Golgi Spectrin: Identification of an Erythroid β-Spectrin Homolog Associated with the Golgi Complex

Kenneth A. Beck, Jo Ann Buchanan, Vivek Malhotra,* and W. James Nelson

Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, California 94305-5426; and *Department of Biology, University of California San Diego, La Jolla, California 92093

Abstract. Spectrin is a major component of a membrane-associated cytoskeleton involved in the maintenance of membrane structural integrity and the generation of functionally distinct membrane protein domains. Here, we show that a homolog of erythrocyte β-spectrin (βIΣ*) co-localizes with markers of the Golgi complex in a variety of cell types, and that microinjected β-spectrin codistributes with elements of the Golgi complex. Significantly, we show a dynamic relationship between β-spectrin and the structural and functional organization of the Golgi complex. Disruption of both Golgi structure and function, either in mitotic cells or following addition of brefeldin A, is accompanied by loss of β-spectrin from Golgi membranes and dispersal in the cytoplasm. In contrast, perturbation of Golgi structure without a loss of function, by the addition of nocodazole, results in retention of β-spectrin with the dispersed Golgi elements. These results indicate that the association of β-spectrin with Golgi membranes is coupled to Golgi organization and function.

THE segregation of specific subclasses of membrane proteins and lipids into discrete membrane domains is a fundamental aspect of cell structure and function. On one level, specialized cells, such as transporting epithelia and neurons, require functionally distinct membrane domains to regulate vectorial transport of ions and solutes or unidirectional propagation of electrical stimuli, respectively Rodriguez-Boulan and Nelson, 1989; Rodriguez-Boulan and Powell, 1992). On another level, membrane domains are important in constitutive, dynamic membrane events that occur in all cell types. For example, in the secretory pathway membrane proteins are transported through different membrane compartments, each of which are capable of maintaining its own distinctive composition of resident proteins while, at the same time, allowing a continual flux of nonresident proteins (Machamer, 1991). Thus, the formation of membrane domains is essential not only in cell surface organization of polarized cells, but also for the balance of membrane protein retention and transport that facilitates the genesis of distinct organellar membranes.

Membrane protein organization can be regulated through clustering by a membrane-associated cytoskeletal system. A good candidate for such a system is the spectrin-based membrane skeleton (for review see Bennett, 1990*a*). In erythrocytes, the membrane skeleton is composed of a number of proteins including spectrin, which is comprised of two distinct subunits termed α and β. Spectrin self-associates to form tetramers ($[\alpha\beta]_2$), which in turn are cross-linked by short actin polymers and other proteins into an extensive two-dimensional filamentous meshwork. This meshwork is tightly coupled to the plasma membrane through specific interactions with membrane proteins (Bennett, 1990*a*,*b*). Genetic studies show that defects in membrane skeleton proteins reduce the structural integrity of the plasma membrane of erythrocytes (Agre et al., 1985; Takakuwa et al., 1986). In addition to this structural role, extensive self-assembly of membrane skeleton components coupled to membrane protein binding also leads to a restriction in the mobility and, potentially, cell surface distribution of associated membrane proteins (Sheetz et al., 1980; Tsuji and Ohnishi, 1986; Tsuji et al., 1988).

The identification of protein isoforms of the membrane skeleton in nonerythroid cells, and the subsequent analysis of their distributions and functional properties has further defined the role of the membrane skeleton in the formation of membrane domains. In polarized epithelial cells and neurons, multiple isoforms of membrane skeleton proteins are expressed, and each of these isoforms localizes to different domains of the plasma membrane where they interact with domain-specific membrane proteins (Lazarides and Nelson, 1983; Nelson and Veshnock, 1986, 1987; Zagon et al., 1986; Morrow et al., 1989; Hammerton et al., 1991). There is evidence that the membrane skeleton can directly facilitate the domain-specific accumulation of these membrane proteins by retention in the membrane and, hence, exclusion from internalization (Hammerton et al., 1991; Seimers et al.,

Address all correspondence to K. Beck, Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA 94305-5426. Ph: (415) 725-7553. Fax: (415) 725-8021.

1993). This suggests that the membrane skeleton can be viewed as a membrane protein sorting apparatus which facilitates the retention of a specific subset of membrane proteins within a given membrane domain by effectively sequestering them away from the endocytic machinery.

The requirements for membrane structural integrity and membrane domain organization are not limited to the plasma membrane. The Golgi apparatus is a highly organized organelle composed of spatially and functionally distinct compartments responsible for the processing and sorting of newly synthesized membrane and secreted proteins (for review see Pfeffer and Rothman, 1987; Mellman and Simons, 1992; Rothman and Orci, 1992). Each compartment maintains a unique membrane protein composition under conditions that also allow continuous, sequential transit of newly synthesized proteins. This implies the existence of a molecular machinery that can distinguish and segregate resident and transported membrane proteins. While much is known about the interrelationship between the structural and functional organization of the Golgi complex, several key aspects remain to be resolved. Specifically, little is known about the maintenance of overall Golgi morphology, the retention of resident proteins within the various Golgi compartments and the sorting of newly synthesized membrane proteins within the TGN.

In this study we demonstrate the existence of an isoform of spectrin homologous to erythroid β-spectrin which localizes to the Golgi apparatus of nonerythroid cells. We show that perturbation of Golgi structure and function results in a corresponding change in the distribution of the Golgi-localized β-spectrin. Significantly, under conditions which disrupt both the structure and function of the Golgi complex, β-spectrin is dissociated from Golgi membranes. We suggest that, in addition to a structural role, β-spectrin may participate in, or directly facilitate, the retention of resident Golgi proteins and the sorting of newly synthesized membrane proteins.

Materials and Methods

Antibody Preparation

Erythrocyte spectrin (βIΣ1) was isolated from canine whole blood (Pel-Freez, Rogers, AK) according to the method of Bennett (Bennett, 1983). For antibody production, low salt extracts of washed erythrocyte ghosts were electrophoresed on preparative (12 × 18 × 0.3 cm) 12.5% SDS-polyacrylamide gels (Nelson et al., 1983). Bands corresponding to the spectrin β subunit were excised and electroeluted (Isco, Lincoln, NE). New Zealand White rabbits were inoculated with electroeluted β-spectrin emulsified in Freunds adjuvant (Sigma Chemical Co., St. Louis, MO). Bovine lens α-spectrin was prepared from purified bovine lens plasma membranes (Nelson et al., 1983) by preparative electrophoresis as described above for β-spectrin. The excised portion of the gel was ground up, mixed with Freunds adjuvant, and injected directly into rabbits. Antisera obtained from immunized rabbits were treated with 35% saturated ammonium sulfate, and the precipitated immunoglobulin fraction was resuspended in phosphate buffered saline containing 1 mM sodium azide (PBS-azide) and stored at −70°C. Monoclonal anti-β-tubulin was from Amersham Corp. (Arlington Heights, IL). A mouse monoclonal antibody directed against Golgi membranes (Lee, S. L., V. Malhotra, D. I. Tai, M. Mori, S. Bao, J. D. Song, Q. An, and L. B. Chen. 1991. *J. Cell Biol.* 115:409a) was generously provided by Dr. Lan Bo Chen (Dana-Farber Institute, Boston, MA). Polyclonal anti-β-COP and monoclonal anti-mannosidase II were generously provided by Dr. Jennifer Lippincott-Schwartz (NIH/NICHD, Bethesda, MD). Mouse monoclonal anti-cation independent mannose phosphate receptor (86f7) and anti-lysosomal glycoprotein (35c1) were generously provided by Dr. Donald Messner (University of Rochester, Rochester, NY). A polyclonal antiserum directed against a 15-amino acid peptide that corresponds to residues 6-20 of the mouse β-spectrin gene[1] were kindly provided by Dr. Steven Goodman (University of South Alabama School of Medicine, Mobile, AL).

Affinity Purification

A spectrin-Sepharose affinity resin was prepared by coupling purified canine erythrocyte spectrin to Sepharose CL-4B. Erythrocyte spectrin derived from a low salt extract of canine erythrocyte ghosts was purified further by centrifugation (40,000 *g*, 15 h, SW-40 rotor) on 5–20% sucrose gradients (wt/vol, 20 mM KCl, 5 mM sodium phosphate, pH 7.5, 1 mM sodium azide, 0.2 mM dithiothreitol; Bennett, 1983). This resulted in the removal of contaminating low molecular weight material and gave rise to a spectrin preparation that was >90% pure as assessed on Coomassie stained SDS-polyacrylamide gels. Spectrin was coupled to cyanogen bromide-activated Sepharose CL-4B (Sigma Chemical Co.) at a concentration of 1 mg/ml bed volume according to the manufacturers instructions. The resin was blocked by washing with 10 bed volumes of PBS-azide containing 1 mg/ml bovine serum albumin (fraction V, heat shock; Sigma Chemical Co.). BSA-Sepharose was prepared under identical conditions using bovine serum albumin fraction V (cold alcohol precipitation; Sigma Chemical Co.). The affinity purification procedure was carried out as follows. 1 ml of βspec-1 antiserum was mixed with 1 ml of affinity resin (equilibrated in PBS-azide) and diluted to a volume of 8 ml with PBS-azide. This suspension was rocked 12–15 h at 4°C and then poured into a 1 × 10 cm column. The nonbound material was eluted (flow rate = 0.5 ml/min) and saved (flow-through fraction). The resin was washed with 25 ml of PBS-azide, 25 ml of low salt wash buffer (10 mM sodium phosphate, pH 7.5) and 25 ml of high salt wash buffer (0.5 M NaCl, 10 mM sodium phosphate, pH 7.5). Bound antibody was eluted with 6 ml 0.1 M glycine-HCl, pH 2.5 (acid eluate) followed by 6 ml 0.1 M triethylamine, pH 11.7 (base eluate). The acid eluate was neutralized with 0.6 ml 1 M Tris base and pooled with the base eluate. The pooled eluate fractions, as well as the flow-through fraction, were dialyzed 12–15 h against 2 liters of PBS-azide and concentrated by ultrafiltration (Centriprep-30; Amicon Corp., Beverly, MD) to the original serum volume of 1 ml.

Cell Culture and Microscopy

Madin-Darby canine kidney (MDCK), Madin-Darby bovine kidney (MDBK)[2] epithelial cells and normal rat kidney cells (NRK) were grown in high glucose DME (Sigma Chemical Co.) containing, 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA) and 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL, Gaithersburg, MD). Cultures were maintained at 37°C with 5% CO_2 in air. Microtubule disruption experiments were performed by incubating MDCK cells in culture media containing 33 μM nocodazole (Sigma Chemical Co.) for 30 min at 4°C. The cells were then washed briefly with fresh media (37°C) and further incubated in media containing 33 μM nocodazole at 37°C. In experiments with BFA, MDBK cells were incubated for various periods of time in media containing 5 μg/ml BFA (Epicentre Technologies, Madison, WI) at 37°C. For BFA wash-out experiments, BFA-treated cells were washed with PBS (37°C) and further incubated in BFA-free media (37°C).

For immunofluorescence experiments, cells were plated for 12–15 h on collagen-coated glass cover slips. The cells were fixed either with cold methanol (−20°C, 5 min), 1.9% formaldehyde in PBS (22°C, 10 min) or with a solution of 1% paraformaldehyde, 100 mM lysine 10 mM sodium *m*-periodate, and 0.1% saponin in PBS (PLP). Formaldehyde fixed cells were permeabilized by treatment with 0.01% saponin (Sigma Chemical Co.) for 10 min. All subsequent steps were conducted in the presence of 0.01% saponin. Fixed and permeabilized cells were blocked with PBS containing 0.2% BSA, 50 mM ammonium chloride, 0.5% goat serum (GIBCO BRL, Gaithersburg, MD) and 5 μg/ml DNAse (Boehringer Mannheim Biochemicals, Indianapolis, IN). The cells were then washed with PBS containing 0.2% BSA (PBS-BSA) and incubated for 1–2 h with primary antibody

1. Clark, M. B., M. Yupo, M. Bloom, J. Barker, I. Zagon, W. Zimmer, and S. Goodman, manuscript submitted for publication.

2. *Abbreviations used in this paper*: BFA, brefeldin A; CI-MPR, cation-independent mannose phosphate receptor; lgp, lysosomal membrane glycoprotein; Man II, mannosidase II; MDBK, Madin-Darby bovine kidney; NRK, normal rat kidney; WGA, wheat germ agglutinin.

diluted in PBS-BSA as indicated in the figure legends. Following this incubation, cells were washed for 15 min with PBS-BSA and incubated with rhodamine- or fluorescein-conjugated goat anti-mouse or rabbit IgG (Boehringer Mannheim Biochemicals) diluted 100-fold in PBS-BSA. Excess secondary antibody was washed away with PBS-BSA and the coverslips were mounted in 70% glycerol (vol/vol in PBS) containing 10 mg/ml *n*-propyl gallate (Sigma Chemical Co.). Double labeling experiments were performed by sequential incubations with both primary antibodies followed by incubation with a mixture of both secondary antibodies. Cells double stained with βspec-1 and β-COP were first incubated with anti-β-COP followed by fluorescein-conjugated goat anti-rabbit IgG. The cells were then wash extensively with PBS-BSA (4×, 10 min) and incubated with rhodamine-conjugated (see below) affinity purified βspec-1. DNA was stained with the fluorescent DNA intercalating agent YOYO-1 (Molecular Probes, Eugene, OR), which was stored as a 1 mM stock in 1:4 DMSO/H_2O and diluted 10,000-fold in PBS-BSA prior to incubation with cells for 30 min.

Plastic embedded sections of rat liver were prepared using the Polysciences Immuno-Bed kit (Polysciences Inc., Warrington, PA). Sections were prepared and fixed as described (Avner et al., 1992). All other steps in the immunofluorescence staining protocol were the same as described above except for the primary antibody incubation which was carried out for 12 h at 4°C.

Stained cells were observed by epifluorescence using a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY) and photographed with either Kodak Tri-X PAN 400 or Ektachrome 400 HC film (Eastman Kodak, Rochester, NY). Alternatively, some specimens were observed with a laser scanning confocal microscope (Cell Sciences Imaging Facility, Stanford University, Stanford, CA).

Golgi-enriched membranes from rat liver were prepared for electron microscopy as follows. Membranes (30–50 μg protein) were pelleted by brief (15 min) high speed centrifugation (100,000 *g*; Beckman TL-100 ultracentrifuge; Beckman Instruments, Palo Alto, CA). The pellet was fixed in 1% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, pH 7.4, 0.2 M sucrose) and postfixed in 0.5% osmium tetroxide with 1% potassium ferricyanide (in cacodylate buffer). The pellet was suspended in 1.5% agar to facilitate processing. The sample was en bloc stained with 2% uranyl acetate and embedded in Embed 812 (Electron Microscopy Sciences, Ft. Washington, PA). Ultrathin sections were post-stained with uranyl acetate and lead citrate and examined at 60 kV in a Philips 410 electron microscope.

Microinjection

For microinjection studies, purified canine erythrocyte spectrin was labeled with rhodamine isothiocyanate as follows. Spectrin (1 mg/ml in 150 mM sodium bicarbonate, pH 9.0) was incubated for 1 h at 22°C in the presence of a 30-fold molar excess of rhodamine isothiocyanate (Molecular Probes). The reaction was terminated by the addition of hydroxylamine, pH 8.0 to a final concentration of 0.15 M, and subsequent incubation for 1 h. The sample was then fractionated on a 1 × 10 cm column of Sephadex G-25 (Sigma Chemical Co.) equilibrated with microinjection buffer (48 mM K_2HPO_4, 14 mM NaH_2PO_4, 4.5 mM KH_2PO_4, pH 7.2). Fractions containing labeled protein were pooled, dialyzed 18 h against microinjection buffer and concentrated by ultrafiltration (Centricon-30; Amicon Corp.). Before microinjection, the rhodamine spectrin preparation was centrifuged 20 min at 10,000 *g*. MDCK cells grown on collagen coated coverslips were microinjected (at 37°C) according to the method of Graessmann et al. (Graessmann et al., 1980) using an Eppendorf/Zeiss microinjection system. For control experiments, lysine-derivitized rhodamine-dextran (Molecular Probes) was diluted fivefold in microinjection buffer and centrifuged 20 min at 10,000 *g* prior to injection. Cells were injected over a period of 1 h and then incubated for an additional 4 h before they were fixed in −20°C methanol and processed for indirect immunofluorescence as described above.

Immunoblotting, Immunoprecipitations, and Membrane Preparations

SDS-PAGE and immunoblots were performed as described previously (Nelson and Veshnock, 1986). For immunoprecipitation experiments, confluent MDCK cells were incubated 12 h in low calcium (5 mM), methionine-free DME (Nelson and Veshnock, 1987) containing 500 μCi [^{35}S]methionine (New England Nuclear/DuPont, Wilmington, DE). Cells were then extracted for 10 min at 22°C in extraction buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 0.5% [vol/vol] Triton X-100, 0.2 mM leupeptin, 0.2 mM 1,10 phenanthroline, 20 μg/ml pepstatin A, 0.4 mM PMSF), scraped from the surface of the culture plate and centrifuged 10,000 *g* for 20 min. Sodium dodecylsulfate was added to a final concentration of 0.5% and the extract was boiled for 5 min. The sample was then diluted 10-fold with extraction buffer and processed for immunoprecipitation as described previously (Nelson and Veshnock, 1986). Bovine lens plasma membranes were solated from decapsulated lenses as described (Nelson et al., 1983).

Purified Golgi membranes were isolated from rat liver by the method of Malhotra et al. (1989). Fractionation of purified membranes on wheat germ agglutinin agarose was performed with the following protocol. Golgi-enriched membranes were pelleted by centrifugation (100,000 *g*, 15 min; Beckman TLA 100 ultracentrifuge) and resuspended in homogenization buffer (10 mM Tris-HCl, pH 7.3, 0.5 M sucrose, 5 mM EDTA, 5 mM EGTA). Membranes (30 μg membrane protein) were then incubated in a total volume of 400 μl with 100 μl of wheat germ agglutinin agarose (WGA-agarose; Sigma Chemical Co.) for 18 h at 4°C. WGA-agarose beads were pelleted by brief (1 min) low speed centrifugation in a tabletop clinical centrifuge. The supernatant or unbound fraction (300 μl) was decanted and stored at 4°C and the pellet or bound fraction was washed once with buffer and resuspended to a final volume of 300 μl. 5′-Nucleosidase activity was measured by incubating 40 μl of sample with 2.5 μl of 100 mM adenosine monophosphate (Sigma Chemical Co.) and 200 mM $MgCl_2$ for 60 min at 37°C. Inorganic phosphate release was quantitated using the method of Lanzetta et al. (1979). Galactosyltransferase assays were performed as described (Fleicher and Smigel, 1978).

Results

Spectrin Isoforms are Differentially Localized in MDCK Cells

Antisera were raised against α-spectrin (α-fodrin, αIIΣ*; nomenclature based on Malchiodi-Albedi et al., 1993) isolated from the plasma membranes of a nonvascular tissue, lens, and β-spectrin (βIΣ*, β-spectrin) from plasma membranes of purified canine erythrocytes. As shown previously (Nelson and Veshnock, 1986; Morrow et al., 1989), α-fodrin localized to the cytoplasmic surface of the basolateral membrane of confluent monolayers of polarized MDCK cells (Fig. 1 *D, b*). This antiserum recognizes the α-spectrin isoform in lens (α-fodrin), but not the isoform expressed in erythrocytes (Fig. 1 *A*, lanes *e* and *f*). In direct contrast, the erythrocyte spectrin antibody (βspec-1) predominantly recognized a 220-kD protein (β-spectrin) from erythrocyte plasma membranes (Fig 1 *A*, lane *c*), but did not detect α- or β-spectrin isoforms in the extract of lens plasma membranes (Fig. 1 *A*, lane *d*), even though equal amounts of spectrin from both sources were present (Fig. 1 *A*, lanes *a* and *b*). Minor cross-reactivity was detected against α-spectrin from erythrocytes (Fig. 1 *A*, lane *c*). As expected, the βspec-1 antibody strongly stained the erythrocyte plasma membrane (Fig. 1 *C*). Differences in reactivity of the βspec-1 antibody to spectrin isoforms was not species or tissue dependent; cross-reactivity was found in a variety of tissues derived from diverse sources including dog, rat, mouse, chicken, monkey, and cow (not shown).

MDCK cells were analyzed with the βspec-1 antibody for the presence of an erythroid β-spectrin homolog. A polypeptide of the appropriate molecular mass (220 kD) was immunoprecipitated from [^{35}S]methionine-labeled MDCK cells (Fig. 1 *B*, lane *a*); additional minor protein bands were present also in the control immunoprecipitate (Fig. 1 *B*, lane *b*). The antibody also recognized a single polypeptide of identical molecular mass upon western blotting of purified rat liver Golgi membranes (see Fig. 5 *A*). In addition, indirect immunofluorescence revealed a pattern of staining that was

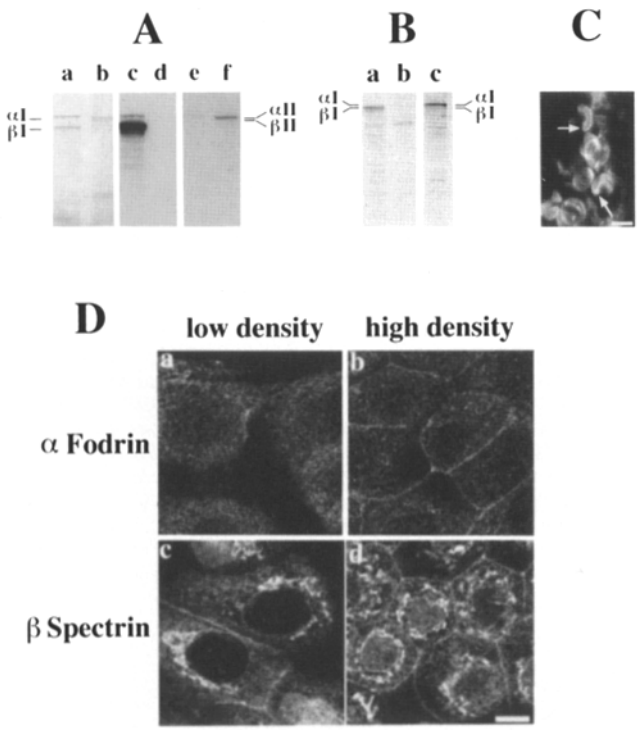


Figure 1. Differential localization of spectrin isoforms in MDCK cells. (*A*) Proteins from purified canine erythrocytes (lanes *a*, *c*, and *e*) and purified bovine lens plasma membranes (lanes *b*, *d*, and *f*) were solubilized in SDS-sample buffer, separated in a 5% SDS-polyacrylamide gel, and either stained with Coomassie blue (*a* and *b*), or transferred to nitrocellulose and immunoblotted with antisera to either erythroid β-spectrin (βspec-1; lanes *c* and *d*) or lens α-spectrin (αF3; lanes *e* and *f*). The electrophoretic mobilities of erythroid α and β subunits (αI and βI) are indicated to the left of lane *a*, whereas those of lens spectrin subunits (*αII* and *βII*) are shown to the right of lane *f*. Note that the lens α and β subunits migrate as a closely spaced doublet (lane *b*). (*B*) [^{35}S]Methionine-labeled polypeptides were immunoprecipitated from a detergent extract of MDCK cells using either the βspec-1 (lane *a*; affinity purified) or αF3 (lane *c*) antibodies. As a control (lane *b*), an identical precipitation was carried out with the eluate fraction from the mock affinity purification procedure (BSA-Sepharose, see Fig. 2 and Materials and Methods). Immunoprecipitated proteins were separated in a 7.5% SDS-polyacrylamide gel and visualized by fluorography. The mobilities of erythroid αI and βI subunits are shown. (*C*) Erythrocytes residing within a blood vessel of a plastic imbedded section of rat liver were stained with the βspec-1 antisera by indirect immunofluorescence. The image was obtained using a scanning laser confocal microscope. Arrows indicate cells which show plasma membrane staining. (*D*) MDCK cells grown either at confluence (48 h, *b* and *d*) or at subconfluent density (*a* and *c*) were fixed in 1.9% formaldehyde, permeabilized with 0.01% saponin, and stained with affinity purified βspec-1 (*c* and *d*) or αF3 (*a* and *b*) by indirect immunofluorescence and imaged by confocal microscopy. Bars, 5 μm.

different from the subcellular distribution of lens α-fodrin (Fig. 1 *D*). The most prominent feature of this staining pattern was the appearance of numerous reticular structures clustered near the nucleus (Fig. 1 *D*, *c*), which by morphological criterion alone highly resembled the Golgi apparatus (see below). Serial optical sections of cells using a scanning laser confocal microscope revealed that these structures reside beneath the surface of the cell within the cytoplasm, indicating that they represent an intracellular organelle or membrane system rather than a surface membrane organization (not shown). These structures were not observed with the α-fodrin antibody (Fig. 1 *D*, *a* and *b*), even upon examination of serial confocal optical sections (not shown). The presence of perinuclear reticular elements stained with βspec-1 was independent of cell density (Fig. 1 *D*, *c* and *d*). At confluence, faint staining of the lateral membranes was also observed. However, the intensity of this staining was markedly less than that of the reticular elements, indicating that the latter reflects the predominant steady state localization of the immunoreactive protein.

To demonstrate that the βspec-1 antiserum stained β-spectrin in these cells we affinity purified the antibody using a spectrin-Sepharose affinity resin (Fig. 2). The affinity purification procedure (see Materials and Methods) gave rise to two fractions: a flow-through fraction which contained material which did not bind to the column, and an eluate fraction containing the affinity purified antibody. Both of these fractions were analyzed for their ability to blot purified erythrocyte spectrin (Fig. 2 *A*) and stain intracellular reticular structures (Fig. 2 *B*). As a control, an aliquot of βspec-1 immune serum was identically fractionated using a column of BSA-Sepharose affinity resin and subjected to the same analysis.

By Western blotting, the majority of the immuno-reactive antibody was present in the eluate fraction of the spectrin-Sepharose column (Fig. 2 *A*, lane *d*). Only a relatively small amount of activity was present in the nonbound or flow-through fraction (Fig. 2 *A*, lane *c*), probably due to oversaturation of the immobilized antigen with the antiserum. The eluate fraction immunoprecipitated a 220-kD polypeptide that co-migrated with erythrocyte β-spectrin by SDS-PAGE (Fig. 1 *B*, lane *a*). Upon mock affinity purification of the βspec-1 antiserum with BSA-Sepharose (Fig. 2 *A*, lanes *a* and *b*; Fig. 1 *B*, lane *b*), an eluate fraction was obtained which showed no immunoreactivity by either Western blotting (Fig. 2 *A*, lane *b*) or immunoprecipitation (Fig. 1 *B*, lane *b*).

The flow-through and eluate fractions obtained from the affinity purification of βspec-1 antibody were used to stain MDCK cells (Fig. 2 *B*, *c* and *d*). Perinuclear reticular structures were stained strongly with the eluate fraction (Fig. 2 *B*, *d*). In contrast, the flow-through fraction (Fig. 2 *B*, *c*) stained amorphous material devoid of reticular structures. As expected, the eluate fraction from the mock purification (Fig. 2 *B*, *b*) did not stain MDCK cells, demonstrating the specificity of the affinity purification. Staining by the mock flow-through fraction (Fig. 2 *B*, *a*), which is equivalent to the unfractionated antibody, represents an accurate composite of the two fractions obtained from the spectrin-Sepharose affinity column (Fig. 2 *B*, *c* and *d*).

These results show that upon fractionation of the βspec-1 antiserum on spectrin-Sepharose both immunoreactivity toward β-spectrin (Fig. 2 *A*, lane *d*), and staining of perinuclear reticular structures (Fig. 2 *B*, *d*) are highly enriched in the specific eluate fraction, and depleted in the flow-through fraction (Fig. 2 *A*, lane *c*: Fig. 2 *B*, *c*). We conclude that antibody binding to β-spectrin directly correlates with staining of perinuclear reticular structures in MDCK cells. Identical results were obtained with an independently pre-

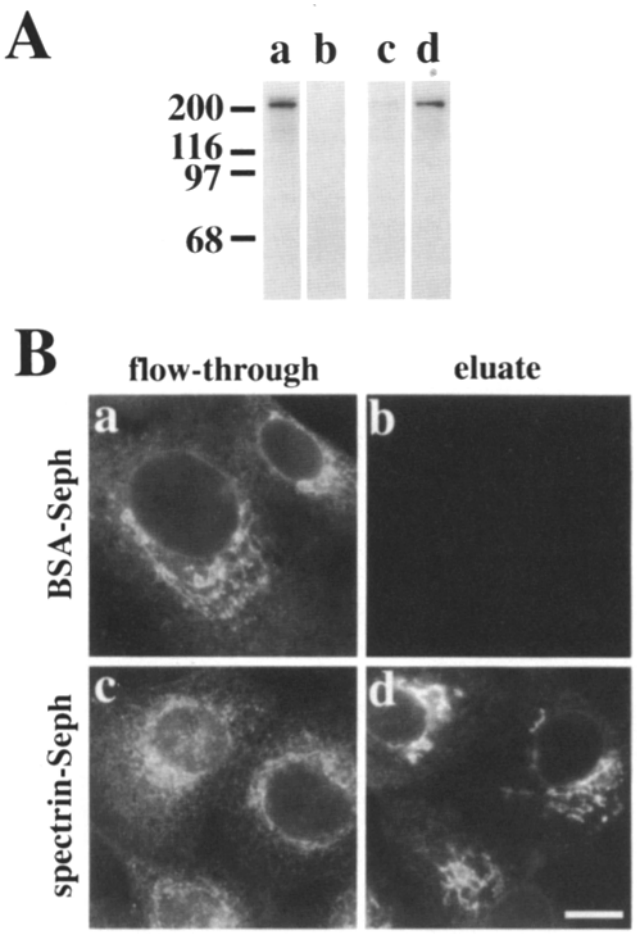


Figure 2. Affinity purification of βspec-1. The βspec-1 antiserum was fractionated by chromatography on either BSA-Sepharose or spectrin-Sepharose. Nonbound (*flow-through*) and specifically eluted (*eluate*) fractions were collected from both columns and assayed by both western blotting and indirect immunofluorescence staining of MDCK cells. (*A*) Immunoblots of purified canine erythrocyte spectrin were performed with the flow-through (*a* and *c*) or eluate fractions (*b* and *d*) from either the BSA-Sepharose (*a* and *b*) or spectrin-Sepharose (*c* and *d*) column. (*B*) MDCK cells stained with the BSA-Sepharose flow-through (*a*) and eluate (*b*) or the spectrin-Sepharose flow-through (*c*) and eluate (*d*). Images were obtained with a Zeiss Axiophot microscope. Bar, 5 μm.

pared β-spectrin antibody (not shown). Moreover, an additional β-spectrin–specific antiserum raised against a 15–amino acid peptide that corresponds to a region of β-spectrin (amino acids 6-20) that is unique to the erythroid isoform[1] also stained perinuclear reticular structures identical to those revealed with the βspec-1 antiserum (Fig. 3, *k* and *l*). In contrast, this staining pattern was not observed with a polyclonal antiserum[1] directed against a specific peptide derived from a non-erythroid β-spectrin (not shown). Hence, three independent antisera specific for erythroid β-spectrin all stained identical intracellular structures, indicating that the staining pattern observed is due to the presence of an erythroid β-spectrin homolog in association with these intracellular sites.

β-Spectrin Localization to the Golgi Apparatus

To determine the identity of the perinuclear reticular structures recognized by the β-spectrin antibody, cells stained with βspec-1 were co-labeled with antibodies specific for marker proteins of different intracellular organelles (Fig. 3). Extensive co-localization was observed with antibodies that recognize Golgi membrane proteins. βspec-1 staining completely overlapped that of a monoclonal antibody directed against Golgi membranes (Fig. 3, *a* and *b*; Lee, S. L., V. Malhotra, D. I. Tai, M. Mori, S. Bao, J. D. Song, Q. An, and L. B. Chen. 1991. *J. Cell Biol.* 115:409a), which in turn exactly co-localized with the Golgi coat protein β-COP (Fig. 3, *c* and *d*). The extent of overlap between β-spectrin distribution and Golgi membranes is shown in a high magnification, double exposure of an NRK cell double stained with βspec-1 and an antiserum directed against the Golgi resident protein mannosidase II (*Man II*, Fig. 4). Overall, the distribution patterns of β-spectrin and Man II co-localized extensively (Fig. 4, *a–c*), demonstrating that both proteins are associated with the same organelle. However, closer examination revealed regions (300–600 nm) enriched in β-spectrin but devoid of mannosidase II (Fig. 4 *d*).

Significant but less extensive co-localization was observed between βspec-1 and an antibody to the cation-independent mannose phosphate receptor (*CI-MPR*, Fig. 3, *g* and *h*). Previous studies have shown that the CI-MPR cycles between the TGN and late endosome compartments (Kornfeld, 1992; Johnson and Kornfeld, 1992). Thus, the overlap of CI-MPR and βspec-1 staining is likely to reflect TGN localization, whereas the punctate staining pattern of CI-MPR located more towards the periphery of the cell, and which did not appear to overlap with the βspec-1 pattern, probably represents late endosomes. Staining with an antibody to a lysosomal membrane glycoprotein (lgp) showed that lysosomes do not co-localize with the structures stained with the βspec-1 antibody (Fig. 3, *i* and *j*). In fact, lysosomes appear to be excluded from the region of the cytoplasm containing the β-spectrin reactive membranes. At present, attempts to localize β-spectrin at the ultrastructural level by immunoelectron microscopy are inconclusive. However, based upon the unequivocal co-localization of βspec-1 and Golgi markers at the light microscope level, we conclude that the perinuclear structures stained with βspec-1 are Golgi membranes.

As further support for the Golgi-localization of spectrin we prepared a membrane fraction from rat liver homogenates that was highly enriched in Golgi membranes (Fig. 5 *C*; and Malhotra, et al., 1989). Immuno-blot analysis of this fraction using the βspec-1 antiserum (Fig. 5 *A*) revealed the presence of an immunoreactive species with a molecular mass equivalent to that of erythroid β-spectrin (220 kD, Fig. 5 *A*); no other bands were detected. To demonstrate that the presence of this cross-reacting polypeptide was not due to contaminating plasma membrane vesicles, we subjected the enriched Golgi membrane preparation to an additional fractionation on wheat germ agglutinin agarose (WGA-agarose; Fig. 5 *B*). We found that plasma membrane vesicles (assayed by 5′-nucleosidase activity) were equally distributed between the bound and unbound fractions (Fig. 5 *B*), presumably due to only half of the plasma membrane vesicles having their lectin-binding sites exposed. The binding of 5′-nucleosidase

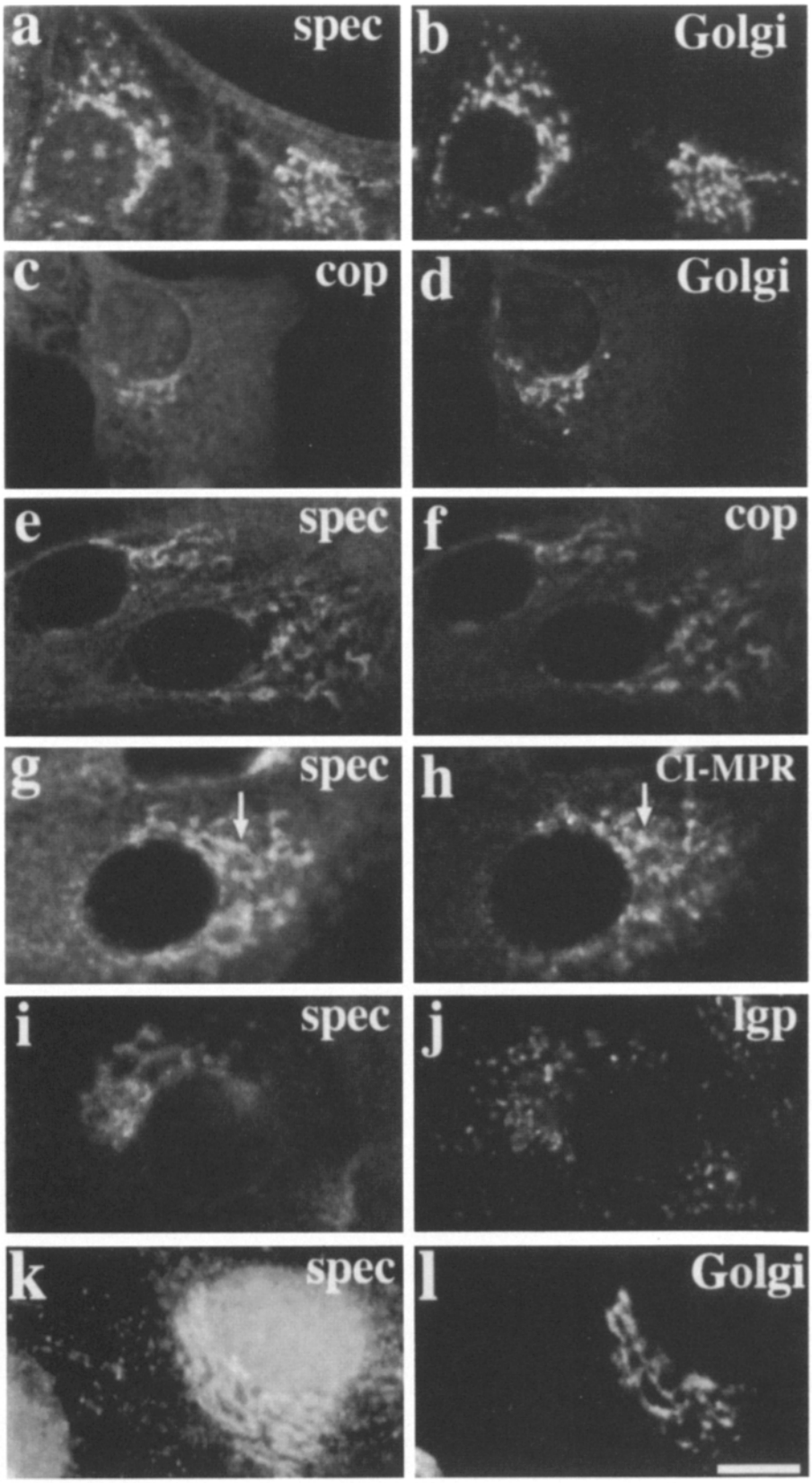


Figure 3. Subcellular localization of β-spectrin. MDCK (*a–f*), MDBK cells (*g–j*), or c_2c_{12} mouse myoblasts (*k* and *l*) were fixed either in methanol (*a* and *b*), 1.9% formaldehyde or PLP (*k* and *l*). Formaldehyde- and PLP-fixed cells were permeabilized with 0.01% saponin. Cells were double stained with antibodies specific for the following antigens: β-spectrin and Golgi membranes (*a* and *b*); β-COP and Golgi membranes (*c* and *d*); β-spectrin and β-COP (*e* and *f*); β-spectrin and CI-MPR (*g* and *h*); β-spectrin and a lysosomal glycoprotein (*i* and *J*); erythroid β-spectrin peptide[1] and Golgi membranes (*k* and *l*). In all cases except e and f (see Materials and Methods) rabbit polyclonal antibodies (*a*, *c*, *g*, and *i*) were visualized with fluorescein-conjugated goat anti-rabbit IgG, and mouse monoclonal antibodies (*b*, *d*, *h*, and *j*) with rhodamine-conjugated goat anti-mouse IgG. In *g* and *h* the arrows show deviations in the colocalization between β-spectrin and CI-MPR. All images were obtained with a laser scanning confocal microscope. Bar, 5 μm.

containing vesicles to WGA-agarose was blocked following preincubation with 1 M *N*-acetyl glucosamine, confirming the specificity of the interaction (not shown).

In contrast to the results observed with the plasma membrane marker, an enzymatic marker for the Golgi apparatus (galactosyltransferase) accumulated exclusively in the unbound fraction following incubation with WGA-agarose (Fig. 5 *B*). This result implies that lectin-binding sites of

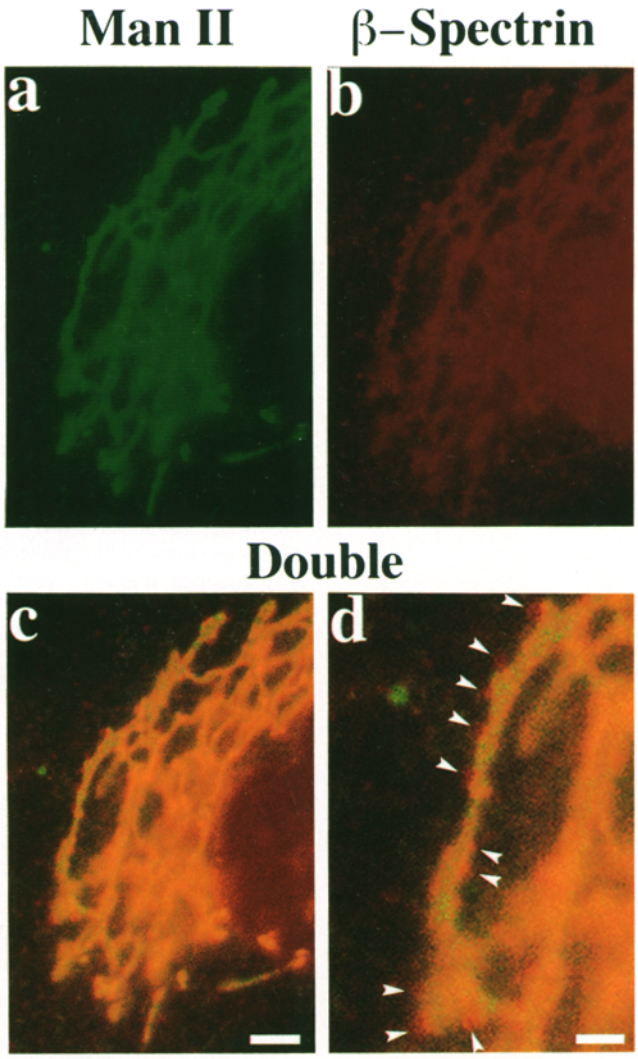


Figure 4. Golgi localization of β-spectrin. A high magnification image of an MDCK cell double stained with βspec-1, followed by a rhodamine-conjugated secondary antibody (*b*), and an anti-mannosidase II monoclonal antibody, followed by a fluorescein-conjugated secondary antibody (*a*). In a double exposure of the rhodamine and fluorescein images, the yellow color indicates the overlap of the two fluorescent antibodies (*c* and *d*). A higher magnification image (*d*) of a region of the cell shown in *c* reveals the presence of numerous knob-like structures stained only with the β-spectrin antibody (*arrowheads*). Images were obtained with a scanning laser confocal microscope. Bars: (*c*) 5 μm; (*d*) 2.5 μm.

Golgi-derived membranes are primarily lumenal and hence are unable to interact with the WGA-agarose beads. Such a topology for purified Golgi membranes has been reported previously (Persson et al., 1991). Examination of the Golgi membrane preparation by electron microscopy (Fig. 5 *C*) revealed an abundance of intact stacks of Golgi cisternae, indicating that normal Golgi morphology and topology was maintained. In addition, protease protection experiments showed that the majority (89% ± 13%, $n = 3$) of the galactosyltransferase activity (a lumenal enzyme) in this preparation was protected from proteolysis except when 0.5% Triton X-100 was added (data not shown). These results show that fractionation on WGA-agarose leads to a differential partitioning of Golgi and plasma membrane derived vesicles and that this is due to a difference in the topology of the membrane vesicles and hence accessibility of lectin-binding sites.

We next determined the distribution of spectrin following WGA-agarose fractionation. We found that, like the Golgi membrane marker, the 220-kD immunoreactive polypeptide was found exclusively in the unbound fraction (Fig. 5 *B*). Hence, a polypeptide of the same molecular weight as spectrin which reacts with a spectrin-specific antiserum co-purifies with Golgi but not plasma membrane–derived vesicles. These results strongly support our conclusion that an isoform of spectrin localizes to the Golgi apparatus.

We also performed an experiment to evaluate the subcellular localization of β-spectrin to the Golgi complex that was independent of βspec-1 antibody reactivity. Purified canine erythrocyte spectrin was covalently labeled with rhodamine and microinjected into living MDCK cells (see Materials and Methods). The cells were then fixed, permeabilized, and stained with the Golgi-specific monoclonal antibody. The microinjected rhodamine-spectrin accumulated primarily as punctate perinuclear structures (Fig. 6, *a*, *d*, and *g*). There was also a faint diffuse signal that could correspond to free cytoplasmic protein. In all cases the punctate perinuclear structures resided in the same region of the cytoplasm as the Golgi apparatus, and overlapped the distribution of reticular Golgi elements identified with the Golgi marker antibody (Fig. 6, *c* and *f*). In contrast, microinjection of rhodamine-labeled dextran produced a diffuse pattern that extended evenly throughout the cytoplasm and did not give rise to localized punctate structures like those observed with microinjected β-spectrin or βspec-1 staining. It is also possible that the punctate peri-nuclear structures labeled with rh-spectrin were lysosomes (Fig. 5, *a*, *d*, and *g*). However, staining of microinjected cells with antibodies against lysosomal membrane proteins showed no colocalization of rh-spectrin with lysosomes (not shown). In addition, rh-spectrin tended to localize to a central region of the Golgi complex (Fig. 6) whereas lysosomes tended to reside more toward the periphery of the cytoplasm (Fig. 3, *i* and *j*). We conclude that these results are consistent with the proposal that β-spectrin associates directly with the Golgi apparatus. Furthermore, since these experiments were totally independent of the anti-spectrin antiserum, we can more confidently rule out the possibility that the βspec-1 recognizes a Golgi resident protein distinct from β-spectrin but which has a cross-reacting epitope.

Dynamics of β-Spectrin Distribution during Disruption of Golgi Structure and Function

The onset of mitosis is accompanied by a rearrangement of cytoplasmic microtubules, a corresponding fragmentation and redistribution of the Golgi apparatus (Lucocq et al., 1989; Lucocq and Warren, 1987), and a temporary cessation of protein processing and secretion (Warren et al., 1983). To establish whether the distribution of Golgi-localized β-spectrin is also affected during the cell cycle, we surveyed cultures of log phase MDCK cells, that had been fixed and stained with βspec-1, for cells captured in different stages of mitosis (Fig. 7). The βspec-1 staining pattern was distinctly different in cells undergoing mitosis compared to interphase

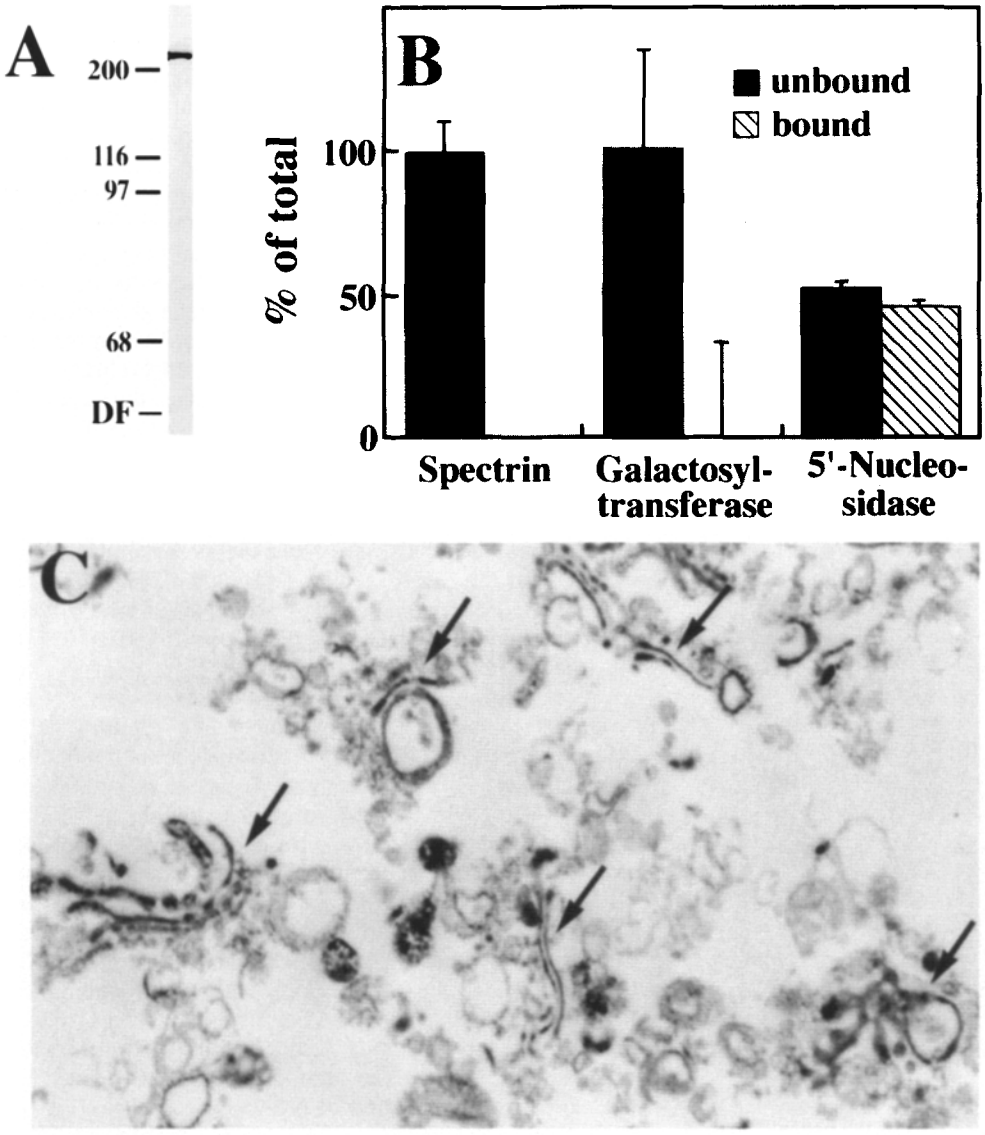


Figure 5. Association of a 220-kD spectrin with rat liver Golgi membranes. (*A*) Purified Golgi membranes were subjected to SDS-PAGE and immunoblotted with βsec-1. Positions of molecular weight standards are indicated to the left (values given are in kD, *DF*, dye front). (*B*) Purified Golgi membranes were fractionated on wheat germ agglutinin agarose as described in the Materials and Methods. Equal amounts of the bound and unbound fractions were assayed for the plasma membrane marker 5'-nucleosidase, the Golgi marker galactosyltransferase, and subjected to SDS-PAGE followed by immunoblotting with affinity purified βspec-1 (error bars represent the mean standard error of quadruplicate measurements). Spectrin could not be detected in the bound fraction. Immunoblots were quantitated by densitometry. (*C*) A representative electron micrograph of pelleted Golgi-enriched membranes before fractionation on WGA agarose. Arrows indicate morphologically distinguishable Golgi cisternae and stacks of cisternae.

cells. Most prominently, we observed a marked increase in diffuse staining throughout the cytoplasm of mitotic cells, as well as a complete loss of staining of reticular elements (Fig. 7, *a* and *b*). Note that interphase cells in the same fields show little or not diffuse cytoplasmic staining compared to mitotic cells. Previous studies (Lucocq et al., 1989) have shown that during mitosis the Golgi apparatus breaks down into multivesicular Golgi clusters and into univesicular structures which are distributed throughout the cytoplasm. Co-localization of β-spectrin with Golgi fragments was not evident (Fig. 7, *c* and *d*). Hence, we conclude that the marked elevation in the intensity of cytoplasmic β-spectrin staining observed in mitotic cells represents the dissociation of β-spectrin from Golgi membranes. In addition to the diffuse cytoplasmic staining, the βspec-1 antibody strongly stained two punctate structures that aligned perpendicular to the plane of the chromosomes (Fig. 7, *e* and *f*). From their morphology and geometric orientation with respect to the chromosomes these structures are centrosomes; the significance of this staining pattern is not known at present.

Depolymerization of microtubules in interphase cells with nocodazole mimics, at least partially, changes in Golgi structure observed during mitosis (Kreis, 1990). Nocodazole-induced microtubule disruption leads to a fragmentation of the Golgi complex resulting in the accumulation of Golgi stacks throughout the cytoplasm. However, in contrast to mitotic cells, the fragmented Golgi stacks remain functional and protein secretion continues under these conditions (Iida and Shibata, 1991). Accordingly, we examined the effects of nocodazole treatment on the distribution of Golgi β-spectrin in NRK cells. Disruption of microtubules by treatment at 4°C in the presence of nocodazole has little or not effect on the morphology of either the Golgi complex or associated Golgi β-spectrin (Fig. 8, *b*, *g*, and *l*). This is consistent with previous studies that have shown that dispersion of the Golgi complex occurs only after cells are warmed above a threshold temperature (34°C; Turner and Tartakoff, 1989), indicating that microtubule depolymerization alone is not sufficient for alterations in Golgi morphology or β-spectrin distribution. Upon warming to 37°C, the Golgi complex be-

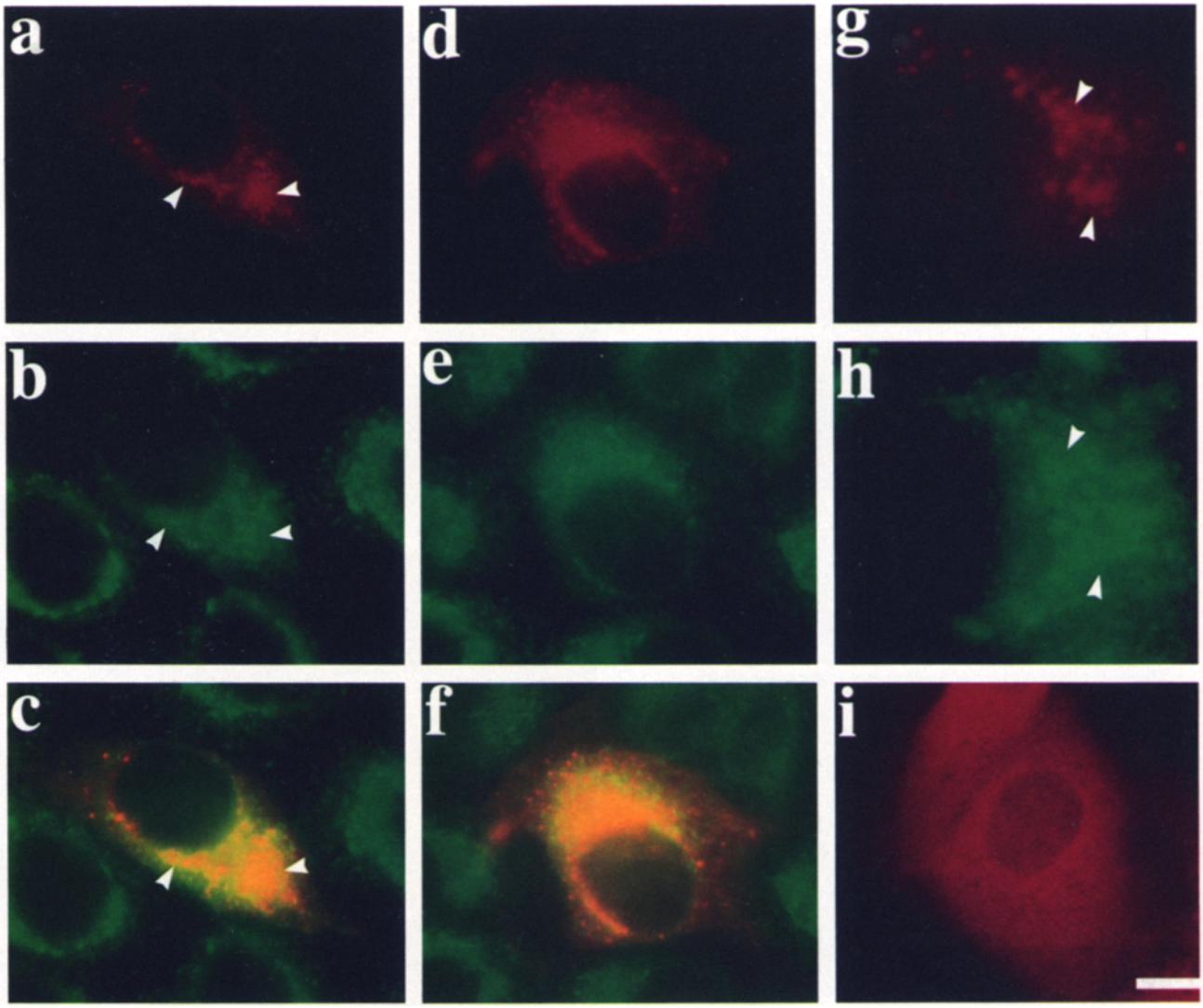


Figure 6. Microinjection of rhodamine-spectrin. Rhodamine-spectrin or rhodamine-dextran were microinjected into living MDCK cells. The cells were then fixed in methanol and stained with the Golgi-specific monoclonal antibody (*a–h*). single cells injected with rhodamine-spectrin were examined for rhodamine fluorescence (*a*, *d*, and *g*), to reveal the distribution of injected spectrin; and fluorescein fluorescence (*b*, *e*, and *h*), to reveal Golgi distribution. Double exposures of rhodamine and fluorescein fluorescence (*c* and *f*) shows that Golgi and rh-spectrin distributions co-localize throughout a large region of the cytoplasm. Discrete features stained by both rh-spectrin and the Golgi monoclonal antibody can be resolved (*arrowheads*). In contrast to the distribution of rh-spectrin, microinjected rh-dextran (*i*) gave a staining pattern indicative of a diffuse cytoplasmic distribution. Images were obtained with a Zeiss Axiophot microscope. Bar, 5 μm.

gan to disperse; fragmentation of the Golgi complex was partial after 20 min (Fig. 8, *c*, *h*, and *m*), extensive within 40 min (Fig. 8, *d*, *i*, and *n*) and complete after 12 h (Fig. 8, *e*, *j*, and *o*). The pattern of β-spectrin distribution differed slightly from that of the Golgi membranes under these conditions. After 20 min, the majority of the β-spectrin signal remained associated with larger perinuclear structures (Fig. 8 *c*), whereas much of the Golgi marker was found associated with small peripheral punctate Golgi stacks (Fig. 8 *m*). While spectrin staining was observed on only a minority of these peripheral Golgi fragments (Fig. 8 *m*), there was no evidence for a general dissociation of spectrin from Golgi membranes. Longer incubations (40 min) resulted in an increase in the number of peripheral punctate structures which were stained with both β-spectrin and Golgi marker antibodies (Fig. 8 *n*). These structures persisted following prolonged incubation of cells at 37°C in nocodazole (12 h, Fig. 8 *o*).

The structural integrity of the Golgi apparatus is also greatly perturbed in the presence of the fungal metabolite brefeldin A (BFA). Previous studies have shown that within a few minutes of BFA treatment, Golgi membranes form an extensive network of tubules which are thought to be involved in the retrograde transport of membranes from the Golgi to the endoplasmic reticulum (for review see Klausner et al., 1992). Longer incubations with the drug results in a complete loss of Golgi structure and an accumulation of Golgi markers within the ER (Lippincott-Schwartz et al., 1990). Similar events occur within the TGN (Wood et al., 1991; Reaves and Banting, 1992). We stained MDBK cells with βspec-1 following treatment for 10 min with BFA (Fig.

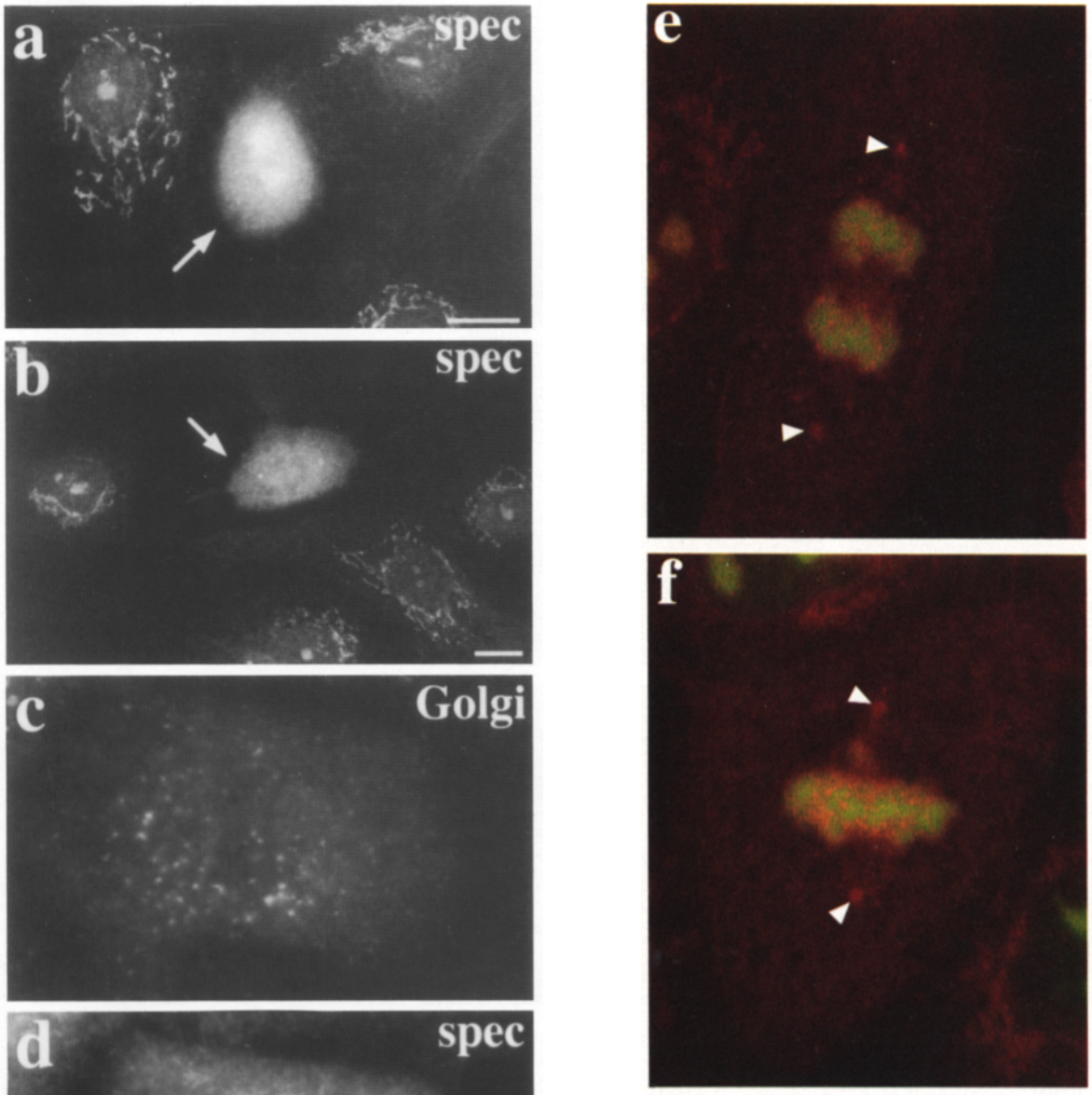


Figure 7. β-Spectrin in mitotic cells. Two separate fields (*a* and *b*) of βspec-1–stained MDCK cells each containing a single mitotic cell (*arrows*) are shown. Note the marked difference in the intensity of diffuse cytosolic staining in the mitotic cells compared to the surrounding interphase cells. A cell which was double-stained with βspec-1 (*d*) and the Golgi monoclonal antibody (*c*) shows that the diffusely distributed staining pattern seen with βspec-1 does not correspond to the distribution of mitotic Golgi fragments localized throughout the cytoplasm. *e* and *f* show mitotic MDCK cells double stained with βspec-1 (*red*) and the DNA intercalating dye YOYO-1 (*green*). It can be seen that the βantibody stained two punctate structures (*arrowheads*) that were aligned perpendicularly to the plane defined by the chromosomal DNA. Bars: (*a* and *b*) 5 μm; (*c* and *d*) 2.5 μm.

9); BFA treatment of MDCK cells has been shown to result in few changes in Golgi morphology (Hunziker et al., 1991). Under these conditions, we detected membranous tubules emanating from both the Golgi (Fig. 9 *f*) and the TGN (Fig. 9 *e*) that were stained with the Golgi-specific and CI-MPR antibodies, consistent with previous reports (Lippincott-Schwartz et al., 1990; Wood et al., 1991; Reaves and Banting, 1992). Co-staining with βspec-1 revealed that β-spectrin was diffusely distributed throughout the cytoplasm under these conditions (Fig. 9 *d*); little or no β-spectrin staining was detected on the membranous tubules, suggesting that β-spectrin had dissociated from Golgi membranes. This

effect was completely reversible; upon removal of BFA, β-spectrin rapidly reassociated with the Golgi membranes coincident with the restoration of steady state Golgi morphology (Fig. 9, *g–i*). This behavior of Golgi β-spectrin in the presence of BFA mirrored that of the Golgi coat protein β-COP, which also undergoes a reversible, BFA-dependent, net loss from Golgi membranes (Donaldson et al., 1990; Orci et al., 1991). Significantly, examination of the time course of the BFA effect shows that both β-spectrin and β-COP dissociate from the Golgi as early as 2 min after the addition of BFA (Fig. 10, *b* and *j*). Thus, there appears to be both a temporally and spatially co-ordinate rapid, net loss of these proteins from Golgi membranes.

In summary, these results show that under conditions where there is a dramatic alteration in Golgi morphology and a complete loss of Golgi function (mitosis and BFA treatment), β-spectrin becomes dissociated from Golgi membranes and appears diffuse throughout the cytoplasm. In contrast, under conditions where Golgi structure is perturbed but without a loss of function (nocodazole treatment), β-spectrin distribution more closely correlates with that of fragmented Golgi membranes. These results imply that the association of β-spectrin with Golgi membranes, and Golgi function are coupled.

Discussion

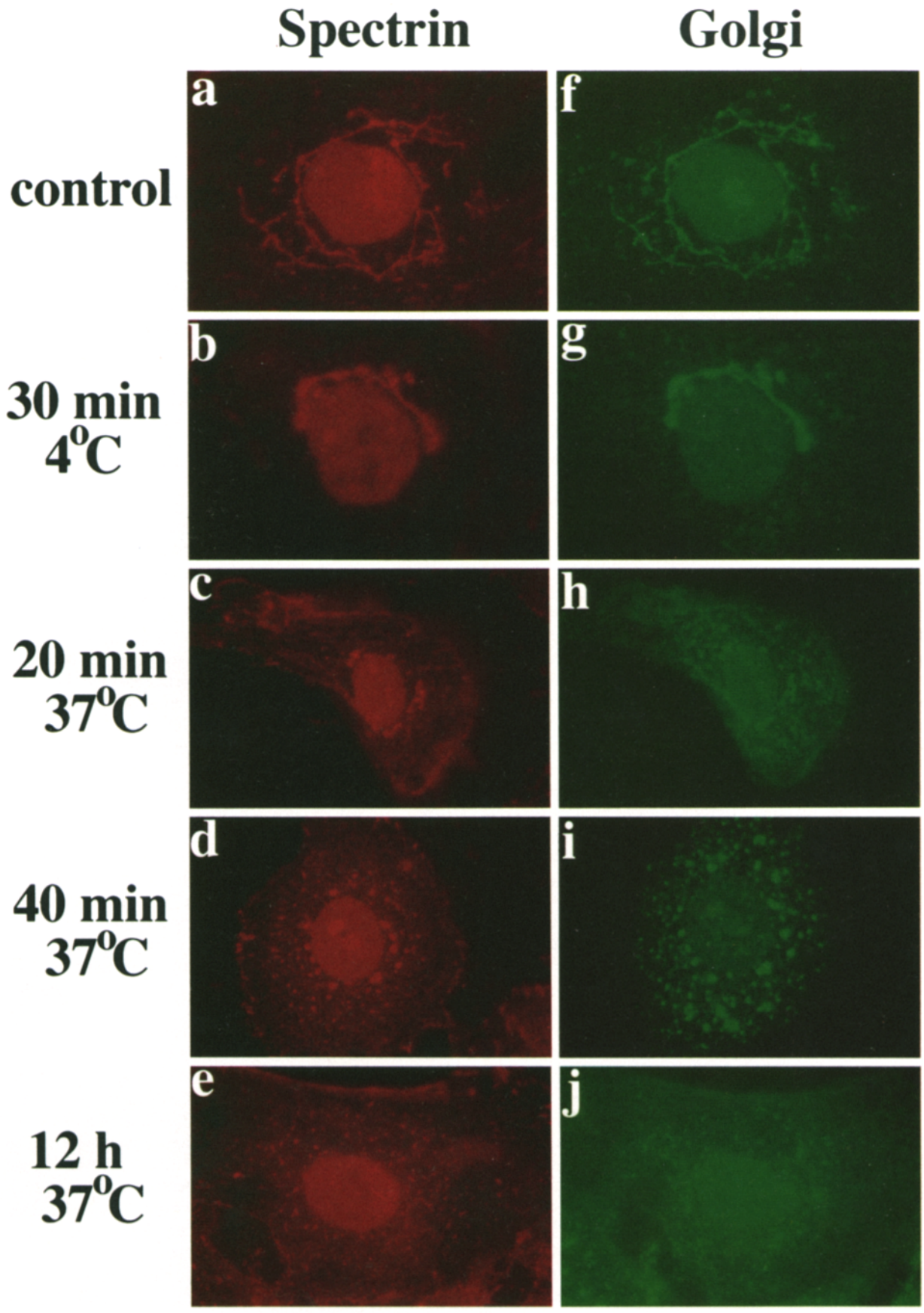
At the level of the plasma membrane, the spectrin-based membrane skeleton plays fundamental roles in the maintenance of the structural integrity of the cell surface and in the formation of discrete membrane domains (Bennett, 1990; Nelson et al., 1990). Neither of these functions, however, are unique to the plasma membrane; intracellular organelles also have a requirement for membrane structural integrity and membrane domain formation. In addition to the localization of spectrin isoforms in plasma membrane domains, we have now identified a homolog of the erythroid isoform of β-spectrin that localizes to the Golgi apparatus of nonerythroid cells, indicating that this family of structural proteins plays a broad role in membrane organization throughout the cell.

Since spectrin was initially identified as a component of a cytoskeletal network which resides and functions at the plasma membrane, it is necessary to consider two important questions related to the distribution of spectrin with Golgi membranes: (*a*) does the antibody βspec-1 recognize a spectrin in nonerythroid cells, and not an unrelated protein with a cross-reactive epitope; and (*b*) are the structures stained by this antibody representative of the Golgi complex? To address the first question, it can be argued that the βspec-1 antiserum was specific for spectrin. First, the antibody was raised against electrophoretically pure β-spectrin which was derived from an already highly purified preparation of spectrin. Second, the spectrin was isolated from erythrocytes, a tissue which lacks a Golgi apparatus and, hence, does not contain Golgi resident proteins that could contaminate the spectrin preparation. Third, the antibody stained erythrocyte plasma membranes (Fig. 1 *C*), and reacted with erythrocyte β-spectrin but not lens fodrin (FIg. 1 *A*), indicating that it is mono-specific for the antigen to which it was raised. Fourth, the antibody immunoprecipitated purified erythrocyte β-spectrin (not shown) and a 220-kD protein from MDCK cells that co-migrated with β-spectrin in SDS–polyacrylamide gels (Fig. 1 *B*). Finally, we prepared a second β-spectrin antiserum which gave identical results to those obtained with the βspec-1 antibody used in these studies (not shown).

That the βspec-1 staining pattern observed in MDCK cells is due to βspec-1 specificity toward an endogenous β-spectrin homolog was first demonstrated by affinity purification of the antibody on spectrin-Sepharose. The affinity purified antibody strongly stained Golgi membranes (Fig. 2), indicating that antibody staining of Golgi membranes and binding to spectrin are directly correlated. In support of this conclusion we found that two independently prepared anti-erythroid β-spectrin antisera, one that was prepared in parallel to βspec-1 (not shown) and a second[1] that was raised against a short peptide corresponding to a unique region of erythroid β-spectrin (Fig. 3, *k* and *l*), also stained the Golgi apparatus. The antigen recognized by the βspec-1 antiserum was found to dissociate from Golgi membranes under different experimental conditions, indicating that βspec-1 recognizes a protein that is peripherally associated with the cytoplasmic face of Golgi membranes, as expected for a spectrin-like molecule. It is interesting to note that in mitotic cells plasma membrane localized spectrin, like Golgi localized β-spectrin, rapidly dissociates from membrane binding sites (Fowler and Adam, 1992). Finally, from preliminary studies we have been able to identify a Golgi-localized ankyrin by immunofluorescence staining and by expression of recombinant ankyrin domains (Beck, K., and W. Nelson, unpublished observations). Taken together these results argue strongly that the staining pattern observed with the βspec-1 antiobdy does in fact reveal an isoform of spectrin that associates with the Golgi complex.

A Golgi localized, 200-kD peripheral membrane protein of unknown function has recently been identified (de Almeida et al., 1993; Narula et al., 1992). Like the Golgi localized spectrin reported here, this protein (p200) becomes rapidly dissociated from the Golgi complex following treatment with BFA (de Almeida et al., 1993; Narula et al., 1992), raising the possibility that p200 and Golgi spectrin are identical. However, this seems unlikely due to the difference in molecular mass between the two proteins as well as the fact that BFA induced dissociation of p200 from Golgi membranes is blocked by pretreatment with aluminum fluoride (de Almeida et al., 1993), whereas we have found that aluminum fluoride has no effect on the sensitivity of Golgi β-spectrin to BFA (data not shown). Moreover, preliminary sequence data for p200 has revealed no sequence homology with spectrin (Jenifer Stow, personal communication). Interestingly, Pimplikar et al. (1994) have identified several high molecular weight proteins in TGN-derived transport vesicle isolates from MDCK cells. These proteins have been proposed to be part of the sorting machinery for membrane proteins, but their relationship to spectrin is unknown.

The intracellular staining pattern of βspec-1 was compared with that of marker proteins of several intracellular compartments which localize to the perinuclear region of the cytoplasm (Golgi complex, TGN, late endosomes and lysosomes, Figs. 3 and 4). Extensive co-localization was observed only with antibodies to Golgi markers (Figs. 3 and 4), and was seen even under conditions when the Golgi was dispersed to the periphery of the cytoplasm by disruption of


Spectrin
Golgi
control
30 min 4°C
20 min 37°C
40 min 37°C
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a
b
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Figure 8.

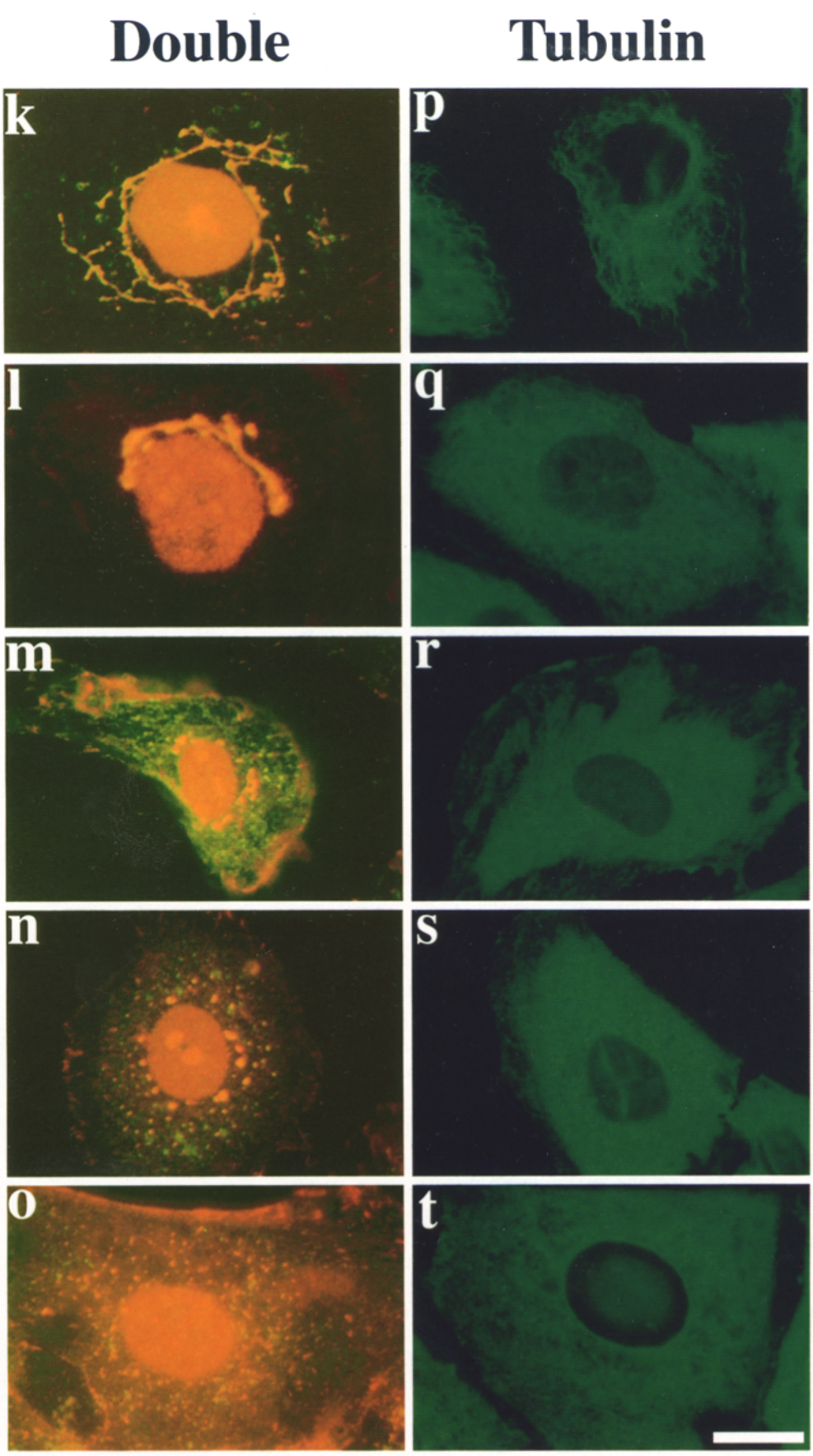

Figure 8. β-Spectrin distribution in nocodazole-treated MDCK cells. Subconfluent cultures of NRK cells were either untreated (*control*; *a*, *f*, *k*, and *p*) or treated with nocodazole (33 μM) for 30 min at 4°C to disrupt microtubules. This was followed by incubation for either 30 min at 4°C (*b*, *g*, *l*, and *q*), 20 min at 37°C (*c*, *h*, *m*, and *r*), 40 min at 37°C (*d*, *i*, *n*, and *s*) or 12 h at 37°C (*e*, *j*, *o*, and *t*) in the continuous presence of nocodazole. The cells were fixed in 1.9% formaldehyde and double stained with affinity purified βspec-1 (*a*, *b*, *c*, *d*, and *e*) and the Golgi monoclonal antibody (*f–j*), or stained independently with a monoclonal antibody directed against β-tubulin (*p–t*). Double exposures for each of the double-stained cells are also shown (*k–o*). Note that the βspec-1 antibody gave significant nuclear staining in this experiment (*a*, *d*, *g*, and *j*). Nuclear staining, which was often observed with this antibody, was dependent on fixation conditions and could frequently be reduced by treatment with DNAse I. Images were obtained using a Zeiss Axiophot microscope. Bar, 5 μm.

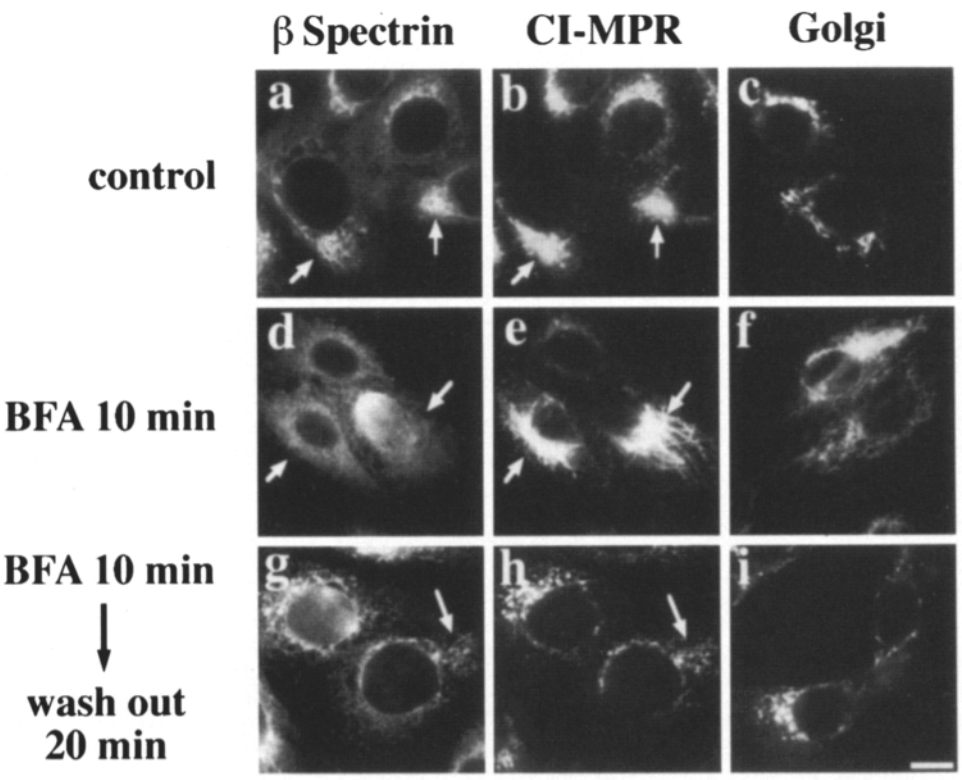

Figure 9. β-Spectrin distribution in MDBK cells treated with BFA. Subconfluent cultures of MDBK cells were either untreated (*a–c*), treated for 10 min with 5 μg/ml BFA (*d–f*), or treated for 10 min with BFA followed by a subsequent incubation for 30 min in the absence of BFA (*g–i*). The cells were then fixed either in ice cold methanol (*c*, *f*, and *i*), or 1.9% formaldehyde followed by extraction in 0.01% saponin (*a*, *b*, *d*, *e*, *g*, and *h*), and stained with the Golgi monoclonal antibody (*c*, *f*, and *i*), or double stained with affinity purified βspec-1 (*a*, *d*, and *g*) and the CI-MPR monoclonal antibody (*b*, *e*, and *h*). Treatment for 10 min in BFA was sufficient to cause extensive tubularization of both Golgi (*f*) and TGN membranes (*e*). The arrows indicate regions of the double-labeled cells which contain abundant TGN and Golgi membranes. Note that in cells treated for 10 min with BFA (*d* and *e*) there is no evidence for an accumulation of β-spectrin (*d*) in these regions. Images were obtained using a Zeiss Axiophot microscope. Bar, 5 μm.

microtubules (Fig. 7). The Golgi localization of β-spectrin was confirmed by Western blot analysis of purified rat liver Golgi membranes which revealed an immunoreactive, 220-kD species that co-purified with the Golgi membrane fraction (Fig. 5). Independent evidence for an association of spectrin with the Golgi was also obtained by microinjection of rhodamine-spectrin which accumulated as punctate perinuclear structures that co-localized with Golgi membranes (Fig. 6). Since our initial attempts to localize β-spectrin by immuno-electron microscopy have been unsuccessful, we were not able to identify the precise Golgi compartment involved in β-spectrin binding. However, it is worth noting that in our high magnification images of double-stained NRK cells (Fig. 4 *d*) we found regions of the Golgi complex that were stained with βspec-1 but not with the anti-mannosidase II antibody. Although the exact identity of these regions can not be specified as yet, their existence indicates that spectrin may not be uniformly distributed throughout the Golgi complex.

While our results represent the first definitive demonstration of Golgi-localized spectrin, earlier studies established the precedent for the association of spectrin isoforms with other intracellular membrane systems, including: chromaffin granules, synaptic vesicles, endoplasmic reticulum, nuclear membrane, mitochondria, as well as other unidentified vesicles (Zagon et al., 1986; Aunis and Perrin, 1984). In lymphocytes, a large cytoplasmic aggregate of spectrin is located in the perinuclear region of the cytoplasm, but not in association with morphologically distinguishable Golgi cisternae (Black et al., 1988). Finally, in rat spermatids, an erythroid isoform of spectrin was found in the perinuclear region (De Cesaris et al., 1989), but double-labeling experiments were not performed to definitively establish co-localization of spectrin with Golgi markers.

β-Spectrin and Golgi Structure and Function

Our studies indicate that β-spectrin is likely to play a fundamental role in Golgi structure and function. We have found that βspec-1 stains the Golgi apparatus of a variety of tissue culture cell lines including MDCK, MDBK, NRK, cos, and L cell fibroblasts (Figs. 1–4 and not shown). This association of β-spectrin with the Golgi complex in different species and tissues is consistent with a conserved role for the protein in Golgi function.

Examination of the behavior of Golgi β-spectrin under conditions where Golgi structure and function was perturbed (mitosis, BFA, nocodazole) demonstrated a correlation between spectrin dissociation from the Golgi and disruption of Golgi structure and function. When both steady state Golgi structure and function were abolished (mitotic cells and cells treated with BFA), we found that β-spectrin was dissociated from Golgi membranes and appeared to be diffusely distributed in the cytoplasm. In contrast, when the Golgi was fragmented without a loss of function (nocodazole), β-spectrin association with Golgi membranes was ultimately maintained. These results imply that the interaction of β-spectrin with the Golgi is tightly coupled to normal Golgi structure

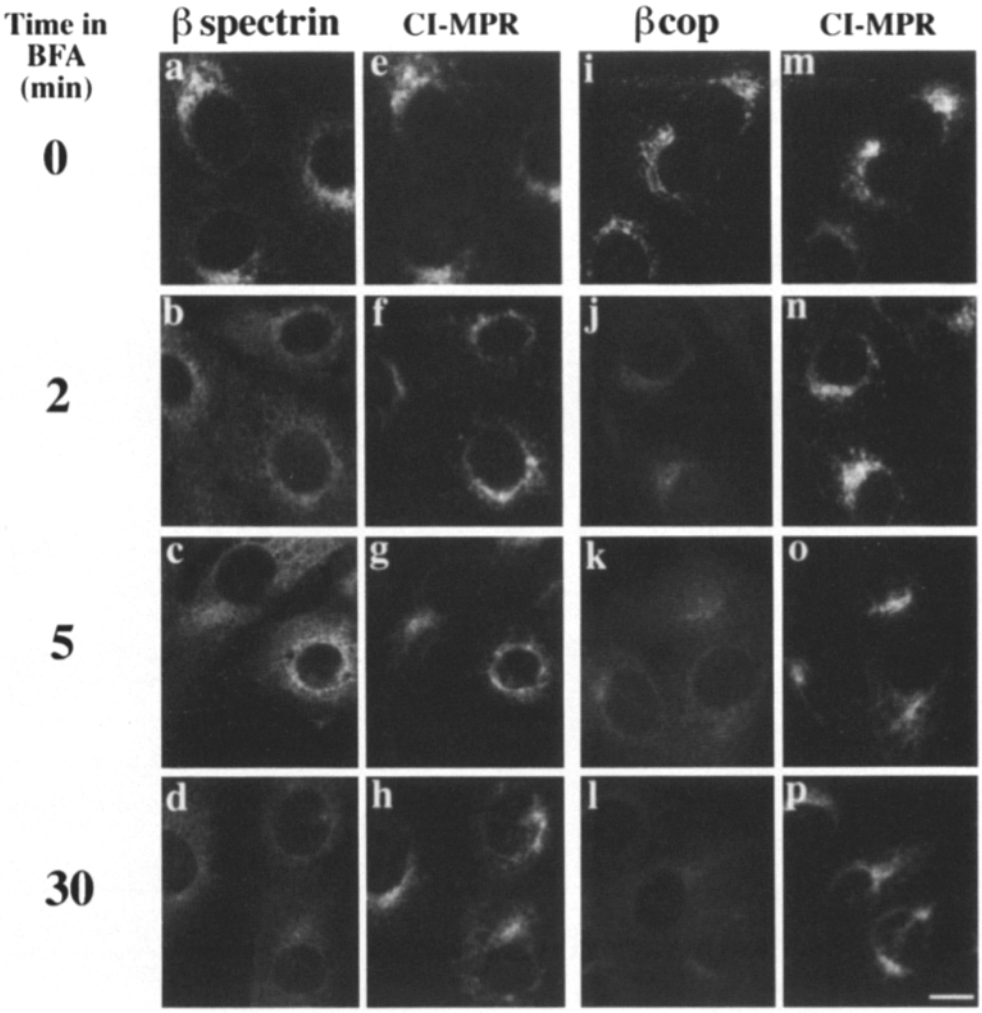

Figure 10. Time course of β-spectrin and β-COP redistribution following BFA treatment. MDBK cells were treated with 5 μg/ml BFA for 0 min (*a*, *e*, *i*, and *m*), 2 min (*b*, *f*, *j*, and *n*), 5 min (*c*, *g*, *k*, and *o*), or 30 min (*d*, *h*, *l*, and *p*). Following fixation in 1.9% formaldehyde and permeabilization in 0.01% saponin, the cells were double stained either with affinity-purified βspec-1 (*a–d*) and CI-MPR antibody (*e–h*), or with anti-β-COP (*i–l*) and anti-CI-MPR (*m–p*). Note that as early as 2 min following BFA treatment there is evidence for dissociation of both β-spectrin and β-COP from Golgi membranes revealed by a decrease in the intensity of Golgi staining relative to an increase in diffuse cytoplasmic staining. The images were obtained with a scanning laser confocal microscope. Bar, 5 μm.

and function. While this correlation is consistent with several roles for β-spectrin at the level of the Golgi, consideration of our present understanding of functions already proposed for the plasma membrane–localized membrane skeleton can help to specify which of these are likely.

In erythrocytes, spectrin self-associates and binds to specific soluble and integral membrane proteins to form an extensive cytoskeletal meshwork that is tightly associated with the plasma membrane (Bennett, 1990*a*,*b*). Based upon these properties, it has been proposed that a major function of spectrin is to provide structure integrity to the plasma membrane. Evidence in support of a structural role for spectrin at the level of the Golgi apparatus is supported by our observations of spectrin distribution in mitotic MDCK cells. We found that β-spectrin dissociated from Golgi membranes at the time when the Golgi complex is extensively fragmented and dispersed (Fig. 7). Similarly, treatment with BFA, which also resulted in an extensive alteration in Golgi morphology, was accompanied by the dissociation of Golgi β-spectrin (Figs. 9 and 10). These results support the view that under steady state conditions β-spectrin may form a cytoskeletal meshwork, analogous to the one found on the erythrocyte plasma membrane, which associates with Golgi membranes and serves to maintain the structural integrity of the Golgi complex. Thus, dissociation of this meshwork, revealed by the accumulation of spectrin in the cytoplasm, would be expected to coincide with the loss of morphological integrity of the Golgi complex and subsequent fragmentation as observed in our experiments.

In the erythrocyte, the spectrin-based membrane skeleton has also been shown to restrict the mobility of membrane proteins in the plane of the membrane (Sheetz et al., 1980; Tsuji and Ohnishi, 1986; Tsuji et al., 1988). In nonerythroid cells, this function serves to restrict membrane protein distribution resulting in the formation of membrane domains (Hammerton, 1991; Seimers et al., 1993). It is possible, therefore, that Golgi β-spectrin may serve a similar function within the Golgi stacks by selectively binding to resident Golgi membrane proteins and restricting their mobility and hence their access to the vesicular transport apparatus. In the context of this function, it is interesting that BFA treatment results in a loss of retention of resident Golgi and TGN marker proteins (Lippincott-Schwartz et al., 1990; Wood et al., 1991; Reaves and Banting, 1992). There is also evidence for a loss of Golgi membrane protein retention during mitosis, where Golgi markers have been found to accumulate in fragments of the endoplasmic reticulum (Thyberg and Moskalewski, 1992). Our results demonstrate that under two different conditions, the dissociation of β-spectrin from Golgi membranes is accompanied by loss of retention of resi-

dent Golgi proteins, suggesting a role for β-spectrin as a Golgi membrane protein retention system.

It is also possible that β-spectrin may play a role in direct sorting of proteins into the vesicular transport pathway. After very short incubations in BFA (2 min), we found a temporally and spatially coordinate net loss of β-spectrin and β-COP from Golgi membranes. β-COP is a constituent of the coatomer protein complex that mediates vesicular transport between the ER and Golgi, and within the Golgi stack (Serafini et al., 1991). Although the molecular mechanism of coatamer function has not been completely defined, it is likely that it functions in a way analogous to clathrin-coated pits of the plasma membrane and the TGN (Rothman and Orci, 1992). Interestingly, the clathrin-associated protein AP-1, which localizes to coated pits of the TGN (Ahle et al., 1988), is also dissociated from the Golgi complex in the presence of BFA (Wong and Brodsky, 1992). Clathrin and associated proteins bind to specific membrane proteins and oligomerization of the clathrin lattice results in the formation of a discrete membrane domain enriched in proteins destined for transport (Brodsky, 1988; Keen, 1990). Coat proteins, therefore, appear to direct the sorting of membrane proteins through their ability to perform two key functions: selective membrane protein binding and coat protein oligomerization. Both of these functions are characteristic of the spectrin-based membrane skeleton. Hence, the spectrin membrane skeleton, Golgi coatamers and clathrin-coated pits all share, in addition to a sensitivity to BFA, basic structural features characteristic of membrane protein sorting machines.

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