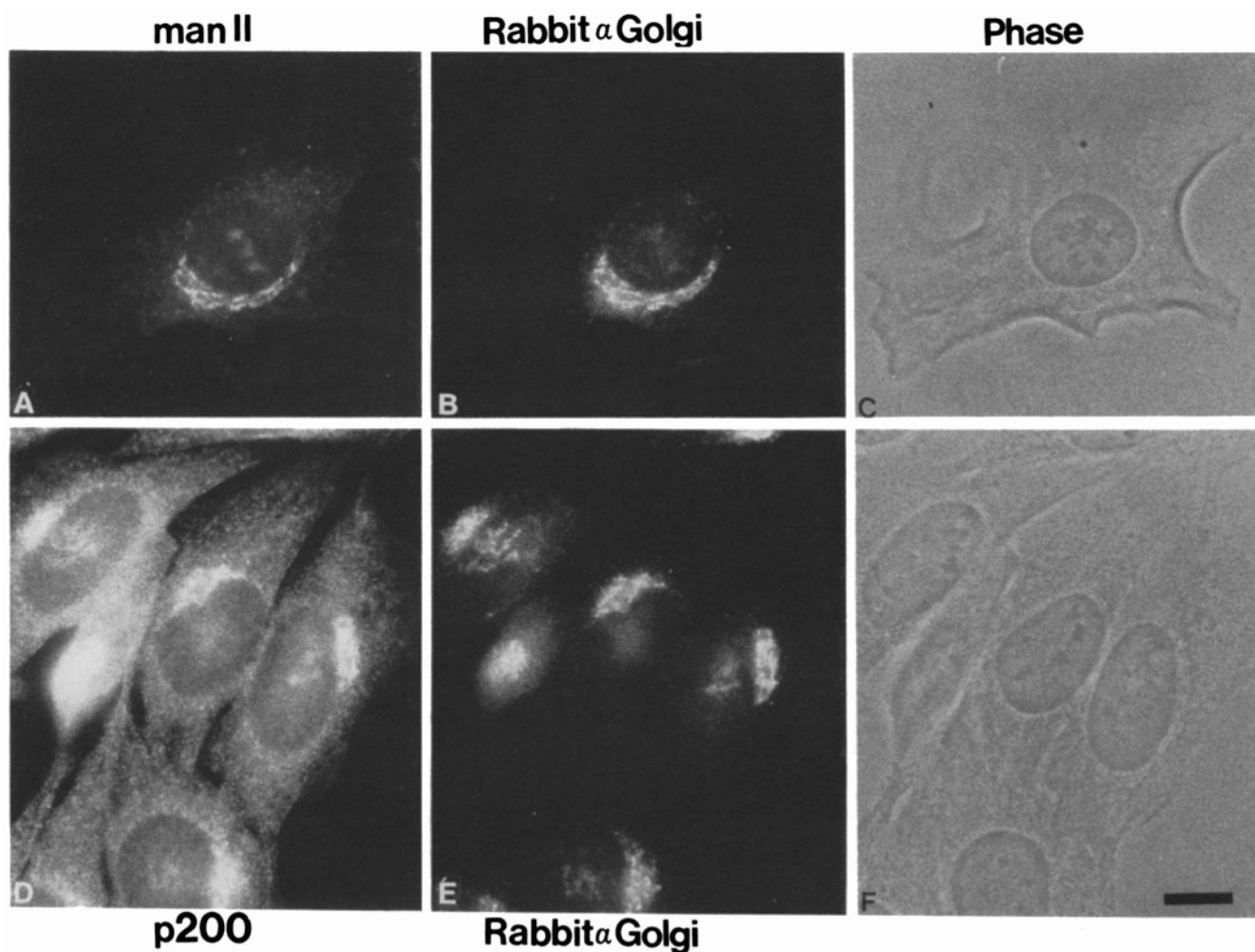


Figure 3. Colocalization of the p200 and other Golgi proteins in NRK cells. NRK cells were double labeled by simultaneous incubations of cells with a rabbit anti-Golgi serum (*B*, *E*) together with either a monoclonal mannosidase II antibody (*A*) or AD7 (*D*). These antibodies were then detected with TRITC-anti-rabbit IgG (*B*, *E*) and FITC-anti-mouse IgG (*A*, *D*), respectively. The top panels show colocalization of mannosidase II (*A*) and rabbit anti-Golgi serum (*B*) in the perinuclear Golgi complex of an NRK cell, which is shown in phase contrast (*C*). Staining with both antibodies coincides over the same perinuclear cluster of Golgi stacks. The bottom panels show colocalization of the AD7 antibody to p200 (*D*) and the rabbit anti-Golgi serum (*E*) in the perinuclear Golgi complex of the group of NRK cells, which are also shown by phase contrast (*F*). The p200 staining is colocalized with the rabbit anti-Golgi staining in the perinuclear Golgi stacks. Bar, 10 μ m.

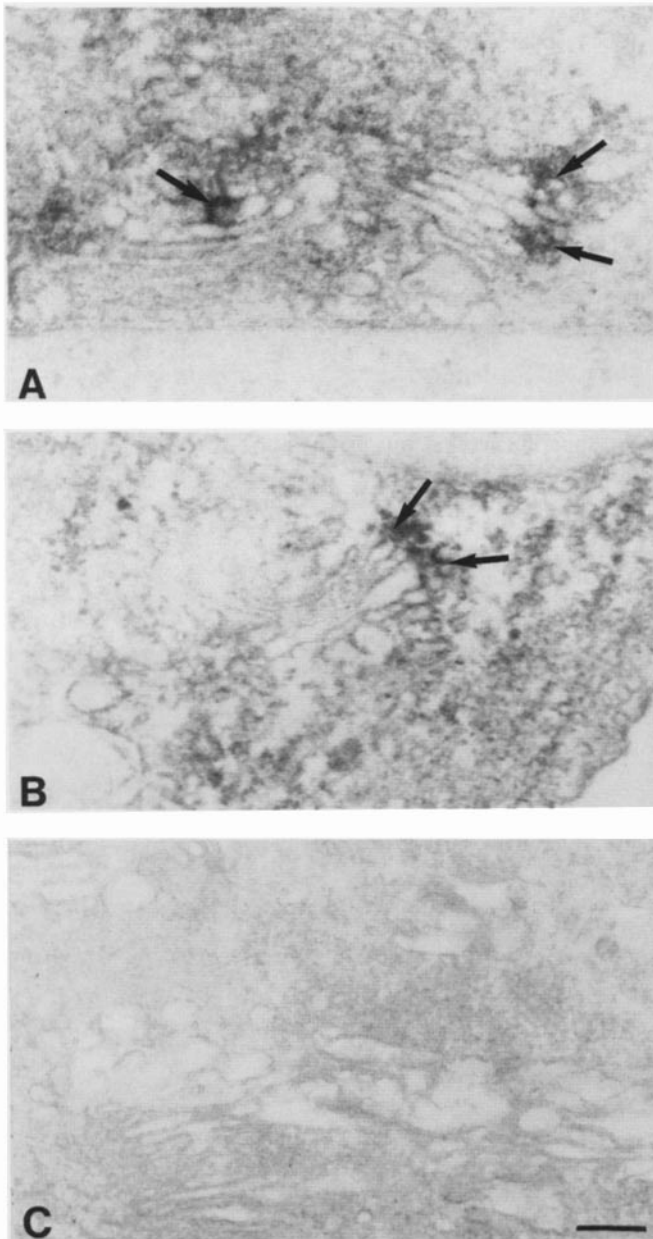


Figure 4. Ultrastructural localization of p200. PtK₁ cells were prepared for immunoperoxidase staining and EM as described in the text. (A and B) Incubations of these cells with the AD7 antibody, then with biotinylated anti-mouse IgG and avidin HRP, resulted in deposition of DAB reaction product over the dilated rims of the Golgi cisternae and in some vesicular structures at the ends of the Golgi stacks. The black reaction product is deposited on the cytoplasmic face of the Golgi membranes and there is no reaction product in the lumens of the cisternae. Because of the presence of soluble p200 in the cells, some diffuse reaction product can also be seen in the cytoplasm. A and B show two large Golgi complexes with AD7 staining concentrated at the ends of cisternae in each stack (arrows). (C) In control cells, reacted with the second antibody-HRP reagents only, there is no specific deposition of DAB reaction product on membranes or in the cytoplasm. Bar, 0.3 μm .

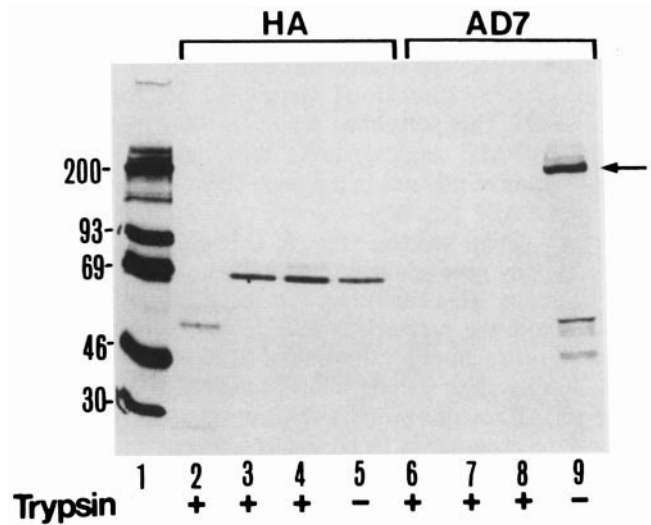


Figure 5. Accessibility of p200 in cell homogenates to trypsin digestion. MDCK cells were labeled overnight with [³⁵S]methionine and infected with influenza virus. The newly synthesized influenza hemagglutinin was labeled with a 20-min pulse of [³⁵S]methionine. Postnuclear supernatants prepared from these cells were then either treated with trypsin alone (lanes 3, 4, 7, and 8) or with trypsin in the presence of detergent to lyse vesicles (lanes 2 and 6) or with protease inhibitors only (lanes 5 and 9) for 15 min at 0°C. The p200 and hemagglutinin proteins were immunoprecipitated sequentially using the appropriate antibodies (indicated as AD7 and HA). Both the endo H-sensitive (ER) form of hemagglutinin (faster migrating bands in lanes 3–5) and the endo H-resistant (Golgi) form (slower migrating band in lanes 3–5) were immunoprecipitated. In the presence of detergent and trypsin, both hemagglutinin (lane 2) and p200 (lane 6) were digested. In the absence of detergent, hemagglutinin was protected inside intact membranes and was not cleaved (lanes 3 and 4), while the p200 was completely digested by trypsin (lanes 7 and 8). In the absence of trypsin, neither hemagglutinin (lane 5) nor p200 (lane 9) was digested.

with influenza virus. The newly synthesized influenza hemagglutinin was labeled with a 20-min pulse of [³⁵S]methionine. Postnuclear supernatants prepared from these cells were then either treated with trypsin alone, with trypsin in the presence of detergent to lyse vesicles, or with protease inhibitors alone (Fig. 5). The p200 and influenza hemagglutinin proteins were then immunoprecipitated sequentially using appropriate antibodies. Since the half time of hemagglutinin transport to the cell surface is ~50–60 min, both the rough ER and Golgi complex would be expected to contain labeled hemagglutinin after a 20-min pulse of [³⁵S]methionine. This is, in fact, demonstrated in Fig. 5 by the immunoprecipitation of two closely spaced polypeptides representing the endo H-resistant (slower migrating) and endo H-sensitive (faster migrating) forms of the hemagglutinin found in the ER and Golgi complex, respectively (Fig. 5, lanes 3–5). In the presence of trypsin, the p200 was completely digested (Fig. 5, lanes 6–8), while in the same microsomes, the hemagglutinin was not digested (Fig. 5, lanes 3–5). Hemagglutinin was only digested by trypsin after detergent permeabilization of microsomes (Fig. 5, lane 2). Taken together, these results suggest that p200 is present on the cytoplasmic face of membrane vesicles while the extracellular domain of viral hemagglutinin is sequestered in

the lumens of these vesicles. The cytoplasmic location of the p200 on the membranes is consistent with its simultaneous presence in the cytosol.

p200 Is Phosphorylated

It is possible that the distribution of p200 on membranes and in the cytosol is regulated by a secondary modification such as phosphorylation. To test this, NRK cells were metabolically labeled with $^{32}\text{PO}_4$ and detergent extracts of the cells were used for immunoprecipitation with the AD7 antibody or with nonimmune serum. The control immunoprecipitate, where nonimmune serum was used, contained no labeled proteins when analyzed by SDS-PAGE and autoradiography (Fig. 6, lane 1). The AD7 immunoprecipitate contained a single protein, the p200 protein which was ^{32}P labeled (Fig. 6, lane 2). Incubation of the immunoprecipitated extracts with alkaline phosphatase before electrophoresis resulted in removal of the ^{32}P label from the p200 band (Fig. 6, lane 3). These results indicate that the p200 peripheral membrane protein is phosphorylated, which again is consistent with its location both on the cytoplasmic face of membranes and in the cytosol.

Golgi-associated p200 Protein Rapidly Redistributes in the Presence of BFA

Previous studies have shown that the β -COP coat protein is dissociated from the Golgi membranes upon treatment of cells with the toxin BFA and that this dissociation precedes further reorganization of Golgi membranes (9, 22). Immunofluorescence staining was performed on BFA-treated NRK cells to determine the effect of BFA on the localization of the p200 protein. Short treatments (1 min) of NRK cells with BFA (5 $\mu\text{g}/\text{ml}$) were not sufficient to induce a redistribution of Golgi structure as determined by the perinuclear mannosidase II staining pattern (Fig. 7, E and F). These conditions did, however, cause a major redistribution of the p200 resulting in a complete disappearance of perinuclear staining, leaving only finely punctate cytoplasmic staining in the BFA-treated cells (Fig. 7, A and B). Thus, the redistribution of the p200 preceded any change in the mannosidase staining pattern (Fig. 7, E and F) and was also more rapid than the redistribution of the β -COP protein. Cells treated

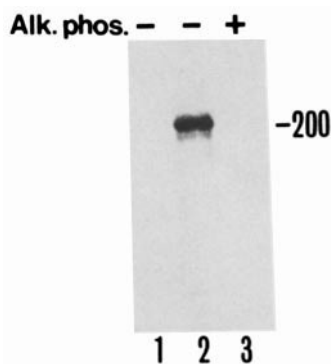


Figure 6. Phosphorylation of p200. NRK cells were metabolically labeled with $^{32}\text{PO}_4$ for 4 h, postnuclear supernatants were prepared and p200 was then immunoprecipitated with the AD7 antibody. Immunoprecipitates were incubated in buffer with or without alkaline phosphatase (+ or -). Immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography. (Lane 1) a control immunoprecipitation with nonimmune serum. (Lane 2) the immunoprecipitate with AD7 contains the ^{32}P -labeled p200 band. (Lane 3) alkaline phosphatase digestion removed the $^{32}\text{PO}_4$ from the p200 band.

in an identical fashion with BFA and stained with the β -COP antibody showed increased cytoplasmic staining but there was still some perinuclear Golgi complex staining (Fig. 7, C and D). In several experiments using very short exposures (<1 min) of NRK cells to BFA, the dissociation of the p200 was consistently found to be more rapid and more complete than β -COP.

The distribution of p200 in the continued presence of BFA was also examined. NRK cells were treated for longer periods (30 min–2 h) with BFA, which induced a reorganization of Golgi membranes as described by others (22, 23, 31) resulting in a dispersal of mannosidase II staining (Fig. 8, C and D). In these cells treated with BFA (30 min–2 h), there was diffuse cytoplasmic staining, but no perinuclear staining with the AD7 antibody, showing that the p200 remained in the cytoplasm in the continued presence of BFA (Fig. 8, A and B).

The effect of BFA on p200 was completely reversible. NRK cells treated for 30 min with BFA (Fig. 9 A) showed diffuse cytoplasmic staining with AD7, but upon washout of the BFA, the perinuclear AD7 staining reappeared (Fig. 9 B). Repeated experiments established that significant amounts of p200 reassociated with the perinuclear Golgi soon after removal of the BFA (within 5 min) and was complete (i.e., the relative amounts of Golgi and cytoplasmic staining were the same as in control cells) by 45 min (Fig. 9 B). The reassociation of p200 with the Golgi membranes was more rapid and complete than reassociation of the β -COP and also preceded restoration of the normal mannosidase II staining pattern (not shown).

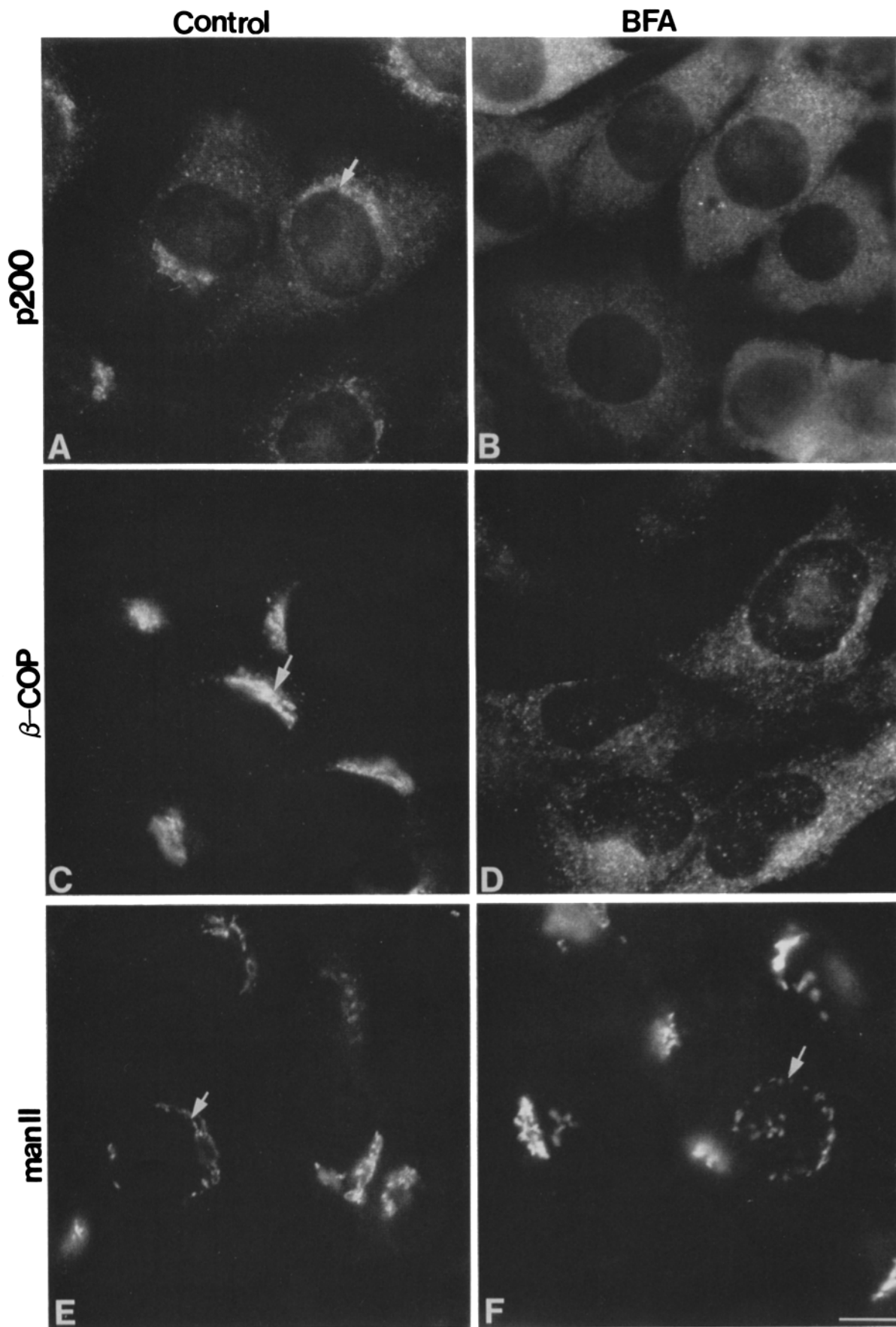
The p200 Protein Is Resistant to BFA in PtK₁ Cells

The kangaroo rat kidney-derived PtK₁ cells have been shown to be resistant to the effects of BFA (21). We therefore examined the response of the p200 protein to BFA treatment in these cells. PtK₁ cells were stained with the AD7 antibody to determine the distribution of p200 in control cells and in cells treated with BFA. Control PtK₁ cells showed a similar AD7 staining pattern to other cells, including the NRK and other cells, with intense perinuclear staining and weaker cytoplasmic staining (Fig. 10 A). After either short (<2 min) or longer (>30 min) treatments of cells with BFA (5 $\mu\text{g}/\text{ml}$) there was no change in the localization of p200 (Fig. 10 B). In the presence of BFA, the amount of perinuclear staining and cytoplasmic staining of AD7 was the same as in untreated cells.

The p200 Protein Is Resistant to BFA in PtK₁ Cells

Discussion

We have identified a peripheral membrane protein which is associated with the Golgi complex, using a mAb raised against canine liver Golgi membrane extracts. Antibody from the AD7 clone recognizes a 200-kD protein (p200) in a variety of cells tested including NRK, LLC-PK₁, PtK₁, and MDCK cells. The p200 protein is found in both soluble and membrane-attached forms in cells, where it is associated with the cytoplasmic face of membranes. Taken together, our data show that p200 is a peripheral membrane phosphoprotein, which also resides in the cytosol, and perhaps exists in dynamic equilibrium between membrane-bound and soluble forms.



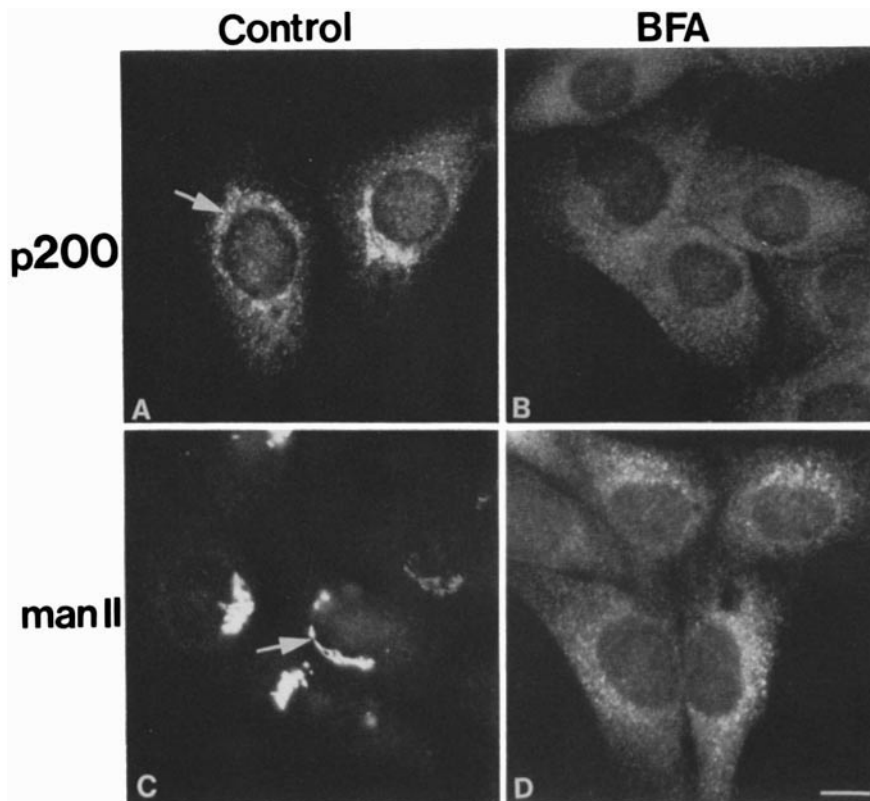


Figure 8. Immunofluorescence staining of NRK cells after longer treatments of BFA. NRK cells treated for 30 min with BFA, or control, untreated cells, were fixed and stained by immunofluorescence. (A and B) Cells stained with the AD7 antibody show typical perinuclear Golgi staining (arrow) (A), which is dispersed after longer treatments of BFA, leaving diffuse punctate staining in the cytoplasm (B). (C and D) Cells stained with the antibody to mannosidase II show typical Golgi complex staining (C). The Golgi structure is disrupted after longer treatments of BFA giving more vesicular staining of mannosidase II (D). Bar, 10 μ m.

Localization of p200 in the juxtannuclear Golgi complex and cytoplasm of cultured cells by immunofluorescence corroborates the detection of p200 in membrane and cytosol fractions by immunoprecipitation and Western blotting. The localization of the p200 on Golgi membranes was confirmed by colocalization with known Golgi markers. The immunofluorescence staining of p200 most closely resembles that of the coat protein, β -COP (9). At the ultrastructural level, β -COP has been previously localized by immunogold staining on Golgi vesicles and on the dilated rims of Golgi cisternae (12). Immunoperoxidase staining showed that, indeed, the p200 is similarly concentrated at the dilated rims of the Golgi cisternae and on vesicles scattered in the Golgi region, suggesting that it is associated with vesicles or profiles of budding membranes, rather than with the bulk of the Golgi cisternae. Thus, the localization of the p200 protein is similar to that of the 110-kD β -COP protein which is part of the large multimeric, "coatomer" complex on Golgi vesicles.

The p200 and β -COP proteins also share a number of biochemical properties that can be summarized as follows: (a)

both proteins are found in soluble and membrane-attached forms; (b) both the p200 (see Fig. 6) and β -COP (Allen, V. J., E. Karsenti, and T. E. Kreis. 1988. *J. Cell Biol.* 107: 759a) are phosphorylated, peripheral membrane proteins; (c) in NRK cells, β -COP and p200 are rapidly dissociated from Golgi in the presence of BFA; and (d) both proteins are resistant to the effects of BFA in PtK₁ cells. Studies by Ktistakis et al. (21), have shown that the PtK₁ cells, of marsupial origin are uniquely resistant to the effects of BFA. Treatment of PtK₁ cells with BFA does not perturb Golgi structure, it does not disrupt the secretion of proteins nor cause redistribution of β -COP (21). Resistance to BFA is believed to be conferred by a nondiffusible factor associated with Golgi membranes in these cells (21). In the present study we have now shown that, as with β -COP, there is no effect of BFA on Golgi-associated p200 in PtK₁ cells, suggesting that the susceptibility or resistance of the Golgi complex to BFA may involve multiple proteins.

The association/dissociation of β -COP on Golgi membranes is also known to be regulated by guanine nucleotides; the uncoating of β -COP and the coatomer from vesicles is

Figure 7. Rapid disruption of p200 staining after short exposure to BFA in NRK cells. NRK cells were treated with BFA for 1 min and then fixed and stained by immunofluorescence. Control cells were fixed without BFA treatment. (A and B) Cells were stained with the AD7 antibody. In control cells (A) the p200 protein is found in the perinuclear Golgi area (arrow) and in the cytoplasm. Immediately after BFA treatment (B) the p200 protein staining disappears from the Golgi area and diffuse cytoplasmic staining throughout the cytoplasm is seen. (C and D) Cells stained with the antibody to the β -COP protein show similar perinuclear Golgi staining (arrow) in control cells (C). This staining also begins to disappear after BFA treatment (D), but some of the β -COP staining remains on the Golgi after this short treatment. (E and F). Mannosidase II is localized in the Golgi complex (arrow) in control cells (E) and after this short treatment of BFA there is no disruption of the mannosidase II staining in the Golgi (arrow) (F). Bar, 10 μ m.

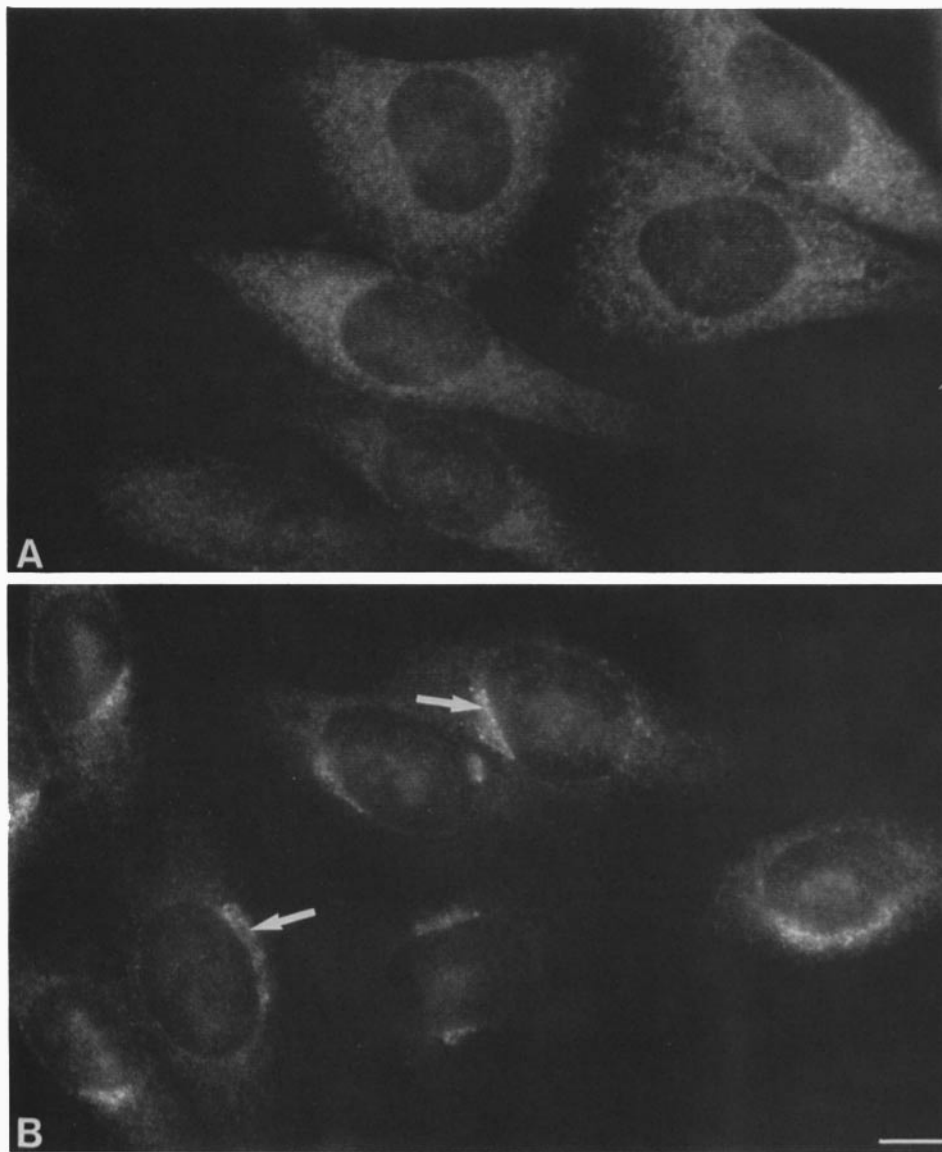


Figure 9. Reversibility of BFA treatment in NRK cells. NRK cells were treated for 30 min with BFA and fixed (*A*) or treated with BFA for 30 min, then washed for 1 h after removal of the BFA (*B*) and fixed. Cells were stained with the AD7 antibody by immunofluorescence. (*A*) In the presence of BFA the AD7 staining is dispersed throughout the cytoplasm. (*B*) After washout of the BFA, the p20 protein reassociates with the Golgi membranes and the perinuclear staining is seen (*arrows*). Bar, 10 μ m.

inhibited in the presence of GTP γ S (28, 31, 36) and, in turn, the effect of BFA on β -COP is blocked by GTP γ S or aluminium fluoride (10). Preliminary studies have shown that the dissociation of p20 from the Golgi in the presence of BFA is also blocked by GTP γ S and aluminium fluoride, suggesting a role for G proteins in its membrane association (J. Doherty and J. L. Stow, manuscript in preparation).

Despite these similarities in localization and behavior, p200 and β -COP are not identical proteins. They are obviously different in size, 110 versus 200 kD, and mAbs to either protein do not cross react. There is a difference in the sensitivity of p200 and β -COP to BFA since the dissociation/association of p200 on the Golgi, upon addition or washout of BFA, occurs more rapidly than β -COP. The p200 protein is distinct, in terms of its molecular weight, from other coat proteins, adaptins, or Golgi membrane proteins that have been reported to date. The 227-kD, SEC 7 protein in yeast is also associated with the Golgi complex and it apparently shares some similarities to p200 since they are both

peripheral membrane phosphoproteins (15). It is possible that the p200 protein in mammalian cells and the SEC 7 protein in yeast may be related, but a direct comparison has not yet been made. Vesicle-associated coat proteins identified to date, include the α -COP (160 kD), β -COP (110 kD), γ -COP (98 kD), and δ -COP (61 kD) proteins on nonclathrin-coated vesicles (39) and the 100–120-kD α , β , and γ adaptins in the adaptor complexes HA-1/API or HA-2/AP2, on clathrin-coated vesicles (20, 34, 35). The above evidence leads us to propose that the p200 protein is possibly a new member of the coat protein family.

It is not known whether p200 resides on the same vesicles as other coat proteins, such as the α , β , γ and δ COPs, or whether it is on a distinct vesicle population. Colocalization of β -COP and p200 on Golgi vesicles has not been done since attempts to localize p200 by immunogold staining have not so far been successful. Immunofluorescence staining and confocal scanning laser imaging of p200 suggests that it is not likely to be on clathrin-coated vesicles in the endocytic

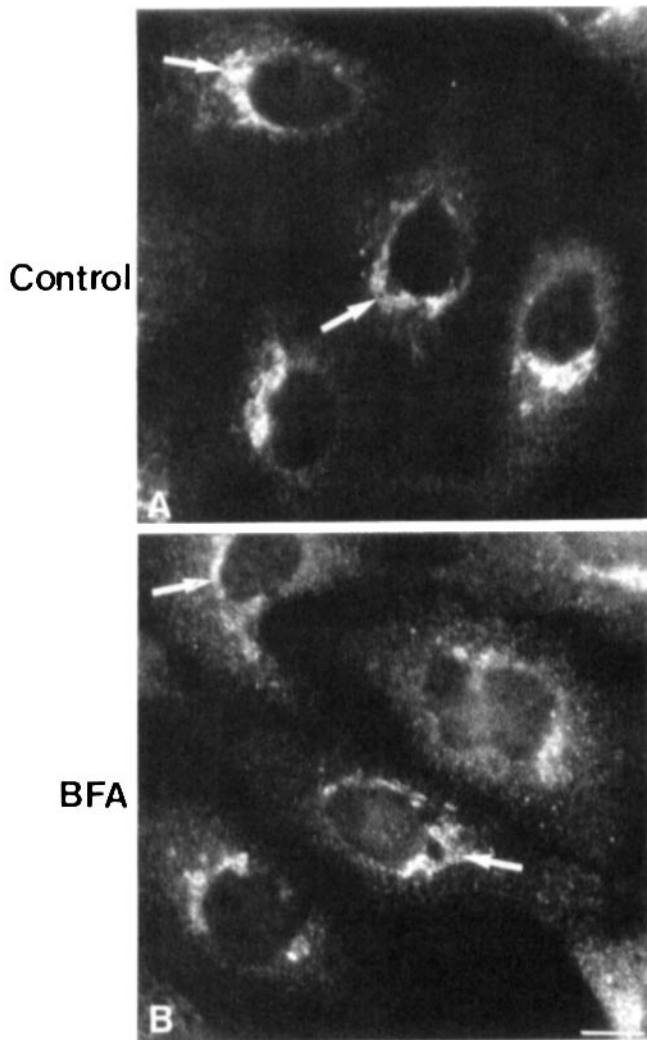


Figure 10. Immunofluorescence staining of PtK₁ cells. PtK₁ cells were fixed and stained with the AD7 antibody by immunofluorescence (A), or cells were treated with BFA for 30 min before fixation and staining (B). (A) There is prominent perinuclear staining with the AD7 antibody in these cells (arrows), in addition faint cytoplasmic staining is visible. (B) After BFA treatment there is no change in the distribution of the p200 as seen by the unchanged perinuclear staining (arrows). Bar, 10 μ m.

pathway, since it is more concentrated in the juxtannuclear region and is not present at the cell surface (data not shown). No immunoperoxidase staining of endocytic vesicles or the plasma membrane was found at the ultrastructural level. However, colocalization of p200 with clathrin or adaptin proteins will be required to completely clarify this point.

Although the function of the p200 protein is not yet known, its properties and behavior, especially with respect to its BFA sensitivity, are provocative. Further insight into the potential role of p200 in vesicle trafficking, and its relationship to other Golgi proteins, will be achieved through a complete molecular description of the protein and through the use of *in vitro* functional assays. Efforts in these directions are currently underway in our laboratories.

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