The Golgi-associated Hook3 Protein Is a Member of a Novel Family of Microtubule-binding Proteins

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Abstract. Microtubules are central to the spatial organization of diverse membrane-trafficking systems. Here, we report that Hook proteins constitute a novel family of cytosolic coiled coil proteins that bind to organelles and to microtubules. The conserved NH_2-terminal domains of Hook proteins mediate attachment to microtubules, whereas the more divergent COOH-terminal domains mediate the binding to organelles. Human Hook3 bound to Golgi membranes in vitro and was enriched in the cis-Golgi in vivo. Unlike other cis-Golgi–associated proteins, however, a large fraction of Hook3 maintained its juxtanuclear localization after Brefeldin A treatment, indicating a Golgi-independent mechanism for Hook3 localization. Because overexpression of Hook3 caused fragmentation of the Golgi complex, we propose that Hook3 participates in defining the architecture and localization of the mammalian Golgi complex.

Key words: membrane trafficking • Hook protein • endosomes • brefeldin A • Golgi complex

Introduction

The positioning, trafficking, and architecture of various membrane compartments rely on microtubules (Schroer, 2000). For example, the microtubule organizing center (MTOC)[1] specifies the characteristic perinuclear position of secretory and endocytic pathway components (Matteoni and Kreis, 1987; Ho et al., 1989; Bloom and Goldstein, 1998). Within each pathway, vesicular transport between compartments is organized along microtubules: trafficking from early to late endosomes depends on microtubules, as does retrograde and anterograde trafficking between the Golgi complex and the ER (Gruenberg et al., 1989; Strous et al., 1991; Aniento et al., 1993; Lippincott-Schwartz, 1998). Furthermore, after the dispersion of the Golgi complex during mitosis, appropriate reformation of the juxtanuclear Golgi ribbon requires microtubules (Lippincott-Schwartz, 1998; Shima et al., 1998). This functional relationship between microtubules and Golgi membranes is reflected in their extensive interdigitation about the MTOC (Cole and Lippincott-Schwartz, 1995).

Two families of motor proteins mediate organelle transport along microtubules (Hirokawa, 1998). Different members of the Kinesin family carry specific membranous cargos along microtubules (Goldstein and Philp, 1999). Dynein family members carry cargo from the cell periphery towards the juxtanuclear minus ends of microtubules located at the MTOC. Interference with dynein function results in the mislocalization of elements of the secretory and the endocytic compartments toward the cell periphery (Burkhardt et al., 1997; Presley et al., 1997; Itin et al., 1999). In cells lacking cytoplasmic dynein, however, mislocalized organelles are still attached to microtubules (Harada et al., 1998), suggesting the existence of additional linker proteins.

Such a function in organelle positioning has been suggested for the family of cytoplasmic linker proteins (CLIPs; for review see Schroer, 2000). CLIPs share a microtubule-binding domain, and, as these proteins lack a motor domain, their function may be comparable to the molecular tethers identified in vesicle fusion events (Pfeffer, 1999). The founding member of this family, CLIP-170, binds to endosomes and to microtubules (Pierre et al., 1992). Similarly, CLIP-115 is a brain-specific protein that links a unique membrane compartment, the dendritic lamellar body, to microtubules (De Zeeuw et al., 1997). Another CLIP-like protein, GMAP210, exhibits Golgi specificity (Infante et al., 1999). The number of CLIPs assigned to specific compartments is very limited, however, suggest that other proteins with similar function exist (Schroer, 2000).

In this report, we show that Hook proteins constitute a novel family of microtubule-binding proteins that may link membrane compartments to microtubules. The *Drosophila* Hook protein (dHK) is a cytosolic endosome-asso-

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[1]*Abbreviations used in this paper:* aa, amino acids; BfA, Brefeldin A; CLIP, cytoplasmic linker protein; dHK, *Drosophila* Hook protein; hHK1, human Hook1 protein; hHK2, human Hook2 protein; hHK3, human Hook3 protein; MTOC, microtubule organizing center; MVB, multivesicular body; NZ, nocodazole; RT, room temperature.

ciated protein necessary for normal trafficking of endocytosed ligands (Krämer and Phistry, 1996, 1999). Our analysis of *Drosophila* mutants lacking Hook protein revealed that dHK functions to assemble or stabilize mature multivesicular bodies (MVBs), an intermediate compartment in the endocytic pathway (Sunio et al., 1999). Here, we introduce three human Hook proteins, which exhibit different compartmental specificities. Although the Hook proteins localize to distinct organelles, they share a novel microtubule-binding domain. Through its interaction with microtubules, the human Hook3 protein (hHK3) may serve in the positioning of the mammalian Golgi complex.

Materials and Methods

Molecular Cloning and Sequencing of hHK3

The human EST database (dbEST, GenBank) was searched for Hook homologues using the BLAST program (Altschul et al., 1990). Among the I.M.A.G.E. Consortium cDNA clones (Lennon et al., 1996), this search revealed several overlapping cDNA clones (2662038, 1662556, 379556, and hp0259) representing the same ORF encoding human Hook3. The full-length human Hook3 sequence was obtained by 5′ and 3′ RACE reactions from a human placental cDNA library (CLONTECH Laboratories, Inc.). The sequence of hHK3 has been deposited in Genbank (sequence data available from GenBank/EMBL/DDBJ under accession number AF241830).

Expression Constructs and Transfections

For expression in mammalian cells, PCR-generated cDNAs encoding full-length hHK3 or the COOH-terminal truncations of ΔC-hHK$1_{1–555}$, ΔC-hHK$2_{1–548}$, and ΔC-hHK3_{1-555}, which were all tagged with a COOH-terminal Myc epitope, were inserted between the Asp718 and XhoI sites of pCDNA3.1 (Invitrogen). Other hHk3 truncations and fusions with dHk (either amino acids [aa] 6–562 or 287–679) were generated in pCDNA3.1 with a NH_2-terminal hemagglutinin epitope tag. HEK293, Cos7, or Vero cells were transfected using Lipofectamine (GIBCO BRL).

Cell Lines and Antibodies

HEK293, HeLa, Hep2, normal rat kidney, and Vero cells were obtained from the American Type Culture Collection and cultured using standard techniques. Antibodies used were directed against FTCD (previously 58K; Bashour and Bloom, 1998), β-COP (Pepperkok et al., 1993), GM130 (Transduction Laboratories), LAMP-1 (Chen et al., 1985), transferrin receptor (Boehringer), *Drosophila* Hook (Krämer and Phistry, 1996), ERD2/KDEL receptor (Majoul et al., 1998), clathrin (Brodsky, 1985), LBPA (Kobayashi et al., 1998), M6PR (Boker et al., 1997), calnexin (Affinity BioReagents, Inc.), TGN46 (Serotec), ERGIC-53 (Schindler et al., 1993), α- or γ-tubulin (Sigma-Aldrich), syntaxin5 (Rowe et al., 1998), and Cox1 (Molecular Probes).

For the generation of antibodies against human Hook proteins, glutathione *S*-transferase fusion proteins of aa 438–728 from hHK1, 427–719 from hHK2, and 423–630 from hHK3 were purified from bacteria and injected into rabbits or mice. The corresponding purified His_6-tagged fusion proteins were used for preabsorbing specific antibodies in control experiments and for affinity purification (Harlow and Lane, 1988). For multiple tissue Western blots, tissues were harvested from mice, homogenized in SDS loading buffer at 25 μg wet tissue/μl, separated by SDS-PAGE, and probed by Western analysis as described (Sevrioukov et al., 1999).

Immunofluorescence Labeling and Imaging

Immunofluorescence staining was essentially as described (Krämer and Phistry, 1996), after fixing cells using methanol at −20°C or 4% paraformaldehyde. Endogenous human Hook proteins were detected using affinity-purified antibodies raised in rabbits. Secondary antibodies were coupled to Alexa488 or Alexa568 (Molecular Probes). When double labeling hHK3-overexpressing cells with rabbit anti-KDEL receptor antibodies (Majoul et al., 1998), we used anti-hHK3 sera raised in mice, anti-Myc, or antihemagglutinin antibodies.

For double labeling with two primary antibodies raised in rabbits (hHK3 and syntaxin5), hHK3 antibodies were labeled using a digoxigenin labeling kit (Molecular Probes). Cells were first stained with anti-syntaxin5 antibodies and goat anti–rabbit Fab fragments (Alexa488), followed by digoxigenin-labeled anti-hHK3 antibodies, mouse anti-digoxigenin antibodies (The Jackson Laboratory), and, finally, goat anti–mouse antibodies (Alexa568).

Images were captured using a MRC1024 confocal laser-scanning microscope (Bio-Rad Laboratories) or a cooled ORCA charge-coupled device camera (Hamamatsu); images were prepared for publication using Adobe Photoshop. For nocodazole (NZ) or Brefeldin A (BfA) treatments, cells were incubated for 1 h in normal growth media supplemented with 10 μg/ml NZ or 10 μg/ml BfA. To bundle microtubules in vivo, cells were treated for 1 h with 10 μg/ml NZ, which was washed out and replaced for 2–4 h with media supplemented with 40 μM taxol, after which cells were processed for immunofluorescence imaging.

Cross-linking and Immunoprecipitation

Three 10-cm dishes of HEK293 cells (~75% confluent) were harvested and dounce homogenized. Nuclei and cell debris were sedimented at 1,000 *g* at 4°C. The postnuclear supernatant collected was adjusted to 1% Triton X-100. For cross-linking, BS^3 (Pierce Chemical Co.) was added to 100 μM and incubated for 30 min at room temperature (RT) with gentle mixing. To quench reactive groups, ethanolamine was added to 100 mM and incubated 30 min at RT. Samples were immunoprecipitated by incubation for 1 h at 4°C with the appropriate antibody and protein–A agarose and were analyzed by Western blotting (Sevrioukov et al., 1999).

For binding assays, cytosol was prepared from HEK293, *Drosophila* S2 cells, and the transfected cell lines HEK293:ΔC-hHK1_{1-555}, HEK293:ΔC-hHK2_{1-548}, or HEK293:ΔC-hHK3_{1-555}, as described (Ktistakis et al., 1996). Protein concentrations were 7–13 mg/ml for all preparations; 50-μl aliquots were stored at –80°C.

Microtubule-binding Assays

Microtubule spin-down assays were performed using the microtubule-associated protein spin-down kit (Cytoskeleton) according to the manufacturer's instructions. In brief, microtubules were assembled from purified bovine brain tubulin for 20 min at 35°C in the presence of GTP and stabilized with taxol. Assembled microtubules (10 μg) were incubated with 30 μg of cytosolic proteins in total volume of 50 μl for 20 min at RT. Microtubules and associated proteins were pelleted at 100,000 *g* through a 40% glycerol cushion. Pellets were dissolved in 10 μl SDS-loading buffer and compared with 10 μl from the supernatant by Western analysis.

To test for direct binding to microtubules, the His_6-tagged fusion proteins His_6–hHK3N$1_{1–164}$, hHK3N$2_{1–224}$, and hHK3C$C_{423–630}$ were bound to Ni^{2+}–agarose beads at a concentration of 6–8 mg/ml. For the binding assay, stabilized microtubules were prepared as follows: 2.5 μl microtubule cushion buffer (PEM [80 mM Pipes, pH 7.0, 1 mM EGTA, 1 mM Mg^{2+}, 1 mM GTP] plus 40% glycerol) was added to 20 μl tubulin (5 mg/ml) and incubated 20 min at 35°C. Taxol was added to 180 μl G-PEM (PEM plus 1 mM GTP) to a final concentration of 40 μM. After 20 min of microtubule assembly, 180 μl prewarmed taxol–G-PEM (35°C) was added, and the reaction was stored at RT. 20-μl beads coupled to recombinant proteins were washed once with 100 μl G-PEM and mixed with 50-μl stabilized microtubules. After 2 h at RT, beads were pelleted (1,000 *g* for 5 min at RT), and a 10-μl aliquot was collected from the supernatant for analysis. The beads were washed three times with 500 μl G-PEM, and bound tubulin was eluted with 10 μl SDS loading buffer. Relative amounts of tubulin were compared by Western analysis.

For testing for the binding of dHK to endogenous microtubules, 100 μg *Drosophila* cytosolic proteins were adjusted to 20 μl with G-PEM (+) or PEM (−). 32.5 μl PEM was added to the sample (−) and applied to 100 μl cushion buffer without taxol. 5 μl of cushion buffer was added to the sample (+) and incubated 20 min at 35°C. 30 μl G-PEM plus 80 μM taxol was added to the (+) sample and incubated at RT for 20 min, and the sample was applied to 100 μl of cushion buffer (20 μM taxol). Both samples were spun 40 min at 100,000 *g* at 25°C. 10 μl were collected from the supernatants remaining above the cushion. 50 μl were aspirated, and the cushions were rinsed with 50 μl G-PEM. 110 μl were aspirated from the remaining cushion, and the pellets were harvested in SDS-loading buffer and analyzed by Western blotting. Blots were probed for both dHK and tubulin after cutting the blot at ~67 kD, and were then reassembled for chemiluminescent detection.

Binding to Golgi Membranes

Highly enriched Golgi membranes were a gift from Michael G. Roth (University of Texas Southwestern, Dallas, TX; Ktistakis et al., 1995). This Golgi membrane fraction had been purified from CHO cells. By electron microscopy, half the membranes were identifiable Golgi stacks and they were 20-fold–enriched in mannosidase II activity compared with whole cell membranes (Ktistakis et al., 1996). 120 μl Golgi membranes (200 μg protein/ml) were diluted with 800 μl buffer B (25 mM Hepes, pH 7.3, 25 mM KCl, 2.5 mM $MgSO_4$, 1 mM DTT, 0.2 M sucrose, protease inhibitors), incubated 5 min on ice, and then pelleted (15,000 *g* for 9 min at 4°C). Membranes were resuspended in 120 μl buffer B plus 0.1 mg/ml BSA (to a final concentration of 0.2 mg/ml Golgi protein). 10 μl membranes were incubated with ~30 μg cytosolic proteins in a total volume of 50 μl for 20 min at 37°C. The reaction was cooled on ice for 5 min, and then spun at 15,000 *g* for 9 min at 4°C. A 10-μl aliquot was taken from the supernatant and combined with 5 μl SDS-loading buffer. The pellet was resuspended in 20 μl buffer B and spun through 100 μl 20% sucrose cushion for 30 min at 15,000 *g* at 4°C in a swinging bucket rotor. 30 μl the supernatant was aspirated, and the cushion washed with 50 μl buffer B. The pellet containing membranes was collected with 10 μl SDS-loading buffer (5% SDS, 50 mM Tris/HCl, pH6.8) for Western analysis.

Electron Microscopic Immunocytochemistry

Vero cells were fixed in 3% paraformaldehyde, 0.2% glutaraldehyde in PBS (pH 7.4) for 20 min at RT, permeabilized in 0.1% saponin/PBS for 20 min, blocked in 1% normal goat serum, 1% BSA in PBS for 30 min, and then incubated with anti-hHK3 antibodies (purified IgG fraction, dilution 1:200) in blocking buffer for 24 h at 4°C. Primary antibodies were detected with Nanogold–Fab conjugates (Nanoprobes) for 48 h at 4°C; silver enhancement was performed with IntenSE M kit (Amersham Pharmacia Biotech) for 10 min at RT. The cells were postfixed with 0.5% OsO_4 for 30 min, dehydrated with an ascending series of ethanol ≤100%, embedded in Poly/Bed 812 media (Polysciences, Inc.), and polymerized at 60°C for 48 h. Ultrathin sections (60 nm) were cut with Leica Ultracut UCT microtome and stained with 5% uranyl acetate before observation.

Results

A Family of Human Hook Proteins

The unique role of dHK in endocytic trafficking (Sunio et al., 1999) prompted us to explore the functions of mammalian Hook proteins. The cloning of hHK1 and hHK2 has been previously described (Krämer and Phistry, 1999). Further screening of the human EST database revealed a cDNA encoding a fragment of hHK3. A clone encoding the full-length hHK3 protein of 718 aa was obtained by 5′ and 3′ RACE reactions (Materials and Methods). Comparison of homology across the Hook family defined several domains of similarity (Fig. 1 A). The highest degree of sequence identity is found in the acidic NH_2-terminal globular domain. An extended central coiled coil motif, which mediates homodimerization in dHK (Krämer and Phistry, 1996; Sevrioukov et al., 1999), is also conserved. The basic COOH terminus, which is predicted to be globular in structure, is the most divergent segment in these proteins.

To begin characterization of these proteins, we affinity purified specific antisera against each of the three human Hook proteins. When tested on crude lysates from HEK293 cells, the antisera against hHK1, hHK2, and hHK3 detected endogenous proteins that migrated slightly slower than predicted from their molecular masses of 85 kD for hHk1, 83 kD for hHK2, and 83 kD for hHK3 (Fig. 1 B, lanes 1, 3, and 5). The signals were abolished by preincubation with an excess of the respective His_6–Hook fusion proteins (Fig. 1 B, lanes 2, 4, and 6), confirming the specificity of the antibodies.

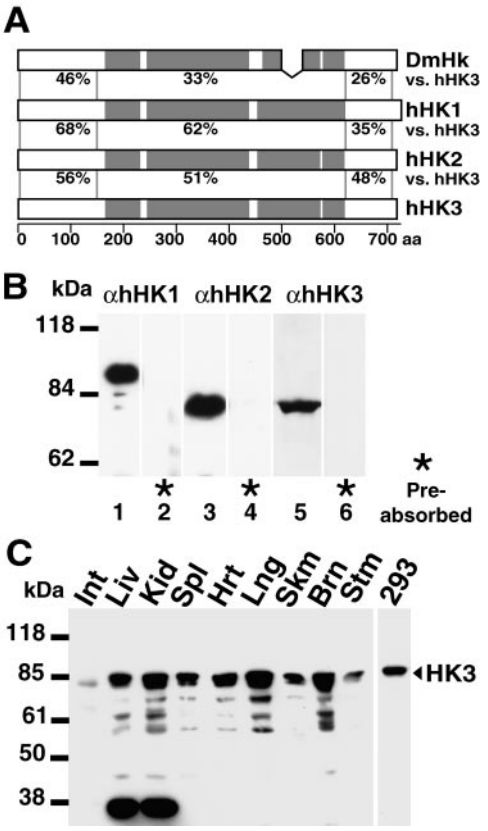


Figure 1. The Hook family of proteins has a conserved NH_2-terminal domain and central coiled coil with a divergent COOH-terminal domain. (A) Hook proteins are schematically aligned, and percent identities are indicated for the NH_2- and COOH-terminal globular domains and the central coiled coil (gray) compared with hHK3. The sequence of the hHK3 protein was deposited in Genbank (sequence data available from GenBank/EMBL/DDBJ under accession number AF241830). (B) Endogenous Hook proteins in HEK293 cell lysates were detected with affinity-purified antisera. Proteins close to the predicted size for hHK1 (lane 1), hHK2 (lane 3), and hHK3 (lane 5) were specifically recognized. Preabsorption of antibodies with 10 μg/ml of a corresponding His_6 fusion protein abolished detection (lanes 2, 4, and 6). (C) Expression of Hook3 protein was detected in all murine tissues examined, 293 cells, and all human and monkey cell lines tested (data not shown). We do not know whether the bands of ~36 kD detected in liver and kidney cells correspond to degradation products, alternative splice forms, or cross-reacting proteins. Int, intestine; Liv, liver; Kid, kidney; Spl, spleen; Hrt, heart; Lng, lung; Skm, skeletal muscle, Brn, brain; Stm, stomach; 293, HEK293 cells.

Hook Family Members Exhibit Distinct Subcellular Localizations

Because of the similarity of coiled coil domains between the three human Hook proteins, we used a combination of cross-linking and immunoprecipitation experiments to determine whether Hook proteins form heterodimers. Postnuclear supernatants from HEK293 cells were immunoprecipitated with antibodies specific for each Hook protein, either directly or after cross-linking with 100 μM BS^3. Western analysis of the immunoprecipitates revealed that the endogenous Hook proteins exist as members of three separate protein complexes (Fig. 2). All three hu-

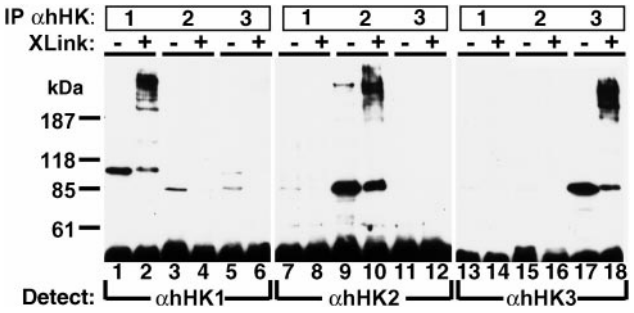


Figure 2. Endogenous human Hook proteins function in distinct complexes. HEK293 postnuclear supernatants were subjected to immunoprecipitation using the indicated hHK antisera (IP αhHK), either directly (−) or after cross-linking with 100 μM BS^3 (+). Precipitated Hook proteins were detected with the respective hHK antiserum by Western blotting. Each hHK antiserum is able to specifically immunoprecipitate its respective endogenous protein from noncross-linked samples (hHK1, lane 1; hHK2, lane 9; hHK3, lane 17). In each of the cross-linked samples, high molecular weight complexes are detected with the specific anti-hHK antibodies used for their immunoprecipitation (hHK1, lane 2; hHK2, lane 10; hHK3, lane 18) but not the other two anti-hHK antibodies (lanes 4, 6, 8, 12, 14, and 16). The identity of the bands at ~85 kD in lanes 3 and 5 has not been determined.

man Hook proteins were predominantly found in complexes larger than expected for Hook dimers, but putative additional components of these complexes have not yet been identified. When complexes immunoprecipitated with one antibody were probed for the presence of the two other human Hook proteins, none was detected (Fig. 2, lanes 4, 6, 8, 12, 14, and 16). These results demonstrate that endogenous Hook proteins do not heterodimerize and suggest separate functions for the three human Hooks.

A distinct role for each of the Hook proteins was also supported by their different subcellular localizations. Endogenous Hook proteins were localized using confocal microscopy and double immunofluorescence labeling with well-characterized markers in Hep2 and Vero cells (Fig. 3; and data not shown). hHK1 (Fig. 3 A) and hHK2 (Fig. 3 D) localize to discrete punctate subcellular structures. Especially in the case of hHK2, the structures appeared often closely associated with microtubules. This was most easily visualized in the thin layer of cytosol just above the nucleus (Fig. 3, G–I). The identity of these structures has been elusive thus far: neither hHK1 nor hHK2 colocalized with antibodies labeling early endosomes (anti-EEA1 or antitransferrin receptors), MVBs (6C4), late endosomes (anti-M6PR), lysosomes (Lamp1), the ER (Calnexin), Golgi complexes (FTDC), or mitochondria (Cox1).

Hook3 Protein Associates with Golgi Complex

By contrast to hHK1 and hHK2, endogenous hHK3 was primarily detected in a perinuclear pattern reminiscent of the Golgi complex. When cells were double labeled for hHK3 and markers specific to the cis- and medial-Golgi compartments (Pepperkok et al., 1993; Bashour and Bloom, 1998), a striking coincidence was observed (Fig. 3, J–L, Fig. 4; and data not shown). A fraction of the hHK3 protein is found outside the Golgi complex; this staining

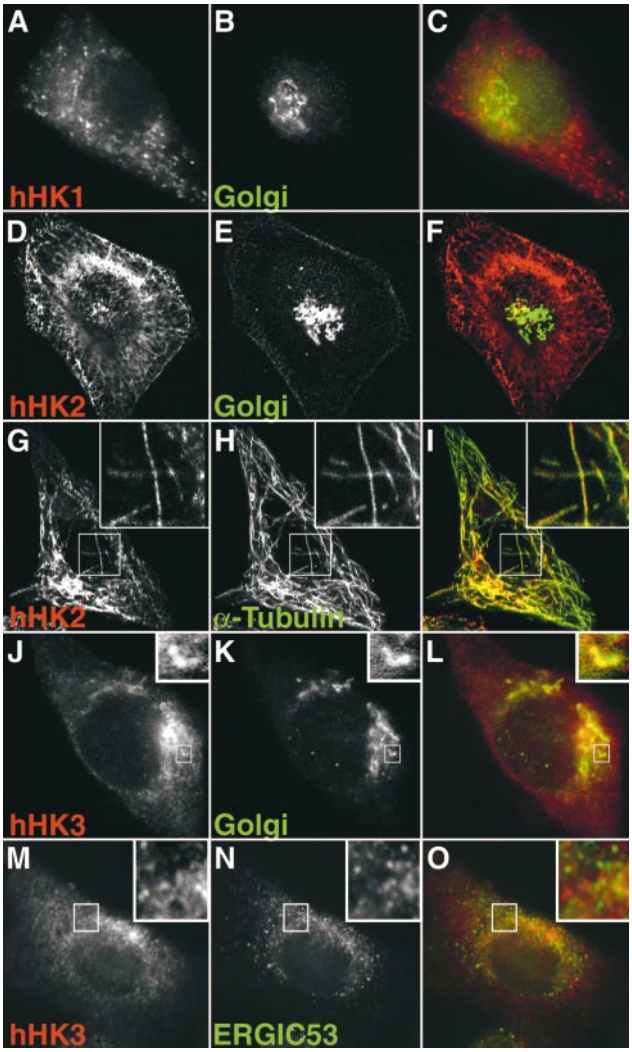


Figure 3. Endogenous hHK3 localizes to the Golgi complex. The localization of endogenous Hook proteins in Hep2 cells (A, D, G, J, and M) was compared with the Golgi marker FTCD (B, E, and K), microtubules (H), or ERGIC-53 (N). hHK1 localized to discrete unidentified subcellular structures (A) that do not significantly overlap with the Golgi complex (B). hHK2 localized to discrete subcellular structures that were often observed in linear tracks (D and G), which colocalized with microtubules (H and I). Much of hHK3 staining (J) precisely colocalized with a marker of the cis- and/or medial-Golgi (K), FTCD (Bashour and Bloom, 1998). hHK3 labeling detected outside the Golgi (M–O, inset) did not colocalize with ERGIC-53, a marker for ER-to-Golgi intermediates (M–O). In the merged images (C, F, I, L, and O), staining for FTCD, microtubules, or ERGIC-53 is shown in green; and the Hook proteins, in red.

does not colocalize with markers for ER-to-Golgi intermediates (Fig. 3, M–O, ERGIC-53). A function of hHK3 in a ubiquitous cellular compartment such as the Golgi complex is consistent with its expression in many tissues and cell lines, including HEK293, Vero, Cos7, and CHO cells (Fig. 1 C; and data not shown).

The close colocalization between hHK3 and FTCD (Fig. 3, J–L), a peripheral membrane protein associated with the cis-Golgi and earlier compartments of the secretory pathway (Bashour and Bloom, 1998; Gao et al., 1998), sug-

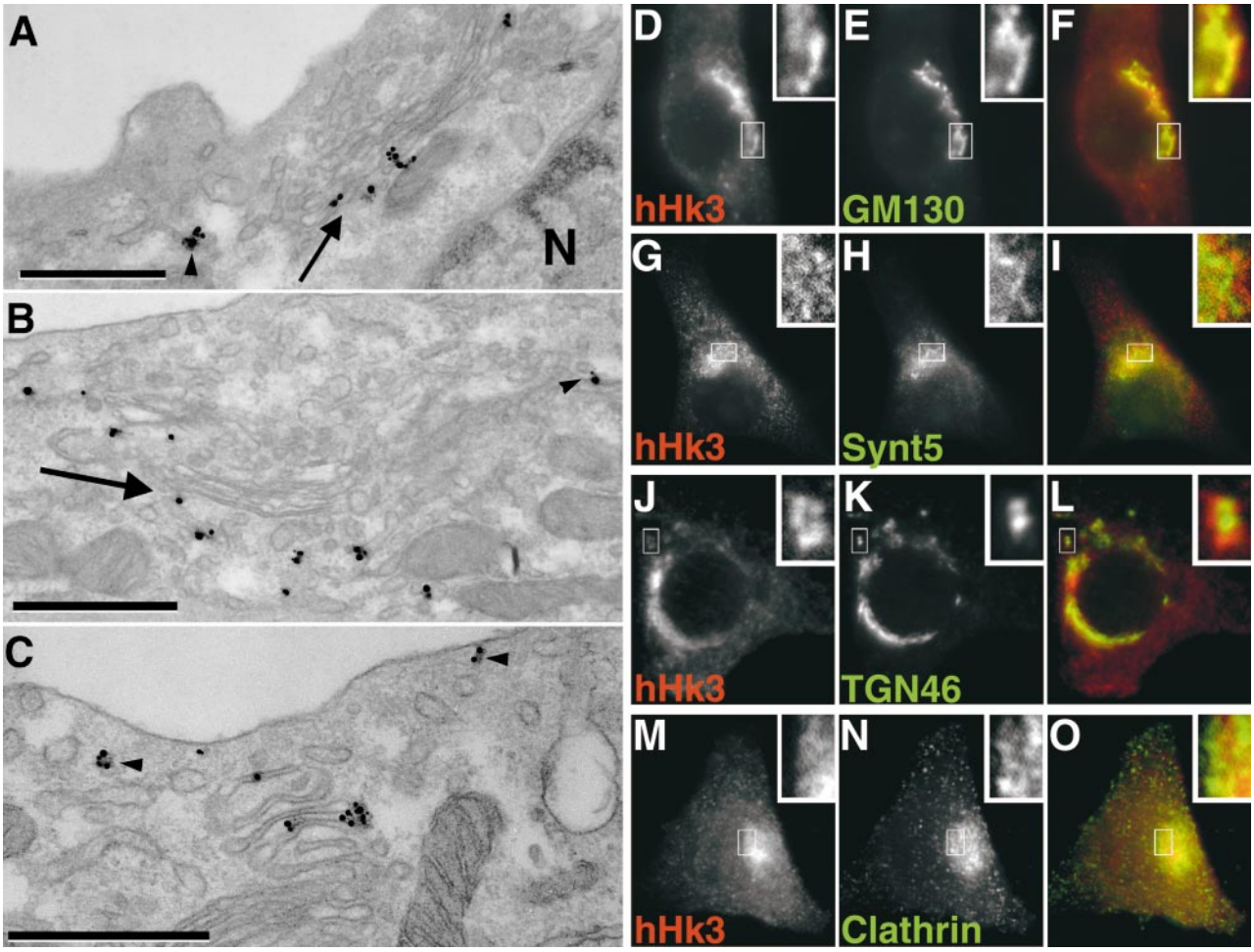


Figure 4. Endogenous hHK 3 localizes to the cis-face of the Golgi complex. (A–C) The localization of endogenous Hook3 in Vero cells was analyzed by preimbedding immunogold electron microscopy. The panels show three representative examples of the range of staining observed. In the Golgi area, the labeling was polarized to one face of the Golgi complex (A and B, arrows), which, based on the electron micrographs and the immunofluorescence colocalization experiments (D–O), was identified as the cis-face of the Golgi complex. Labeling of the TGN was negligible in all cells observed. Outside the Golgi complex, vesicles of unknown identity were labeled (arrowheads). (D–O) Localization of hHK3 was compared with several Golgi markers by double immunofluorescence staining in Vero cells: the cis-Golgi marker GM130 (D–F), the intermediate compartment marker syntaxin5 (G–I), the trans-Golgi markers TGN46 (J–L), and clathrin (M–O). The insets show details of the staining in higher magnification. In the merged images (F, I, L, and O), hHK3 staining is shown in red; and the Golgi markers, in green. N, nucleus. Bar, 0.5 μm.

gested that hHK3 localizes to the cis-face of the Golgi complex. Immunoelectronmicroscopy was used to further analyze the location of hHK3 within the Golgi complex of Vero cells. Immunogold labeling of hHK3 revealed a polarized distribution of hHK3 in the Golgi complex, consistent with labeling of the cis-face of the Golgi (Fig. 4, A–C). Gold particles were detected on the cis-most Golgi stacks (Fig. 4 A) but also the pleiomorphic vesicular–tubular clusters cis to the Golgi stacks (Fig. 4 B). We cannot rule out that a lack of antibody accessibility prevents the detection of hHK3 between Golgi stacks, but it is important to note that the more easily accessible area of the TGN closer to the cell surface was devoid of staining in the vast majority of cells examined.

Because of the absence of double labeling in these immunoelectron micrographs, we wanted to further address hHK3 localization in the Golgi complex by comparison to well-defined Golgi markers using immunofluorescence microscopy. Consistent with its colocalization with FTCD (Fig. 3, J–L), hHK3 also exhibited close colocalization with the cis-Golgi matrix protein GM130 (Nakamura et al., 1995). The close colocalization was most obvious at high magnifications (Fig. 4, D–F, insets). At low magnification, hHK3 colocalized with several Golgi markers in the general area of the Golgi complex, such as the intermediate compartment marker syntaxin5 (Fig. 4, G–I; Rowe et al., 1998), and the TGN markers TGN46 (Fig. 4, J–L; Prescott et al., 1997), and clathrin (Fig. 4, M–O; Brodsky, 1985). At higher magnification, however, it was apparent that hHK3 staining was distinct from each of these three markers (Fig. 4, G–O, insets). The combination of these light and electron microscopy localization data suggested that hHK3 is enriched on the cis-face of the Golgi complex.

The drugs BfA and NZ were used to further probe the properties of hHK3. Treatment with BfA results in the re-

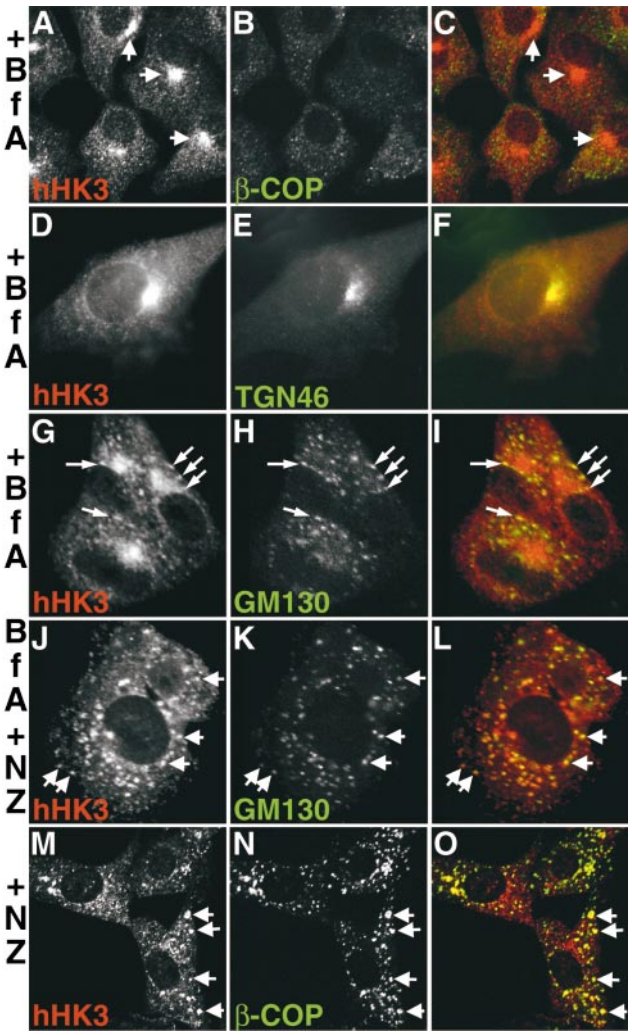


Figure 5. Juxtanuclear localization of hHK3 after BfA treatment. Localization of endogenous Hook3 in Vero (A–L) and HeLa cells (M–O) was compared with that of β-COP (B and N), TGN46 (E), and GM130 (H and K), after treatment with BfA (10 μg/ml) for 1 h (A–I) or BfA (10 μg/ml) for 1 h, and then BfA and NZ (10 μg/ml) for 1 h (J–L) or NZ (10 μg/ml) alone for 1 h (M–O). After 1 h of BfA treatment, β-COP protein was cytosolic (B), but the majority of hHk3 protein remained in a juxtanuclear position (A, D, and G). In BfA-treated cells, a fraction of hHK3 protein colocalized with the cis-Golgi matrix protein GM130 in distinct punctae (G–I, arrows). The majority of hHK3 protein was released from its juxtanuclear position after NZ was added to the BfA-treated cells; then, the majority of hHK3 localized to the GM130-positive punctae (J–L, arrows). After NZ treatment of HeLa cells, hHK3 (M) was associated with the resultant Golgi fragments (arrowheads), which were identified by β-COP labeling (N). In the merged images, hHK3 is visualized in red; and Golgi markers, in green.

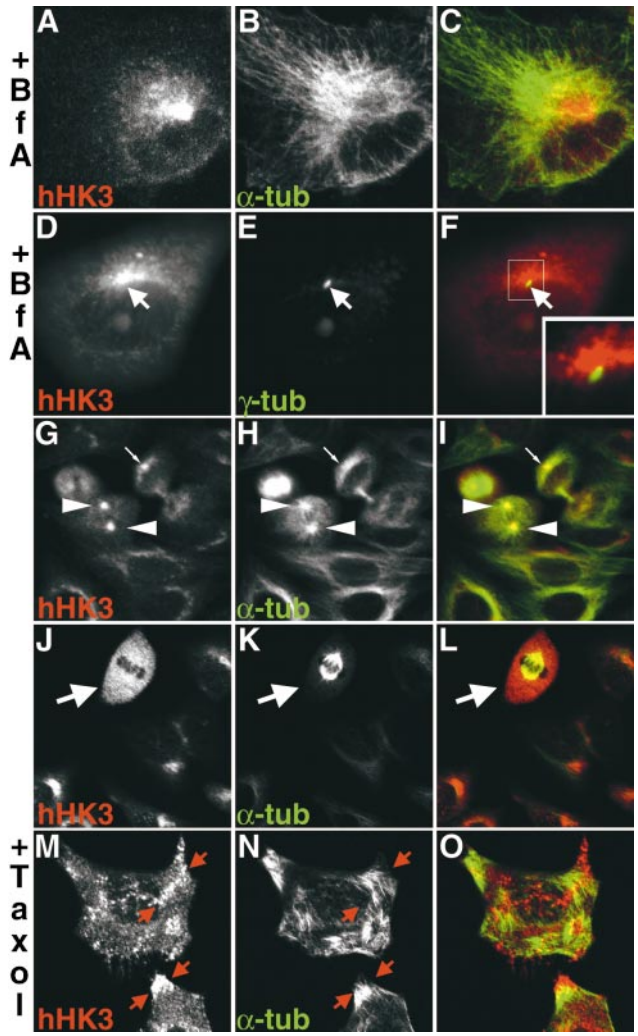


Figure 6. Association of hHK3 with microtubules in vivo. hHK3 localization was compared with α-tubulin (B, H, K, and N) or γ-tubulin (E) in Vero cells that were either treated with BfA (A–F), visualized during mitosis (G-L), or in which microtubules were stabilized with taxol (M–O). During the early stages of mitosis (prophase), hHK3 (G–I) is found associating with the microtubule asters (arrowheads). Later in mitosis (metaphase), hHK3 is found in a more diffuse cytoplasmic pattern (J–L). During cytokinesis, hHK3 is relocalized in a tight juxtanuclear focus (G–I, arrow). It is important to notice that images acquired from rounded mitotic cells lie above the focal plane of the surrounding, flattened interphase cells. As a result, fluorescence intensities are not directly comparable between these two populations of cells. When depolymerized microtubules were bundled in vivo in the presence of taxol (40 μM), hHK3 protein (M) was enriched near the ends of microtubule bundles (M–O, arrows).

lease of β-COP proteins into the cytosol (Fig. 5 B) and the disruption of the Golgi complex (for review see Chardin and McCormick, 1999). The strongest staining for hHK3, however, remained in a juxtanuclear accumulation even after a 1-h treatment with 10 μg/ml BfA (Fig. 5, A–I). Under these conditions, the cis-Golgi matrix protein GM130, localizes to dispersed punctate structures (Fig. 5 H; Nakamura et al., 1995; Seemann et al., 2000). A significant fraction of hHK3 labeling in BfA-treated cells colocalized with these GM130-positive punctae (Fig. 5, G–I, arrows).

Similar to TGN46 under these conditions (Fig. 5, D–F), hHK3 accumulated close to the MTOC (Fig. 6, A–F). Microtubules were necessary for this juxtanuclear localization of hHK3. When such BfA-treated cells were subsequently treated with 10 μg/ml NZ to disrupt microtubules, in the continued presence of BfA, hHK3 protein was found to redistribute to the peripheral GM130-positive punctate structures (Fig. 5, J–L). In cells treated with NZ only, much of hHK3 was observed associated with the scattered Golgi fragments, similar to other peripheral Golgi proteins (Fig. 5, M–O; Ho et al., 1989).

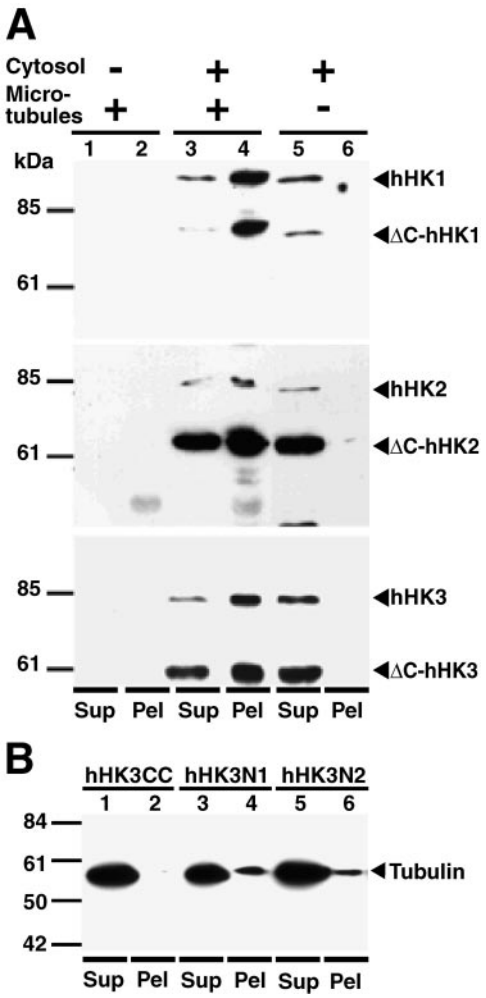


Figure 7. Hook proteins bind microtubules through their conserved NH_2-terminal domains. (A) Microtubule spin-down assays were performed using cytosol from HEK293 cells containing endogenous hHK1, hHK2, and hHK3 and expressing the COOH-terminal truncations ΔC-hHK1$_{1–555}$, ΔC-hHK2$_{1–548}$, and ΔC-hHK3$_{1–555}$. Samples from these assays were analyzed by Western blots using the indicated anti-Hook antibodies. hHK1, hHK2, and hHK3 are detected in the top, middle, and bottom blots, respectively. None of the human Hook proteins was detected among the purified microtubules (lanes 1 and 2). When no microtubules were added, all human Hook proteins were found in the supernatant (lane 5) but not in the pellet (lane 6). When cytosol and microtubules were combined, all full-length endogenous human Hook proteins as well as COOH-terminal truncations of each Hook protein were found copelleted with microtubules (lane 4). (B) To test for direct binding of hHK3 to microtubules, agarose beads coupled to the purified fusion proteins His$_6$–hHK3N1$_{1–164}$, His$_6$–hHK3N2$_{1–224}$, or His$_6$–hHK3CC$_{423–630}$ were tested for binding to stabilized microtubules. Excess tubulin is found in the supernatant of all samples (lane 1, 3, and 5). Fusion proteins containing the NH_2-terminal 164 aa of hHK3 can bind microtubules (lanes 4 and 6), whereas a fusion protein from the hHK3 coiled coil does not (lane 2).

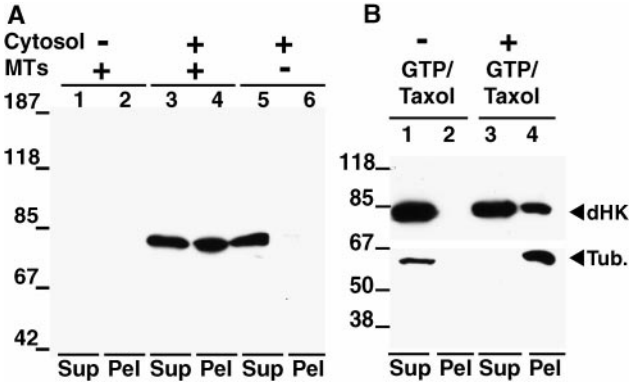


Figure 8. The dHKs bind microtubules. (A) Cytosol from *Drosophila* tissue culture cells was used in microtubule spin-down assays to test the ability of dHK to associate with bovine microtubules. dHK was found in the pellet associating with microtubules (lane 4) only when *Drosophila* cytosol and taxol-stabilized microtubules were combined. (B) dHK protein also bound to endogenous *Drosophila* microtubules when they were stabilized by the addition of GTP–taxol. Tubulin and dHK were pelleted in the presence of taxol–GTP (lane 4) but not in their absence (lane 2).

Hook Proteins Bind Microtubules

The requirement for microtubules in localizing hHK3 to its juxtanuclear position prompted us to further examine their association in vivo. During mitosis, hHK3 was localized to the microtubule asters in prophase (Fig. 6, G–I, arrowheads). When the mitotic spindle is evident in metaphase, hHK3 is found in a more diffuse cytoplasmic distribution, indicating that hHK3 function may be mitotically regulated (Fig. 6, J–L). A close association between hHK3 and microtubules in vivo was also evident in cells in which microtubules first had been depolymerized with NZ (10 μg/ml) for 1 h, and then bundled by the addition of taxol (40 μM) for 2–4 h. In such cells, hHK3 was enriched near the end of microtubule bundles (Fig. 6, M–O).

To determine whether hHK3 associates with microtubules in vitro, we used an in vitro microtubule spin-down assay (Fig. 7). Microtubules were assembled from purified tubulin. Taxol-stabilized microtubules were incubated with cytosol from HEK293 cells, or HEK293 cells expressing COOH-terminally truncated forms of the different human Hook proteins. Microtubules and associated proteins were then sedimented by high speed centrifugation. The pellets were probed for each of the human Hooks by Western blotting. All three human Hook proteins associated with microtubules (Fig. 7 A, lane 4). Furthermore, COOH-terminal truncations of each of the Hook proteins were also found to associate with microtubules. Although these experiments demonstrated the association of Hook proteins with microtubules, this assay did not exclude a requirement of other cytosolic partners in the microtubule-binding reaction.

To assess whether hHK3 binds directly to microtubules, we purified a set of His$_6$-tagged recombinant portions of hHK3. These polypeptides were coupled to Ni^{2+}–agarose beads that were then incubated with in vitro–assembled taxol-stabilized microtubules. After the incubation, a low speed spin was used to pellet the hHK3-coupled beads and any bound microtubules. The amount of tubulin in supernatants and pellets was assessed by Western blotting. An NH_2-terminal domain of 164 aa from hHK3 was sufficient to bind directly to microtubules (Fig. 7 B, lane 4), whereas an His$_6$ fusion protein derived from the hHK3 coiled coil region did not bind (Fig. 7 B, lane 2). These experiments indicate direct binding of hHK3 to microtubules and define a novel microtubule-binding domain.

Because this NH_2-terminal domain exhibits the highest similarity to the dHK (Fig. 1 A), we tested the abil-

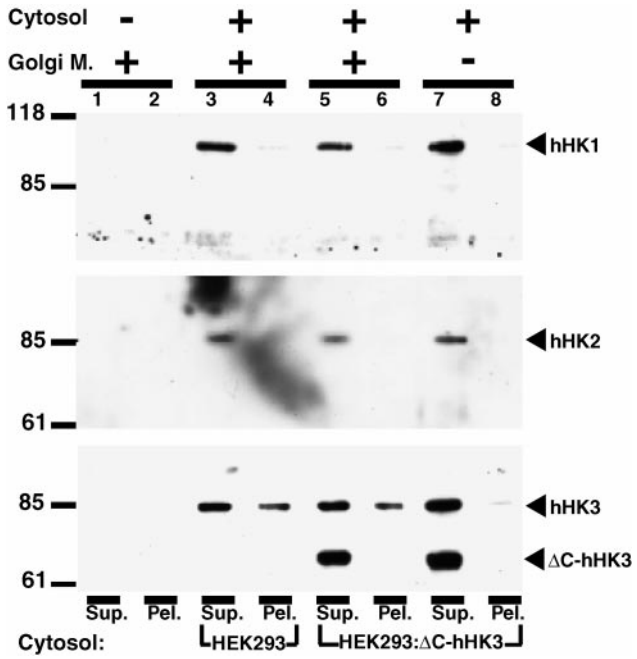


Figure 9. hHK3 binds Golgi membranes through a COOH-terminal domain. Purified Golgi membranes (Ktistakis et al., 1996) were incubated with cytosol from HEK293 or HEK293:ΔC-hHK3$_{1–555}$ cells. Bound proteins were collected by centrifugation through a 20% sucrose cushion, and pellets were probed for the presence of hHK1, hHK2, and hHK3 proteins by immunoblotting. None of the Hook proteins was detected in samples without added cytosol (lanes 1 and 2). In samples combining HEK293 cytosol and purified Golgi membranes, hHK3, but not hHK1 or hHK2, was found in the Golgi-containing pellets (lanes 4 and 6). When HEK293:ΔC-hHK3$_{1–555}$ cytosol was used, endogenous full-length hHK3 was found in the pellet, but ΔC-hHK3$_{1–555}$ was not foundin the pellet (lane 6), and it instead was found entirely in the supernatant (lane 5).

ity of dHK to bind to microtubules. Standard microtubule spin-down assays demonstrated that dHK from *Drosophila* cytosol bound exogenous bovine microtubules (Fig. 8 A). To test whether dHK can also bind to *Drosophila* microtubules, GTP and taxol were used to stabilize endogenous microtubules in *Drosophila* cytosol. When microtubules were sedimented by high speed centrifugation, dHK was found in the microtubule pellet (Fig. 8 B, lane 4). In the absence of stabilized microtubules, all of the dHK protein remained in the supernatant (Fig. 8 B, lanes 1 and 2). These results indicated that Hook proteins constitute a novel family of microtubule-binding proteins.

The COOH Terminus of hHK3 Directs Binding to Golgi Membranes

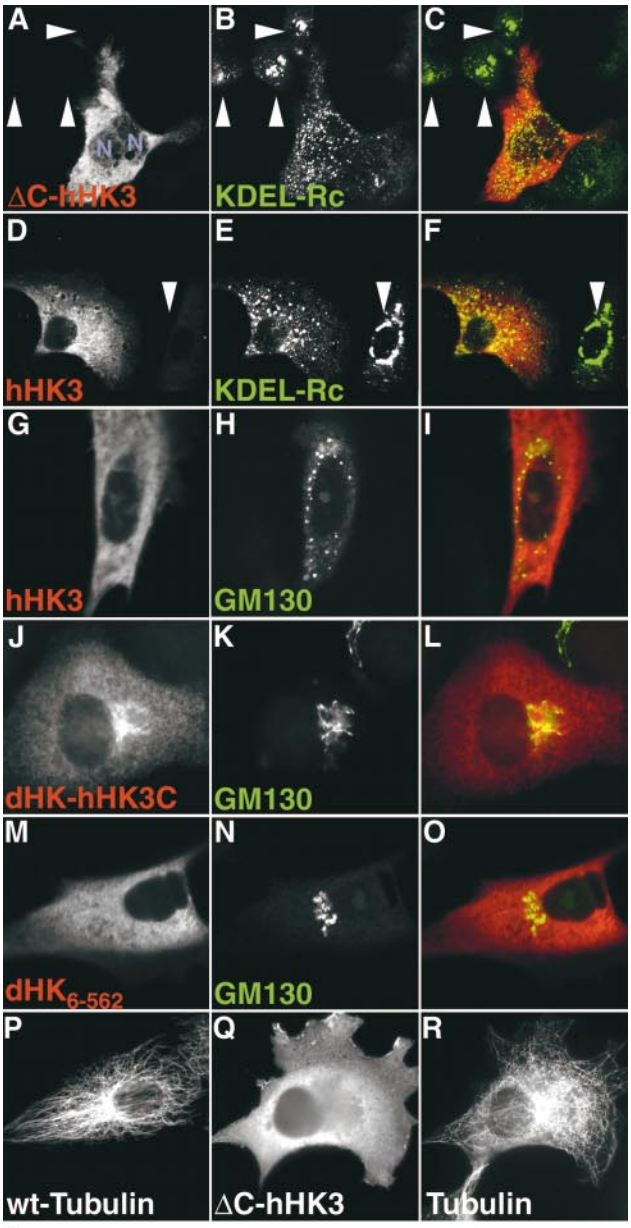
To further test the binding of hHK3 to Golgi membranes, we used an in vitro binding assay, using a highly enriched Golgi membrane fraction purified from CHO cells (Ktistakis et al., 1995). These Golgi membranes were incubated with cytosol, and then sedimented at low speed (15,000 *g*) through a 20% sucrose cushion. Proteins that remained in the supernatant and proteins that pelleted with membranes were analyzed by Western blotting (Fig. 9). No Hook3 was detected in samples containing Golgi membranes with no added cytosol (lanes 1 and 2). This indicated that added cytosol was the only source of hHK3 detected in the assay. In the absence of added membranes, hHK3 remained in the supernatant (Fig. 9, lanes 7 and 8). Endogenous hHK3 from HEK293 cytosol bound to the pelleted membranes (Fig. 9, lanes 4 and 6). However, membrane binding was eliminated by deletion of the COOH-terminal 166 aa (ΔC-hHK3; lane 6). Unlike hHK3, the hHK1 and hHK2 proteins did not pellet in the presence of the Golgi-enriched membranes.

Consistent with these in vitro results, the COOH-terminal 130 aa of hHK3 were sufficient to cause an enrichment of a dHK–hHK3C$_{589–718}$ chimera in the Golgi complex (Fig. 10, J–L). By contrast, the corresponding dHK protein was evenly distributed in the cytosol (Fig. 10, M–O). These experiments corroborated the specific binding of hHK3 to Golgi membranes, which we had observed in vivo (Fig. 4). Furthermore, they suggest that the domain-conferring membrane specificity to Hook proteins maps to their most divergent part, the COOH-terminal domain.

hHK3 Overexpression Disrupts Golgi Morphology

The binding of hHK3 to both microtubules and Golgi membranes suggested a role for hHK3 in the microtubule-dependent positioning of the Golgi complex. To test this possibility, we interfered with endogenous hHK3 function in transient overexpression experiments. In *Drosophila*, the expression of a COOH-terminal truncation of dHK mimics the *hook* loss-of-function phenotype (Krämer and Phistry, 1999). Overexpression of a similarly truncated hHK3 construct (ΔC-hHK3$_{1–555}$) dramatically disrupted Golgi morphology in Vero cells (Fig. 10). All Golgi markers that we characterized, including antibodies against the KDEL receptor, FTCD, β-COP, and TGN46, displayed the same scattered fragmented Golgi appearance (Fig. 10, A–C; and data not shown). The absence of the COOH-terminal domain of overexpressed ΔC-hHK3$_{1–555}$ was not critical for its Golgi-disrupting effect. Overexpression of the full-length hHK3 caused a similar scattered Golgi morphology (Fig. 10, D–I).

In addition to the changes in Golgi localization, hHK3 overexpression also disrupted the microtubule network. Microtubules appeared less well organized; for example, they often crossed each other in the cell periphery (Fig. 10 R; and data not shown), an occurrence rarely seen in untransfected cells (Fig. 10 P). Microtubular disorganization correlated with a significantly increased fraction of cells with two or more nuclei in the populations of cells expressing full-length hHK3 or several hHK3 truncations and chimeras (Fig. 10 S). This disruption of the microtubular network was not sufficient, however, to cause dispersal of the Golgi complex. Cells expressing several hHK3 and dHK truncations and chimeras retained the well-defined and -positioned Golgi complex, although their effects on microtubules were similar to that of full-length hHK3 or ΔC-hHK3$_{1–555}$ (Fig. 10, J–O and S). Together, these experiments are consistent with a direct role of hHK3 in the positioning of the Golgi complex.



S

hHK3 truncations	Golgi dispersion	cells with multiple nuclei
hHK3	+++	52%
ΔC-hHK3$_{1-555}$	+++	47%
ΔN-hHK3$_{168-718}$	-	20%
hHK3$_{1-280}$-dHkC	-	38%
dHk-hHK3$_{589-718}$	-	30%
dHk-hHK3$_{537-718}$	-	61%
dHk-hHK3$_{461-718}$	-	33%
dHk$_{6-562}$	-	9%
- Control	-	4%

Figure 10. Overexpression of hHK3 proteins disrupts Golgi morphology. Transfected cells overexpressing ΔC-hHK3$_{1–555}$ (A–C, Q, and R), full-length hHK3 (D–I), a dHK–hHK3C$_{589–718}$ chimera (J–L), or a dHK$_{6–562}$ truncation (M–O) were detected by the high levels of Hook proteins. Cells overexpressing ΔC-hHK3$_{1–555}$ (A) or hHK3 (D and G) exhibited a severely disrupted Golgi morphology, as indicated by the KDEL receptor (B and E) or GM130 (H) localizations. Arrowheads in A–F point to the well-defined Golgi complexes of untransfected cells. The COOH-terminal domain of hHk3 was sufficient to direct the dHK–hHK3C$_{589–718}$ chimera to the Golgi complex (J–L) but did not noticeably disrupt Golgi morphology (K). By itself, the dHK$_{6–562}$ part of the chimera was evenly distributed in the cytoplasma (M–O). All Hook chimeras and truncations listed in S, but not an unrelated control plasmid, caused a disruption of the microtubular network. Microtubules in transfected cells appeared noticeably disordered (R), often crossing in the cell periphery, a rare occurrence in untransfected cells (P). The disruption of the microtubular network paralleled an increase in the number of cells with multiple or multilobed nuclei in transfected cells (S; e.g., two nuclei in A are labeled N). In S, the gray bars indicate the dHK portion of chimeras.

Discussion

Hook proteins may constitute a novel family of microtubule linker proteins. Although motor proteins of the dynein and kinesin families catalyze the directed movement of membranous organelles along microtubules, there is growing evidence that nonmotor linker proteins are necessary to properly position and organize these cellular compartments (for review see Allan and Schroer, 1999). The results presented here, together with the genetic analysis of the *Drosophila hook* gene (Krämer and Phistry, 1996, 1999; Sunio et al., 1999), support a model in which Hook proteins are microtubule-binding proteins that interact with specific compartments of the secretory and endocytic pathways.

A comparison of the subcellular localization of different Hook proteins suggests roles in separate membrane-trafficking pathways. Previously, we demonstrated that the dHK is localized to endosomes (Krämer and Phistry, 1996). Here, we document the localization of a large fraction of hHK3, but not hHK1 or hHK2, to Golgi membranes (Figs. 3–5). The COOH-terminal domain of hHK3 is required (Fig. 9) for binding to Golgi membranes and is sufficient to redirect dHK to the mammalian Golgi complex (Fig. 10, J–L). This domain is the least conserved between different Hook proteins; its diversity may therefore be responsible for the distinct subcellular localizations of Hook proteins.

Although Hook proteins interact with different membranes, they all share the ability to bind to microtubules. This interaction was demonstrated by several in vivo and in vitro experiments. In vivo, we showed the taxol-induced enrichment of hHK3 at the ends of bundled microtubules (Fig. 6, M–O). In vitro, spin-down assays using exogenous purified brain microtubules (Figs. 7 and 8 A) or endogenous microtubules (Fig. 8 B) confirmed the interaction of Hook proteins with microtubules.

Our in vitro assays with purified Hook proteins revealed the direct binding of hHK3 to microtubules and mapped its microtubule-binding site to its NH_2-terminal domain (Fig. 7 B), which is highly conserved among Hook proteins (Fig. 1). It is unlikely that this interaction reflected nonspecific binding to negatively charged brain microtubules. The microtubule-binding domain encompassing the NH_2-

terminal 164 aa of hHK3 has an isoelectric point of 4.3, arguing against nonspecific electrostatic interactions. Furthermore, Hook proteins also bound to endogenous non-brain-derived microtubules (Fig. 8 B; Walenta, J.H., and H. Krämer, unpublished data). Although this NH_2-terminal domain is well-conserved among Hook proteins, it is not similar to any previously described microtubule-binding domain for other linker (De Zeeuw et al., 1997; Infante et al., 1999; Scheel et al., 1999) or motor proteins (Gee et al., 1997; Kozielski et al., 1997; Mandelkow and Johnson, 1998; Kikkawa et al., 2000). The changing extent to which hHk3 colocalizes with microtubules in different stages of the cell cycle (Fig. 6) indicates its binding to microtubules is dynamically regulated in vivo.

Microtubule binding may also explain the localization of hHK3 in BfA-treated cells. BfA interferes with the formation of coat protein I coats and eventually causes the redistribution of resident cis- and medial-Golgi enzymes to the ER (for review see Chardin and McCormick, 1999). Under these conditions, a small fraction of hHK3 localizes to punctate structures, which are marked by the Golgi matrix protein GM130 (Fig. 5, G–I; Seemann et al., 2000). The majority of hHk3, however, maintains a localization near, but distinct from, the MTOC in the absence of the Golgi complex (Figs. 5 and 6, A–F). This localization is dependent on microtubules, because, when NZ is added to BfA-treated cells, the vast majority of hHk3 relocates to the GM130-positive structures. This behavior is distinct from that of other cis- or trans-Golgi proteins after BfA treatment (Fig. 5; Lippincott-Schwartz et al., 1991; Nakamura et al., 1995; Füllekrug et al., 1999), but is not surprising for a protein that binds to Golgi membranes and microtubules and suggests the possibility that hHK3 may play an active role in the localization of the Golgi complex close to the MTOC.

Such a model is consistent with the results of hHK3 overexpression experiments. Overexpression of hHK3 or a COOH terminally truncated version dramatically dispersed the Golgi complex (Fig. 10), consistent with a proposed role of hHK3 in specifying the position of the Golgi complex. Dispersal of the Golgi complex can be achieved, however, by different manipulations based on very different mechanisms. One class of pharmacological or overexpression experiments interferes with membrane fission or fusion events, such as protein exit from the ER (e.g., Shima et al., 1998; Zaal et al., 1999) or interference with Golgi membrane fusion (Hatsuzawa et al., 2000). Golgi morphology can also be disrupted by a second class of manipulations that interferes with the transport of ER-to-Golgi intermediates along microtubules (Bloom and Goldstein, 1998). This can be achieved by the NZ-induced dissociation of microtubules, which results in the appearance of distinct Golgi units at the location of ER exit sites (Storrie et al., 1998). Similar changes in Golgi morphology are caused by the inactivation of cytoplasmic dynein, either genetically (Harada et al., 1998) or by the overexpression of the dynamitin subunit (Burkhardt et al., 1997; Presley et al., 1997).

The effect of hHK3 overexpression on the Golgi complex appears to be more similar to that second class of microtubule-related manipulations. In hHK3-overexpressing cells, all tested Golgi markers (including GM130, TGN46, β-COP, and KDEL receptor) were found in discrete punctate structures rather than distributed in the ER membrane (Fig. 10; data not shown). As in NZ-treated cells (Bloom and Goldstein, 1998), hHK3 overexpression did not appear to inactivate Golgi function. HEK293 cells overexpressing hHK3 and displaying the dispersed Golgi morphology could be grown for several weeks under G-418 selection. Furthermore, green fluorescent protein–tagged VSV-G protein was secreted to the plasma membrane in Vero cells overexpressing hHK3 (Walenta, J.H., and H. Krämer, unpublished observation). These consequences of overexpressing hHK3 protein are remarkably different from that of another Golgi-associated microtubule-binding protein, GMAP-210 (Infante et al., 1999). Unlike hHK3, overexpression of GMAP-210 resulted in an enlargement of the juxtanuclear Golgi complex, indicating distinct roles in Golgi morphology for these two proteins.

Similar to the effect caused by inactivating dynein (Quintyne et al., 1999), overexpression of hHK3 not only resulted in the dispersal of Golgi membranes but also a disruption of the microtubule network (Fig. 10). This raises the possibility that overexpression of human Hook3 proteins disrupts the Golgi complex by indirectly interfering with dynein-mediated transport of ER-to-Golgi intermediates. Two observations argue against such an indirect effect: (a) overexpression of several other hHK3 truncations or dHK-hHK3 chimeras caused very similar defects in the microtubule network, but did not disperse the Golgi complex (Fig. 10 S), and (b) hHK3 proteins in vivo and in vitro bind to Golgi membranes consistent with a direct effect. The results on hand, however, do not permit us to unequivocally resolve this issue. Future hHk3 loss-of-function studies will be necessary to further test the proposed role of hHK3 as a Golgi-specific linker protein.

A function of Hook proteins as microtubule linker proteins is also consistent with the analysis of *hook* loss-of-function mutations in endocytic trafficking in *Drosophila* (Krämer and Phistry, 1996, 1999). In the endocytic pathway of *Drosophila* and mammalian cells, mature MVBs are intermediates in the normal delivery of cargo to late endosomes (Gruenberg and Maxfield, 1995; Futter et al., 1996; Gu et al., 1997; Sunio et al., 1999) and serve as an important compartment in the sorting of endocytic and biosynthetic cargo (Felder et al., 1990; Hirst et al., 1998). The assembly of such mature MVBs is dramatically reduced in *hook* mutant cells (Sunio et al., 1999). This specific destabilization of an endocytic organelle is distinct from the effects described for interfering with microtubules or dynein function (Gruenberg et al., 1989; Aniento et al., 1993). In those studies, the pharmacological disruption of microtubules or depletion of dynein resulted in the accumulation of cargo in an intermediate compartment before delivery to late endosomes. However, delivery of endocytic cargo to late endosomes is not blocked in cells lacking Hook. In the absence of mature MVBs, delivery to late endosomes might proceed through the direct fusion of the small, immature MVB fragments observed in *hook* mutants (Sunio et al., 1999). From these studies, it appears that Hook function in *Drosophila* is not necessary for internalization of cargo or its delivery to late endosomes. Rather, dHK appears to be responsible for the assembly or stability of mature MVBs, analogous to the proposed role of hHK3 in the mammalian Golgi complex.

Finally, it is interesting to consider Hook proteins in their evolutionary context. Yeast, which have fragmented Golgi and do not use microtubules for secretion (Jacobs et al., 1988), do not have Hook family members. With increasing cellular and organismal complexity, we find an increasing number of Hook proteins: *Drosophila* has a single Hook family member and here we have identified three human Hook proteins. Our results suggest that Hook proteins are an evolutionary response to the increased cellular complexity of higher organisms. This increased complexity necessitates precise organization of membrane compartments and assembly of intermediates for efficient trafficking over long distances. Our knowledge of microtubule-dependent motor proteins provides a solid framework for understanding the transport of organelles along microtubules. However, the model of microtubule linkers necessary for the anchoring of different organelles in their characteristic positions is still rapidly evolving (Pierre et al., 1992; Echard et al., 1998; Nielsen et al., 1999; Schroer, 2000). The four Hook proteins discussed here expand on this theme. The identification of additional Hook proteins and their membrane receptors will further enhance our understanding of the interactions between microtubules and specific organelles.

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