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Golgi membrane skeleton: identification, localization and oligomerization of a 195 kDa ankyrin isoform associated with the Golgi complex

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SUMMARY

To extend our finding of a Golgi-localized form of the membrane skeleton protein spectrin, we have identified an isoform of ankyrin that associates at steady state with the Golgi complex. Immuno-light and -electron microscopy show that this ankyrin isoform localizes to the perinuclear cytoplasm on tubular vesicular structures that co-stain with Golgi marker proteins. An antiserum raised against erythrocyte ankyrin, which was used to identify the Golgi ankyrin, recognized three prominent polypeptides of 220, 213 and 195 kDa in MDCK cells. Affinity purification of this antiserum against each of these MDCK cell ankyrins revealed that only an antibody specific for the 195 kDa form retained the ability to stain the Golgi complex; affinity purified antibody preparations specific for both the 220 and 213 kDa forms stained punctate and reticular cytoplasmic structures distinct from the Golgi complex. Antibody specific for the 195 kDa ankyrin did not recognize a recently identified 119 kDa ankyrin that is also localized to the Golgi. The 195 kDa Golgi ankyrin binds purified erythrocyte spectrin, and rapidly co-sediments with Golgi β-spectrin during brief, low speed centrifugation of Triton X-100 extracts of MDCK cells. Golgi ankyrin and β-spectrin are retained on tubular vesicular 'Golgi ghosts' following extraction of cultured cells with Triton X-100. Significantly, Golgi ghost tubules containing ankyrin/spectrin are co-linear with individual microtubules, suggesting a role for both Golgi membrane skeleton and microtubules in spatial localization of the Golgi. Golgi ankyrin dissociates from Golgi membranes during mitosis and in cells treated with brefeldin A, indicating that Golgi ankyrin has a dynamic assembly state similar to that of Golgi spectrin and other Golgi membrane coat proteins.

Key words: Membrane skeleton, Golgi, Ankyrin, Spectrin, Membrane protein, Cytoskeleton, Membrane trafficking

INTRODUCTION

Compartmental organization of cellular functions depends on biogenesis and stabilization of structurally and functionally distinct intracellular organelles. Organelle biogenesis is dependent on identification, segregation and targeting of distinct cohorts of proteins; stabilization requires maintenance of membrane structural integrity and retention of compartment-specific proteins in the face of membrane turnover and continuous protein trafficking through the compartment.

The cellular machinery required for these processes is thought to involve cytoplasmic coat proteins. For example, COP protein complexes (COP-I and COP-II) are involved in vesicle transport between different membrane compartments (Kreis and Pepperkok, 1994; Rothman, 1994; Schekman and Orci, 1996). However, little is known about the structural organization of these complexes, and it is not yet clear whether they play roles in regulating the membrane protein composition of transport vesicles. Another candidate machinery is the spectrin-based membrane skeleton (Beck and Nelson, 1996). This ubiquitous, cytoskeletal matrix comprises an extensively cross-linked, oligomeric protein complex localized in close apposition with the cytoplasmic surface of cellular membranes; these properties are the basis for proposed functions of the membrane skeleton in structural support of membranes and formation of membrane protein domains (for review, see Bennett and Gilligan, 1993). Studies of the structural integrity of erythrocytes deficient in membrane skeleton proteins revealed that the membrane skeleton provides structural support to the plasma membrane (Palek and Lambert, 1990), and limits protein diffusion in the plane of the membrane (Sheetz et al., 1980). Studies of polarized epithelial cells and neurons have shown that the membrane skeleton regulates the formation of functionally distinct plasma membrane domains (Lazarides and Nelson, 1983; Nelson and Veshnock, 1987; Morrow et al., 1989; Kordeli et al., 1990). For example, in Madin Darby canine kidney (MDCK) epithelial cells the membrane skeleton: (1) is restricted in its distribution to a distinct cell surface domain (basal-lateral; Nelson and Veshnock, 1987; Morrow et al., 1989); (2) binds classes of membrane proteins that have restricted cell surface distributions in these cells (ion transporters and channels, and cell adhesion molecules; see Nelson and Veshnock, 1987; Morrow et al., 1989; Bennett, 1990); and (3) forms an extensive, submembranous oligomeric protein complex in which protein assembly is regulated temporally and spatially with development of cell surface polarity (Nelson and Veshnock, 1987; Morrow et al., 1989). Thus, the membrane skeleton can be

viewed as a membrane protein sorting machine that collects specific classes of proteins and limits their diffusion in the membrane, thereby creating a functionally and structurally distinct membrane domain (Beck and Nelson, 1996).

Like the plasma membrane, the Golgi complex has a requirement for these functions of the membrane skeleton: structural stability and formation of membrane domains. Recently, we discovered an isoform of β-spectrin that localizes to the Golgi complex (Beck et al., 1994). Immunofluorescence microscopy revealed co-localization of β-spectrin with Golgi marker proteins, although a lack of complete spatial overlap with the Golgi marker mannosidase II indicated a discrete distribution of β-spectrin within the Golgi complex. Golgi spectrin displayed a dynamic association with Golgi membranes: Golgi spectrin dissociated from the Golgi complex during mitosis and following treatment with brefeldin A. The nature of the interaction of β-spectrin with Golgi membranes or other cytoskeletal components was not addressed.

To determine if β-spectrin is part of a Golgi membrane cytoskeletal complex, we sought evidence for a Golgi localized form of ankyrin. Ankyrin plays a critical linkage role in membrane skeleton function at the plasma membrane by binding simultaneously to β-spectrin and the cytoplasmic domains of specific integral membrane proteins (Bennett, 1992). In this report, we provide evidence for Golgi-localized ankyrin by immuno-light and -electron microscopy. Furthermore, we show that Golgi ankyrin and β-spectrin exist as a large oligomeric complex that exhibits a dynamic assembly state on Golgi membranes. Ultrastructural studies reveal that this complex localizes to the *trans*-Golgi network, which is responsible for sorting of classes of membrane proteins to different membrane organelles and the plasma membrane.

MATERIALS AND METHODS

Protein and antibody preparation

Erythrocyte spectrin and ankyrin were isolated from canine whole blood (Pel-Freeze, Rogers, AK) according to the method of Bennett (1983). For production of erythrocyte ankyrin antibody, purified ankyrin (diethylaminoethyl-Sepharose peak 3; Bennett, 1983) was electrophoresed on preparative (12×18×0.3 cm) 12.5% SDS-polyacrylamide gels (Nelson et al., 1983) and electro-eluted (Isco, Lincoln, NE). Rabbits were inoculated with electro-eluted ankyrin emulsified in Freunds adjuvant (Sigma, St Louis, MO). Serum obtained from immunized rabbits was treated with 35% saturated ammonium sulfate, and the precipitated immunoglobulin fraction was resuspended in Dulbecco's-phosphate buffered saline containing 1 mM sodium azide (PBS-azide) and stored at –70°C. Affinity purified anti-canine erythrocyte β-spectrin antibody (βSpec-1) was prepared as described previously (Beck et al., 1994). A mouse monoclonal antibody to mannosidase II (53FC3) was from BabCo (Richmond, CA), and monoclonal antibody to β-tubulin was from Amersham (Arlington Heights, IL). Polyclonal anti-TGN 38/41 was generously provided by Dr Kathryn Howell (University of Colorado School of Medicine, Denver, CO). Mouse monoclonal antibody to clathrin heavy chain was kindly provided by Dr Frances Brodsky (University of California, San Francisco, CA). Fluorescein- and rhodamine-labeled goat anti-rabbit IgG, and peroxidase anti-peroxidase soluble immune complexes were from Jackson Laboratories (West Grove, PA).

Cell culture and immunofluorescence

Madin Darby canine kidney cells (MDCK, strain J; Mays et al., 1995), human embryonic kidney cells (293) and normal rat kidney cells (NRK) were grown in high glucose DMEM containing 10% fetal bovine serum, 100 i.u./ml penicillin and 100 mg/ml streptomycin (Gibco-BRL, Gaithersburg, MD). Cultures were maintained at 37°C with 5% CO_2 in air. For experiments with brefeldin A (Epicentre Technologies, Madison, WI), NRK cells were incubated for various times with medium containing 5 μM brefeldin A at 37°C. For brefeldin A wash-out experiments, brefeldin A-treated cells were washed with PBS (37°C) and further incubated in brefeldin A-free medium. Treatments with nocodazole were performed by incubating MDCK cells in culture medium containing 33 μM nocodazole (Sigma, St Louis, MO) for 60 minutes at 4°C. Cells were then washed briefly with fresh medium (37°C), and then incubated for 60 minutes with medium containing 33 μM nocodazole at 37°C. Cells were treated with 50 μM colchicine in culture medium for 60 minutes at 4°C.

For immunofluorescence experiments, cells were plated for 12-15 hours on collagen-coated glass coverslips. Fixation was performed either with methanol (–20°C, 5 minutes; Figs 2 and 5), or 2% formaldehyde in PBS (22°C, 10 minutes; Figs 1, 3, 4, 6 and 7). Formaldehyde fixed cells were permeablized with 0.5% Triton X-100 in PBS for 10 minutes. For pre-fixation extraction experiments, cells were washed 3 times in PBS, followed by two brief washes in extraction medium without 0.5% Triton X-100. Washed cells were then incubated in MEMA buffer (100 mM sodium MES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM sodium azide) containing 0.5% Triton X-100 for 10 minutes at 22°C. A freshly prepared cocktail of protease inhibitors containing 22 μg/ml aprotinin, 20 μg/ml pepstatin A, 100 μM phenlymethylsulfonyl fluoride and 10 μM leupeptin (all from Sigma, St Louis, MO) was included in the incubation. Alternative extraction buffers are indicated in the figure legends. After extractions, cells were fixed for 10 minutes with 2% formaldehyde diluted in extraction buffer without Triton X-100 (see Fig. 4), or with –20°C methanol for 5 minutes (see Fig. 5). Fixed and permeabilized cells were blocked, and then incubated with primary and secondary antibodies as described previously (Beck et al., 1994). Labeling of cells with C_5-DMB-ceramide (Bodipy-ceramide, Molecular Probes, Eugene, OR) was performed as described (Lipsky and Pagano, 1985). Cells were observed by epifluorescence using a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY), and photographed with Kodak Ektachrome 400 HC color slide film (Eastman Kodak, Rochester, NY). Alternatively, some specimens were viewed with a Molecular Dynamics laser scanning confocal microscope (Cell Sciences Imaging Facility, Stanford University, Stanford, CA).

Electron microscopy

Immuno-electron microscopy studies were performed on NRK cells grown on collagen coated coverslips. Cells were fixed in a solution of 1% paraformaldehyde, 100 mM lysine, and 10 mM sodium *m*-periodate in PBS (McLean and Nakane, 1974). Fixed cells were then cryoprotected by successive incubations at 4°C for 10 minutes each in 10, 20 and 30% sucrose (w/v) in PBS. Cells were then freeze-thaw permeabilized by quickly dipping the coverslips in liquid nitrogen. After two rounds of freeze-thaw, cells were blocked as described above and incubated with primary antibody for 2 hours at 22°C, followed by incubation with peroxidase coupled secondary antibody (PAP; Sternberger, 1986). Cells were then fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 10 minutes, rinsed with 0.1 M cacodylate buffer with 0.14 M sucrose, and peroxidase precipitate was developed as described (Pickel et al., 1975). Following post-fixation (McDonald, 1984), the coverslips were embedded and further processed as described previously (Buchanan et al., 1989). Silver sections were cut with a Reichert Ultracut E and examined unstained at 60 kV with a Phillips 410 electron microscope.

Cell fractionation, membrane preparation and immunoblotting

For cell extracts, 150 cm^2 culture plates of confluent MDCK cells

were washed 3 times with PBS at 4°C followed by two brief washes in extraction medium without Triton X-100. The washed monolayers were then scraped with a rubber policeman in 2 ml of either PBS or MEMA extraction buffer containing 0.5% Triton X-100, incubated for 20 minutes at 4°C, and then centrifuged for 15 minutes at 10,000 ***g***. A freshly prepared cocktail of protease inhibitors containing 22 μg/ml aprotinin, 20 μg/ml pepstatin A, 100 μM phenlymethylsulfonyl fluoride and 10 μM leupeptin (all from Sigma, St Louis, MO) was included in the extraction buffer. In some cases, the extract was sonicated with a Branson Sonifier 250 (three 1 second pulses on setting # 3) before centrifugation. Velocity centrifugation was performed by loading 250 μl of MDCK cell extract on 4 ml 5-20% linear sucrose gradients in PBS. Gradients were centrifuged for 5 hours in a Beckman Sw60 rotor (Beckman Instruments, Palo Alto, CA) at 60,000 rpm, and fractionated using a peristaltic pump (265 μl/fraction). Pelleted material was resuspended in 1% SDS preheated to 70°C. Gradient fractions were electrophoresed on 5% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore).

Affinity purification of ankyrin antibodies

Ankyrin antisera were affinity purified from strips of Immobilon-P membranes derived from electrophoretic transfers of MDCK cell extracts using a modification of the procedure of Olmsted (1981). A 0.5% TX-100 extract derived from a single 150 cm^2 plate of MDCK cells was prepared as described above, diluted in SDS-sample buffer, loaded into a single 10 cm well and subjected to electrophoresis on a 13×10 cm 5% SDS-polyacrylamide gel followed by electrophoretic transfer to Immobilon-P. The edges of the Immobilon P membrane were excised and stained with the erythroid ankyrin specific antiserum (EAnk-2) to identify the positions of the top (220 kDa), middle (213 kDa) and bottom (195 kDa) immunoreactive ankyrin isoforms. The edges were then re-aligned with the remaining portion of the Immobilon-P membrane and strips of membrane corresponding to each of the three ankyrin bands were excised and incubated for 15 hours at 4°C with 1 ml of EAnk-2 diluted 1:13 in 100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20 (TTBS). The strips were then subjected to six 10 minute washes in 2 ml of TTBS and bound antibody was stripped from the membrane by incubation for 2 minutes with 1 ml 0.2 M glycine-HCl, pH 2.8, 0.02% gelatin, followed by a second 2 minute incubation with 100 mM triethylamine pH 11.0 (Kodak, Rochester, NY). The acid and base eluates were pooled and dialyzed at 4°C for 15 hours against phosphate buffered saline containing 1 mM sodium azide. The dialyzed antibody preparations were then concentrated by ultrafiltration with Centriprep-30 concentrators (Amicon, Beverly, MA).

RESULTS

A Golgi-localized isoform of ankyrin

Since Golgi β-spectrin was identified with an antibody raised to canine erythroid β-spectrin, and pure erythroid β-spectrin localized to the Golgi when microinjected into cells (Beck et al., 1994), we sought to identify a Golgi-localized ankyrin using an antiserum (EAnk-2) raised against electrophoretically pure canine erythrocyte ankyrin (see Materials and Methods). The antibody reacted strongly with purified erythrocyte ankyrin (Fig. 1A, lane a), but did not react with purified erythrocyte spectrin (Fig. 1A, lane b). Likewise, an antibody to erythrocyte β-spectrin did not recognize ankyrin (Fig. 1A, lane c), but reacted with purified spectrin (Fig. 1A, lane d). Immunoblots of whole MDCK cell extracts with EAnk-2 identified three prominent immuno-reactive polypeptides of 220, 213 and 195 kDa (Fig. 1B).

To determine whether these three immunoreactive polypeptides were indeed ankyrins we incubated an MDCK cell extract with purified erythrocyte spectrin immobilized on Sepharose beads and examined bound material by immunoblotting with the EAnk-2 antiserum (Fig. 1C). All three major ~200 kDa ankyrins, and a protein of ~120 kDa bound spectrin-Sepharose (Fig. 1C, lane a). Ponceau S staining of the immunoblot filters revealed that the majority of cellular proteins did not bind immobilized spectrin (not shown). Binding of MDCK cell ankyrins to spectrin was reduced by 1 M salt (Fig. 1C, lane b), consistent with properties of other known ankyrin/spectrin interactions (Bennett, 1983; Nelson et al., 1983).

We compared the sedimentation properties of these ankyrin isoforms recognized by EAnk-2 with that of the plasma membrane (fodrin) skeleton. While the majority of EAnk-2 reactive ankyrins are detergent insoluble, a small amount of material (<10%) can be solubilized in 0.5% Triton X-100 and analyzed by sucrose gradient centrifugation. MDCK cell extracts were separated in 5-20% sucrose gradients as described previously (Nelson and Hammerton, 1989) for plasma membrane-associated ankyrin and spectrin (fodrin). EAnk-2 recognized three high molecular mass proteins (195-220 kDa) that co-sedimented in fractions 6-9 (Fig. 1D, ankyrin), although each of the ankyrins appear to have slightly different sedimentation properties; the peak of the 220 kDa form was in fraction 8, the 213 kDa form was in fractions 8 and 9, and the 195 kDa form was evenly distributed in fractions 7, 8 and 9. The sedimentation profile of EAnk-2 reactive proteins was compared to that of plasma membrane fodrin, which sedimented with a distinctly different profile in fractions 8-12 (Fig. 1D, fodrin). Note, we have extensively characterized the sedimentation profiles of the plasma membrane skeleton of ankyrin-fodrin-Na,K-ATPase, and showed that these proteins co-sediment in the same fractions shown here for fodrin (Nelson and Hammerton, 1989). These results demonstrate that EAnk-2 reactive ankyrin isoforms have sedimentation properties distinctly different from that of the plasma membrane-associated membrane skeleton.

The subcellular distribution of ankyrin isoforms detected with EAnk-2 was examined by indirect immunofluorescence microscopy of cultured NRK cells. EAnk-2 antiserum reacted with perinuclear, reticular elements morphologically reminiscent of the Golgi complex (Fig. 1E, a); specific plasma membrane staining was not observed. Double labeling revealed that these perinuclear structures stained with an antibody specific for the Golgi enzyme, mannosidase II (Fig. 1b). Specific Golgi staining was not observed with pre-immune antisera (data not shown), nor with antibodies specific for non-erythroid ankyrin isoforms (Ank-2 and Ank-3, data not shown), which stain the plasma membrane (Nelson and Veshnock, 1986; Morrow et al., 1989). Golgi localization of the erythroid ankyrin homolog was also observed in MDCK cells and human embryonic kidney cells 293 (data not shown).

In order to determine which of the three ankyrins recognized by the EAnk-2 antiserum is localized to the Golgi we affinity purified the antiserum against the 220, 213 and 195 kDa MDCK cell ankyrins using immunoblots of MDCK cell extracts (see Materials and Methods). An antibody affinity purified against the 220 kDa isoform stained both the 220 kDa and the 213 kDa MDCK cell ankyrins (Fig. 2A, lane 2), whereas an antibody affinity purified against the 213 kDa form

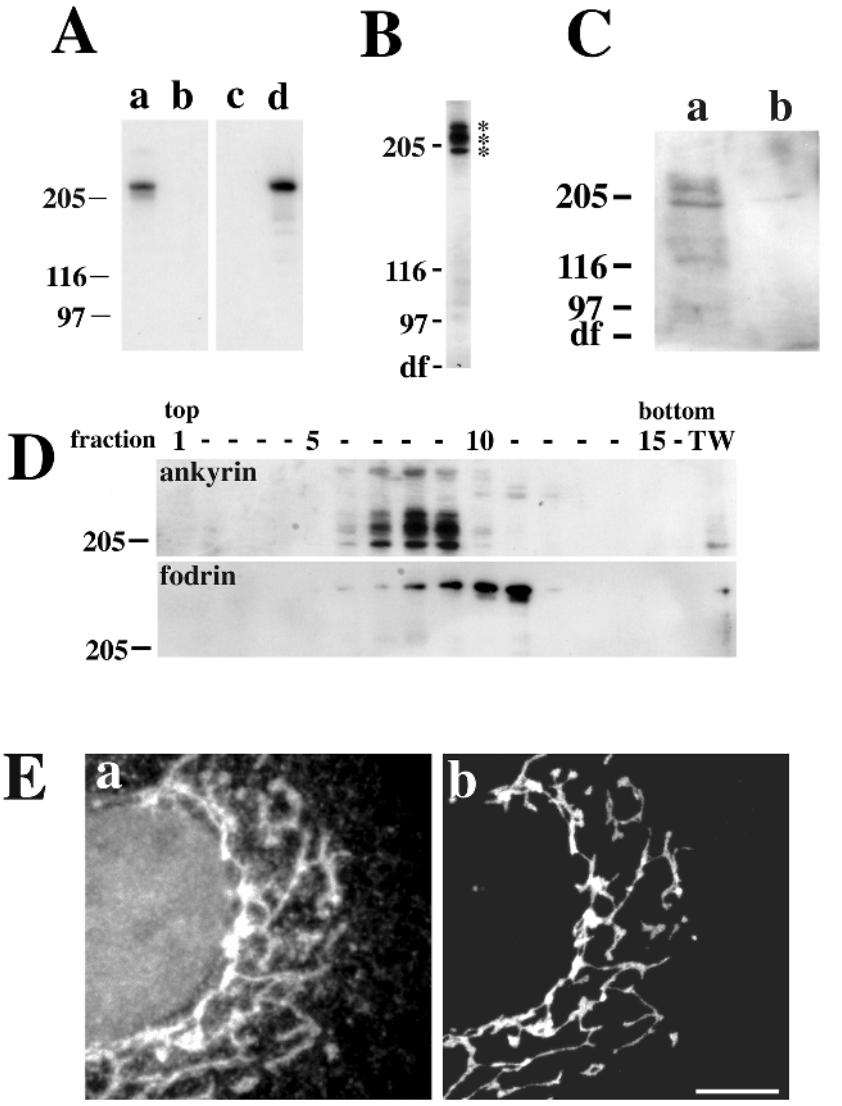


Fig. 1. Erythroid ankyrin-specific antiserum identifies a Golgi-localized ankyrin. (A) Purified canine erythrocyte ankyrin (lanes a and c) and β-spectrin (lanes b and d) were subjected to electrophoresis in a 5% SDS-polyacrylamide gel, transferred to nitrocellulose and blotted with antisera specific for canine erythrocyte ankyrin (EAnk-2, a and b) or erythroid β-spectrin (βspec-1, c and d). Migrations of molecular mass standard proteins are indicated on the left of the panel. (B) An immunoblot of a Triton X-100 extract of MDCK cells was probed with EAnk-2. Three prominent polypeptides of 220 kDa, 213 kDa, and 195 kDa were identified in the MDCK cell extract (asterisks). (C) A 0.5% Triton X-100 extract of MDCK cells was sonicated briefly and incubated with canine erythrocyte spectrin-Sepharose (see Materials and Methods). Spectrin-Sepharose was then washed extensively with PBS followed by an additional 3 washes with either PBS (a) or PBS with 1 M NaCl (b). Bound proteins were solubilized with 1% SDS and processed for immunoblotting with EAnk-2. (D) A 0.5% Triton X-100 extract of MDCK cells was sonicated briefly and subjected to velocity gradient centrifugation (see Materials and Methods). Gradient fractions were immunoblotted with antibodies specific for erythroid ankyrin (ankyrin) and non-erythroid spectrin (fodrin). Fraction numbers arranged from top (left) to bottom (right) are indicated at the top of the figure. TW, pelleted material. (E) NRK cells were fixed in 0.5% formaldehyde, permeabilized with buffer containing 0.5% Triton X-100 and double stained with EAnk-2 (rabbit polyclonal, a), and an antibody specific for the medial Golgi marker mannosidase II (mouse monoclonal, b). Both antisera stained identical reticular perinuclear structures. Bar, 5 μm.

stained all three ankyrin species (Fig. 2A, lane 3). Significantly, antibody affinity purified against the 195 kDa ankyrin reacted with only the 195 kDa isoform (Fig. 2A, lane 4). Thus, the 195 kDa ankyrin is not a proteolytic product of either the 220 or 213 kDa forms. All three of these affinity purified antibody preparations reacted with comparable affinity to erythrocyte ankyrin (Fig. 2B, lanes 2-4), confirming that all three affinity purified antibodies recognize ankyrin homologs. Note that we did not detect any staining of the previously reported 119 kDa Golgi localized ankyrin (Devarajan et al., 1996) with the 195 kDa ankyrin specific antiserum (Fig. 2A, lane 4). The three antibody preparations differed in their ability to stain the Golgi complex by indirect immunofluorescence (Fig. 2C). Only the antibody preparation specific for the 195 kDa ankyrin gave strong staining of the Golgi complex (Fig. 2C, g and h). Affinity purified antibody against the 213 kDa form which recognized all three polypeptides stained the Golgi complex with an intensity (Fig. 2C, e and f) similar to that of the unfractionated antibody (Fig. 2C, a and b). Antibody that recognized both the 220 and 213 kDa ankyrins showed staining of punctate and reticular cytoplasmic structures and little or no Golgi staining (Fig. 2C, c and d). These results demonstrate that ankyrin antibody staining of the Golgi complex correlates with specific antibody recognition of the 195 kDa ankyrin form.

We next sought to determine the subcellular localization of ankyrin at the ultrastructural level by immunoperoxidase staining and electron microscopy (Fig. 3). An ankyrin antibody-specific peroxidase precipitate was observed on membranes in the perinuclear cytoplasm by both light and electron microscopy (Fig. 3, a and c); peroxidase precipitate was not observed with a pre-immune serum (Fig. 3, b and d). At the electron microscope level, staining was observed on morphologically identifiable Golgi cisternae (Fig. 3c); staining of the plasma membrane, mitochondria and endoplasmic reticulum was not observed.

We observed two structures of the Golgi complex that were stained by the ankyrin antibody. First, numerous, vesicular structures localized on one side of Golgi stacks, often distal to the nucleus, were stained (Fig. 3e; arrows). These vesicles appeared morphologically distinct from clathrin coated vesicles which were devoid of peroxidase reaction product (Fig. 3e, cv). Further evidence for unique distributions of ankyrin/spectrin containing vesicles and clathrin coated vesicles was obtained by double immunofluorescence

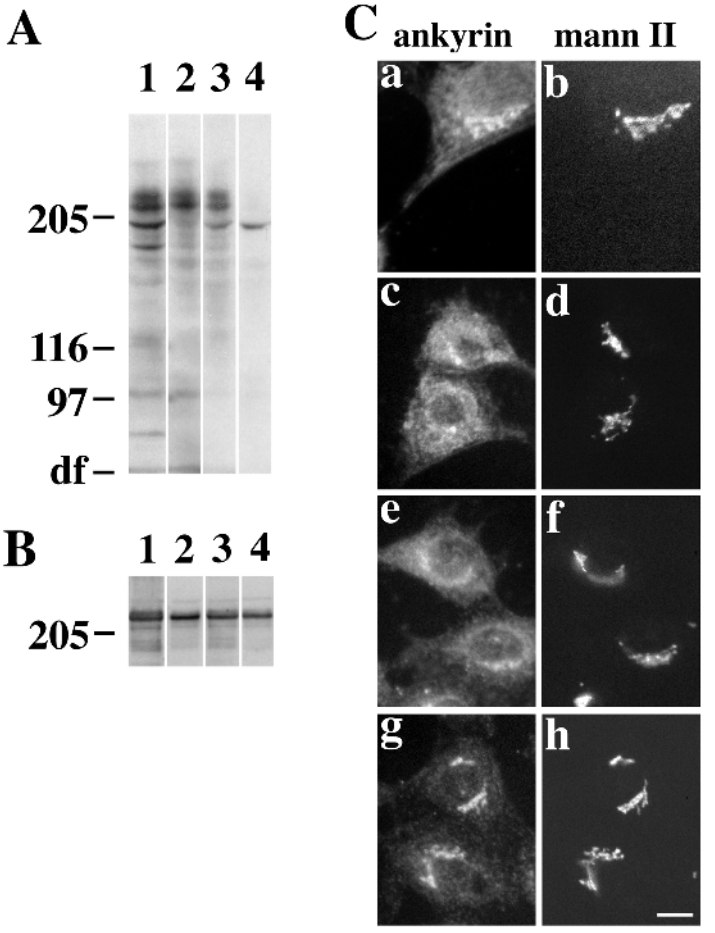


Fig. 2. Golgi localization is unique to the 195 kDa ankyrin isoform. (A) An extract of MDCK cells was subjected to immunoblot analysis with either the unfractionated EAnk-2 antiserum (lane 1) or antibody preparations affinity purified against the 220 kDa (lane 2), 213 kDa (lane 3) or 195 kDa (lane 4) MDCK cell ankyrins (see Materials and Methods for affinity purification procedures). df, dye front. (B) Extracts of canine erythrocytes were immunoblotted with the same antibody preparations as described for A: lane 1, unfractionated antibody; lane 2, affinity purified with 220 kDa ankyrin; lane 3, affinity purified with 213 kDa ankyrin; lane 4, affinity purified with 195 kDa ankyrin. (C) NRK cells were double stained by indirect immunofluorescence with an anti-mannosidase II antibody (b,d,f,h) and with ankyrin antibody preparations: (a) unfractionated antibody; (c) affinity purified with 220 kDa ankyrin; (e) affinity purified with 213 kDa ankyrin; (f) affinity purified with 195 kDa ankyrin. Bar, 10 μm.

microscopy. Fig. 3, f and g shows confocal sections of NRK cells double stained with Golgi ankyrin and clathrin antibodies. The patterns of staining of these two proteins are different and there appears to be little or no co-localization except in the perinuclear region of cells fixed prior to extraction (Fig. 3f); the later staining is probably due to the superimposition of membranes from different optical planes. A lack of co-localization was particularly evident in cells that had been extracted prior to fixation (Fig. 3g). Similar results were obtained with NRK cells double stained with antibodies to Golgi β-spectrin and the γ-subunit of the Golgi-specific clathrin associated protein AP-1 (data not shown). We also observed specific ankyrin staining of fenestrated tubular structures associated with cisternae that were peeling off Golgi stacks (Fig. 3h).

Assembly state of Golgi ankyrin

Following brief low speed centrifugation (10,000 ***g***, 10 minutes) of 0.5% Triton X-100 extracts of MDCK cells, greater than 90% of the EAnk-2 reactive ankyrins, together with a majority (>90%) of Golgi β-spectrin, are found in the pellet fraction (data not shown). While this result does not definitively demonstrate an interaction of Golgi ankyrin with Golgi spectrin, it does indicate that both molecules are constituents of a higher order detergent insoluble protein complex. Whether such a complex of spectrin and ankyrin exists at the level of the Golgi apparatus was determined by examining β-spectrin and ankyrin localization in detergent extracted NRK cells. NRK cells were extracted for 10 minutes in buffers containing 0.5% Triton X-100 prior to fixation and staining with Golgi ankyrin and β-spectrin specific antisera (Fig. 4). To evaluate the efficiency of the extraction conditions, we examined the extractability of bodipy-ceramide that was incorporated into glycosphingolipids and sphingomyelin in the *trans*-Golgi (Fig. 4a and b). Treatment with Triton X-100 was sufficient to extract all bodipy-ceramide (Fig. 4b). These extraction conditions also gave rise to efficient extraction of TGN 38/41, an integral membrane protein of the TGN (compare Fig. 4c and e). Despite efficient extraction of proteins and lipids under these conditions, ankyrin- and β-spectrin-specific antisera reacted with Triton X-100 insoluble, perinuclear, tubulo-reticular structures (Fig. 4i and m, respectively) that appeared morphologically similar to the Golgi complex prior to extraction (Fig. 4g and k, respectively). Importantly, these structures co-stained with mannosidase II antibody (Fig. 4j and n). We refer to these structures as 'Golgi ghosts.' The localization of ankyrin and spectrin to detergent insoluble Golgi ghosts indicates that both are constituents of a higher order oligomeric protein complex associated with the Golgi apparatus.

Association of spectrin/ankyrin Golgi ghosts with a stable population of microtubules

Cytoplasmic retention of cellular structures following

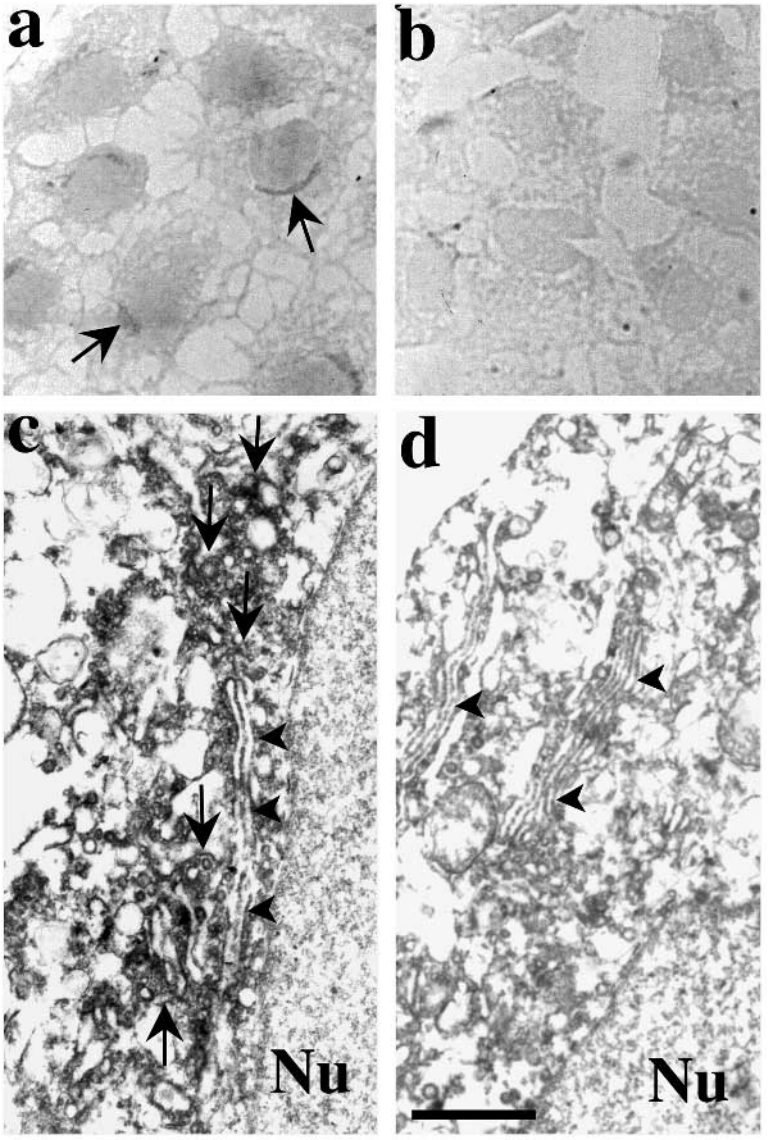
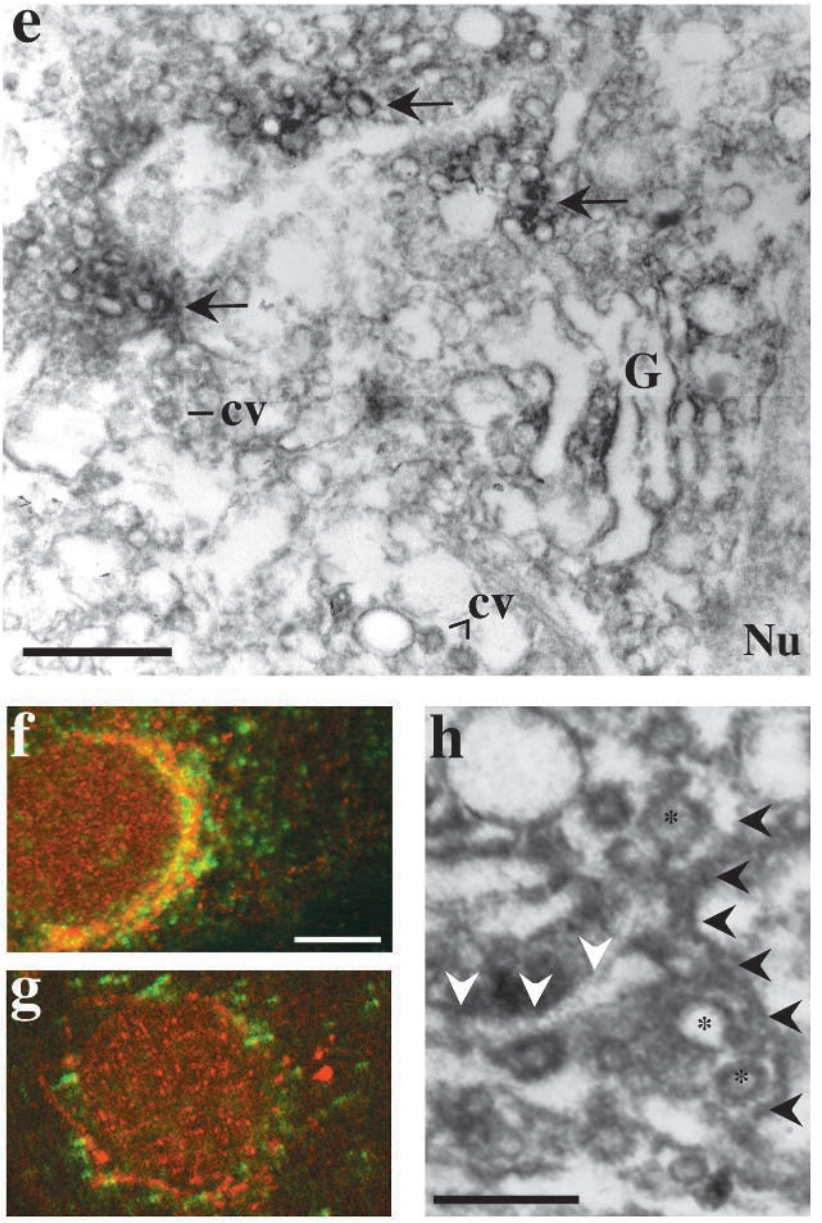


Fig. 3. Ultrastructural localization of ankyrin within the Golgi complex. NRK cells were immunostained either with the EAnk-2 antiserum (a,c,e,h) or with a pre-immune serum (b and d), and visualized by phase contrast microscopy (a and b) or transmission electron microscopy (c,d,e,h). At the level of the light microscope, peroxidase precipitate was observed associated with perinuclear Golgi complexes (arrows, a). Low magnification electron micrographs revealed an accumulation of peroxidase reaction product (arrows, c) in the proximity of Golgi stacks (arrowheads, c). A precipitate was not observed associated with the Golgi in cells treated with the pre-immune serum (b; arrowheads, d). Nu, nucleus. Bar, 1 μm. Higher magnification images revealed staining of vesicular structures (50-80 nm, e, arrows) that were distal to the Golgi complex (G), and fenestrated tubular structures (h, black arrowheads, asterisks indicate holes in the tubular network) associated with Golgi cisternae (h, white arrowheads). Coated vesicles were not stained (e, cv). Bars, 500 nm. (f and g) NRK cells were either fixed immediately (f) or extracted in MEMA buffer (see Materials and Methods and Fig. 4) containing 0.5% Triton X-100 (g) before fixation, and double staining with antibodies to erythroid ankyrin (with rhodamine-conjugated secondary) and clathrin (with fluorescein-conjugated secondary). Staining was visualized by laser scanning confocal microscopy. Double exposure images reveal incomplete overlap between Golgi ankyrin and clathrin. Bar, 5 μm.

detergent extraction implies an association with cytoskeletal elements. Microtubules are good candidates for anchoring the ankyrin/spectrin Golgi ghost; subcellular localization and structural organization of the Golgi complex are dependent on microtubules (for review see Kreis and Pepperkok, 1994; Rothman, 1994; Schekman and Orci, 1996), and ankyrin binds with high affinity to tubulin (Bennett and Davis, 1981). Under specific extraction conditions (100 mM NaMES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.5% Triton X-100), abundant stable microtubules were retained within cells, which could serve to anchor ankyrin/spectrin Golgi ghosts (Fig. 5b). Treatment with microtubule destabilizing agents also revealed an association of Golgi ghosts with a sub-population of microtubules (Fig. 5e and h). Incubation with either colchicine or nocodazole, combined with low temperature, disrupted the majority of cytoplasmic microtubules, but left behind small numbers of drug- and temperature-stable microtubules (Fig. 5e and h). An antibody specific for glu-tubulin (Gurland and Gundersen, 1995) failed to react with this stable population of microtubules (data not shown), indicating that they had not been detyrosinated, a secondary modification that correlates with stability of a subset of microtubules (Schulze et al., 1987).

Golgi ghosts were retained within microtubule-disrupted and detergent extracted cells (Fig. 5d and g). Close inspection of colchicine-treated NRK cells double stained with antibodies to erythroid ankyrin and β-tubulin revealed that individual tubulo-reticular elements of Golgi ghost structures are precisely juxtaposed and often co-linear with individual microtubules (Fig. 5d-f, arrowheads). In a few cases, short segments of ankyrin-stained Golgi ghosts meandered away from associated microtubules (Fig. 5d-f, arrows), indicating Golgi ghosts are free standing cytoskeletal structures and that microtubules are required for spatial localization of the Golgi rather than stability. Treatment of NRK cells with nocodazole also resulted in retention of Golgi ghosts although these structures were often condensed within the perinuclear cytoplasm (Fig. 5g-i). In the presence of nocodazole, microtubule organization was more severely perturbed that with colchicine. However, short microtubule fragments were often found associated with



discrete punctate sources that also co-stained with Golgi ankyrin antibody (Fig. 5g).

Dynamics of Golgi ankyrin

To assess the potential for a dynamic association of ankyrin with Golgi membranes, we examined the distribution of ankyrin in cells treated with the fungal metabolite brefeldin A. Brefeldin A affects the assembly dynamics of a variety of peripheral Golgi coat proteins including Golgi coatomer complexes COP I (Orci et al., 1991); clathrin (Wong and Brodsky, 1992); p200 (Narula et al., 1992); and Golgi β-spectrin (Beck et al., 1994). Both the structure and function of the Golgi complex are greatly perturbed in the presence of brefeldin A, which causes a complete blockage of anterograde protein transport, as well as tubularization and dissolution of the Golgi and TGN (Klausner et al., 1992). Following 10 minutes treatment with brefeldin A, we observed a loss of co-localization with Golgi membrane markers (Fig. 6c and d). Instead, Golgi ankyrin was diffusely distributed throughout the cytoplasm, consistent with a net dissociation of ankyrin from Golgi membranes. This effect was reversible upon washout of brefeldin A (Fig. 6e).

We also examined ankyrin distribution during mitosis when extensive fragmentation of Golgi membranes accompanies a complete loss of Golgi function. Mitotic cells stained with the EAnk-2 antibody characteristically displayed elevated cytoplasmic staining compared with that in adjacent interphase cells (Fig. 7a and b). Co-localization of ankyrin with Golgi fragments was not observed in mitotic cells (not shown), indicating that ankyrin redistributes to the cytoplasm during mitosis. Often, we found evidence for ankyrin localization to punctate structures associated with mitotic spindles (Fig. 7c and d), which has been reported previously for ankyrin (Bennett and Davis, 1981) as well as for β-spectrin (Beck et al., 1994).

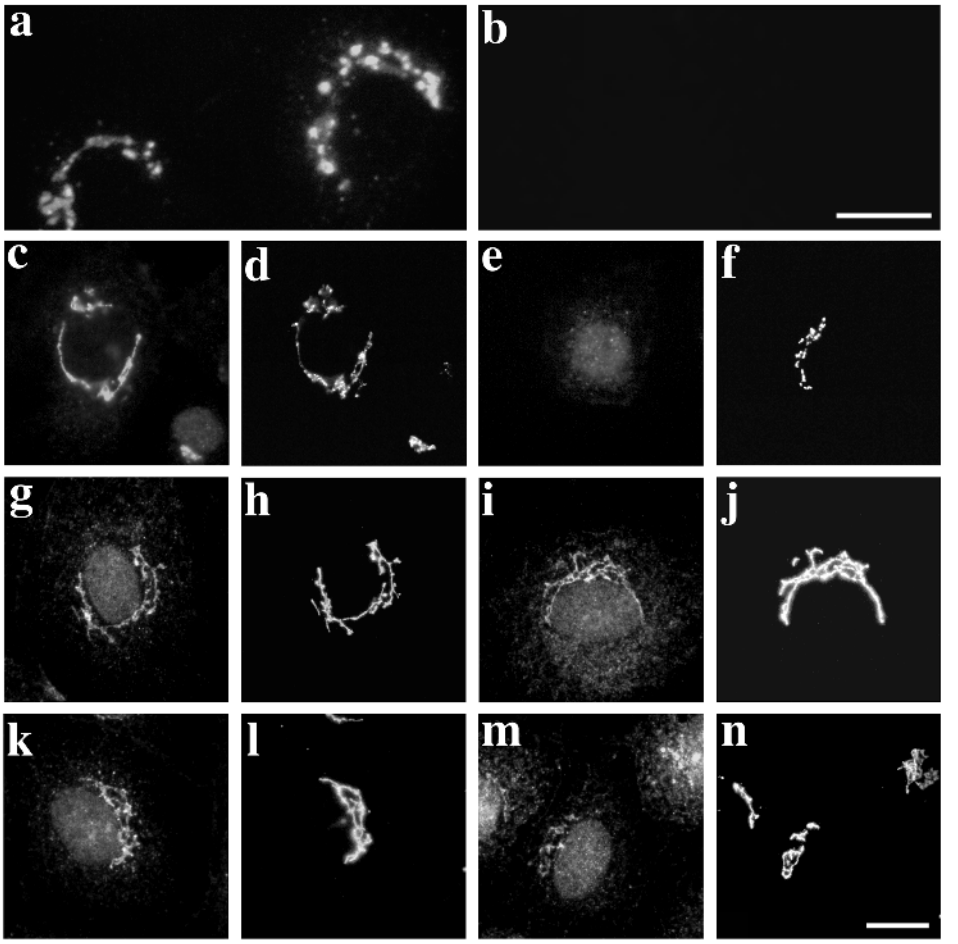


Fig. 4. Localization of ankyrin and β-spectrin to a detergent insoluble Golgi ghost. NRK cells were extracted in MEMA buffer containing 0.5% Triton X-100 (0.1 M NaMES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM sodium azide) either before (b,e,f,i,j,m,n) or after (a,c,d,g,h,k,l) fixation with 2% formaldehyde and processed for immunofluorescence microscopy. Fixed cells were incubated with Bodipy-ceramide (a and b), or double stained with a mouse monoclonal antibody to mannosidase II (d,h,l,f,j,n), and either anti-TGN 38/41 (c and e), or anti-ankyrin (g and i), or anti-β-spectrin (k and m) rabbit polyclonal antisera. Cells were stained with rhodamine anti-rabbit or fluorescein anti-mouse secondary antibodies and visualized by epifluorescence microscopy. Bars, 10 μm.

DISCUSSION

Ankyrin links plasma membrane proteins to the underlying spectrin/actin cytoskeleton in erythrocytes, epithelial cells and neurons, and these interactions contribute to membrane structural integrity and formation of membrane domains (Nelson and Veshnock, 1987; Morrow et al., 1989; Nelson and Hammerton, 1989; Davis and Bennett, 1990; Bennett, 1992; Davis and Bennett, 1994). In this report, we provide evidence demonstrating that an isoform of ankyrin, together with β-spectrin, localizes to the Golgi. Ankyrin localization to the Golgi was observed at both the light (Fig. 1D) and electron (Fig. 3) microscope levels using an antibody raised against canine erythrocyte ankyrin. This localization is in contrast to that of non-erythroid ankyrins which show a predominant plasma membrane localization (Nelson and Veshnock, 1986; Morrow et al., 1989). The EAnk-2 antibody reacted with polypeptides in MDCK cells and purified Golgi membranes with high molecular masses (220, 213 and 195 kDa) similar to that of canine erythrocyte ankyrin (210 kDa, Fig. 1B). All three of these immunoreactive polypeptides bound to purified erythrocyte spectrin in a salt dependent manner (Fig. 1C), consistent with their identity as ankyrins. Furthermore, the sedimentation profile of Golgi ankyrins was distinct from that of the plasma membrane ankyrin-fodrin complexes (Fig. 1D). Upon affinity purification of the erythroid ankyrin antiserum against these three ankyrins, only an antibody specific for the 195 kDa isoform stained the Golgi (Fig. 2C, g and h). In contrast, an antiserum that recognized both the 220 and 213 kDa ankyrins gave little or no detectable Golgi staining, but rather stained cytoplasmic punctate and reticular structures. The identity of these latter structures is not known at this time, but the staining pattern raises the possibility that additional ankyrin isoforms are localized to other internal membrane compartments.

During the course of this work, Devarajan et al. (1996) found a cDNA encoding a 119 kDa ankyrin homolog, and subsequently found that antibodies to the encoded protein reacted with the Golgi. Immunoblots with our ankyrin antiserum reveal cross-reaction with an approximately 119 kDa polypeptide (Fig. 2A) and staining of Golgi and cytoplasmic reticular structures. However, affinity purification of the antiserum against the 195 kDa ankyrin resulted in strong reactivity with only the 195 kDa form, and no cross-reactivity with a 119 kDa protein. Significantly, the 195 kDa ankyrin affinity purified antibody stained Golgi strongly. Thus Golgi staining observed with our erythroid ankyrin antibody does not correlate with cross-reactivity of the antibody with the 119 kDa ankyrin. We also note that the 119 and 195 kDa ankyrins differ in their solubility in buffers containing 0.5% Triton X-100; the 195 kDa ankyrins are largely insoluble, whereas the 119 kDa ankyrin is fully solubilized (Devarajan et al., 1996). In addition, Golgi localization of 119 kDa ankyrin was prominent in subconfluent cells, but was punctate throughout the cytoplasm in confluent cells, even though the Golgi remains localized to the perinuclear

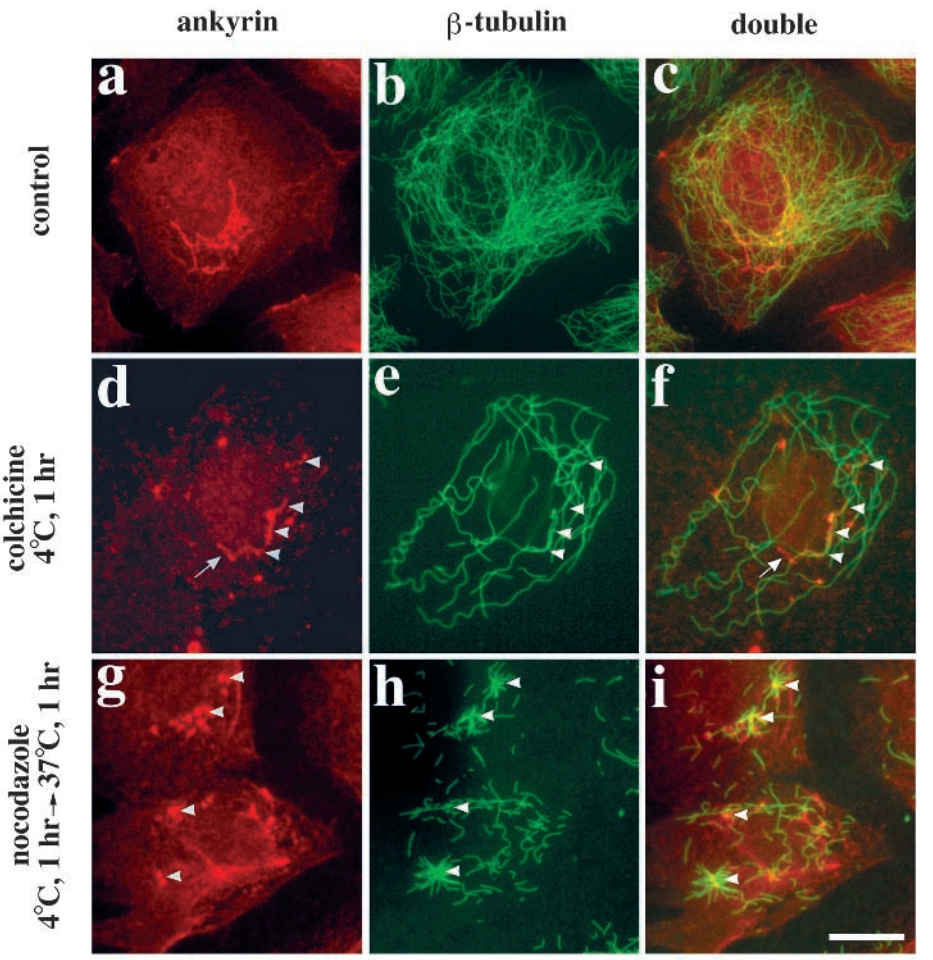


Fig. 5. Co-localization of ankyrin with microtubules is revealed in drug and low temperature treated cells. NRK cells were untreated (a-c), treated at 4°C in the presence of 50 μM colchicine for 1 hour (d-f), or treated at 4°C for 1 hour followed by treatment with 33 μM nocodazole for 1 hour at 37°C (g-i). Cells were extracted with 0.5% Triton X-100 in MEMA buffer for 10 minutes, fixed and double stained with anti-ankyrin (a,d,g) and β-tubulin (b,e,h) antibodies followed by rhodamine and fluorescein-conjugated secondary antibodies, respectively; double exposure images (c,f,i). Golgi ghosts co-localize with microtubules (arrowheads). Bar, 10 μm.

cytoplasm. Note that both Golgi β-spectrin (Beck et al., 1994) and the ankyrin isoforms detected with the EAnk-2 antibody localize to the Golgi independently of cell culture density. Thus, we conclude that MDCK cells posses at least two distinct Golgi localized ankyrin isoforms: a 195 kDa erythroid ankyrin homolog revealed in this report and the smaller 119 kDa ankyrin identified by Devarajan et al. (1996). It is possible that these two Golgi ankyrins serve distinct functions within the Golgi complex.

The known functions of the membrane skeleton are dependent on the assembly of membrane skeleton components into an oligomeric protein cytoskeletal lattice. By several independent criteria we have shown that Golgi β-spectrin and ankyrin associate in a higher order oligomeric protein complexes: Golgi ankyrins bind to purified erythrocyte spectrin (Fig. 1C); Golgi ankyrins and Golgi β-spectrin are rapidly pelleted together during brief low speed centrifugation of 0.5% Triton X-100 extracts of MDCK cells (data not shown); and extraction of NRK cells with buffers containing 0.5% Triton X-100 results in retention of a detergent insoluble Golgi ghost that reacts with both Golgi β-spectrin and ankyrin antisera (Fig. 4).

We do not yet have direct evidence of function(s) of the Golgi membrane skeleton, but they may be similar to those of the plasma membrane skeleton (see above). In the Golgi complex, the membrane skeleton may be involved in maintaining the structural organization of Golgi compartments. Both Golgi β-spectrin (Beck et al., 1994) and Golgi ankyrin (present study) dissociate from Golgi membranes under conditions that are accompanied by disruption of Golgi structure: e.g. mitosis (Fig. 7) and brefeldin A treatment (Fig. 6). To some extent, the structural integrity of the Golgi is maintained through interactions with microtubules; disruption of microtubules results in fragmentation and dispersion of Golgi membranes (Kreis, 1990). Our results indicate that the Golgi membrane skeleton is co-linear with a sub-population of microtubules (Fig. 5). Although we do not know if this interaction is direct, it is noteworthy that ankyrin has been shown to bind tubulin in vitro (Bennett and Davis, 1981). Alternatively, a recently reported interaction of Golgi localized β-spectrin with centractin (Holleran et al., 1996), a protein implicated in microtubule based motility, suggests an additional mechanism for membrane skeleton association with microtubules (Holleran et al., 1996; Vallee and Sheetz, 1996). Thus, the membrane skeleton and microtubules may co-operate to maintain Golgi structure and its spatial location in the perinuclear cytoplasm. Alternatively, or perhaps in a concerted fashion, the Golgi membrane skeleton could also directly maintain the stability of Golgi membranes by serving as a membrane coat.

Studies employing real time fluorescence imaging of the Golgi complex in living cells have demonstrated that the Golgi is a highly dynamic organelle (Cole et al., 1996; Cooper et al., 1990). Hence, a Golgi membrane skeleton is expected to possess a corresponding degree of dynamics in its association with Golgi membranes. Two observations provide evidence of

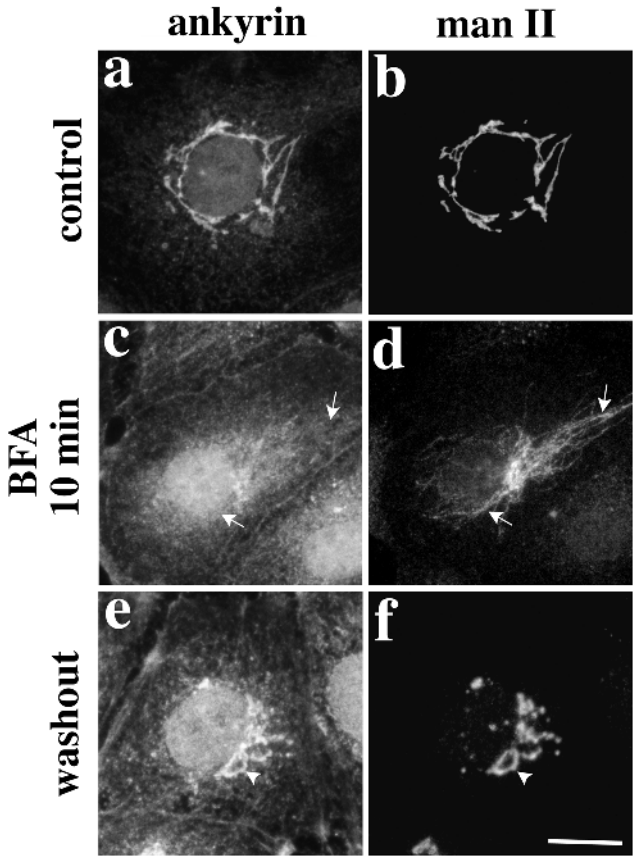


Fig. 6. Effects of brefeldin A on ankyrin distribution. NRK cells were either untreated (a and b), treated with 5 μM brefeldin A for 10 minutes (c and d), or treated for 10 minutes with brefeldin A followed by a subsequent incubation for 30 minutes in the absence of brefeldin A (washout; e and f). Cells were then fixed in 2% formaldehyde and double stained with EAnk-2 antibody (a,c,e) and an antibody specific for mannosidase II (b,d,f). Arrows indicated examples of Golgi tubules that are not stained with the ankyrin antibodies. Bar, 10 μm.

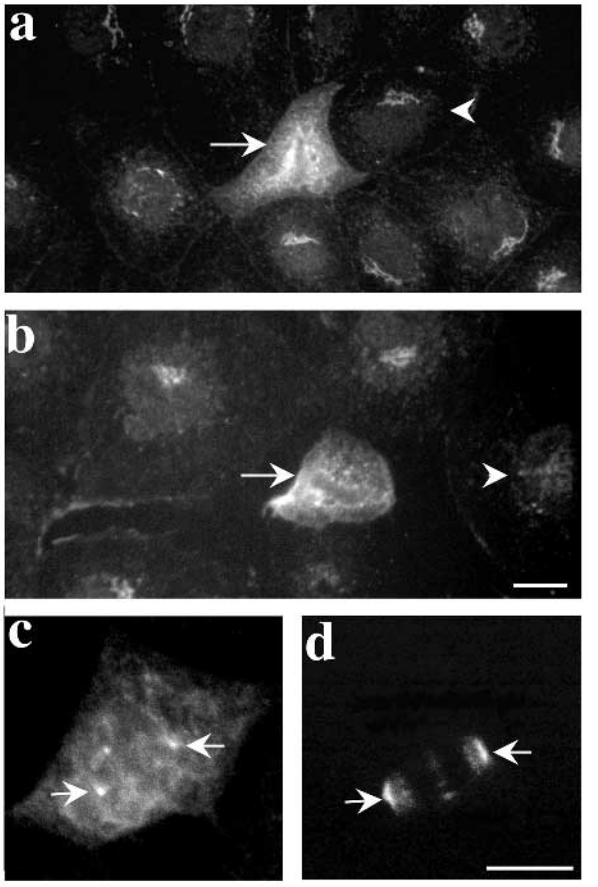


Fig. 7. Distribution of Golgi ankyrin during mitosis. Ankyrin staining in two separate fields of NRK cells having a single mitotic cell (arrows) surrounded by several interphase cells (arrowheads) are shown (a and b). Bar, 10 μm. An NRK cell double stained with EAnk-2 (c) and an antibody specific for β-tubulin (d) reveals localization of ankyrin to punctate structures that co-localize with the mitotic spindle (arrow). Bars, 10 μm.

a dynamic assembly state of the Golgi membrane skeleton. First, Golgi β-spectrin (Beck et al., 1994) and Golgi ankyrin (Fig. 7) dissociate from the Golgi complex during mitosis. Second, Golgi β-spectrin (Beck et al., 1994) and ankyrin (Fig. 6) dissociate from Golgi membranes following treatment with brefeldin A. Note that brefeldin A affects the distribution of other peripheral Golgi coat proteins (coatomer complex, clathrin and p200; Orci et al., 1991; Wong and Brodsky, 1992; Narula and Stow, 1995) by inhibiting coat protein binding to Golgi membranes resulting in a net dissociation of coat proteins (Orci et al., 1991; Donaldson et al., 1992). That brefeldin A induces dissociation of β-spectrin and ankyrin from Golgi membranes with kinetics similar to that of other coat proteins indicates that the Golgi membrane skeleton also undergoes a constitutive cycle of assembly on to, and dissociation from membranes.

The plasma membrane skeleton has also been proposed to facilitate formation of membrane domains by collecting classes of membrane proteins and restricting their distribution within the plane of the lipid bilayer (Nelson, 1992). The Golgi complex also requires the formation of membrane domains to retain resident proteins, transport newly synthesized proteins between different cisternae, and sort proteins in the TGN (Griffiths and Simons, 1986; Geuze and Morre, 1991; Machamer, 1991). Each of these events requires mechanisms that identify specific cohorts of membrane proteins and spatially segregate them from membrane proteins that have different intracellular fates. Ultrastructurally, we have localized ankyrin to small (60 nm) vesicular structures and membranous tubules associated with cisternal elements that are characteristic of the TGN (Rambourg and Clermont, 1990), a Golgi compartment that serves a site for the sorting of a variety of membrane proteins. Ongoing studies of membrane proteins that bind Golgi ankyrin/β-spectrin and regulate Golgi ankyrin/β-spectrin assembly should help to further elucidate the function of the Golgi membrane skeleton.

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