

The Mitotic Phosphorylation Cycle of the cis-Golgi Matrix Protein GM130

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Abstract. The cis-Golgi matrix protein GM130 is phosphorylated in mitosis on serine 25. Phosphorylation inhibits binding to p115, a vesicle-tethering protein, and has been implicated as an important step in the mitotic Golgi fragmentation process. We have generated an antibody that specifically recognizes GM130 phosphorylated on serine 25, and used this antibody to study the temporal regulation of phosphorylation in vivo. GM130 is phosphorylated in prophase as the Golgi complex starts to break down, and remains phosphorylated during further breakdown and partitioning of the Golgi fragments in metaphase and anaphase. In telophase, GM130 is dephosphorylated as the Golgi fragments start to reassemble. The timing of phosphorylation and

dephosphorylation correlates with the dissociation and reassociation of p115 with Golgi membranes. GM130 phosphorylation and p115 dissociation appear specific to mitosis, since they are not induced by several drugs that trigger nonmitotic Golgi fragmentation. The phosphatase responsible for dephosphorylation of mitotic GM130 was identified as PP2A. The active species was identified as heterotrimeric phosphatase containing the B α regulatory subunit, suggesting a role for this isoform in the reassembly of mitotic Golgi membranes at the end of mitosis.

Key words: Golgi • mitosis • GM130 • phosphorylation • protein phosphatase 2A

Introduction

The Golgi complex is characterized by its unique appearance, resulting from the close apposition of flattened cisternae arranged in parallel to form stacks. In mammalian cells, the stacks are connected laterally by tubular connections to form a single compact reticulum often localized adjacent to the nucleus (Rambourg and Clermont, 1997). During cell division, the Golgi complex undergoes a dramatic morphological transformation (Robbins and Gonatas, 1964; Cabrera-Poch et al., 1998). Early in mitosis, during prophase, the Golgi ribbon is broken down to discrete stacks (Colman et al., 1985), which themselves undergo more extensive fragmentation as cells progress into metaphase, yielding clusters of small vesicles and tubules in addition to a variable amount of free vesicles in the cytoplasm (Lucocq and Warren, 1987; Lucocq et al., 1987, 1989; Misteli and Warren, 1995a). The mitotic Golgi clus-

ters are partitioned between the two nascent daughter cells by a mechanism that appears to utilize the microtubules of the mitotic spindle (Shima et al., 1997, 1998). During telophase, the vesicles and tubules of the mitotic clusters fuse to generate stacked cisternae, which then coalesce and connect to reform the Golgi ribbon (Lucocq et al., 1989; Souter et al., 1993).

Assays have been established to mimic the events of mitotic disassembly and reassembly in vitro (Misteli and Warren, 1994; Rabouille et al., 1995b). Incubation of purified rat liver Golgi stacks with mitotic cytosol results in the conversion of stacked cisternae into small vesicles and tubules (Misteli and Warren, 1994), very similar to the breakdown products seen in vivo (Lucocq et al., 1987; Misteli and Warren, 1995a). Two principal fragmentation pathways appear to be responsible for these changes. The major fragmentation pathway, accounting for loss of 60–70% of the cisternal membrane, is the coat protein I (COPI)¹ pathway, which consumes the cisternal rims by continued budding of COPI transport vesicles in the absence of their docking and fusion (Misteli and Warren, 1994). The second COPI-independent pathway converts

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¹Abbreviations used in this paper: COPI, coat protein I; IQ, ilimaquinone; MEK1, mitogen-activated protein kinase kinase 1; NRK, normal rat kidney; PKD, protein kinase D.

the cisternal core into heterogeneous vesicles and tubules by a mechanism that is not yet clear (Misteli and Warren, 1995b). This pathway may be analogous to the specific fragmentation of the Golgi complex mediated by the sea sponge metabolite ilimaquinone (IQ) (Takizawa et al., 1993; Jamora et al., 1999).

Mitotic Golgi fragmentation *in vitro* is dependent upon Cdc2-cyclin B (Misteli and Warren, 1994; Lowe et al., 1998). One of the Cdc2 substrates has been identified as GM130 (Lowe et al., 1998), a protein originally identified as part of a putative structural Golgi matrix (Nakamura et al., 1995). GM130 is predicted to form a coiled-coil rod with a proline-rich domain in the middle that might form a flexible hinge (Nakamura et al., 1995). It is anchored to the cytoplasmic face of the cis-Golgi by its extreme COOH terminus, which interacts tightly with the N-myristoylated Golgi reassembly stacking protein (GRASP) 65 (Barr et al., 1997, 1998). The NH₂ terminus of GM130 binds specifically to the vesicle-tethering protein p115 (Nakamura et al., 1997). p115 was originally identified as an essential factor for intra-Golgi transport (Waters et al., 1992), and recent studies have shown that it can tether COPI vesicles to Golgi membranes *in vitro* (Sönnichsen et al., 1998). p115 is thought to mediate tethering by cross-linking GM130 on the Golgi membrane to giantin on COPI vesicles (Sönnichsen et al., 1998). Giantin is a type II membrane protein predicted to form a coiled-coil rod projecting into the cytoplasm (Linstedt and Hauri, 1993; Seelig et al., 1994). Tethering mediated by p115, in a manner analogous to that demonstrated for the yeast homologue Uso1p (Sapperstein et al., 1996; Cao et al., 1998), is believed to bring the appropriate vesicle- and target-soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein (SNAP) receptor (SNARE) proteins into close proximity and promote the formation of trans-SNARE pairs, which then drive membrane fusion by a mechanism that is not well understood (Mayer, 1999). Cdc2-mediated phosphorylation of GM130 occurs on serine 25, with the effect of potently inhibiting p115 binding (Lowe et al., 1998). This would account for the reduced association of p115 with Golgi membranes seen both *in vitro* and *in vivo* (Levine et al., 1996; Shima et al., 1997), and could explain the COPI fragmentation pathway, since if p115 can no longer bind GM130, then COPI vesicles would no longer be able to dock with Golgi cisternae and so would accumulate. A role for the mitogen activated protein kinase kinase 1 (MEK1) in mitotic Golgi fragmentation has also been proposed, but the putative targets remain to be identified (Acharya et al., 1998).

Postmitotic reassembly of Golgi stacks occurs in two phases: cisternal regrowth and cisternal stacking (Rabouille et al., 1995b). Regrowth of cisternae *in vitro* can be catalyzed by two members of the AAA (ATPases associated with diverse cellular activities) ATPase family, NSF (and its soluble cofactors α -SNAP, γ -SNAP and p115) or p97 (and its soluble cofactor p47), which seem to contribute nonadditively to the growth process (Rabouille et al., 1995a). As cisternae start to grow, they become aligned and dock with each other to form stacks. Recent studies have shown that p115, in addition to its role in NSF-mediated cisternal growth, is also required for the initial alignment and docking of cisternae, acting before the stacking

factor GRASP65 (Shorter and Warren, 1999). The activity of p115 in both cisternal regrowth and cisternal stacking is dependent upon binding to its membrane tethering partners, GM130 and giantin (Shorter and Warren, 1999). The finding that p115 binding to GM130 is inhibited by phosphorylation of GM130 on serine 25 suggests that this residue must be dephosphorylated for Golgi reassembly to occur. The identity of the phosphatases required for the dephosphorylation of GM130, as well as other mitotic Golgi phosphoproteins, such as GRASP65 (Barr et al., 1997), is currently unknown.

Our current understanding of the mitotic regulation of GM130 and its proposed role in mitotic Golgi fragmentation have come from *in vitro* approaches in which the Golgi complex has one of two defined states, interphase and mitotic. To more fully understand the involvement of GM130 phosphorylation in mitotic fragmentation we need to observe the phosphorylation and dephosphorylation events as they occur *in vivo*. To this end, we have generated an antibody that specifically recognizes GM130 phosphorylated on serine 25. Here we report our findings on the timing of GM130 phosphorylation and dephosphorylation, and link these biochemical events to the morphological changes that take place during Golgi disassembly and reassembly in mitosis. We also identify the trimeric form of PP2A with the B α regulatory subunit as the phosphatase responsible for the dephosphorylation of GM130 at the end of mitosis.

Materials and Methods

Antibodies and Reagents

The rabbit anti-phospho-GM130 antibody (anti-phosphoserine 25) was raised against the phosphopeptide CQQKNpSPGVPAG (PS25pep). The peptide was coupled to keyhole limpet hemocyanin (KLH) using glutaraldehyde and meta-maleimidobenzoyl N-hydroxysuccinimide ester and injected into rabbits to raise polyclonal antisera. Antibodies were affinity-purified on the phosphopeptide coupled to Ultralink Iodoacetyl beads (Pierce Chemical Co.). Rabbit polyclonal antibodies to the NH₂-terminal 73 amino acids of GM130 have been described previously (anti-N73pep; Nakamura et al., 1997; Sönnichsen et al., 1998). The monoclonal anti-GM130 antibody used in immunofluorescence experiments was purchased from Transduction Labs. Monoclonal anti-GM130 antibody 5F10 was generated from mice immunized with full-length bacterially expressed GM130. Rabbit antibodies to p115 (MLO-1) were raised against the peptide ETIQKLCDRVASSTL coupled to maleimide-activated KLH (Pierce Chemical Co.) and affinity-purified on the peptide coupled to Ultralink Iodoacetyl beads. Monoclonal anti-p115 antibody (4H1) (Waters et al., 1992) was purified from tissue culture supernatant on protein G-Sepharose. mAbs to rat β -1,4 galactosyltransferase, cyclin B1, and α -tubulin were kindly provided by Drs. Tatsuo Suganuma (Miyazaki Medical College, Japan), Jonathon Pines (Wellcome/Cancer Research Campaign Institute, Cambridge, UK), and Viki Allan (University of Manchester, Manchester, UK), respectively. Rabbit polyclonal antibodies to mannosidase I were kindly provided by Dr. Francis Barr (Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK). Mouse monoclonal anti-vimentin and goat polyclonal anti-lamin B antibodies were purchased from Santa Cruz Biotechnology. Antibodies to PP2A subunits were kindly provided by Drs. Brian Hemmings (Friedrich Miescher Institute, Basel, Switzerland), Ned Lamb and Patric Turowski (Institute of Human Genetics, Centre National de la Recherche Scientifique, Montpellier, France), or purchased from Santa Cruz Biotechnology. HRP, rhodamine, and FITC-conjugated secondary antibodies were purchased from Tago Inc. Texas red- and Alexa 488-conjugated antibodies were purchased from Molecular Probes Inc.

Purified PP1 and PP2A were purchased from Upstate Biotech. Purified PP1 was from rabbit skeletal muscle and contained multiple PP1 isoforms. Purified PP2A was in the trimeric form (called PP2A1) and from rabbit

skeletal muscle. Sf9 cell extracts with expressed PP2A isoforms were kindly provided by Drs. Feng Gu and Gary Thomas (Vollum Institute, Oregon Health Sciences University, Portland, OR). Okadaic acid, inhibitor-2, and microcystin-LR were purchased from Calbiochem. [γ - 32 P]ATP was purchased from ICN Ltd. IQ and nocodazole were purchased from Sigma Chemical Co. Hoechst 33342 and SYTO 13 were purchased from Molecular Probes Inc. All other reagents were purchased from Sigma Chemical Co., Boehringer Mannheim, or BDH Chemicals Ltd., unless otherwise stated.

Incubation of Rat Liver Golgi Membranes with Interphase and Mitotic Cytosols

Rat liver Golgi membranes were purified as in Hui et al. (1998). Interphase and mitotic cytosols were prepared from spinner HeLa cells according to Sönnichsen et al. (1996) and desalted into buffer A (20 mM β -glycerophosphate, 15 mM EGTA, 50 mM KOAc, 10 mM MgOAc, 2 mM ATP, 1 mM DTT, 0.2 M sucrose). Golgi membranes (10 μ g) were incubated with desalted cytosol (8 mg/ml) in a final volume of 50 μ l in the presence of an ATP regenerating system (10 mM creatine phosphate, 20 μ g/ml creatine kinase) for 30 min at 30°C. In some experiments, reactions were terminated at various times by placing on ice. Membranes were reisolated by spinning through a layer of 0.4 M sucrose (in buffer A) for 15 min at 100,000 *g*. Membrane pellets were solubilized in SDS sample buffer containing 5 mM NaF. For experiments where GM130 dephosphorylation and p115 rebinding were analyzed, 10 μ g mitotic Golgi membranes (see below) were incubated with interphase cytosol (2 mg/ml in buffer A) supplemented with 50 ng p115 (to give a p115 concentration similar to that in mitotic cytosol) in a final volume of 50 μ l for various lengths of time at 30°C. Membranes were then reisolated by centrifugation and solubilized in SDS sample buffer containing 5 mM NaF.

Fractionation of Purified Rat Liver Golgi Membranes

50 μ g of purified rat liver Golgi membranes (Hui et al., 1998) was adjusted to 1.6 M sucrose (in 0.1 M KPO₄, pH 6.7, 5 mM MgCl₂) in a volume of 0.5 ml. This was overlaid with 1 ml 1.2 M sucrose, then 1.5 ml 1.0 M sucrose, and finally by 1.5 ml 0.4 M sucrose (all sucrose solutions were made in 0.1 M KPO₄, pH 6.7, 5 mM MgCl₂), and centrifuged for 4 h at 55,000 rpm in an SW55 rotor. Fractions (0.4 ml) were collected from the top, 40 μ g soybean trypsin inhibitor added as a carrier, and proteins precipitated using methanol/chloroform. Precipitated proteins were solubilized in SDS sample buffer and analyzed by SDS-PAGE.

Drug Treatments and Cell Synchronization

Normal rat kidney (NRK) and HeLa cells were maintained in DME containing 10% FCS. Cells were incubated with okadaic acid (1 μ M) for 1 h at 37°C, or with IQ (25 μ M) for 2 h at 37°C. Cells were first chilled to 4°C for 5 min before incubation with nocodazole (5 μ g/ml) for 2 h at 37°C. For enrichment of mitotic NRK cells, 2.5 μ g/ml aphidicolin was added to the medium for 12–14 h to arrest cells at the G1/S boundary. Cells were washed several times to remove the drug and incubated in fresh medium for 5–6 h to allow progression into mitosis.

Preparation of Cell Lysates and Immunoprecipitation of GM130

Mitotic NRK cells were removed from culture dishes by repeated flushing with a Pasteur pipette and collected by centrifugation. Cells were washed with ice-cold PBS and lysed in lysis buffer (20 mM Hepes, pH 7.4, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 5 mM EDTA, 2 mM EGTA, 10 mM NaF, 40 mM β -glycerophosphate, 1 mM sodium orthovanadate) containing protease inhibitors (1 mM PMSF, 0.5 mM 1-10 phenanthroline, 2 μ M pepstatin, 2 μ g/ml aprotinin, 4 μ g/ml aprotinin, 20 μ g/ml chymostatin) for 10 min on ice before centrifugation at 20,000 *g* for 10 min. Clarified lysates were frozen in liquid nitrogen and stored at –80°C. Protein concentration was measured using the BCA method (Pierce Chemical Co.). For analysis by Western blotting, proteins were precipitated with 10% TCA and resuspended in SDS sample buffer. For immunoprecipitation of GM130, 200 μ l of lysate (100 μ g protein) was incubated with 50 μ l of 5F10 hybridoma culture supernatant for 1 h at 4°C, before addition of 20 μ l protein G-Sepharose beads (50% slurry) and incubation for a further 1 h at 4°C. Beads were washed three times with lysis buffer, once with PBS, and proteins eluted by boiling in 2 \times SDS sample buffer.

Fluorescence Microscopy

Cells were grown on glass coverslips and fixed in 100% methanol (–20°C) for 4 min. In some cases, cells were fixed in paraformaldehyde (3.5% in PBS) for 20 min at room temperature, quenched with 10 mM glycine, pH 8.5 (in PBS), and permeabilized with 0.1% Triton X-100 (in PBS) for 5 min at room temperature. Coverslips were incubated with primary antibodies diluted in PBS containing 0.5 mg/ml BSA for 20 min at room temperature, washed with PBS, and incubated with rhodamine-, Texas red-, FITC-, or Alexa 488-conjugated secondary antibodies diluted in PBS containing 0.5 mg/ml BSA for a further 20 min at room temperature. The DNA dye Hoechst 33342 (200 ng/ml) was included in the second incubation to allow identification of mitotic stages. For visualization of DNA with SYTO 13, methanol-fixed cells were incubated with 1 mg/ml DNase-free RNase (in PBS) for 1 h at room temperature before labeling. SYTO 13 was included in the second incubation at 500 nM. Coverslips were mounted in Mowiol, allowed to dry, and viewed by laser scanning confocal microscopy on a Zeiss LSM510 confocal microscope. Serial optical sections (collected at 0.45- μ m intervals) in the z-axis of the cell were collected and overlaid for the final images shown.

Quantitation of images was performed with the Metamorph program (Universal Imaging Corp.). The total cellular fluorescence of GM130-containing structures was calculated using conflated series of sections through entire cells. Background was subtracted and a threshold introduced to exclude diffuse cytoplasmic staining. All pixels above this threshold value were counted as GM130-containing structures in both interphase and mitotic cells. The results were expressed as the mean \pm SD/cell and normalized to interphase cells. The mean fluorescence intensities for GM130 and p115 were measured for a total of 40 different selected Golgi (GM130-positive) areas in four different interphase and metaphase cells. These areas corresponded to the Golgi ribbon in interphase cells and individual mitotic clusters in metaphase cells. The ratio of GM130 to p115 fluorescence was calculated for each area. The ratios were then averaged and expressed as the mean \pm SD, and normalized to interphase cells.

Dephosphorylation Assays

Mitotic Golgi membranes were prepared by incubating rat liver Golgi membranes (1 mg) with mitotic HeLa cytosol (5 ml at 2 mg/ml in Buffer A with an ATP-regenerating system) for 30 min at 30°C. Membranes were reisolated by centrifugation for 20 min at 100,000 *g* in a SW55 rotor (Beckman Instruments) through a layer of 0.4 M sucrose in dephosphorylation buffer (20 mM Hepes, pH 7.4, 50 mM KOAc, 1 mM EGTA, 1 mM DTT), onto a 2 M sucrose cushion. Membranes were resuspended at 2 mg/ml in dephosphorylation buffer containing 0.2 M sucrose and stored at –80°C. 32 P-labeled phosphorylase a was prepared by incubating phosphorylase b (10 mg/ml) with phosphorylase kinase (300 μ g/ml) and 0.4 mM [γ - 32 P]ATP (750 μ Ci/ml) in 75 mM β -glycerophosphate, pH 8.6, 0.2 mM CaCl₂, 2 mM MgOAc, for 1 h at 30°C. Free ATP was removed by spinning through two Biogel P6-DG (Biorad) spin columns previously equilibrated in 50 mM Tris, pH 7.4, and the protein stored at 4°C. All dephosphorylation experiments were carried out in dephosphorylation buffer containing 0.2 M sucrose and 0.2 mg/ml BSA. 5 mM caffeine was included when phosphorylase a was the substrate. 1 μ g N73pepPS25 or 5 μ g mitotic Golgi membranes was incubated with interphase cytosol, purified phosphatase, or Sf9 cell lysate in a final volume of 25 μ l for 20 min at 30°C. Sf9 cell lysates, prepared according to Molloy et al. (1998), were diluted 1:50 before addition to the assay. In experiments with phosphatase inhibitors, 0.01% Brij-35 was included. When phosphorylase a was the substrate, proteins were precipitated with TCA and [32 P]phosphate in the supernatant measured by liquid scintillation counting. In experiments with N73pepPS25, 2 μ M microcystin-LR was added at the end of the incubation and 1 μ l of the reaction mix was spotted onto nitrocellulose. In experiments with Golgi membranes, samples were centrifuged for 15 min at 45,000 rpm in a TLA45 rotor (Beckman Instruments) and the pelleted membranes solubilized in SDS sample buffer containing 5 mM NaF.

SDS-PAGE, Western, and Dot Blotting

Proteins were separated by SDS-PAGE on 8 or 10% gels and transferred to nitrocellulose membrane (Hybond C; Amersham Pharmacia Biotech) by semidry blotting. For dot blots, proteins were pipetted directly onto nitrocellulose. Membranes were blocked in milk buffer (5% non-fat milk powder, 0.2% Tween-20 in PBS) before incubation with primary antibodies diluted in milk buffer. HRP-conjugated goat anti-rabbit or anti-mouse antibodies (1:2,000) were used to detect primary antibodies. Bands were

Results

Antibodies to Mitotically Phosphorylated GM130

We showed previously that GM130 is phosphorylated in mitosis and that phosphorylation inhibits binding to the vesicle-tethering protein, p115 (Nakamura et al., 1997). Mitotic phosphorylation is mediated by Cdc2-cyclin B and takes place on serine 25 (Lowe et al., 1998). To study the phosphorylation of GM130 in more detail, we generated rabbit polyclonal antibodies to a synthetic peptide (PS25pep; CQQKNpSPGVAG) containing residues 21–31 of GM130, in which the serine 25 residue was phosphorylated. Antibodies were purified on the phosphopeptide and used to study phosphorylation of GM130 by Western blotting and immunofluorescence microscopy. The purified antibodies (designated anti-phosphoserine 25 or PS25) detected a single band of ~130 kD on Western blots of mitotically-treated Golgi membranes, whereas no labeling of interphase-treated Golgi membranes was observed (Fig. 1 A, left panel). An ~130-kD band was also detected on Western blots of mitotic but not interphase NRK cell lysates (Fig. 1 A, middle panel). To confirm this was mitotically phosphorylated GM130 and not another cross-reacting 130-kD protein, GM130 was immunoprecipitated from the cell lysates using a specific mAb before analysis by Western blotting. The PS25 antibodies recognized an ~130-kD band in immunoprecipitates from mitotic but not interphase NRK cells (Fig. 1 A, right panel), confirming that the antibodies recognize mitotically phosphorylated GM130. The specificity of the PS25 antibody was further confirmed in competition experiments with the phosphorylated peptide PS25pep or a nonphosphorylated version of the same peptide. PS25pep abolished reactivity of the PS25 antibodies to the ~130-kD GM130 band, whereas the nonphosphorylated peptide had no effect (Fig. 1 B, upper panel). Reactivity of another antibody that recognizes the NH₂ terminus of both nonphosphorylated and phosphorylated GM130 was not affected by either peptide (Fig. 1 B, lower panel).

The PS25 antibody was next tested for its ability to recognize GM130 in mitotic cells by immunofluorescence microscopy. NRK cells were fixed in methanol and labeled with antibodies to phosphoserine 25 and β -1,4 galactosyltransferase, a resident enzyme of the Golgi complex. Mitotic cells were identified using the DNA dye Hoechst 33342. Serial optical sections were sampled using a laser scanning confocal microscope, and the sections overlaid and visualized in two dimensions. As shown in Fig. 2 A, PS25 antibodies labeled the Golgi complex of cells in the prometaphase stage of mitosis, whereas there was no labeling of cells in interphase. Labeling was not dependent upon fixation method, since PS25 antibodies also labeled the Golgi complex in paraformaldehyde-fixed mitotic cells (data not shown). PS25 antibodies also labeled the Golgi complex in mitotic but not interphase HeLa cells (see Fig. 3 B), confirming that phosphorylation of serine 25 is conserved between species. Golgi labeling with PS25 antibodies was efficiently competed by the PS25pep phosphopep-

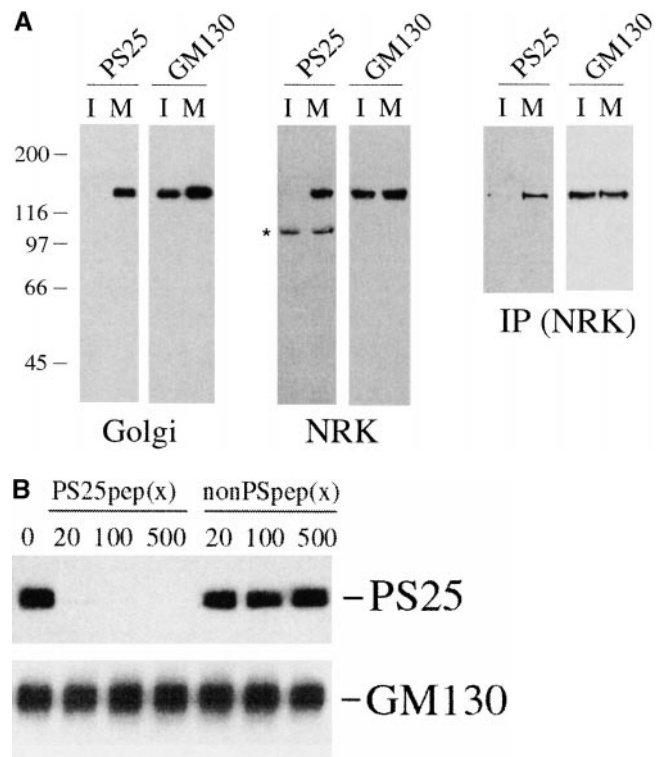


Figure 1. Specificity of the anti-phosphoserine 25 antibodies by Western blotting. (A) Left panel: Rat liver Golgi membranes were incubated with interphase (I) or mitotic (M) cytosol, reisolated by centrifugation, solubilized in SDS sample buffer, and subjected to Western blotting with antibodies against phosphoserine 25 (PS25) or the NH₂ terminus of GM130 (GM130). Middle panel: Interphase (I) or mitotic (M) NRK cells were lysed in buffer containing Triton X-100, and solubilized proteins analyzed by Western blotting with antibodies against phosphoserine 25 (PS25) or the NH₂ terminus of GM130 (GM130). The asterisk denotes a cross-reacting protein of unknown identity found in interphase and mitotic lysates. Right panel: GM130 was immunoprecipitated (IP) from Triton X-100 solubilized interphase (I) and mitotic (M) NRK cells with a monoclonal anti-GM130 antibody before analysis by Western blotting with antibodies against phosphoserine 25 (PS25) or the NH₂ terminus of GM130 (GM130). Molecular mass markers are shown in kD. (B) Mitotic Golgi membranes were analyzed by Western blotting with antibodies to phosphoserine 25 (PS25) or the NH₂ terminus of GM130 (GM130) in the absence or presence of the indicated molar excess of the phosphoserine 25 (PS25pep) or unphosphorylated (nonPSpep) peptide. GM130 appears as a doublet, most likely due to proteolytic cleavage.

tide and not affected by the nonphosphorylated version of this peptide (Fig. 2 A).

Phosphorylation of GM130 at Different Stages of Mitosis

NRK cells at different stages of mitosis were fixed in methanol and double-labeled with antibodies to phosphoserine 25 and the nucleic acid stain SYTO 13. Mitotic stages were identified by the SYTO 13-labeled DNA. Phosphorylation of GM130 occurs early in mitosis, during prophase (Fig. 2, B–D). Counting of prophase cells re-

vealed that 57% ($n = 130$) were positive for phosphoserine 25, suggesting that phosphorylation occurs around the middle of prophase. Labeling intensity for phospho-GM130 appeared to increase in proportion to the level of chromatin condensation, with late prophase cells (with highly condensed chromatin) showing the strongest labeling (Fig. 2 C). Double-labeling with antibodies to lamin B showed that phosphorylation of GM130 occurs before nuclear envelope breakdown (Fig. 3 A). Phosphorylation is maintained as the nuclear envelope disassembles during transit through late prophase into prometaphase (Fig. 3 A). Double-labeling of HeLa cells with antibodies to phosphoserine 25 and cyclin B1 revealed a close temporal relationship between GM130 phosphorylation and translocation of cyclin B1 into the nucleus (Fig. 3 B). 77% of PS25-positive prophase cells ($n = 150$) had cyclin B1 in the nucleus. Cells with nuclear cyclin B1 tended to exhibit stronger labeling with phosphoserine 25 antibodies compared with those cells with predominantly cytoplasmic cyclin B1 (Fig. 3 B), suggesting that phosphorylation continues to occur as cyclin B1 is translocated into the nucleus. The finding that all cells with nuclear cyclin B1 were positive for PS25 ($n = 100$) is consistent with this. Phosphorylation of serine 25 is maintained during prometaphase, metaphase, and anaphase, during which time the Golgi is broken down into smaller dispersed fragments (Fig. 2, B and D). Reactivity against phosphoserine 25 is lost during telophase, coinciding with the congregation of the partitioned Golgi fragments and their coalescence into larger Golgi elements (Fig. 2, B and D). GM130 is therefore phosphorylated in prophase as the Golgi complex starts to break down, and dephosphorylated in telophase as the mitotic Golgi fragments start to reassemble.

Double-labeling with anti-GM130 antibodies showed that almost all the dispersed fragments containing GM130 were phosphorylated on GM130 (Fig. 2 D). The amount of GM130 in these fragments was also quantitated, since it has been suggested that they disperse almost completely during metaphase through early telophase, the dispersed fragments fusing with the ER (Zaal et al., 1999). As shown in Table I, we did find that the amount of GM130 in identifiable fragments fell from metaphase through early telophase, but the level only fell to ~50%, not the 2% reported by Zaal et al. (1999) for β 1,4-galactosyltransferase and other Golgi markers, including GM130.

Phosphorylation of GM130 and Binding of p115 to Golgi Membranes

In vitro experiments have shown previously that mitotic phosphorylation of GM130 inhibits binding to the vesicle-tethering factor p115 (Nakamura et al., 1997). In agreement with this, immunofluorescence analysis showed that p115 was largely absent from mitotic Golgi clusters in HeLa cells (Shima et al., 1997). To investigate the temporal relationship between the phosphorylation of GM130 and the dissociation of p115 from the Golgi complex, cells were double-labeled with antibodies to phosphoserine 25 and p115 (Fig. 4 A). Dissociation of p115 at different mitotic stages was also monitored by double-labeling cells with antibodies to total GM130 and p115 (Fig. 4 B). In interphase cells, p115 gave a typical perinuclear Golgi label-

Table I. Quantitation of GM130-containing Structures at Different Mitotic Stages

Stage	Total cellular fluorescence of GM130-containing structures
Interphase	$(1) \pm 0.22$ ($n = 31$)
Prophase	1.03 ± 0.19 ($n = 10$)
Prometaphase	1.00 ± 0.32 ($n = 7$)
Metaphase	0.52 ± 0.18 ($n = 11$)
Anaphase	0.52 ± 0.27 ($n = 9$)
Early telophase	0.57 ± 0.19 ($n = 8$)
Late telophase/cytokinesis	1.03 ± 0.24 ($n = 8$)

NRK cells were labeled with antibodies to GM130 and imaged by confocal microscopy. Quantitation was performed as detailed in Materials and Methods. The total cellular fluorescence of GM130-containing structures in interphase and mitotic cells is expressed in arbitrary units as the mean \pm SD ($n = 7-31$) and normalized to give an interphase value of 1.

ing in addition to numerous punctate structures corresponding to the intermediate compartment as reported previously (Nelson et al., 1998). As cells enter prophase and GM130 is phosphorylated, the p115 distribution starts to change. There is a marked increase in the number of p115-positive and GM130-negative punctate structures around the Golgi region, and there is an increase in the amount of soluble p115, observed as a diffuse cytoplasmic staining (Fig. 4, A and B). By the time cells reach prometaphase, the amount of Golgi-associated p115 has dramatically reduced, and there is a corresponding increase in diffuse cytoplasmic p115 staining (Fig. 4, A and B). In metaphase, little p115 remains associated with mitotic clusters (Fig. 4 B, compare green [GM130-positive] clusters in the metaphase cell with the yellow [GM130- and p115-positive] Golgi in the adjacent interphase cell), an observation confirmed by quantitation. As shown in Table II, 78% of the p115 found on interphase GM130-containing Golgi membranes had dissociated by metaphase. Since most of the GM130 in the metaphase cells was present in identifiable fragments (72% in this experiment), this means that most of the p115 on interphase membranes had dissociated by metaphase. The small amount of residual p115 binding (22% of that found in interphase) could be to another p115-binding protein on the Golgi complex such as giantin, or it could be to intermediate compartment membranes, which lack GM130, in close proximity to the mitotic Golgi membranes.

The correlation between GM130 phosphorylation and dissociation of p115 from Golgi membranes was also stud-

Table II. Quantitation of Golgi-associated GM130 and p115 in Interphase and Metaphase Cells

Stage	Total cellular fluorescence of GM130-containing structures	Ratio of p115 to GM130 on GM130-containing structures
Interphase	$(1) \pm 0.13$	$(1) \pm 0.21$
Metaphase	0.72 ± 0.10	0.22 ± 0.05

NRK cells were double-labeled with antibodies to GM130 and p115 and imaged by confocal microscopy. Quantitation was performed as detailed in Materials and Methods. The total cellular fluorescence of GM130-containing structures in interphase and metaphase cells is expressed in arbitrary units as the mean \pm SD ($n = 7$) and normalized to give an interphase value of 1. The ratio of p115 to GM130 on GM130-containing structures was determined for interphase and metaphase cells from the mean fluorescent intensities of p115 and GM130 in the same selected areas of GM130-positive structures. Values are expressed in arbitrary units as the mean \pm SD ($n = 40$) and normalized to give an interphase value of 1.

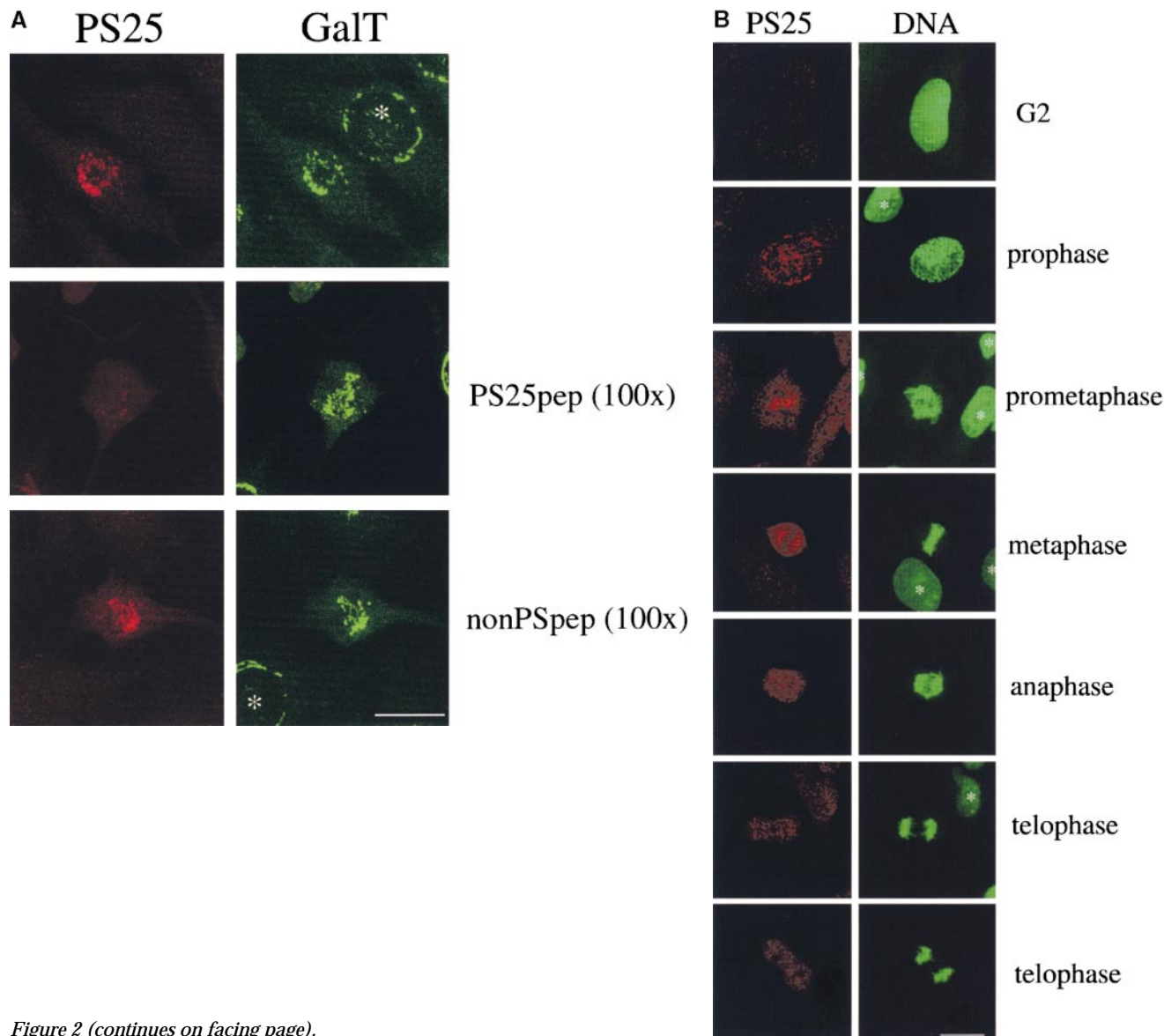


Figure 2 (continues on facing page).

ied in vitro. Rat liver Golgi membranes were incubated for various lengths of time with mitotic cytosol, reisolated through a sucrose cushion, and analyzed by Western blotting for the phosphorylation of serine 25 and the amount of membrane-associated p115. Phosphorylation of GM130 was rapid, already apparent at 2 min, and complete at 5–10 min (Fig. 4 C). Dissociation of p115 proceeded with very similar kinetics, and there was a good inverse relationship between the amount of GM130 phosphorylation and the amount of membrane-associated p115 (Fig. 4 C). No GM130 phosphorylation occurred with interphase cytosol, and although there was some dissociation of p115 from the Golgi membranes, it was much less than that observed during mitotic incubations.

Since GM130 is dephosphorylated during telophase, we might expect rebinding of p115 to occur at this stage, especially if it is important for Golgi reassembly. There are low amounts of p115 bound to mitotic Golgi clusters through anaphase to early telophase (Fig. 4 B). By late telophase, however, p115 binding is restored to interphase levels. As

shown in Fig. 4 A, lower panel, rebinding of p115 correlates well with GM130 dephosphorylation. The telophase cell at the bottom of the field, in which GM130 is still phosphorylated, has a low amount of Golgi-associated p115, whereas the cell at the top, where GM130 has been dephosphorylated, has much more p115 bound. The link between dephosphorylation of GM130 and rebinding of p115 to Golgi membranes was also studied in vitro. Mitotic Golgi membranes with phosphorylated GM130 and little bound p115, were incubated with interphase cytosol for various lengths of time, and the dephosphorylation of GM130 and binding of p115 to the membranes analyzed by Western blotting. As shown in Fig. 4 D, dephosphorylation of GM130 correlated well with membrane binding of p115.

Drug-induced Golgi Fragmentation and Phosphorylation of GM130

The Golgi complex fragments in cells treated with the pro-

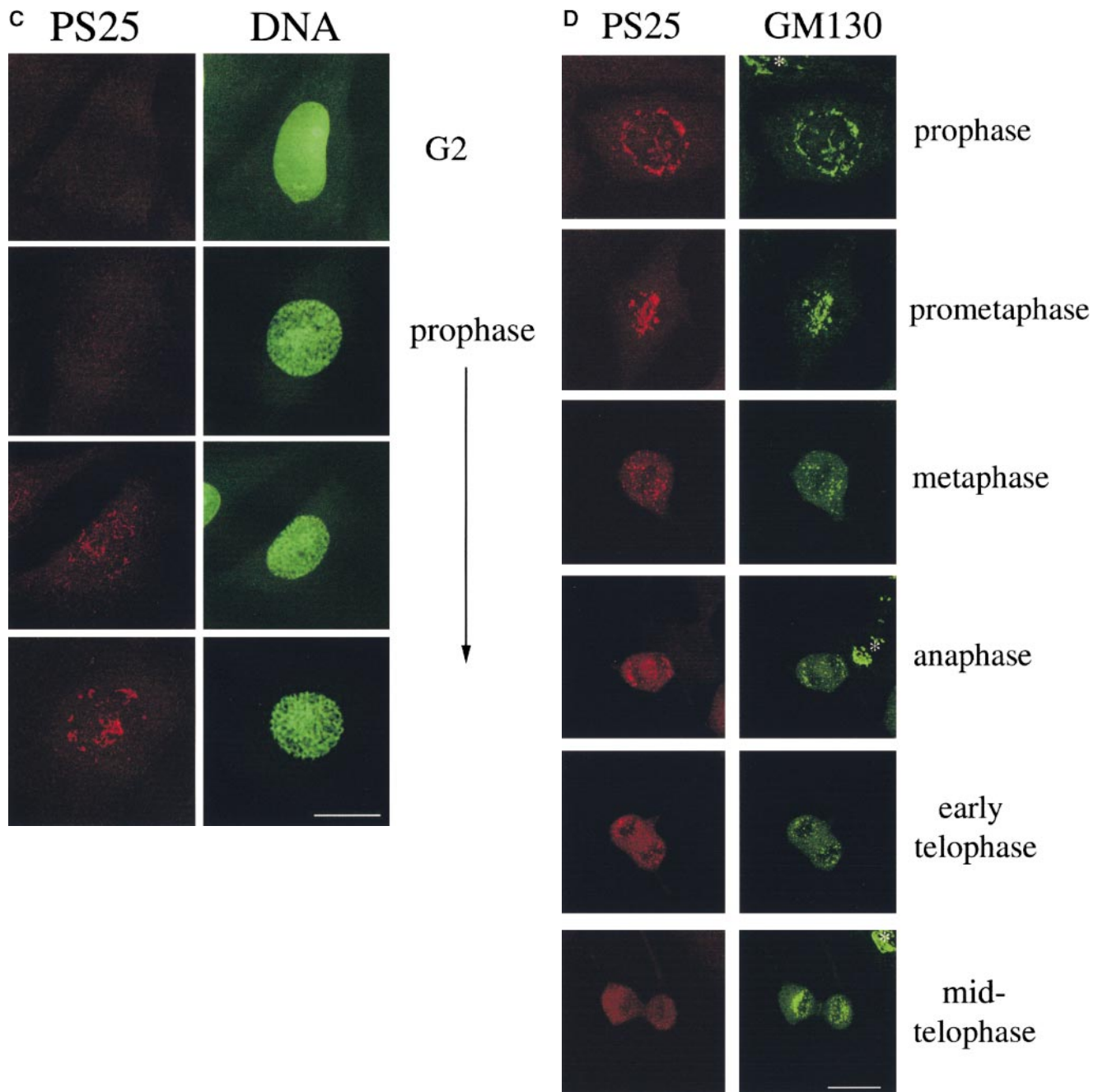


Figure 2. Mitotic phosphorylation of GM130 analyzed by immunofluorescence microscopy. (A) Specificity of the anti-PS25 antibodies. Methanol-fixed NRK cells were double-labeled with antibodies to phosphoserine 25 (PS25) and β -1,4 galactosyltransferase (GalT) in the absence (upper panel) or presence of a 100-fold molar excess of the phosphoserine 25 (PS25pep, middle panel) or unphosphorylated (nonPS25pep, lower panel) peptide. Representative cells in interphase (indicated by an asterisk) and prometaphase (unmarked) are shown. Bar, 20 μ m. (B) Phosphorylation of GM130 at different stages of mitosis. NRK cells were synchronized to enrich for mitotic cells, fixed in methanol, and double-labeled with antibodies to phosphoserine 25 (PS25) and the nucleic acid stain SYTO 13 (DNA) to identify mitotic stages. The cell in the bottom panel is a later stage of telophase than the cell in the panel directly above. Interphase cells are marked by an asterisk. Bar, 20 μ m. (C) GM130 phosphorylation in prophase. Synchronized NRK cells were fixed in methanol and double-labeled with antibodies to phosphoserine 25 (PS25) and the nucleic acid stain SYTO 13 (DNA). The upper panel shows a G2 cell with no chromatin condensation. The middle two panels show prophase cells with condensed chromatin that are either negative or positive for PS25. The bottom panel shows a late prophase cell with highly condensed chromatin which is PS25 positive. Bar, 20 μ m. (D) Phosphorylation of GM130 and Golgi division in mitosis. NRK cells were synchronized to enrich for mitotic cells, fixed in methanol, and double-labeled with antibodies to phosphoserine 25 (PS25) and total GM130 (GM130). Mitotic stages were determined using the DNA binding dye Hoechst 33342. Interphase cells are marked by an asterisk. Bar, 20 μ m.

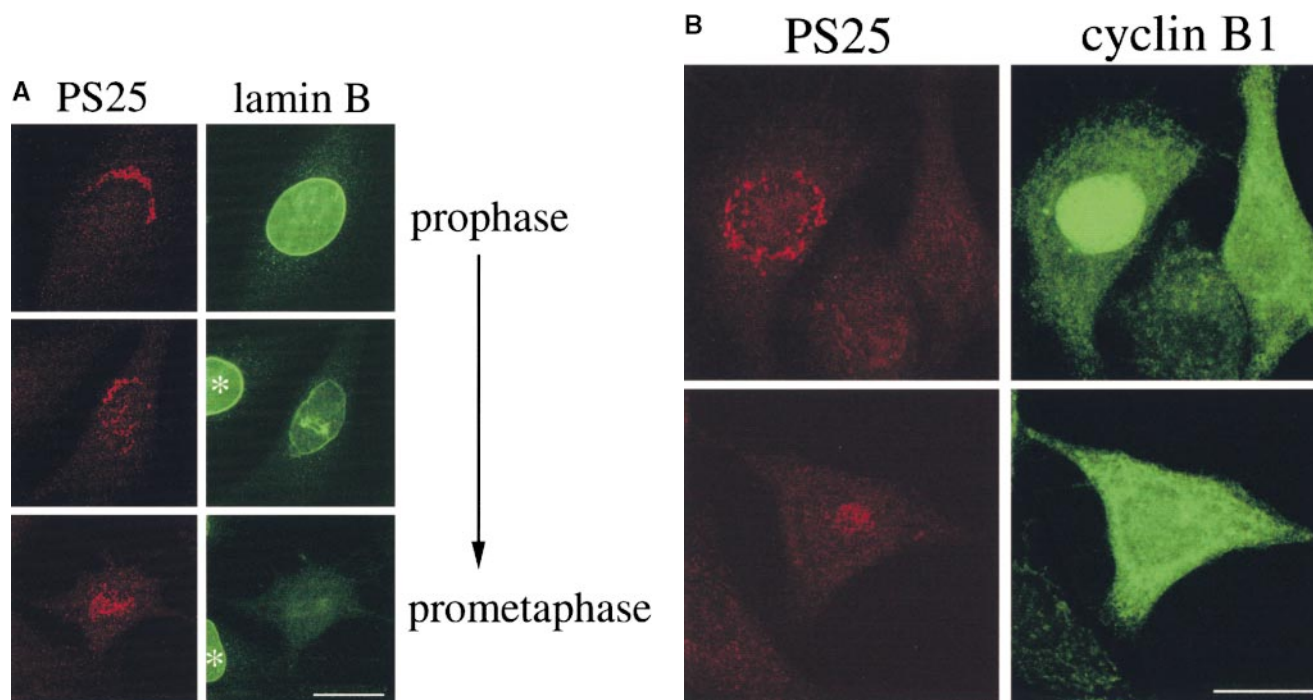


Figure 3. Mitotic phosphorylation of GM130 precedes nuclear envelope breakdown and coincides with nuclear translocation of cyclin B1. (A) Phosphorylation of GM130 before nuclear envelope breakdown. Methanol-fixed NRK cells were double-labeled with antibodies to phosphoserine 25 (PS25) and lamin B. Examples of cells with phosphorylated GM130 and an intact (upper panel), partially disassembled (middle panel), and fully disassembled (lower panel) nuclear envelope are shown. Interphase cells are marked with an asterisk. Bar, 20 μm . (B) Phosphorylation of GM130 and translocation of cyclin B1 into the nucleus. Methanol-fixed HeLa cells were double-labeled with antibodies to phosphoserine 25 (PS25) and cyclin B1. Upper panel: a prophase cell with phosphorylated GM130 and cyclin B1 in the nucleus is shown on the left. A G2 cell, negative for phospho-GM130 and with cytoplasmic cyclin B1 is on the right. Lower panel: a prophase cell that is PS25-positive with predominantly cytoplasmic cyclin B1 is shown. Bar, 20 μm .

tein phosphatase inhibitor okadaic acid, yielding numerous clusters of Golgi-derived vesicles and tubules morphologically very similar to those seen in mitosis (Lucocq et al., 1991). This has led to the proposal that okadaic acid mimics mitotic Golgi fragmentation by activating the same breakdown pathway as that used in mitosis (Lucocq, 1992). We therefore investigated whether okadaic acid induces phosphorylation of GM130 on serine 25 and dissociation of p115 from Golgi membranes. As shown in Fig. 5 A, treatment of NRK cells with 1 μM okadaic acid led to Golgi fragmentation, but GM130 was not phosphorylated on serine 25, and p115 remained bound to the Golgi fragments. Similar results were obtained with HeLa cells, except the Golgi fragments were more numerous and more dispersed (data not shown). Similar results were also obtained in vitro. Purified Golgi membranes were incubated with interphase or mitotic cytosol in the absence or presence or 1 μM okadaic acid, which has been shown to fragment Golgi membranes in vitro (Misteli and Warren, 1994), and analyzed for phosphorylation of GM130 on serine 25 and membrane association of p115. As shown in Fig. 5 B, okadaic acid had no effect upon serine 25 phosphorylation or p115 membrane association. In contrast, PS25 was efficiently phosphorylated by mitotic cytosol and p115 dissociated from the membranes (Fig. 5 B).

IQ triggers Golgi fragmentation through a mechanism involving G $\beta\gamma$ -mediated activation of protein kinase D (PKD) (Takizawa et al., 1993; Jamora et al., 1999). NRK

cells were incubated with 25 μM IQ for 2 h and the phosphorylation of serine 25 analyzed by immunofluorescence microscopy. Golgi fragments generated by IQ were not labeled with anti-phosphoserine 25 antibodies and p115 remained bound to the Golgi fragments (Fig. 5 C). NRK cells were also treated with nocodazole, a drug that depolymerizes microtubules and converts the Golgi ribbon into mini-stacks dispersed throughout the cytoplasm (Thyberg and Moskalewski, 1989). Nocodazole-generated Golgi fragments were not labeled with phosphoserine 25 antibodies, and p115 remained associated with these Golgi fragments as reported previously (Shima et al., 1997) (Fig. 5 C). Phosphorylation of GM130 on serine 25 and dissociation of p115 from Golgi membranes are therefore specific for mitosis, suggesting that Golgi fragmentation in mitosis occurs by a different mechanism, or one acting in parallel to that induced by okadaic acid, IQ, and nocodazole.

Dephosphorylation of Mitotically Phosphorylated GM130 by PP2A

Cdc2-cyclin B has been identified as the mitotic GM130 kinase (Lowe et al., 1998). However, which phosphatase dephosphorylates mitotic GM130 in telophase is not known. To identify the GM130 phosphatase, an assay was developed to measure GM130 dephosphorylation in vitro. Two substrates were used for these experiments: one was a peptide comprising the NH₂-terminal 73 amino acids of

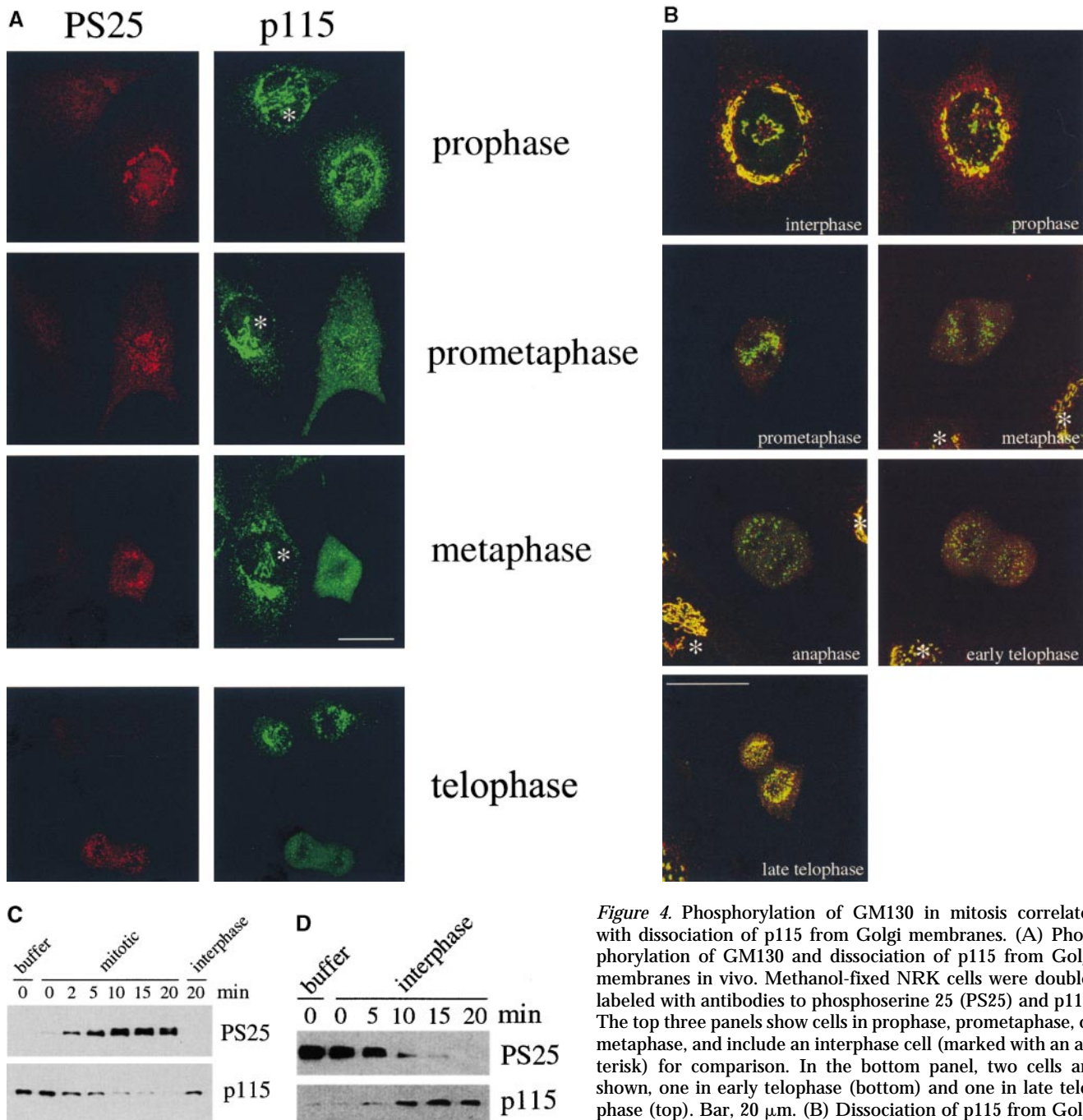


Figure 4. Phosphorylation of GM130 in mitosis correlates with dissociation of p115 from Golgi membranes. (A) Phosphorylation of GM130 and dissociation of p115 from Golgi membranes in vivo. Methanol-fixed NRK cells were double-labeled with antibodies to phosphoserine 25 (PS25) and p115. The top three panels show cells in prophase, prometaphase, or metaphase, and include an interphase cell (marked with an asterisk) for comparison. In the bottom panel, two cells are shown, one in early telophase (bottom) and one in late telophase (top). Bar, 20 μm . (B) Dissociation of p115 from Golgi membranes in vivo. Methanol-fixed NRK cells were double-

labeled with antibodies against total GM130 and p115. Images are depicted as overlays of GM130 (green) and p115 (red), with regions of overlap indicated by yellow. Interphase cells are marked with an asterisk. Bar, 20 μm . (C) Phosphorylation of GM130 and dissociation of p115 from Golgi membranes in vitro. Rat liver Golgi membranes were incubated in buffer alone or in mitotic or interphase cytosol for the times indicated, and reisolated by centrifugation before solubilization in SDS sample buffer and analysis by Western blotting with antibodies to phosphoserine 25 (PS25) or p115. (D) Dephosphorylation of GM130 and reassociation of p115 with Golgi membranes in vitro. Mitotic Golgi membranes were incubated in buffer alone or in interphase cytosol supplemented with 50 ng exogenous p115 for the times indicated, and reisolated by centrifugation before solubilization in SDS sample buffer and analysis by Western blotting with antibodies to phosphoserine 25 (PS25) or p115.

GM130 synthesized with a phosphoserine at position 25 (N73pepPS25), and the second was native mitotically phosphorylated GM130, prepared by incubating Golgi membranes with mitotic cytosol. Each substrate was incubated with cytosol prepared from interphase HeLa cells, and dephosphorylation monitored by loss of reactivity to the

phosphoserine 25 antibodies. The PS25 antibodies reacted strongly with N73pepPS25 phosphopeptide, but not with the same peptide lacking a phosphate group on serine 25 (Fig. 6 A, upper left panel). Reactivity was lost when the phosphopeptide was incubated with increasing concentrations of HeLa cytosol (Fig. 6 A, upper right panel). This

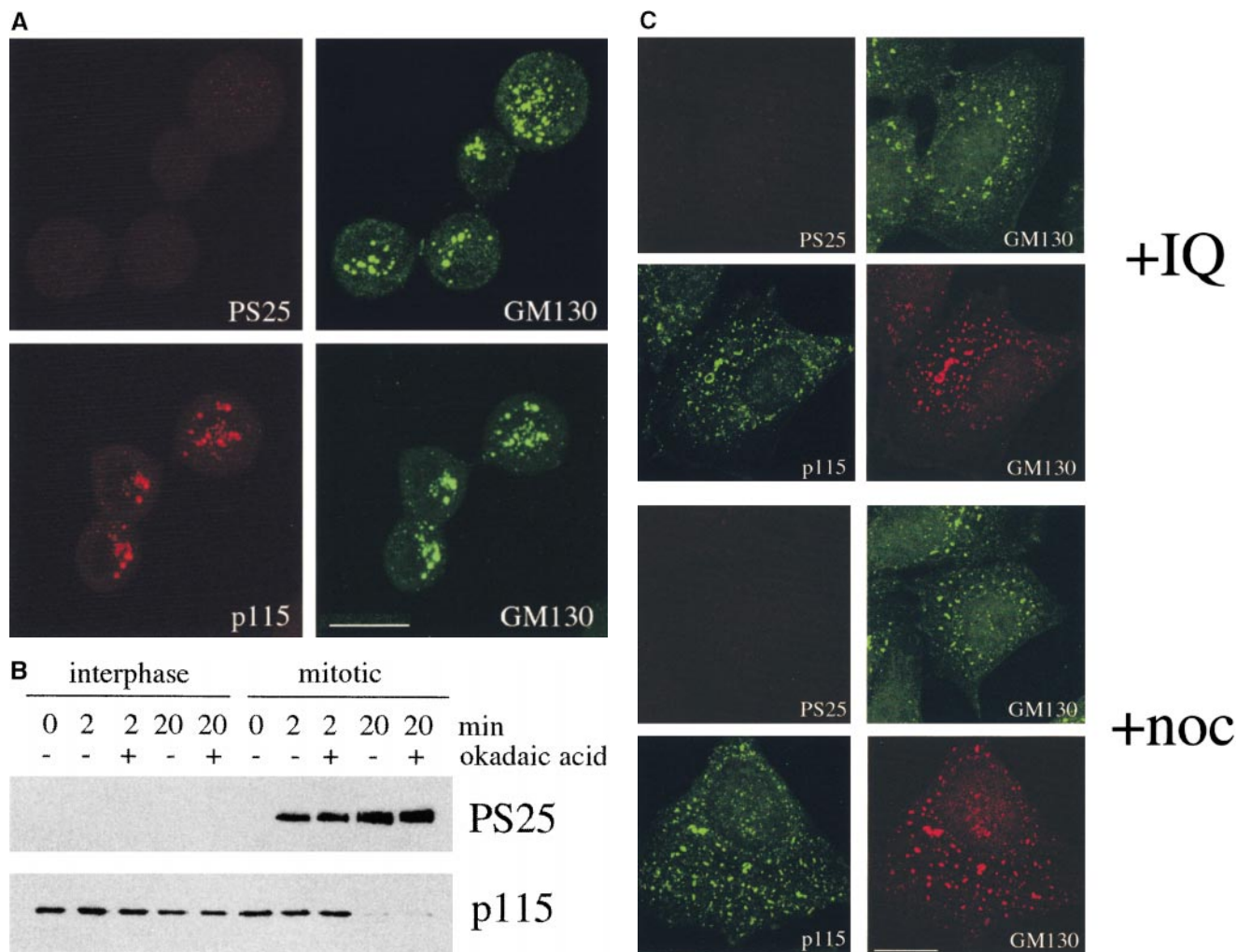


Figure 5. Drug-induced Golgi fragmentation independent of GM130 phosphorylation. (A) Golgi fragmentation with okadaic acid. NRK cells were treated with 1 μ M okadaic acid for 1 h and double-labeled with antibodies to phosphoserine 25 (PS25) and total GM130 (upper panel), or p115 and GM130 (lower panel). Bar, 20 μ m. (B) Immunoblotting of GM130 and p115 after okadaic acid treatment in vitro. Golgi membranes were incubated with interphase or mitotic cytosol for 2 or 20 min at 30°C in the absence or presence of 1 μ M okadaic acid and subjected to Western blotting with antibodies to phosphoserine 25 (PS25) or p115. (C) Golgi fragmentation with IQ or nocodazole. NRK cells were treated with 25 μ M IQ (upper panels) or 5 μ M nocodazole (noc; lower panels) for 2 h and double-labeled with antibodies to PS25 and GM130 or p115 and GM130. Bar, 20 μ m.

was due to dephosphorylation and not degradation, since reactivity to anti-N73pep antibodies was not affected. Similar results were obtained with native GM130 on mitotic Golgi membranes. GM130 was dephosphorylated by interphase HeLa cytosol at similar concentrations to that observed for the phosphopeptide (Fig. 6 A, lower panel).

Four major classes of protein phosphatase have been identified in mammalian cells: PP1, PP2A, PP2B, and PP2C (for reviews see Cohen, 1989; Shenolikar, 1994). Differences in substrate specificity, divalent cation requirement, and sensitivity to various inhibitors can be used to distinguish between these phosphatases (Cohen, 1991). Inhibitor studies with microcystin and okadaic acid have shown that most of the serine/threonine phosphatase activity in the cell is attributable to PP1 and PP2A (Cohen, 1991). Interestingly, both phosphatases have been linked to mitotic events (Axton et al., 1990; Kinoshita et al., 1990; Mayer-Jaekel et al., 1993; Tournebize et al., 1997). We

therefore decided to investigate whether PP1 or PP2A can dephosphorylate mitotic GM130. Both PP1 and PP2A are inhibited by okadaic acid, but with different sensitivities (Bialojan and Takai, 1988; Cohen, 1991). PP2A is inhibited at a 100-fold lower okadaic acid concentration than PP1, with an IC_{50} (concentration yielding 50% inhibition) of 0.1 nM compared with an IC_{50} of 10–15 nM for PP1. As shown in Fig. 6 B, dephosphorylation of the N73pepPS25 phosphopeptide was sensitive to low concentrations of okadaic acid (IC_{50} of \sim 0.1 nM). Similar results were obtained with native phospho-GM130 on mitotic Golgi membranes (IC_{50} of \sim 0.1–0.5 nM), suggesting that PP2A dephosphorylates GM130. To characterize this further, experiments were performed with protein phosphatase inhibitor-2, which inhibits PP1 with an IC_{50} of 2 nM but has no effect upon PP2A (Cohen, 1991). Dephosphorylation of both the GM130 phosphopeptide and native GM130 was insensitive to inhibitor-2 at concentrations up to 500

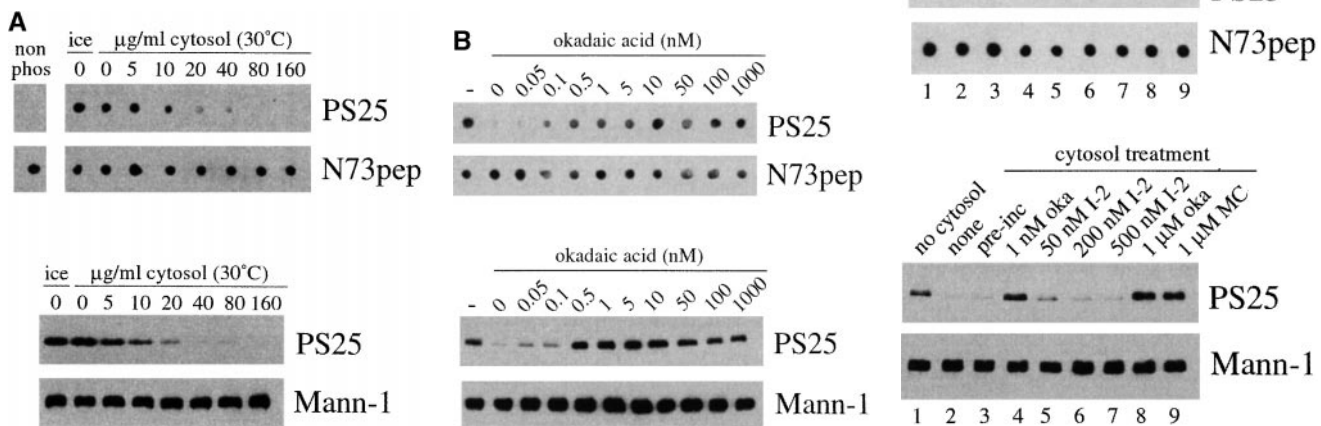


Figure 6. Dephosphorylation of mitotic GM130 is sensitive to low concentrations of okadaic acid. (A) Dephosphorylation of GM130 by interphase cytosol. Upper panel: N73pepPS25, the NH₂-terminal GM130 peptide with phosphoserine at position 25, was incubated with increasing amounts of interphase cytosol at 30°C, before spotting onto nitrocellulose and probing with antibodies against phosphoserine 25 (PS25) or the whole NH₂-terminal peptide (N73pep). The panel on the left shows an equivalent spot of the NH₂-terminal peptide with a nonphosphorylated serine at position 25. Lower panel: Mitotically-treated Golgi membranes were incubated with increasing amounts of interphase cytosol, solubilized in SDS sample buffer, and analyzed by Western blotting with antibodies to phosphoserine 25 (PS25) or mannosidase I (Mann-1). (B) Sensitivity of the GM130 phosphatase to okadaic acid. Upper panel: The N73pepPS25 peptide was incubated in the absence of cytosol (-) or with 20 μg/ml interphase cytosol in the presence of increasing amounts of okadaic acid. Dephosphorylation was monitored by blotting with antibodies to phosphoserine 25 (PS25) or the whole peptide (N73pep). Lower panel: Mitotic Golgi membranes were incubated in the absence of cytosol (-) or with 20 μg/ml interphase cytosol in the presence of increasing amounts of okadaic acid. The membranes were then solubilized in SDS sample buffer and analyzed by Western blotting with antibodies to phosphoserine 25 (PS25) or mannosidase I (Mann-1). (C) The GM130 phosphatase is insensitive to inhibitor-2. The N73pepPS25 peptide (upper panel) or mitotic Golgi membranes (lower panel) were incubated in the absence of cytosol (no cytosol) or with interphase cytosol in the absence (none) or presence of the inhibitors indicated (oka, okadaic acid; I-2, inhibitor-2; MC, microcystin-LR) before analysis by blotting with antibodies to phosphoserine 25 (PS25), the NH₂-terminal peptide (N73pep) or mannosidase I (Mann-1). In some cases the cytosol was preincubated for 15 min in the absence (lane 3) or presence of increasing amounts of inhibitor-2 (lanes 5–7) before addition of the peptide or membranes. The activity of each inhibitor was confirmed by testing their ability to inhibit the phosphatase activity of cytosol or purified PP1 and PP2A using phosphorylase a as control substrate (data not shown).

nM (Fig. 6 C, lanes 5–7), whereas it was completely inhibited by 1 nM okadaic acid in the same experiment (Fig. 6 C, lane 4), suggesting again that the GM130 phosphatase is PP2A and not PP1. To obtain additional evidence that PP2A is the GM130 phosphatase, dephosphorylation reactions were carried out with purified PP1 and PP2A. Equal amounts of phosphatase activity (assayed using phosphorylase a as the substrate) were added in each case. As shown in Fig. 7, only PP2A dephosphorylated the GM130 phosphopeptide and native mitotic GM130 on Golgi membranes.

PP2A exists as a trimeric complex *in vivo*, with a constant dimeric core of a 36-kD catalytic (PP2Ac or C) subunit and a 65-kD (PR65 or A) subunit bound to a third, variable B subunit (Mayer-Jaekel and Hemmings, 1994; Millward et al., 1999). Three classes of B subunit have so far been identified: B/PR55, B'/PR61, and B''/PR72, PR130, or PR59. The B subunits have been shown to act as positive regulators to enhance catalytic activity toward particular substrates both *in vitro* and *in vivo* (Ferrigno et al., 1993; Mayer-Jaekel et al., 1994; Sontag et al., 1996; Zhao et al., 1997; Turowski et al., 1999). Furthermore, the distinct subcellular localization of the B subunits may act

to target isoforms of PP2A to particular locations in the cell (Sontag et al., 1995; McCright et al., 1996; Turowski et al., 1999). To test whether the B subunit of PP2A plays a role in modulating the activity of PP2A towards GM130, the strategy of phosphatase isoform reconstitution by baculovirus infection was employed (Kamibayashi et al., 1994). Sf9 cells were coinfecting with baculovirus recombinants expressing combinations of the C, A, and one of the variable B subunits, and lysates assayed for their ability to dephosphorylate GM130 *in vitro*. There was some GM130 phosphatase activity in uninfected cell lysates (Fig. 8 A, none), most likely due to endogenous PP2A. This was not increased by expression of the AC dimer (Fig. 8 A). Coexpression of the B α subunit with AC dramatically increased the GM130 phosphatase activity (Fig. 8 A). Activity towards the phosphopeptide was increased ~15-fold and activity towards GM130 on Golgi membranes increased ~10-fold. In contrast, coexpression of the B' α subunit with AC did not increase GM130 phosphatase activity. In fact, the B' α subunit slightly inhibited basal activity towards GM130 (Fig. 8 A), most likely by displacing endogenous Sf9 cell B family subunits from holoenzyme complexes (Tehrani et al., 1996). Each lysate exhibited similar

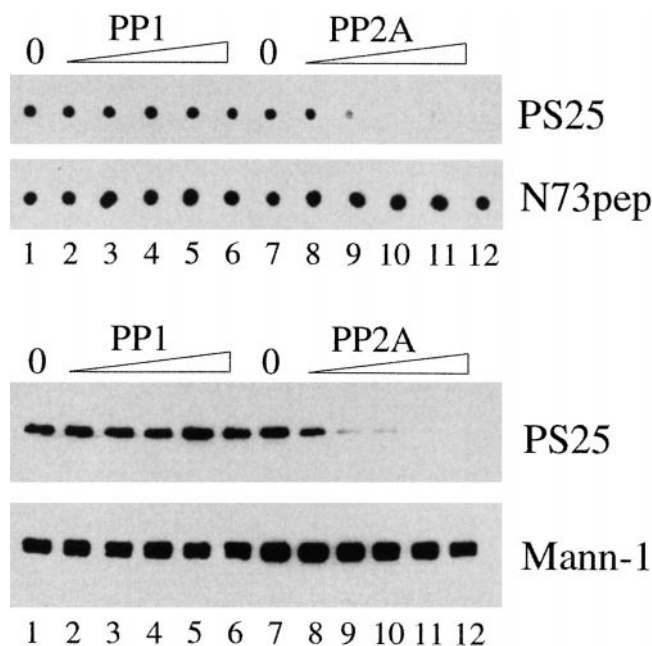


Figure 7. Dephosphorylation of GM130 by purified phosphatases. The N73pepPS25 peptide (upper panel) or mitotic Golgi membranes (lower panel) were incubated with increasing amounts of purified PP1 or PP2A before immunoblotting with antibodies to phosphoserine 25 (PS25), the NH₂-terminal peptide (N73pep) or mannosidase I (Mann-1). Equivalent amounts of PP1 or PP2A were added in each case, as assayed using the control substrate phosphorylase a (data not shown). Relative phosphorylase phosphatase activities added were 1 (lanes 2 and 8), 2 (lanes 3 and 9), 4 (lanes 4 and 10), 8 (lanes 5 and 11), and 16 (lanes 6 and 12).

activity towards phosphorylase a, and Western blotting confirmed that the appropriate subunits were indeed expressed by the recombinant baculoviruses and that similar levels of catalytic subunit were expressed in each combinatorial infection (Fig. 8 B). The dramatic changes in GM130 dephosphorylation are therefore not due to differential stabilization of the catalytic subunit, but rather reflect the inherent ability of the B α subunit to facilitate the recognition of GM130 by PP2A holoenzyme.

Localization of the B α Regulatory Subunit of PP2A to Golgi Membranes

The B α subunit of PP2A has been localized previously by immunofluorescence microscopy to the cytoplasm, intermediate filaments, and microtubules (Sontag et al., 1995; Turowski et al., 1999). To determine whether the B α subunit is also localized to the Golgi complex, Golgi membranes were purified from rat liver and analyzed by Western blotting with antibodies to PP2A subunits. The B α subunit was present in the Golgi fraction at a similar concentration to that in total rat liver homogenate (Fig. 9 A). The catalytic subunit was also present in the Golgi fraction, although it was depleted compared with homogenate. In contrast, no B' α subunit could be detected in the Golgi fraction. Neither vimentin nor α -tubulin, which were present in the homogenate, could be detected in the Golgi fraction, suggesting that B α is not present on contaminat-

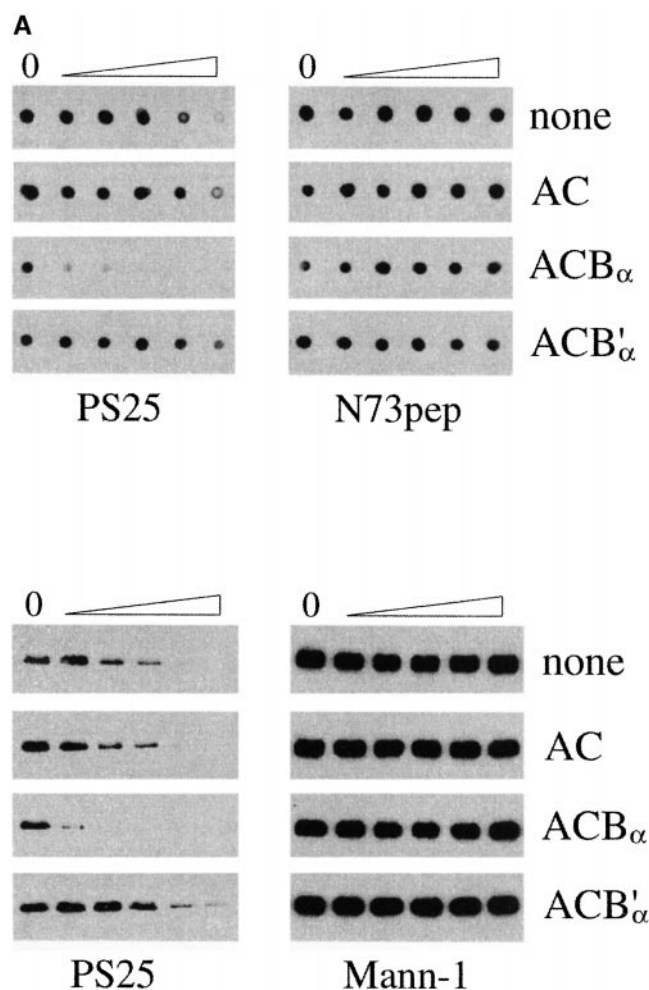


Figure 8. Isoform-specific dephosphorylation of GM130 by PP2A. (A) Dephosphorylation of GM130 by PP2A isoforms. Baculovirus recombinants were used to express PP2A subunits in various combinations in insect cells. Cells were harvested 64–72 h after infection and lysates were tested for their ability to dephosphorylate the N73pepPS25 peptide (upper panel) or mitotic GM130 on Golgi membranes (lower panel). Amounts of each lysate added were (from left to right): 1, 2, 4, 8, and 16 μ l. Each lysate had a similar phosphorylase a as the substrate. Relative phosphorylase a phosphatase activities were 1, 1.6, 1.6, and 0.95 for none, AC, ACB α , and ACB' α lysates, respectively. Noninfected Sf9 cells (none) had a detectable level of GM130 phosphatase activity, which was not increased by expression of the AC subunits alone or in combination with the B' α subunit. In contrast, the B α subunit significantly increased activity directed against phospho-GM130. (B) Subunit expression analyzed by Western blotting. Equivalent amounts of lysates from uninfected Sf9 cells (none) or cells infected with recombinants expressing the AC, ACB α , or ACB' α subunits of PP2A were subjected to SDS-PAGE and Western blotting with antibodies specific for the catalytic subunit (C), the A subunit, or the B α or B' α regulatory subunits.

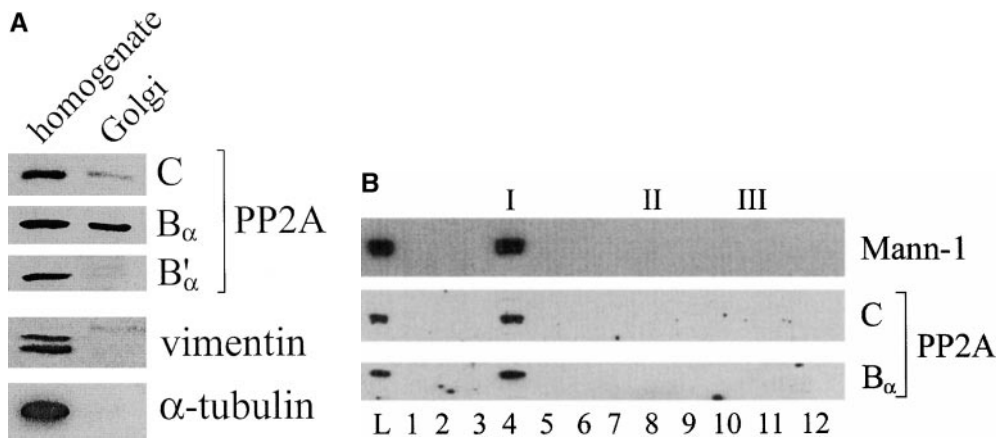


Figure 9. Localization of the B α regulatory subunit of PP2A to Golgi membranes. (A) Equal amounts (10 μ g) of rat liver homogenate or purified rat liver Golgi membranes (purified 200-fold over homogenate) were subjected to SDS-PAGE and Western blotting with antibodies specific for the PP2A catalytic subunit (C), PP2A B α or B' α regulatory subunits, vimentin, or α -tubulin. (B) Purified rat liver Golgi membranes were further fractionated by flotation on a

discontinuous sucrose gradient. Fractions were collected from the top, and proteins precipitated with methanol/chloroform before analysis by SDS-PAGE and Western blotting with antibodies specific for mannosidase I (Mann-1), or the catalytic (C) or B α subunits of PP2A. L, load. The positions of the 0.4–1 M sucrose (I), 1–1.2 M sucrose (II), and 1.2–1.6 M sucrose (III) interfaces are shown.

ing cytoskeletal elements. To address the possibility that B α might be on other membranes contaminating the Golgi fraction, another round of purification using flotation on a sucrose gradient was performed. The Golgi membranes floated from the bottom of the gradient to the 0.4–1 M sucrose interface (fraction 4), as determined by Western blotting for mannosidase I (Fig. 9 B). All of the B α present in the starting material floated to the same position as the Golgi membranes, and none was detected at the 1–1.2 M and 1.2–1.6 M sucrose interfaces, where heavier membranes such as the ER migrate (data not shown). The catalytic subunit of PP2A also floated to the position of the Golgi membranes (Fig. 9 B). Together, these findings suggest B α -containing PP2A is localized to the Golgi complex, and that it is enriched there relative to other PP2A isoforms.

Discussion

We previously used a biochemical approach to identify GM130 as a mitotic phosphoprotein (Nakamura et al., 1997; Lowe et al., 1998). We could show that GM130 is phosphorylated on serine 25, and that phosphorylation inhibits binding to the vesicle-tethering factor p115 *in vitro*. However, the temporal link between GM130 phosphorylation, p115 dissociation, and Golgi breakdown *in vivo* was unclear. We therefore raised antibodies to phosphorylated GM130 to study the timing of these events in cells. Phosphorylation of GM130 on serine 25, which occurs in mid-prophase, coincides with the dissociation of p115 from Golgi membranes, which is apparent already during prophase and maximal by the time cells reach prometaphase. Experiments in living cells have shown that the Golgi ribbon breaks down to large fragments (1–3 μ m) arranged around the nucleus early in prophase (Shima et al., 1998). These fragments most likely correspond to discrete stacks (Colman et al., 1985), which have been identified as early mitotic breakdown intermediates by EM (Lucocq and Warren, 1987; Misteli and Warren, 1995a). Quantitative EM analysis has shown that the cross-sectional length of the stacks decreases as cells progress through prophase. This occurs before unstacking and is accompanied by the

accumulation of small 50–70-nm vesicles in the vicinity of the stacks (Misteli and Warren, 1995a). By prometaphase, no stacks remain, being replaced entirely by tubulo-vesicular clusters (Misteli and Warren, 1995a; Lucocq et al., 1987). Phosphorylation of GM130 on serine 25 and dissociation of p115 therefore occur at the same time as Golgi cisternae are converted into small vesicles. This is consistent with the proposal that GM130 phosphorylation is responsible for the inhibition of COPI vesicle tethering.

Biochemical studies strongly suggested that GM130 is phosphorylated by Cdc2-cyclin B (Lowe et al., 1998). Cdc2-cyclin B1 is thought to be activated during mid to late prophase at about the time it is translocated from the cytoplasm to the nucleus (Furuno et al., 1999; Hagting et al., 1999). Translocation is a rapid event, preceding nuclear envelope breakdown by \sim 10 min (Hagting et al., 1999). This timing is consistent with the proposed role for Cdc2 in phosphorylating nuclear lamins and triggering nuclear envelope breakdown (Peter et al., 1990). Our results, which suggest that GM130 is phosphorylated around the time cyclin B1 starts to translocate to the nucleus, are not inconsistent with Cdc2-cyclin B1-mediated phosphorylation of GM130. However, it seems more likely that cyclin B2 is the relevant kinase partner, since this cyclin has been localized to the Golgi complex in interphase cells (Jackman et al., 1995). In prometaphase, cyclin B2 disperses from the Golgi region and becomes cytoplasmic (Jackman et al., 1995). Cdc2-cyclin B2 could therefore phosphorylate GM130 (and perhaps other Golgi substrates) during prophase to initiate Golgi complex disassembly before its dissociation from the Golgi membranes.

Dephosphorylation of GM130 in telophase coincides with the rebinding of p115 to mitotic Golgi fragments and the congregation and coalescence of these fragments into larger structures. EM analysis has shown that during this time, mitotic Golgi clusters increase in size by accretion of vesicles, and that these vesicles fuse with each other to form cisternae that undergo stacking (Lucocq et al., 1989; Souter et al., 1993). GM130 dephosphorylation therefore coincides temporally with the accretion of Golgi vesicles and their conversion into stacked cisternae, consistent with the proposed role for p115 in the initial tethering of

Golgi vesicles during cisternal regrowth and in the initial alignment of cisternae during stacking.

An alternative view of Golgi partitioning has recently been put forward by Zaal and colleagues (1999). These authors presented evidence suggesting that fragmentation of the Golgi apparatus is followed, during metaphase, by fusion of these fragments with the ER. This persists until late telophase, when Golgi stacks are rebuilt at peripheral ER exit sites. In this model, the partitioning of the Golgi apparatus is not an autonomous function of this organelle but rather a function of the ER that now contains it. The key observation was the almost complete disappearance of Golgi fragments during metaphase through early telophase, using a variety of Golgi markers, including GM130. This observation conflicted with our earlier fluorescence data (Shima et al., 1997, 1998), which we have now confirmed by quantitating the distribution of GM130 during mitosis. We find that GM130-containing Golgi fragments are present throughout. Interestingly, we did observe a drop in the amount of GM130 in these mitotic fragments from metaphase through early telophase, the same period observed by Zaal and colleagues (1999). But the drop was never >50%, much less than the 98% reported by Zaal and colleagues (1999) for β 1,4-galactosyltransferase. One could attribute this 50% drop to fusion of some mitotic fragments with the ER. However, we prefer our original interpretation, based on extensive stereological analysis, that mitotic fragments shed vesicles into the surrounding cytoplasm and these would be below the resolution of light microscopy (Lucocq et al., 1989). This interpretation is also in line with more recent data showing that mitotic Golgi membranes can be separated from mitotic ER (Jesch and Linstedt, 1998) and that Golgi enzyme activity cannot be detected in the ER during mitosis (Farmaki et al., 1999). Further work is obviously needed to examine the fate of those Golgi components that are no longer found in fragments. However, it is important to note that the presence of a substantial pool of Golgi fragments throughout mitosis precludes the ER as the sole means of partitioning the Golgi apparatus.

Golgi fragmentation induced by okadaic acid occurs without GM130 phosphorylation on serine 25 or dissociation of p115 from Golgi membranes. Treatment of cells with okadaic acid leads to an increase in the activity of many protein kinases, resulting in a dramatic increase in the overall cellular protein phosphorylation (Haystead et al., 1989; Millward et al., 1999). However, in most cell types there is little activation of Cdc2 (Ghosh et al., 1998). Consistent with this, we found only slight effects upon histone H1 kinase activity of NRK and HeLa cells (1.7-fold increase in activity after okadaic acid treatment; data not shown), in agreement with previous results (Lucocq et al., 1991; Ajiro et al., 1996). Furthermore, in the majority of okadaic acid-treated cells, we could not detect nuclear envelope breakdown or spindle formation (data not shown). The lack of GM130 phosphorylation on serine 25 in okadaic acid-treated cells suggests that this residue is extremely specific for Cdc2. It also demonstrates that although okadaic acid can mimic mitotic fragmentation morphologically, the biochemical mechanisms involved may be different. Clearly, kinases other than Cdc2 can trigger Golgi fragmentation, and they may even do so in

mitosis, but to what extent okadaic acid activates the kinases physiologically relevant for mitotic breakdown is unclear. Two kinases with demonstrated roles in regulation of Golgi structure are MEK1 and PKD. MEK1 is activated in okadaic acid-treated cells (Gomez and Cohen, 1991), and could therefore be responsible for some of the changes that occur during okadaic acid-induced Golgi breakdown. Interestingly, it has been proposed that MEK1 mediates Golgi fragmentation in mitosis (Acharya et al., 1998). PKD is activated by IQ and is responsible for bringing about the fragmentation of the Golgi complex induced by this drug (Jamora et al., 1999). Since both okadaic acid and IQ did not induce phosphorylation of GM130, it is unlikely that MEK1 and PKD have a role in the COPI mitotic Golgi fragmentation pathway. However it is possible that they could play a role in the COPI-independent pathway, and drugs such as okadaic acid and IQ might mimic this pathway by activating these kinases. Further work is necessary to determine if this is the case.

Several lines of evidence strongly suggest that PP2A is the GM130 phosphatase. First, dephosphorylation of a synthetic GM130 phosphopeptide (N73pepPS25) as well as native mitotically phosphorylated GM130 was inhibited by okadaic acid with an IC_{50} of ~ 0.1 nM. PP2A is the only known phosphatase that is sensitive to okadaic acid at this concentration (Bialojan and Takai, 1988; Cohen, 1991). Second, dephosphorylation was insensitive to protein phosphatase inhibitor-2, a potent inhibitor of PP1 that does not inhibit PP2A (Cohen, 1991). Third, purified PP2A efficiently dephosphorylated both the phosphopeptide and native phospho-GM130, whereas PP1 at similar levels had no effect. Fourth, recombinant baculovirus-expressed PP2A dephosphorylated the phosphopeptide and native phospho-GM130.

Dephosphorylation of GM130 by PP2A was dependent upon the presence of the B α regulatory subunit. This subunit has been demonstrated to have an important role in the dephosphorylation of several other cyclin-dependent kinase substrates (Ferrigno et al., 1993; Mayer-Jaekel et al., 1994). Cdc2-phosphorylated HMG-1Y (high mobility group protein-1Y), histone H1, and caldesmon were efficiently dephosphorylated by PP2A *in vitro*, but only when the B subunit was present. Experiments using phosphopeptide substrates have shown that the proline residue located at the COOH-terminal side of Cdc2 phosphorylation sites is a negative determinant for dephosphorylation by the PP2A catalytic subunit (Donella Deana et al., 1990). The B subunit appears to overcome this inhibition, making the Cdc2 site a preferred substrate for the holoenzyme (Ferrigno et al., 1993; Mayer-Jaekel et al., 1994). Whereas other mitotic substrates of PP2A are bound to exist, it is interesting to note that those described here are all structural proteins involved in maintenance of chromatin (HMG-1Y and histone H1), cytoskeleton (caldesmon), and Golgi (GM130) architecture. The B subunit of PP2A could therefore have an important role in reversing many of the morphological changes brought about by activation of Cdc2 in mitosis. Studies of PP2A function *in vivo* support a role for the B subunit in proper mitotic progression. Strains of budding yeast that carry a mutation in the B subunit gene lack a functional spindle assembly checkpoint and are defective in cytokinesis (Minshull et al.,

1996; Wang and Burke, 1997), whereas mutations of the corresponding gene in *Drosophila* cause abnormal sister chromatid separation (Mayer-Jaekel et al., 1993). These effects are likely a result of impaired dephosphorylation of specific substrates by PP2A.

The B α subunit of PP2A is present on purified rat liver Golgi membranes, suggesting it may play a role in targeting of the catalytic subunit to the Golgi complex in vivo. This would be consistent with previous work demonstrating a role for this subunit in determining PP2A subcellular localization (Sontag et al., 1995; Turowski et al., 1999). The reason why PP2A is active against GM130 only in telophase and not earlier in mitosis is not clear. The B α subunit may dissociate from Golgi membranes during the early stages of mitosis and reassociate during telophase, or B α -containing PP2A may become activated during telophase. Previous work has failed to reveal dramatic changes in overall PP2A localization or activity during the cell division cycle (Ferrigno et al., 1993; Mayer-Jaekel et al., 1994), but it cannot be excluded that subtle changes in distribution or levels of activity of particular PP2A isoforms could modulate phosphatase activity towards distinct substrates. In fact, it has been reported that the activity of microtubule-associated PP2A is regulated in a cell cycle-dependent manner (Sontag et al., 1995). Further work is clearly necessary to elucidate the regulatory mechanisms that control the correct timing of GM130 dephosphorylation.

We would like to thank the Warren lab for support and helpful comments; Joachim Seemann, Laurence Pelletier, and David Shima for critically reading the manuscript; Drs. Feng Gu and Gary Thomas for kindly providing the Sf9 cell extracts; Lynn Spruce for peptide cross-linking; and Drs. Jonathon Pines, Francis Barr, Tatsuo Suganama, Viki Allan, Brian Hemmings, Ned Lamb, and Patric Turowski for generously providing antibodies.

N.K. Gonatas was supported by a Javits Neuroscience investigator award (NS 05572-33).

Submitted: 14 October 1999

Revised: 18 February 2000

Accepted: 6 March 2000

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