Cytoplasmic Dynein Participates in the Centrosomal Localization of the Golgi Complex

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Abstract. The localization of the Golgi complex depends upon the integrity of the microtubule apparatus. At interphase, the Golgi has a restricted pericentriolar localization. During mitosis, it fragments into small vesicles that are dispersed throughout the cytoplasm until telophase, when they again coalesce near the centrosome. These observations have suggested that the Golgi complex utilizes a dynein-like motor to mediate its transport from the cell periphery towards the minus ends of microtubules, located at the centrosome. We utilized semi-intact cells to study the interaction of the Golgi complex with the microtubule apparatus. We show here that Golgi complexes can enter semi-intact cells and associate stably with cytoplasmic constituents. Stable association, termed here "Golgi capture," requires ATP hydrolysis and intact microtubules, and occurs maximally at physiological temperature in the presence of added cytosolic proteins. Once translocated into the semi-intact cell cytoplasm, exogenous Golgi complexes display a distribution similar to endogenous Golgi complexes, near the microtubule-organizing center. The process of Golgi capture requires cytoplasmic tubulin, and is abolished if cytoplasmic dynein is immunodepleted from the cytosol. Cytoplasmic dynein, prepared from CHO cell cytosol, restores Golgi capture activity to reactions carried out with dynein immuno-depleted cytosol. These results indicate that cytoplasmic dynein can interact with isolated Golgi complexes, and participate in their accumulation near the centrosomes of semi-intact, recipient cells. Thus, cytoplasmic dynein appears to play a role in determining the subcellular localization of the Golgi complex.

A fundamental question in cell biology is how organelles achieve their distinct cytoplasmic localizations. A large body of data indicates that cytoskeletal proteins play an important role in these processes. Microtubules have long been known to mediate the saltatory movements of certain membrane-bound organelles in eukaryotic cell cytoplasms (Freed and Lebowitz, 1970; Bhisey and Freed, 1971; Rebhun, 1972). However, it has recently become clear that all classes of membrane-bound, cytoplasmic organelles maintain an intimate and dynamic association with the microtubule apparatus (see Kelly, 1990; McIntosh and Porter, 1989; Vale, 1990; Schroer and Sheetz, 1991*a*; Vallee, 1991). High resolution microscopic techniques have revealed clear connections between microtubules and the ER (Terasaki et al., 1986; Lee and Chen, 1988), the Golgi complex (Ho et al., 1989; Cooper et al., 1990; Kreis, 1990), the intermediate compartment between the ER and Golgi (Lippincott-Schwartz et al., 1990), endosomes (Herman and Albertini, 1984; Parton et al., 1991) and lysosomes (Collot et al., 1984; Matteoni and Kreis, 1987; Swanson et al., 1987; Heuser, 1989; Hollenbeck and Swanson, 1990). In addition, mitochondria have also been shown to interact with the microtubule-based cytoskeleton (see Hirokawa, 1982; Ball and Singer, 1982).

The dynamic nature of organelle–microtubule interactions is underscored by the cell cycle–dependent redistribution of the Golgi complex (reviewed by Kreis, 1990). Interphase cells are believed to contain a single Golgi complex (Rambourg and Clermont, 1990), located in close proximity to the microtubule-organizing center (MTOC),[1] on one side of the nuclear envelope. During mitosis, the Golgi vesiculates into smaller units which disperse throughout the cytoplasm (Lucocq et al., 1987; 1989). At telophase, the vesicles coalesce and return to a pericentriolar location (Ho et al., 1989). Postmitotic Golgi vesicle movement is directed towards the minus ends of microtubules, which are located at the centrosome. This feature has led many investigators to propose a role for cytoplasmic dynein in Golgi redistribution, because dynein has the capacity to drive organelle movements towards the minus ends of microtubules (Paschal et al., 1987; Schroer et al., 1989; Schnapp and Reese, 1989).

In interphase cells, disruption of cytoplasmic microtubules by nocodazole, for example, is sufficient to fragment the Golgi into "mini" Golgi stacks that distribute throughout

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1. *Abbreviations used in this paper*: MAP, microtubule-associated protein; MTOC, microtubule-organizing center.

the cytoplasm (Robbins and Gonatas, 1964; Pavelka and Elinger, 1983; Thyberg and Moskalewski, 1985; Ho et al., 1989). If nocodazole is removed, microtubules repolymerize, and the Golgi regains its perinuclear distribution, eventually re-forming a single organelle. These experiments demonstrate that the mechanisms by which the Golgi achieves its localization are operational within interphase cells. Moreover, they indicate that Golgi localization should be amenable to analysis in permeabilized cells.

We show here that isolated Golgi complexes can interact with cytoplasmic components of recipient gently broken "semi-intact" cells, and accumulate in the vicinity of the centrosome. This process requires ATP hydrolysis, the presence of intact microtubules, and is entirely dependent upon the microtubule-based motor, cytoplasmic dynein.

Materials and Methods

Materials

Chemicals and enzymes were from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. CHO clone 15B cells (Gottlieb et al., 1975) were obtained from Dr. Jim Rothman (Sloan Kettering, New York, NY). Phosphocellulose-purified bovine brain tubulin was kindly provided by Dr. Tim Mitchison (University of California, San Francisco, CA); Biotin X-NHS and Texas red-streptavidin were obtained from Calbiochem-Behring Corp. (La Jolla, CA) and Cell-Tak from Collaborative Research Inc. (Bedford, MA). Hexokinase and GTPγS were purchased from Boehringer Mannheim, Indianapolis, IN. Ovalbumin fraction V $Man_5(GlcNAc)_2$-asparagine glycopeptide was from Biocarb (Accurate Chemical, Westbury, NY). UDP-^{3}H-N-acetylglucosamine was purchased from NEN (Boston, MA). Taxol was supplied by the Drug Synthesis & Chemistry Branch, Division of Cancer Treatment (NIH).

Antibodies

Affinity-purified antibodies directed against chicken brain dynein heavy chain, and antidynein intermediate chain mAb 70.1 were the generous gifts of Dr. Trina Schroer (Johns Hopkins University, Baltimore, MD) and Dr. Michael Sheetz (Duke University, Durham, NC), respectively. Polyclonal anti-"pericentrin" antibodies were provided by Drs. Stephen Doxsey and Marc Kirschner (University of California, San Francisco, CA; manuscript in preparation). These antibodies recognize a well-characterized centrosomal antigen, first identified using sera from patients with linear scleroderma (also obtained from Doxsey, S. J., and M. W. Kirschner, see Karsenti et al., 1984). Monoclonal anti-mannosidase II antibodies (53FC3) were the gift of Dr. Brian Burke (Harvard University, Cambridge, MA); monoclonal anti-β tubulin antibody was from Amersham Corp. (Arlington Heights, IL); FITC-conjugated second antibodies were from Jackson Immunoresearch, Inc. (West Grove, PA). Rabbit anti-β-COP "EAGE" peptide antibodies were raised in this laboratory as described by Duden et al. (1991).

Preparation of Golgi Membranes and CHO Cytosol

Wild-type Golgi membranes were purified from rat liver according to Tabas and Kornfeld (1979), yielding preparations with protein concentrations of 1.7 to 2.5 mg/ml. For certain experiments, wild-type Golgi membranes were biotinylated with 3.5 mM NHS-LC-Biotin in 25 mM Hepes (pH 7.4), 50 mM KCl for 15 min on ice. The reaction was stopped by the addition of 10 mM Tris-HCl (pH 7.4). Biotinylated Golgi membranes were then loaded onto a sucrose gradient as described by Clary and Rothman (1990) and recovered at the 25–35% sucrose interface. CHO cytosol was prepared as described by Balch et al. (1984). Suspension cells were broken by six passages through a steel ball bearing homogenizer; cytosol was desalted into 25 mM Tris-HCl, pH 8.0, 50 mM KCl using a column of Bio-Gel P6DG (Bio-Rad Laboratories, Palo Alto, CA) and was frozen in liquid nitrogen in small aliquots as soon as possible. Most preparations had protein concentrations of 5–7 mg/ml, as determined by the procedure of Bradford (1976) using Bio-Rad reagent and BSA as standard.

Preparation of Intact and Semi-Intact Cells

CHO clone 15B cell monolayers (10-cm dishes) were maintained in α-MEM plus 7.5% FBS (Gibco Laboratories, Grand Island, NY) and grown to a density of 1–2 × 10^7 cells per plate. When intact cells were employed, they were grown in suspension; cells harvested from monolayers by gentle trypsinization gave identical results (data not shown). Semi-intact cells were prepared using the swelling technique of Beckers et al. (1987). Briefly, a metal block was placed on ice, and covered with a very wet paper towel. Dishes were transferred to the towel-covered blocks, and washed three times with ice-cold 10 mM Hepes/KOH, pH 7.2, 15 mM KCl. Cells were then swollen in the same buffer for 10 min on ice. The plates were drained, and then held vertical for 30 s to facilitate aspiration of any trace of remaining buffer. Reaction mix was then added directly to the dishes and the cells were scraped with a trimmed, inflexible rubber policeman. Scraping was accomplished using a firm, squeaking, horizontal, left-to-right motion, beginning at the top of the plate. The plates were scraped four times, turning the plate 90° between each scrape. The dish was then tilted up, and the cells were collected at the bottom edge by scraping. A uniform suspension of semi-intact cells was generated by pipetting up and down three times through a 1-ml plastic pipet tip; the extract was then transferred to a tube on ice.

Golgi Capture Assay

Each assay (200 μl) contained semi-intact CHO clone 15B cells (110–120 μl), 25 mM Hepes-KOH (pH 7.2), 115 mM KCl, 1.5 mM $Mg(OAc)_2$, 0.24 U/ml Aprotinin, 10 μg/ml leupeptin, 1 μM pepstatin, 1× ATP-regenerating system (1 mM $MgCl_2$, 1 mM ATP, 15 mM creatine phosphate, 21 U/ml rabbit muscle creatine phosphokinase), 20–40 μl of cytosol, and 30 μl of wild-type Golgi membranes (~50 μg). When energy dependence was tested, the ATP-regenerating system was omitted and replaced by an ATP-depleting system (10 mM glucose, 56 U/ml hexokinase). Incubations were generally carried out for 1 h at 37°C and stopped by centrifugation through a 1 ml cushion of 1 M sucrose, 10 mM Tris-HCl (pH 7.4) for 2 min at 12,000 *g*. Unbound Golgi complexes stayed at the top of the sucrose cushion; cell-associated Golgi complexes pelleted with the cells. The sucrose was carefully aspirated, tube walls were dried with a Kimwipe-covered Q-tip, and pellets were then frozen in liquid nitrogen. The extent of Golgi capture was measured by assaying thawed cell pellets for GlcNAc transferase I activity (Dunphy and Rothman, 1983). Specifically, we measured the transfer of ^{3}H-GlcNAc to $Man_5(GlcNAc)_2$-asparagine (ovalbumin glycopeptide V). Tubes containing cell pellets received 0.02 ml of 1 M sucrose, 10 mM Tris-HCl (pH 7.4); assays were carried out for 30 min at 37°C in 0.1 M Mes (pH 6.5), 10 mM $MnCl_2$, 0.2% Triton X-100, 2 mM ATP, 0.5 mM UDP-GlcNAc, 0.5 μCi UDP-[^{3}H]GlcNAc, and 1–10 nM of ovalbumin glycopeptide V ($Man_5(GlcNAc)_2$-asparagine) in a final volume of 0.05 ml. At the end of the reactions, the labeled glycopeptide was separated from the radioactive precursor by binding and specific elution from Con A-Sepharose.

Preparation of Microtubule-depleted CHO Cytosol

Aliquots of CHO cytosol were treated with 0.5 μM taxol at 22°C for 10 min in the presence of bovine brain tubulin, added to a final concentration of 0.5 mg/ml. Mixtures were incubated for an additional 10 min at 37°C with 10 μM taxol before being centrifuged at 400,000 g_{max} for 5 min at 22°C. Supernatants (microtubule-depleted cytosol) were dialyzed for 4 h at 4°C against 25 mM Hepes-KOH (pH 7.2), 1.5 mM $Mg(OAc)_2$, 25 mM KCl.

UV Photocleavage and Immunodepletion of Dynein

Samples of CHO cytosol in 35 mM Pipes (pH 7.4), 5 mM $MgSO_4$, 5 mM EGTA, 0.5 mM EDTA were brought to 2 mM ATP and 100–200 μM vanadate and irradiated on ice at 365 nm for 1 h, 6 cm from the UV light source (UVP Transilluminator TL 33; UVP Inc., San Gabriel, CA). The extent of photocleavage was estimated on 6% polyacrylamide gels and confirmed by Western blotting (Burnette, 1981) using an affinity-purified, antichicken cytoplasmic dynein antibody (gift of Dr. T. Schroer) and a chemiluminescence detection technique (ECL; Amersham Corp.). Dynein was immunodepleted from CHO cytosol as described by Goda and Pfeffer (1991) using either 50 ml of clone 70.1 hybridoma cell-conditioned media, or an equal volume of culture media alone, prebound at 4°C overnight to anti-IgM agarose (Sigma Chemical Co.).

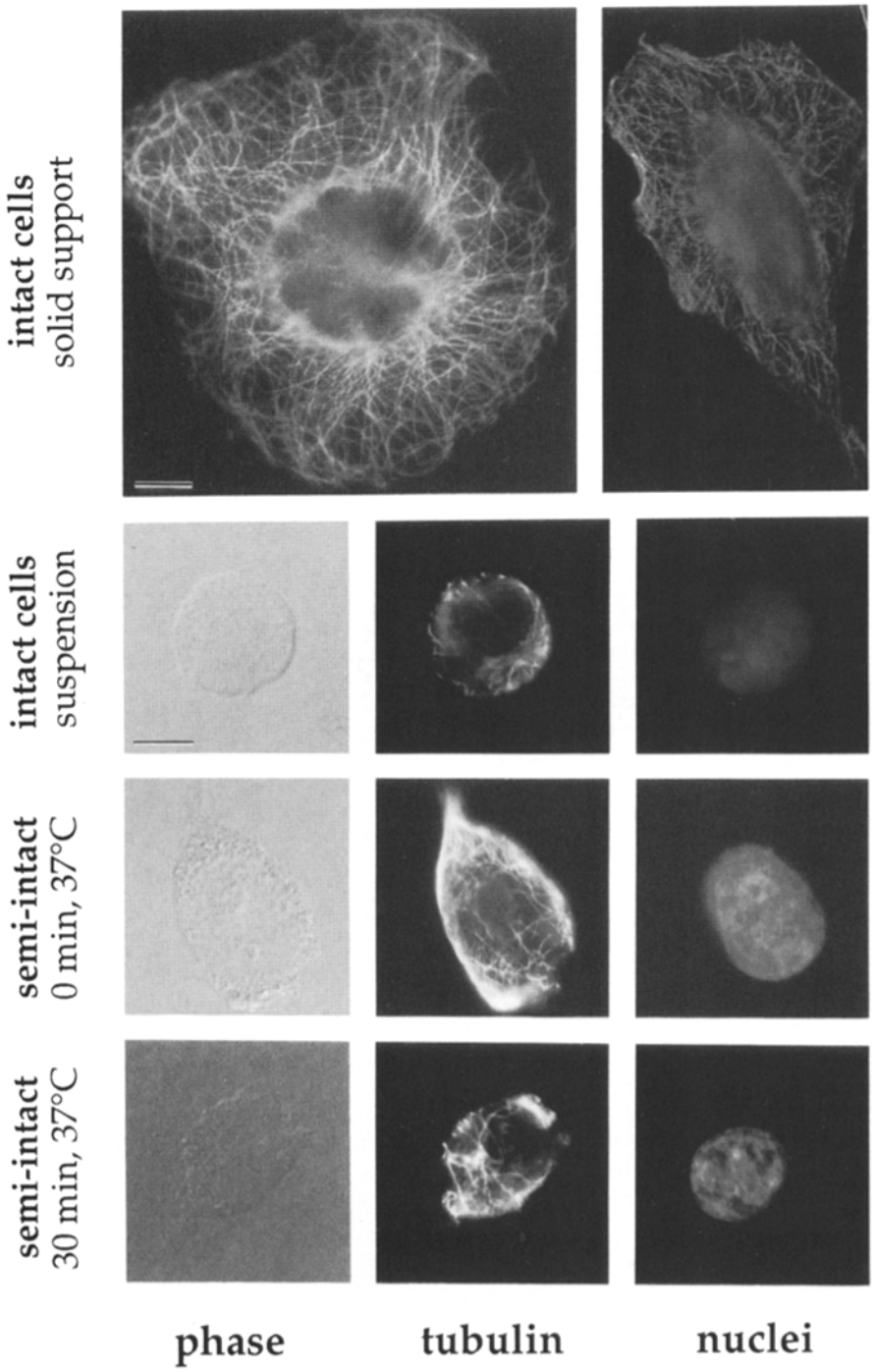

Figure 1. Immunofluorescent detection of microtubules in intact and semi-intact cells. Intact cells were grown either on coverslips ("solid support") or in suspension and permeabilized with 0.5% Triton X-100 to provide antibodies access to the cytoplasm. Semi-intact cells were analyzed after 0 or 30 min at 37°C in the presence of cytosol, ATP, and an ATP regenerating system, without permeabilization. All cells were labeled for tubulin using a mouse anti-β-tubulin antibody, followed by fluorescein-conjugated sheep antimouse antibody. Nuclei were labeled with DAPI. Bar, 10 μm.

Purification of CHO Cytoplasmic Dynein

Dynein was purified according to the procedure of Lye et al. (1987) with slight modification. A CHO cell postnuclear supernatant was centrifuged at 100,000 *g* for 60 min to remove particulate material, dialyzed into PMEG buffer lacking glycerol overnight, and spun at 50,000 rpm in a TLA 100.3 rotor for 5 min to remove aggregates. Cytosol (19 ml) was supplemented with bovine brain tubulin to 100 μg/ml before ATP depletion and microtubule polymerization, the latter of which was carried out for 15 min at 37°C. ATP release was carried out in a volume of 2 ml. Sucrose gradient fractionation of the ATP release-fraction was carried out without prior concentration in 35 mM Pipes, pH 7, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM EDTA, and 1 mM DTT according to Paschal et al. (1987): 1-ml samples were applied to 10 ml, 5–20% linear sucrose gradients, which were poured above a 1-ml, 30% sucrose cushion and centrifuged in an SW41 rotor for 17 h at 30,000 rpm. 0.75-ml fractions were collected from the top of the tube.

Immunofluorescence

Intact cells grown in suspension and semi-intact cells were prefixed for 30 min in 3.7% buffered formaldehyde and 0.02% glutaraldehyde, and subsequently spun onto Cell-Tak–coated coverslips at 100 *g* for 3 min and fixed again for 10 min. Immunofluorescence was carried out as described by Warren et al. (1984). Intact cells were permeabilized with 0.5% Triton X-100 before labeling, to provide antibodies access to the cytoplasm; semi-intact cells were processed without Triton. Antibodies and Texas red-streptavidin were used at a 1:1,000 dilution. Nuclei were labeled with 0.1 μg/ml DAPI. Slides were viewed using a Zeiss Axiophot microscope, equipped with

DAPI, FITC, and Texas red filters set up for quantitative analysis (Omega Optical Inc., Brattleboro, VT), with a 100× Plan Neofluar, 1.3 oil immersion objective; images were recorded on Kodak T-MAX P3200 film.

Quantitation of Immunofluorescence Micrographs

To ensure the statistical significance of possible colocalization of Golgi and centrosomes, only cells containing a centrosome located on the coverslip to one side of the cell were counted. Centrosomes were readily visible in ~50% of the semi-intact cells; in other cases, they were either masked by a superimposed nucleus and difficult to see, or more rarely, were washed out of the semi-intact cells. Cells were divided into grids of nine squares each, representing 0.33 × 0.33 cell diameters. Colocalization was scored when the bulk of fluoresence was present in the same quadrant as the centrosome plus one or two adjacent squares in the grid. Examples of colocalization are shown in Figs. 5 and 6.

Results

The ability of gently broken, "semi-intact" CHO cells (Beckers et al., 1987) to sustain a variety of vesicular transport events suggested that these cells might provide an excellent system in which to investigate microtubule-directed Golgi localization. We first used indirect immunofluorescence to examine the integrity of the microtubule apparatus in semi-intact cells (Fig. 1). When CHO cells are grown and fixed directly on coverslips, they display extensive, stellar microtubule arrays. If the same cells are instead grown in suspension culture and then centrifuged onto the surface of coverslips, the round cells are much smaller because they are not attached to a substratum (Fig. 1, row 2). The nuclei of such suspension-grown cells occupy a large proportion of the cytoplasm; microtubule arrays appear to surround the nuclei, as determined by comparing the DAPI staining with the antitubulin fluorescence in multiple focal planes. As shown in the lower portion of Fig. 1, semi-intact cells retain a great deal of cytoskeletal structure. They appear swollen relative to intact cells, and their microtubule arrays are more easily detected. Detergent was not needed to permeabilize the semi-intact cells for immunofluorescence; tubulin antibodies displayed full access to the entire cytoplasm.

The microtubules seen in freshly prepared semi-intact cells resembled those present in the intact, round, CHO suspension cells. After a 37°C incubation in the presence of crude cytosol, ATP, and an ATP regenerating system, microtubule arrays were still readily detected, although their fluorescence intensity was somewhat decreased. This suggests that microtubules are slowly depolymerizing during the incubation, probably due to dilution of cytosolic tubulin below the critical concentration for microtubule maintenance. As will be shown below, a large proportion of the semi-intact cells could be shown to contain a centrosome, even after 60 min of incubation (see below).

In addition to retaining an organized cytoskeleton, semi-intact cells efficiently retain their Golgi complexes. Control experiments showed that semi-intact CHO wild type cells retained >95% of their Golgi complexes, after 60 min at 37°C in the presence of ATP and cytosol, as measured by their content of the medial Golgi-specific enzyme, N-acetylglucosamine (GlcNAc) transferase I (data not shown; Dunphy et al., 1985). However, endosomes, detected by preloading for 30 min with ^{125}I-lactoperoxidase, were much less efficiently retained: ~50% of the lactoperoxidase was lost in parallel experiments (data not shown).

Fig. 2 outlines the assay we devised to study the interaction of Golgi complexes with the microtubule apparatus. The system utilizes semi-intact CHO clone 15B cells which lack GlcNAc transferase I (Gottlieb et al., 1975). Semi-intact cells are incubated with isolated, wild type Golgi complexes at 37°C, in the presence of a crude cytosol fraction, ATP and an ATP-regenerating system. The association of wild type Golgi complexes with the mutant CHO cells is then measured after differential centrifugation: semi-intact cells are sedimented through a sucrose cushion, and cell-associated Golgi complexes are detected by assaying the pellet for GlcNAc transferase-I enzyme activity.

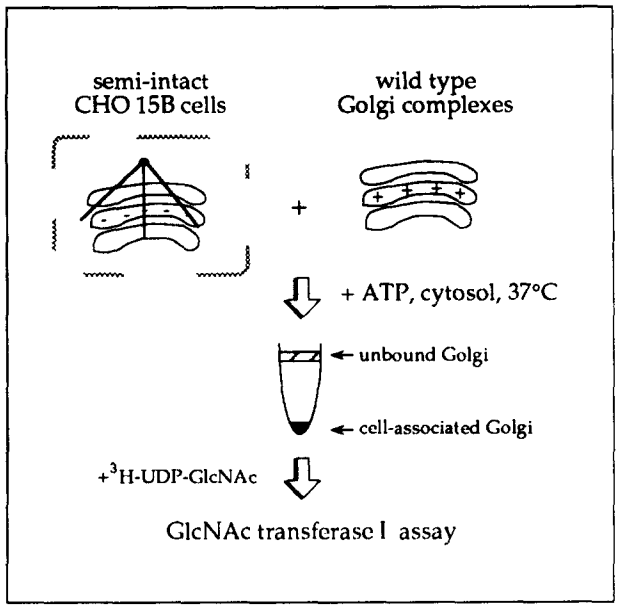

Figure 2. Complementation scheme to detect association of exogenous Golgi complexes with semi-intact cells. Semi-intact CHO clone 15B cells, which lack the medial Golgi enzyme, GlcNAc transferase I, are incubated with wild-type Golgi membranes, cytosol, ATP, and an ATP regenerating system at 37°C. At the end of the reaction, unbound Golgi are separated from cell-associated Golgi by differential centrifugation through a sucrose cushion. The presence of cell-associated Golgi complexes is then detected by assaying the pellet fractions for GlcNAc transferase I activity.

Stable Association of Exogenous Golgi Complexes with Semi-intact Cells

As shown in Fig. 3, when reactions were carried out at 37°C, Golgi complexes associated with cells with linear kinetics for ~1 h (Fig. 3). Considerably less GlcNAc transferase I activity became cell-associated at 0°C. To demonstrate that Golgi complexes were not binding nonspecifically to the outer surfaces of the cells, we compared the association of Golgi complexes with intact and semi-intact CHO cells. As shown in Fig. 3 *B*, cell association was only observed when Golgi complexes were incubated with semi-intact cells; reactions containing intact cells showed the same background level of sedimenting GlcNAc transferase activity as reactions which lacked cells altogether. This experiment led us to term the stable association of Golgi complexes with cytoplasmic constituents of semi-intact cells as "Golgi capture."

Fig. 3 *C* confirmed that the presence of a crude cytosol fraction in standard reactions enhanced the rate and the ex-

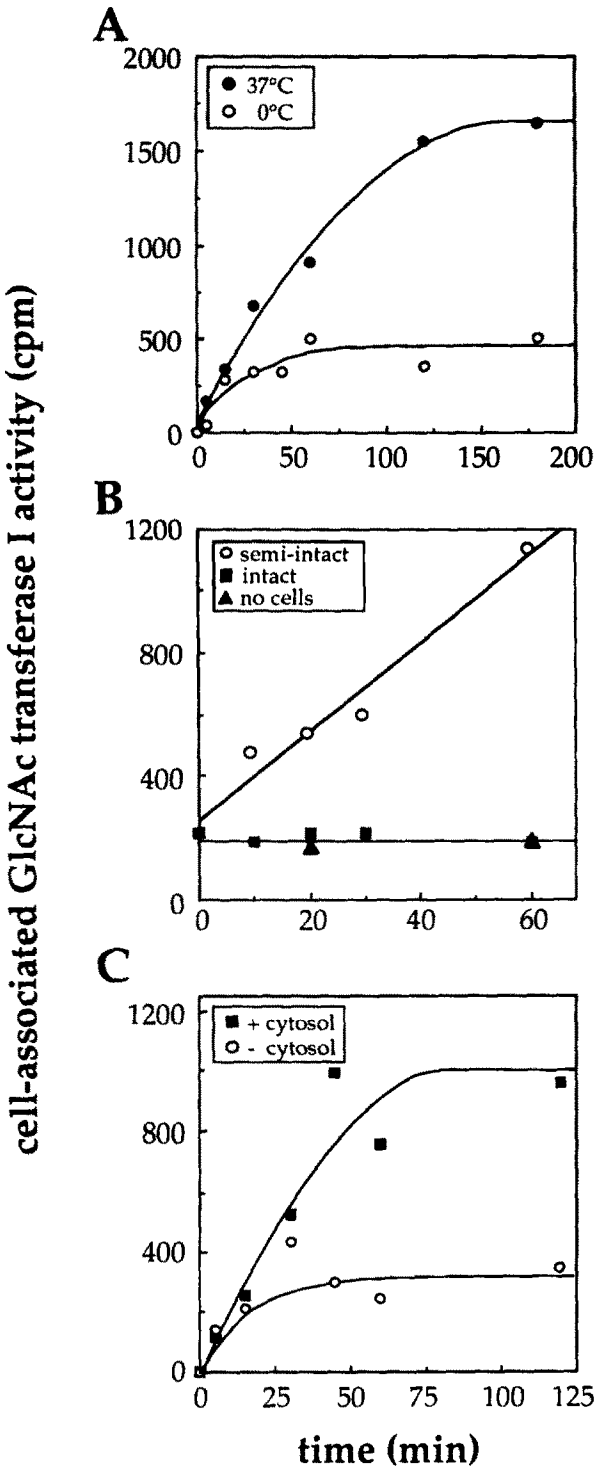

Figure 3. Golgi complexes associate with semi-intact, but not intact cells, in a temperature- and cytosol-dependent manner. (*A*) Standard assays were carried out for increasing times at the indicated temperatures as described in Materials and Methods. The background obtained in the absence of cells (180 cpm) was subtracted from each point. (*B*) Reactions were carried out using either semi-intact cells, intact cells, or no cells, as indicated. (*C*) Reactions were performed in the presence ("standard assay" conditions) or absence of 0.5 mg/ml cytosol for the times shown; the background cpm obtained in the absence of cells (186 cpm) was subtracted from each point. A value of 1,000 cpm for GlcNAc transferase activity represents 57 pmol GlcNAc incorporated into ovalbumin glycopeptide in 30 min. In *A–C* data points represent single determinations at each time point.

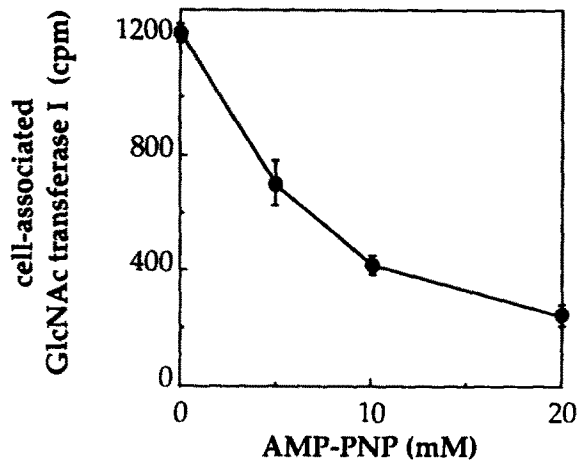

Figure 4. Golgi capture requires ATP hydrolysis. Reactions contained the indicated concentrations of AMP-PNP in addition to 100 μM ATP and an ATP regenerating system. The arithmetic mean of duplicates (± SD) is shown.

tent of Golgi capture. The level of Golgi capture was directly proportional to the amount of cytosol added (data not shown). Under optimal conditions, Golgi capture was very efficient; up to 30% of the added Golgi complexes associated with the semi-intact cells.

A comparison of the solid symbols shown in Fig. 3 (*A* and *C*) reflects the day-to-day variability in the assay. This variability is probably due to differences in the state of confluency of the cells, and therefore, the efficiency with which they are broken. Nevertheless, considering that the assay measures the uptake of entire Golgi complexes into broken cells, its reproducibility is very good.

In addition to cytosol, Golgi capture also required ATP. Little or no capture (<5–10%) was observed when hexokinase and glucose were included in the reaction mixtures to deplete ATP (not shown). Moreover, ATP hydrolysis seemed to be required because AMP-PNP, a nonhydrolyzable ATP analogue, was a potent inhibitor of the Golgi capture reaction (Fig. 4). The apparent requirement for ATP hydrolysis suggested that Golgi capture reflected a dynamic process, rather than a simple binding reaction.

Golgi complexes obtained from CHO cells or rabbit liver were fully functional in the Golgi capture assay (data not shown), suggesting that capture uses evolutionarily conserved components. In addition, bovine brain cytosol could substitute for CHO cytosol.

Captured Golgi Complexes Cluster Near the Centrosome

Indirect immunofluorescence was used to visualize directly the localization of exogenous Golgi complexes. For this purpose, we utilized biotinylated Golgi complexes in a Golgi capture reaction. Briefly, cells were broken gently and a standard reaction was performed at 37°C. Fixative was then added to the reactions for 30 min, after which the semi-intact cells were centrifuged onto the surface of "Cell Tak"-coated coverslips and processed for immunofluorescence (Fig. 5).

Centrosomes were visualized using a polyclonal antibody that is specific for a centrosomal protein termed pericentrin (Doxsey et al., 1991). In each cell, centrosomes were located on one side of the nucleus, which was detected by DAPI

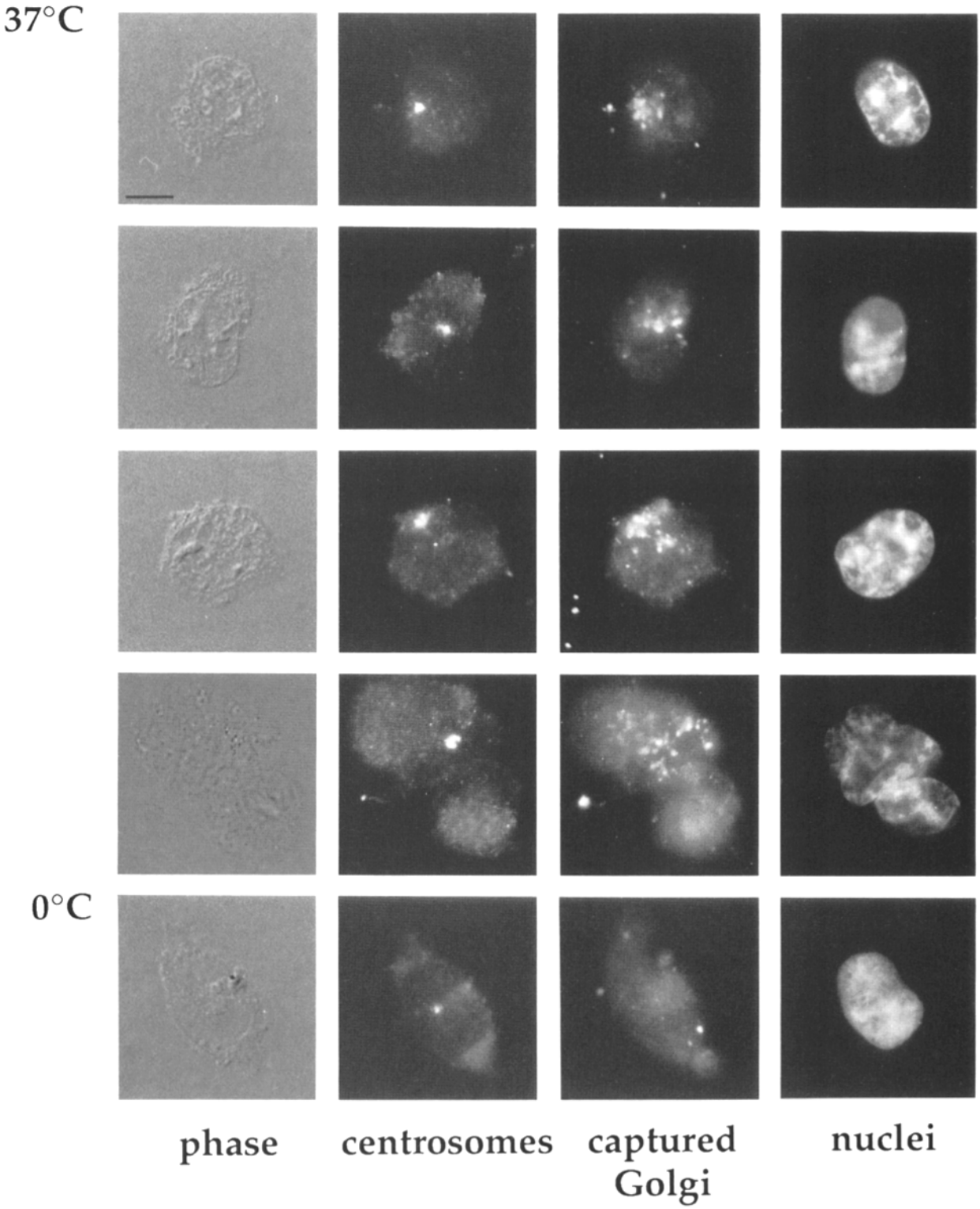

Figure 5. Localization of centrosomes and exogenous Golgi in semi-intact cells. Reactions were carried out for 60 min using biotinylated Golgi membranes. Semi-intact cells were fixed, centrifuged onto coverslips, then triple-labeled with rabbit anti-pericentrin antibodies and fluorescein-conjugated goat antirabbit to localize centrosomes, Texas red streptavidin to detect biotinylated Golgi complexes, and DAPI to identify nuclei. The upper four rows represent reactions carried out at 37°C; the lower row shows a reaction held at 0°C. Bar, 10 μm.

staining. When exogenously added, biotinylated Golgi complexes were visualized on the same coverslips using streptavidin-Texas red, there was a striking colocalization of the Golgi complexes with the centrosomes (Fig. 5). Quantitative analysis revealed that 86% of the cells displayed detectable Golgi capture (65 cells counted). In cells containing exogenous Golgi complexes, 93% of the Golgi complexes were either directly adjacent to the centrosome (as shown in Fig. 5;

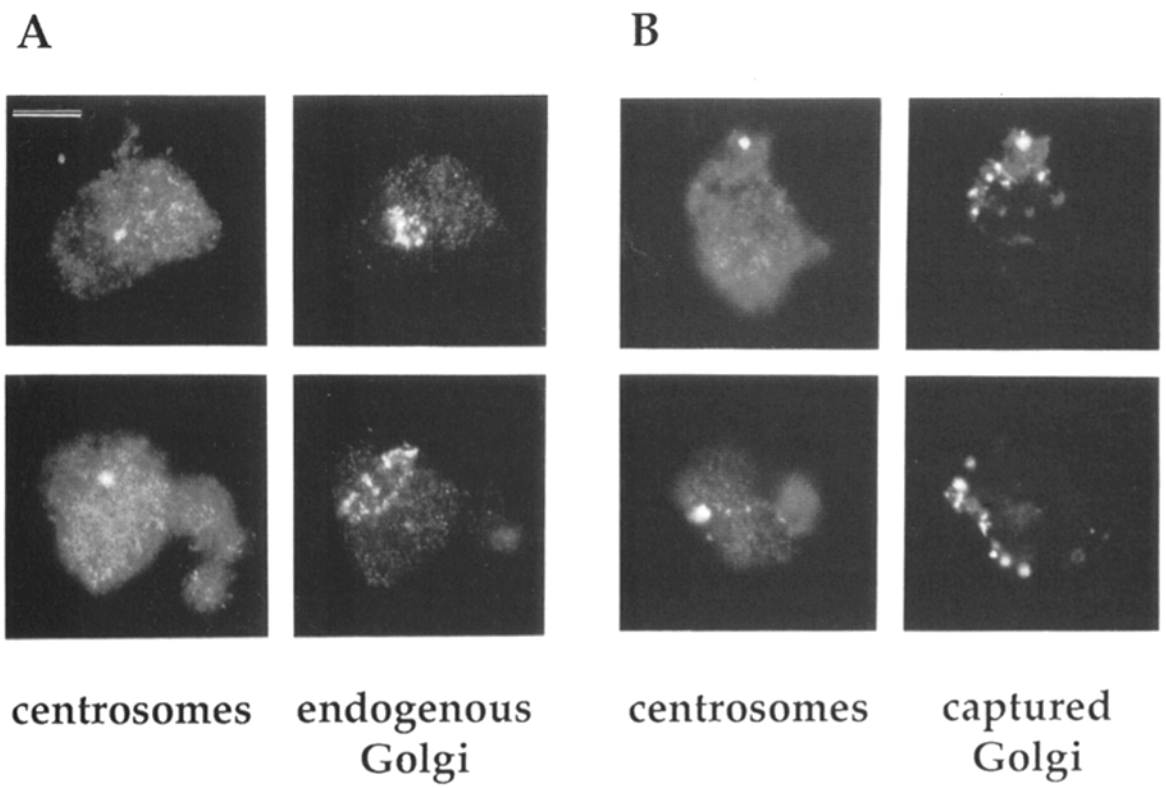

Figure 6. Both endogenous and captured Golgi complexes colocalize with centrosomes. (*A*) Reactions were carried out in the absence of exogenous Golgi for 60 min. After fixation and centrifugation onto coverslips, semi-intact cells were double-labeled with human scleroderma serum and rhodamine-conjugated goat anti-human IgG to localize centrosomes (*left column*) and rabbit anti-β-COP antibodies and fluorescein-conjugated goat anti-rabbit IgG to detect endogenous CHO cell Golgi complexes (*right column*). (*B*) Reactions were performed as in *A* in the presence of exogenous rat liver Golgi membranes. Exogenous Golgi complexes were detected selectively using a monoclonal anti-mannosidase II antibody that does not react with the host cell Golgi, and fluorescein-conjugated goat anti-mouse IgG (*right column*). Centrosomes were detected with rabbit anti-pericentrin antibodies and Texas red-conjugated goat anti-rabbit IgG (*left column*). For *B* only, semi-intact cells were permeabilized with 0.2% Triton X-100 after fixation and coverslip attachment. Bar, 10 μm.

55 cells counted) or appeared to be radiating away from it (Fig. 5, row *4*). This is precisely the distribution we observed for endogenous Golgi complexes, identified using antibodies to the Golgi coat protein, β-COP (Duden et al., 1991), in control semi-intact cells (Fig. 6 *A*).

When parallel reactions were carried out on ice, biotin-labeled Golgi complexes failed to colocalize with centrosomes, and the intensity of the Golgi fluorescence was decreased significantly (Fig. 5, *bottom* row). Specifically, only 25% of cells containing a centrosome displayed any Golgi fluorescence. Of those cells which were labeled (20 cells counted), 60% displayed a completely random Golgi localization; 15% of the cells contained exogenous Golgi complexes located at the centrosome; and 25% contained Golgi complexes in the same half of the cell as the centrosome. These experiments demonstrate that exogenous Golgi complexes interact with cytoplasmic components to achieve a peri-centrosomal distribution. In addition, they suggest that the centrosomal localization of exogenous Golgi complexes is not accomplished by simple binding to a component of the centrosome, since the majority of exogenous Golgi complexes are randomly distributed over the cytoplasm at 0°C.

Additional control experiments were performed to ensure that biotinylated structures represented Golgi complexes, rather than contaminating organelles. First, we used an antimannosidase II mAb that is specific for the Golgi of rodent cells, and fails to react with CHO Golgi complexes (Burke et al., 1981). This reagent enabled us to examine the distribution of exogenous rat liver Golgi complexes without interference from the endogenous host cell organelles. As shown in Fig. 6 *B*, antimannosidase II–stained, exogenous Golgi complexes codistributed with centrosomes of recipient cells, as seen for the biotinylated Golgi preparations (Fig. 5).

Golgi Capture Does Not Represent Conventional Vesicular Transport

To rule out the possibility that we were measuring the vesicular transfer of GlcNAc transferase I between exogenous and endogenous Golgi complexes (which are also located at the centrosome of the recipient cells), we tested whether Golgi capture was sensitive to the nonhydrolyzable analogue of GTP, GTPγS. Melançon and co-workers (1987) have shown that the vesicular transport of proteins between Golgi cisternae is readily inhibitable by GTPγS. However, unlike inter-Golgi transport, Golgi capture was not inhibited by up to 2 mM GTPγS; in fact, the process was stimulated 1.5–2-fold by GTPγS, when compared with control reactions containing either no GTP or 100 μM GTP (data not shown).

Captured Golgi Complexes Are Functional

It was of interest to determine whether the exogenous, cap-

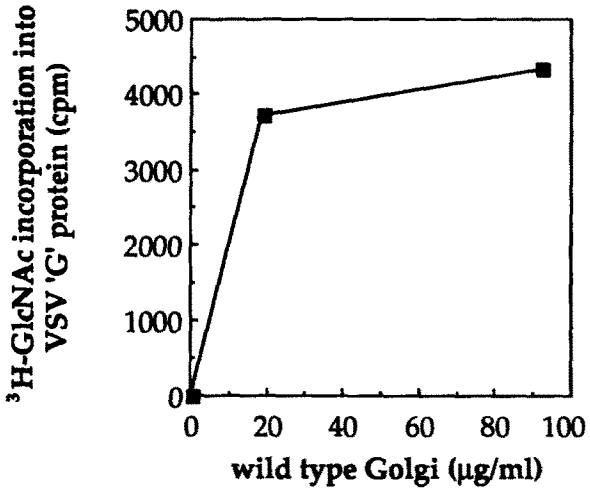

Figure 7. Golgi capture does not reflect conventional vesicular transport, but leads to capture of a transport-competent organelle. The ability of captured Golgi to serve as an acceptor of vesicular transport from the endogenous Golgi complex was monitored by a modification (Goda and Pfeffer, 1988) of the procedure of Balch et al. (1984). VSV-infected CHO clone 15B cells were used as hosts for Golgi capture; the transfer of VSV G protein from the host cell Golgi to the wild type Golgi was monitored by the incorporation of ^{3}H-GlcNAc into G protein at the end of the capture reaction. Reactions were carried out for 1 h at 37°C with increasing amounts of wild type Golgi membranes; the G glycoprotein was immunoprecipitated and ^{3}H-G protein was detected by scintillation counting. The background cpm obtained in the absence of added Golgi complexes was 2,487 cpm. This level of background radioactivity is only seen in intra-Golgi transport reactions carried out using semi-intact cells (as opposed to reactions containing only purified Golgi complexes) and probably reflects the incorporation of ^{3}H-GlcNAc into core oligosaccharides occurring in the ER of semi-intact cells. Data points represent single determinations from a representative experiment. The values presented reflect accurately the incorporation of ^{3}H-GlcNAc into VSV 'G' glycoprotein, as verified by densitometry of autoradiograms obtained after SDS-PAGE of samples obtained from parallel incubations.

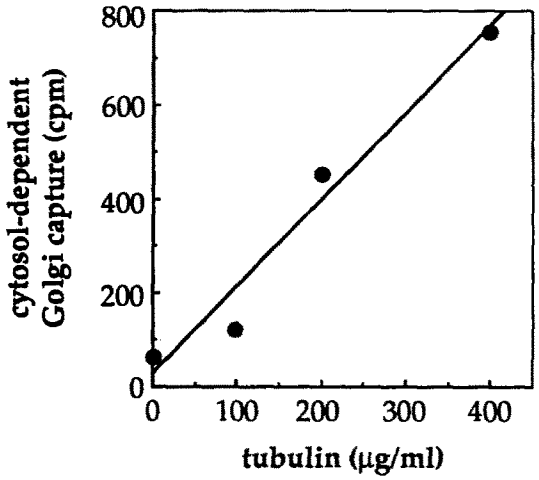

Figure 8. Golgi capture requires cytosolic tubulin. Crude cytosol was treated with taxol in the presence of 0.5 mg/ml purified tubulin, and then centrifuged to yield a microtubule-depleted fraction. The tubulin-depleted cytosol was subsequently dialyzed and used at 0.9 mg/ml in a 90-min Golgi capture assay. Purified tubulin was added back to the reaction at the indicated concentrations. The level of cytosol-dependent Golgi capture is shown; cpm observed in the absence of cytosol, but in the presence of tubulin were subtracted from each point (886, 896, 805, and 644 cpm in reactions containing 0, 100, 200, and 400 μg/ml tubulin, respectively). Data points represent single determinations of a representative experiment.

tured Golgi complexes retained their capacities to serve as acceptors for inter-Golgi vesicular transport and to mediate oligosaccharide maturation. For this purpose, we tested whether a glycoprotein present in the recipient cells could be glycosylated by the captured, wild type Golgi. CHO 15B cells were infected with vesicular stomatitis virus under conditions which lead to the production and transport of the vesicular stomatitis virus 'G' glycoprotein to the host cell Golgi complex (Balch et al., 1984). We then monitored the vesicular transport of the 'G' protein from the host cell Golgi to the medial cisternae of captured, wild type Golgi, by measuring the incorporation of ^{3}H-GlcNAc into G protein. Because only captured, wild type Golgi complexes contain functional GlcNAc transferase I activity, this assay provides a measure of vesicular transport from host cell organelles to the captured Golgi complexes. As shown in Fig. 7, G protein present in host cell organelles was transported to the captured, wild type Golgi complex, as evidenced by its incorporation of ^{3}H-GlcNAc from UDP-^{3}H-GlcNAc added to reaction mixtures (Fig. 7). This experiment demonstrates that captured Golgi complexes were functional in their ability to serve as an "acceptor" of vesicular transport and in oligosaccharide maturation.

Golgi Capture Is Microtubule-dependent

The cytoplasmic localization of Golgi complexes depends upon the integrity of the microtubule apparatus (Kreis, 1990). We therefore investigated the role of cytosolic tubulin in the Golgi capture process. CHO cytosol was depleted of tubulin by treatment with taxol, in the presence of a small amount of brain tubulin to seed the polymerization process. After high speed centrifugation to remove polymerized microtubules, the cytosol was tested for its ability to drive Golgi-capture reactions. As shown in Fig. 8, cytosol depleted of tubulin was incapable of facilitating the Golgi capture reaction. However, when purified brain tubulin was re-added in concert, Golgi capture was restored in a concentration-dependent manner. It is important to note that tubulin alone was not sufficient to support Golgi capture, thus, other cytosolic factors must also be required.

Immunofluorescence microscopy revealed that microtubules were substantially depolymerized in semi-intact cells that were incubated at 37°C in the absence of cytosol. It therefore seems most likely that cytosolic tubulin is needed to maintain the concentration of tubulin monomer at a high enough level to preserve the endogenous, semi-intact cell microtubules in a polymerized state.

Taxol treatment might be expected to deplete cytosol of microtubule-associated proteins (MAPs), in addition to tubulin. If MAPs are important for Golgi capture, they must be provided by the residual cytosol and semi-intact cell microtubules, since tubulin subunits were sufficient to restore activity to tubulin-depleted cytosol. For the experiments described below, it should also be noted that tubulin-depleted cytosol contained substantial levels of cytoplasmic dynein, as determined by immunoblot analysis, unless the taxol treatment was carried out in the presence of AMP-PNP (data not shown).

Table I. Efficient Golgi Capture Requires Polymerized Microtubules

	Tubulin polymer	Golgi capture
Complete reaction	1.00	1.00
Nocodazole	0.24	0.43
Complete reaction, 0°C	0.10	0.23

Tubulin polymer was determined by quantitative immunoblotting of semi-intact cell pellets after low speed centrifugation. Golgi capture was measured under standard conditions. Nocodazole was employed at 33 μM for 4 h at 37°C before semi-intact cell preparation; reactions were carried out for 120 min in the continued presence of 33 μM nocodazole.

To test further whether intact microtubules were required, we examined the effect of nocodazole on Golgi capture. Cells were pretreated with nocodazole for 4 h before semi-intact cell preparation, and nocodazole was also present during the capture reaction. Immunoblot analysis was employed to quantify the percentage of tubulin polymer remaining in treated cells. In the presence of nocodazole, microtubule polymerization was inhibited by ~75%. Under these conditions, Golgi capture was inhibited ~60% (Table I). Tubulin polymerization was more completely disrupted at 0°C: only 10% of the tubulin remained in a polymerized state. Under these conditions, Golgi capture was inhibited ~80%. These data reveal a strong correlation between the amount of tubulin polymer present in cells and the extent to which Golgi complexes stably associate with cytoplasmic components. Taken together with the requirement for cytosolic tubulin, it appears that microtubules participate in the process by which Golgi complexes associate with semi-intact cells and accumulate near the centrosome. This would be consistent with the observation that exogenous Golgi complexes often radiate away from the centrosome, as if bound to underlying microtubules. We do not rule out the possibility that interactions with additional cytoplasmic constituents are also involved.

Cytoplasmic Dynein Mediates Golgi Capture

After mitosis, clusters of Golgi vesicles that are distributed throughout the cytoplasm coalesce in the vicinity of the MTOC. Considering the polarity of cytoplasmic microtubules and their role in Golgi reorganization, it has been postulated that a dynein-like motor is responsible for postmitotic Golgi motility. Dynein-mediated motility is sensitive to lower concentrations of NEM than microtubule motors of the kinesin type (Lye et al., 1987; Paschal and Vallee, 1987), thus, we tested whether Golgi capture was also sensitive to this alkylating agent. Cytosol was treated with 1 mM NEM for 15 min, followed by addition of DTT to quench unreacted NEM. As shown in Fig. 9 *A*, NEM treatment abolished the potential of crude cytosol to enhance the capture reaction. These data demonstrate that NEM-sensitive cytosolic protein(s) are responsible for Golgi capture. However, given the broad reactivity of NEM, these results were only consistent with a potential role for cytoplasmic dynein.

A unique characteristic of cytoplasmic dynein is that it is subject to UV-induced photocleavage: dynein heavy chains are cleaved into two inactive polypeptides ("HUV" and "LUV") when irradiated in the presence of sodium vanadate (Lee-Eiford et al., 1986; Gibbons et al., 1987). We subjected CHO cytosol to UV irradiation in the presence or absence of vanadate. Immunoblot analysis of the resulting fractions showed that this treatment led to complete cleavage of the cytosolic dynein (Fig. 9 *C, right*). Cleavage was only observed in the presence of vanadate, and also required Mg^{++} ions (data not shown).

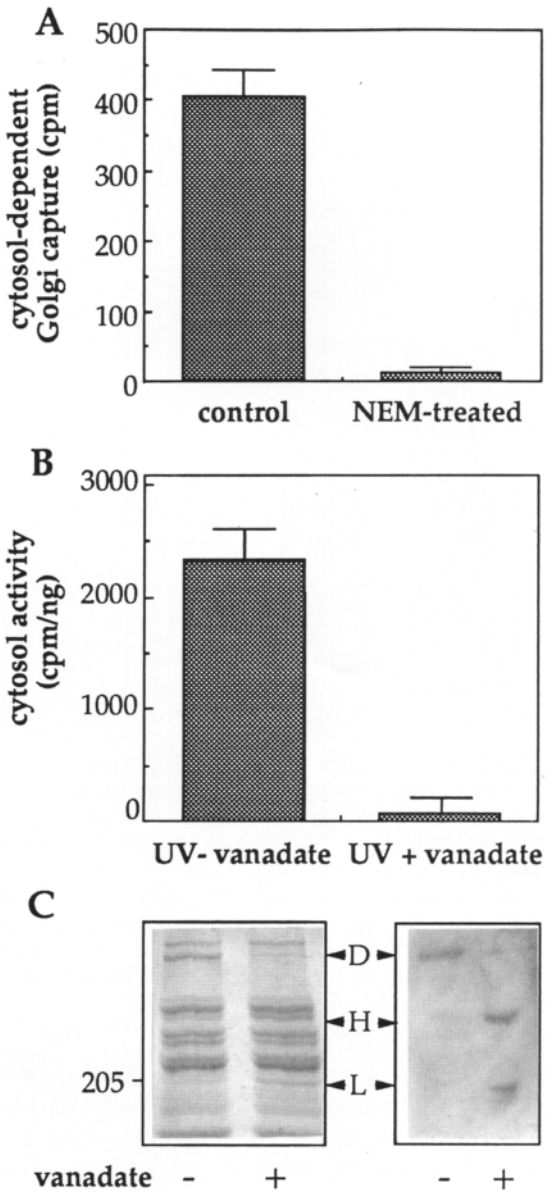

Figure 9. Cytosol activity is inhibited by NEM and UV-vanadate photocleavage. (*A*) Samples of cytosol were treated for 30 min on ice with or without 1 mM NEM. Reactions were then quenched for 15 min on ice with 10 mM DTT before use in a 90-min Golgi capture assay. Cytosol was assayed at 1.4 mg/ml in the reaction. The amount of cell-associated GlcNAc transferase I activity detected without cytosol was 151 cpm. (*B*) Samples of CHO cytosol containing 2 mM Mg-ATP were UV irradiated on ice for 1 h with or without 200 μM vanadate. Cytosols were tested subsequently for their ability to enhance Golgi capture in a standard assay for 1 h at 37°C. In *A* and *B*, the arithmetic mean of duplicates (± SD) is shown. (*C*) Photocleavage of dynein was monitored by SDS-PAGE and Coomassie blue staining (*C, left*) and Western blotting with an anti-chick brain cytoplasmic dynein antibody (*C, right*). The electrophoretic position of a 205K molecular weight marker is indicated, as are the positions of the dynein heavy chain (*D*), and the UV cleavage products HUV (*H*) and LUV (*L*).

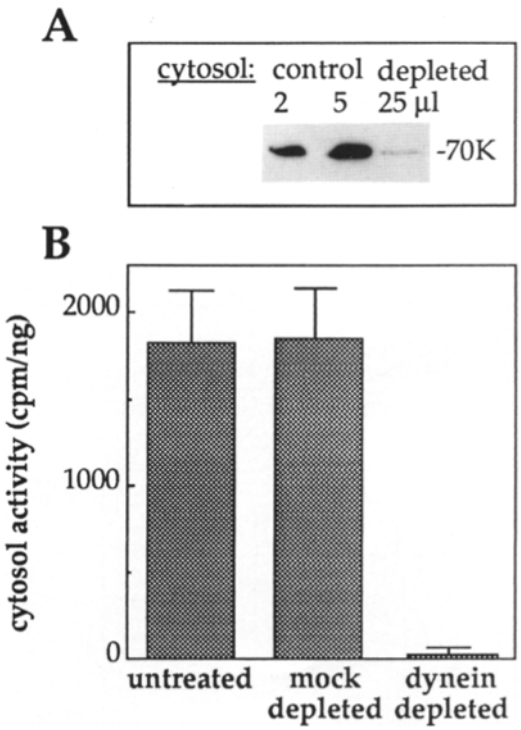

Figure 10. Cytoplasmic dynein is required for Golgi capture. (*A*) Anti-dynein intermediate chain antibodies (coupled to anti-IgM-agarose) deplete >95% of the dynein from cytosol fractions, as determined by monitoring the amount of dynein intermediate chain (~70K) remaining, by immunoblotting. (Lanes *1* and *2*) 2 and 5 µl of control (mock-depleted) cytosol, respectively; (lane *3*) 25 µl of depleted cytosol. (*B*) Standard assays were carried out for 70 min with the indicated cytosol fractions. The ability of cytosol to stimulate capture is shown. The arithmetic mean of triplicates (± SD) is shown.

As shown in Fig. 9 *B*, the cytosol fraction containing photocleaved dynein heavy chains ("UV + vanadate") was unable to support Golgi capture, while a control cytosol, irradiated in the absence of vanadate, retained cytosolic activity. Vanadate treatment in the absence of irradiation did not inhibit the activity of cytosol (data not shown). It is important to note that cytosol and vanadate are diluted 10-fold when assayed. In addition, although purified dynein is inhibited by 5–10 µM vanadate (Lye et al., 1987; Shpetner et al., 1988), there are likely to be multiple vanadate targets in crude cytosol preparations. In summary, these results demonstrate that Golgi capture requires a protein that is sensitive to UV irradiation in the presence of vanadate, analogous to cytoplasmic dynein.

The recent identification of a superfamily of kinesin-like motors (Vale and Goldstein, 1990) raised the possibility that Golgi capture was mediated by a dynein-like motor, rather than cytoplasmic dynein. To resolve this issue, cytoplasmic dynein was immunodepleted from CHO cytosol using a mAb that is specific for the dynein intermediate chain (Steuer et al., 1990). Immunoblot analysis confirmed that at least 95% of the dynein had been depleted from this fraction (Fig. 10 *A*), as determined by monitoring the level of cytoplasmic dynein intermediate chain remaining. When tested in the Golgi capture assay, this depleted cytosol was completely inactive, compared with either a mock-depleted, control cytosol, or untreated cytosol (Fig. 10 *B*).

We next prepared cytoplasmic dynein from CHO cytosol according to established procedures which are based on microtubule affinity and velocity sedimentation (Lye et al., 1987; Paschal et al., 1987). Fig. 11 *A* shows the migration of cytoplasmic dynein upon velocity sedimentation of proteins released from microtubules by ATP. Dynein, as determined by immunoblot analysis with antidynein intermediate chain antibodies (Fig. 11, *solid symbols*), migrated well ahead of the bulk of the protein (Fig. 11, *open circles*), by virtue of its large molecular mass. SDS-PAGE analysis of a typical preparation of cytoplasmic dynein is shown in Fig. 11 *B*. The high molecular weight heavy chain (H) is the most prominent polypeptide constituent. The sucrose gradient fraction also contains polypeptides that could correspond to the multiple intermediate (~70 kD) and light chains (~50–60 kD; Shpetner et al., 1988; Schroer and Sheetz, 1991*a*) and possibly also, the 150-kD dynein-associated polypeptide (Schroer and Sheetz, 1991*b*).

As shown in Fig. 11 *C*, the gradient fraction enriched in cytoplasmic dynein (fraction 10, gradient shown in Fig. 11 *A*) restored ~75% of the Golgi capture activity to a reaction carried out using dynein-immunodepleted cytosol. Addition of 0.7 µg/ml of the dynein fraction provided the amount of capture-stimulating activity present in 450 µg/ml total cytosol. Thus, restorative activity was enriched over 500 fold in the dynein-enriched fraction, and was not detected in other regions of the gradient (data not shown).

It is important to note that the experiment shown in Fig. 11 reflects the ability of a gradient fraction to restore activity to dynein-immunodepleted cytosol. In other words, a protein is present in the dynein-enriched fraction that can restore activity to cytosol missing only dynein and any proteins that may be bound tightly to dynein. This experiment demonstrates that the restorative activity resembles dynein in its tubulin-binding and sedimentation properties.

In summary, we have used four criteria to determine whether cytoplasmic dynein participates in the accumulation of exogenous Golgi complexes at the centrosome. We have shown that this process requires a cytosolic factor that is inactivated by 1 mM NEM, is sensitive to UV-photocleavage in the presence of vanadate, can be immunodepleted with a cytoplasmic dynein-specific mAb, and is enriched at least 500-fold in a purified preparation of cytoplasmic dynein. Taken together, these results demonstrate that cytoplasmic dynein heavy and intermediate chains are likely to be essential components of the process by which exogenous Golgi complexes associate stably with cytoplasmic constituents and accumulate at the centrosome.

Discussion

We have shown here that exogenous Golgi complexes can interact with cytoplasmic constituents of gently broken, "semi-intact" cells and accumulate near the centrosome. This process requires ATP hydrolysis, intact microtubules, and soluble proteins which include cytoplasmic dynein. The assay used here to detect Golgi association with the microtubule-based cytoskeleton requires that isolated Golgi

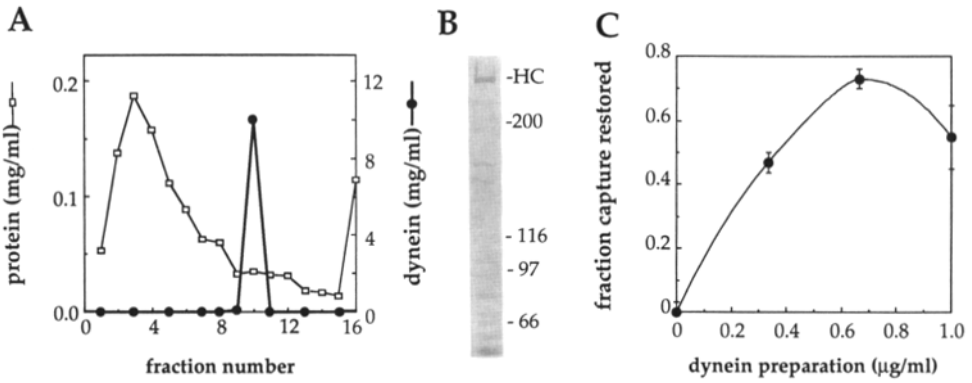

Figure 11. Purified cytoplasmic dynein restores Golgi capture activity to dynein-depleted cytosol. (*A*) Sucrose gradient velocity sedimentation of proteins released from microtubules by ATP. (*Open squares*) total protein; (*closed circles*) cytoplasmic dynein, as determined by immunoblotting for the dynein intermediate chain as in Fig. 9 *A*. The top of the gradient is shown at left. (*B*) SDS-PAGE analysis of a typical CHO cytoplasmic dynein preparation. HC, dynein heavy chain. The values for the molecular mass markers represent kilodaltons. (*C*) Purified cytoplasmic dynein (A, fraction *10*) restores capture activity to reactions carried out with dynein-immunodepleted cytosol (from Fig. 10). Control cytosol activity was 3,300 cpm/ng. The arithmetic mean of triplicates (± SD) is shown.

complexes interact with semi-intact cell contents with high enough affinity to remain stably associated upon sedimentation through sucrose. Our results imply that capture involves both binding of Golgi complexes to intracellular components, as well as microtubule-based movement.

The purified Golgi complexes used in the capture assay are likely to be structurally and functionally comparable to the mini-stacks observed in nocodazole-treated cells. Golgi stacks are isolated at 4°C, conditions that like nocodazole, will destabilize microtubules. In addition, isolated Golgi stacks are similar in size to those generated in nocodazole (see Braell et al., 1984) and are also functional in supporting intercisternal vesicular transport and oligosaccharide maturation (Rothman and Orci, 1990), like those in nocodazole-treated cells (see Rogalski et al., 1984).

Several lines of evidence suggest that the semi-intact cell binding site for Golgi complexes represents polymerized microtubules. Polymerized microtubules are required for capture, as evidenced by the dependence of the reaction on cytosolic tubulin and the sensitivity of the reaction to nocodazole. In addition, indirect immunofluorescence showed that the Golgi capture reaction resulted in the colocalization of Golgi complexes with centrosomes, on one side of the nucleus. Therefore, it seems likely that the initial steps in Golgi capture involve microtubule binding. Simple binding would explain the low but detectable level of Golgi capture observed at 0°C.

We have no information regarding a role for MAPs in Golgi capture. Although tubulin was sufficient to restore activity to cytosol from which tubulin and presumably also MAPs were depleted, MAPs are likely to have been provided by the semi-intact cells. Further experiments will be necessary to address the potential role of MAPs in Golgi capture.

Microtubule-directed motility of Golgi complexes in our system is implied by the requirement for ATP hydrolysis, and more significantly, by the requirement for cytoplasmic dynein, a microtubule-based motor that drives organelles towards the minus ends of microtubules. In addition, at 0°C, the vast majority of exogenous Golgi complexes were distributed randomly throughout the cytoplasm; only at 37°C were they found concentrated near centrosomes. This result supports a model in which exogenous Golgi complexes bind first to microtubule arrays, and are then translocated towards the centrosome by cytoplasmic dynein.

Storrie and colleagues have shown that in fused cells, Golgi complexes coalesce with a half-time of ~1.75 h (Deng et al., 1992). Although we cannot rule out the possibility that we are measuring some coalescence of endogenous and exogenous Golgi complexes, the stable association of Golgi complexes with recipient cells is achieved with much more rapid kinetics than Golgi coalescence in fused cells. If Golgi coalescence does occur, our data would best fit a model in which this process occurred subsequent to microtubule association and movement towards the centrosome.

How does the Golgi complex become localized in the vicinity of the MTOC? One possibility is that a class of microtubule-associated binding proteins retains it there. Because the Golgi complex colocalizes with microtubules rich in detyrosinated tubulin (Thyberg and Moskalewski, 1990), it has been suggested that the Golgi may simply bind preferentially to this class of tubulin (Skoufias et al., 1990). However, the observations that mini-Golgi re-centralization after nocodazole washout is an energy-requiring process (Turner and Tartakoff, 1989) that involves directed movement along microtubule tracks (Ho et al., 1989) lend strong support to the hypothesis that a dynein-like, microtubule-based motor is involved.

Once localized to the centrosomal region, how does the Golgi remain there? One model would be that cytoplasmic dynein continually drives the Golgi complex towards the centrosome. A prediction of this model is that Golgi localization would be energy-dependent. Donaldson et al. (1991) found that the distribution of the Golgi complex was unchanged in energy-depleted cells, at least up to 10 min after deoxyglucose and azide addition. This observation leaves open the possibility that the Golgi is tethered in some way to the MTOC and/or its associated components. It would not be unreasonable for a single copy organelle like the Golgi complex to utilize a limited number of tethering proteins to maintain its perinuclear distribution. A small number of linking sites would also lead to the dispersal of the Golgi complex upon fragmentation into at least 100 mini-Golgi stacks in nocodazole-treated cells.

The fact that the Golgi complex falls apart under conditions of microtubule depolymerization is both provocative

and unexplained. This finding implies that the Golgi is an extremely dynamic organelle that is capable of extensive and continual homotypic fusion and fission reactions (Cooper et al., 1990). Perhaps polymerized microtubules and cytoplasmic dynein increase the frequency of Golgi stack collisions under normal conditions. If collisions increase the probability of membrane fusion and predominate relative to membrane fission, a single organelle will be generated. An interesting and testable prediction of this model would be that cytoplasmic dynein and polymerized microtubules might increase the rate of homotypic Golgi fusion in vitro. Conversely, if membrane fission events predominate, the Golgi will vesiculate. This is precisely the mechanism proposed by Warren (1985) to explain Golgi vesiculation during mitosis.

During mitosis, when the Golgi fragments into clusters of small tubules and vesicles (Lucocq et al., 1987, 1989), the fragments lose their perinuclear localization. This could be due to an alteration in the activity of a tethering protein, a motor protein, and/or a protein that links the Golgi to a motor protein. Allan and Vale (1991) have shown that interphase and metaphase *Xenopus* cytosols contain the same level of cytoplasmic dynein-like motor function. Nevertheless, these workers found that membrane-bound organelle movement in metaphase extracts was inhibited. Thus, the most likely target for cell cycle regulation is the mechanism by which cytoplasmic dynein couples to specific organelles. This result agrees well with the finding that dynein is recruited to the spindle, at mitosis, to participate in chromosome segregation (Pfarr et al., 1990; Steuer et al., 1990).

Previously described organelle motility assays have been based upon microscopic visualization of membranous organelles, without organelle-specific markers. These assays have proven to be exceptionally powerful, and have led to the discovery of novel motor proteins. However, these assays are limited by the fact that organelle preparations are rarely pure; it should be noted that Golgi-enriched fractions are also enriched in endosomes and plasma membrane-derived vesicles. The experimental approach employed here has the distinct advantage of being absolutely specific for the medial Golgi complex, since a Golgi-specific enzyme is monitored.

We have shown that exogenous Golgi complexes can interact with cytoplasmic components and accumulate at the centrosome in the presence of cytoplasmic dynein. These results validate the prediction that cytoplasmic dynein participates in the localization of the Golgi complex (Schroer et al., 1989; Schnapp and Reese, 1989). We have recently found that peripheral membrane proteins on the surface of the Golgi complex are required for Golgi capture (data not shown). It is likely that these proteins link the Golgi to cytoplasmic dynein in a cell-cycle dependent manner.

We thank Drs. Stephen Doxsey and Marc Kirschner for pericentrin antibodies and invaluable advice; Dr. Tim Mitchison for providing tubulin and clone 70.1 cells; and Drs. Trina Schroer, Michael Sheetz, and Brian Burke for very generous gifts of antidynein and antimannosidase II antibodies.

This work was supported by grants to S. R. Pfeffer from the National Institutes of Health (DK-37332) and Smith Kline-Beecham. I. Corthésy-Theulaz received fellowship support from the Swiss National Foundation for Scientific Research and the Fondation du 450ième Anniversaire de l'Université de Lausanne.

Received for publication 8 May 1992 and in revised form 16 June 1992.

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