Identification of a Golgi-associated Protein that Undergoes Mitosis Dependent Phosphorylation and Relocation

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Abstract. By means of a monoclonal antibody (BH3), we have identified a 57-kD protein (p57) that in interphase is restricted largely to the perinuclear region of the cell. Double label immunofluorescence microscopy suggests localization of p57 to the Golgi complex and associated membranous structures. Protease protection experiments and chemical extractability indicate that p57 is a peripheral membrane protein exposed to the cytoplasm. p57 displays unique behavior during mitosis. At the end of G2 or in early prophase, p57 leaves the perinuclear region and accumulates very rapidly within the nucleus, at a time when the nuclear envelope is still intact and before nuclear lamina disassembly. This relocation of p57 coincides with its hyperphosphorylation on serine and threonine residues. After nuclear envelope breakdown p57 becomes uniformly distributed throughout the mitotic cytoplasm until in late telophase when it returns to its perinuclear location and is once again excluded from the nucleus. The behavior of p57 during mitosis suggests that it may play a role in the cellular reorganization evident during mitotic prophase.

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Mitosis in higher eukaryotes is characterized by a profound reorganization of the cytoarchitecture. This includes the restructuring of interphase microtubule arrays to form a mitotic spindle, chromatin condensation and the fragmentation or vesiculation of several other low copy number organelles such as the nuclear envelope, Golgi apparatus, and the endoplasmic reticulum (Robbins and Gonatas, 1964; Zeligs and Wollman, 1979; Warren, 1985). The restructuring of these membranous organelles has been shown to coincide with the inhibition of a number of membrane-mediated processes including endocytosis (Fawcett, 1965; Berlin and Oliver, 1980), receptor recycling (Warren et al., 1984; Sager et al., 1984), and intracellular transport of newly synthesized membrane proteins (Warren et al., 1983; Featherstone et al., 1985).

The mechanisms underlying the vesiculation of these organelles is still a matter of speculation. In the case of the Golgi complex, for instance, it has been suggested that its fragmentation results from a temporary imbalance in the rate of membrane traffic into and out of the organelle (Hesketh et al., 1984; Warren, 1985). The disassembly of the nuclear envelope during prometaphase, on the other hand, seems to occur as a multi-step process (Zeligs and Wollman, 1979). It begins with the disassembly of the nuclear lamina, a major structural component of the nuclear envelope, consisting predominantly of three proteins, lamins A, B, and C, (relative molecular mass of 60–74 kD; for review, see Gerace and Burke, 1988). Fragmentation of the double nuclear membrane succeeds lamina disassembly and commences at regions of the nuclear envelope closest to the asters of the mitotic spindle (Roos, 1973). The nuclear membranes apparently initially form large fragments or cisternae that are subsequently reduced to small vesicles (Roos et al., 1973; Zeligs and Wollman, 1979), probably by membrane budding as suggested by Newport and Spann (1987). The initial fragmentation, however, may be because of mechanical disruption by the cytoskeleton (Bajer and Mole-Bajer, 1969) or to some undescribed mechanism that results in periodic fusion between the extracytoplasmic faces of the inner and outer nuclear membranes. At the end of mitosis, in telophase, these organelles are reassembled and resume their interphase functions. This cycle of fragmentation followed by reassembly appears to provide the cell with a simple mechanism to ensure that each daughter receives a more or less equivalent allotment of each organelle (for review, see Warren, 1985). The numerous vesicles derived from each interphase organelle presumably being free to partition at random between the two cells (Lucocq et al., 1987; Lucocq and Warren, 1987).

While the specific mechanisms underlying these structural rearrangements remain uncertain, the higher level regulation of M-phase events is becoming increasingly well understood. In yeast and in higher organisms, both vertebrate and invertebrate, the same conserved molecular mechanisms appear to be operative (for review, see Cross et al., 1989). The general theme that has emerged from these diverse studies is that entry into mitosis requires the regulated activation of a protein kinase p34, which in yeast is encoded by the *cdc2* gene and which, in higher organisms, comprises a subunit of maturation promoting factor or MPF. Activation of p34

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leads in turn to the triggering of a cascade of reactions (probably involving many other protein kinases, including for instance a lamin kinase), which ultimately result in entry into mitosis. Conversely, exit from mitosis and the beginning of the next cell cycle is likely to involve the action of one or more phosphoprotein phosphatases (Doonan and Morris, 1989; Ohkura et al., 1989; Booher and Beach, 1989).

A number of proteins have been shown to be targets for mitotically activated protein kinases. The best described of these are the nuclear lamins (Gerace et al., 1978; Gerace and Blobel, 1980) whose level of phosphorylation increases by a factor of four- to sevenfold upon entry into mitosis (Ottaviano and Gerace, 1985). This hyperphosphorylation is apparently required for the disassembly of the nuclear lamina during prometaphase (Miake-Lye and Kirschner, 1985; Suprynowicz and Gerace, 1986). Likewise, there is evidence that dephosphorylation of the nuclear lamins during telophase is required for lamina and nuclear membrane reassembly (Burke and Gerace, 1986). It is a reasonable assumption that the structural dynamics during mitosis of the various membranous organelles (e.g., the nuclear membranes, Golgi apparatus, and endoplasmic reticulum) may involve the M-phase specific modification of certain key proteins intimately involved in these rearrangements. By analogy with the lamins, such a modification is most likely to involve phosphorylation. Thus, the identification of proteins associated with these organelles that undergo just such cyclic modification may provide clues as to the mechanisms underlying these M-phase architectural rearrangements.

This paper describes the identification, by means of a monoclonal antibody, of a protein associated with the interphase Golgi apparatus and nearby membranes. This protein, which is a target for a mitotically activated protein kinase, undergoes a dramatic relocation to the nucleus at the onset of mitosis, but apparently before nuclear envelope breakdown. Possible functions for this protein p57 are discussed.

Materials and Methods

Cell Culture and Synchrony

Normal rat kidney (NRK) cells were grown in DME supplemented with 10% FCS, penicillin/streptomycin, and glutamine. CHO cells were maintained in αMEM containing 5% FCS, 5% Serum Plus™ (Hazelton Systems, Inc., Aberdeen, MD), penicillin/streptomycin, and glutamine. CHO cells were synchronized exactly as described by Burke and Gerace (1986) employing an 11-h thymidine block (2 mM thymidine) followed by "shake off" of prometaphase cells in the presence of nocodazole (600 ng/ml).

Monoclonal Antibodies

Hybridoma BH3 was derived from a mouse immunized with a Triton X-114 extract of rat liver nuclear envelopes. In short, spleen cells from a Robertsonian (8.12) 5BnR mouse were fused to FOX NY myelomas (Taggart and Samloff, 1982) using polyethylene glycol as described by Galfre et al. (1977). Hybridomas were distributed into 4 × 96-well microtitration plates and selected in AAT medium consisting of DME (4.5 g/liter glucose) containing 20% FCS, adenine (7.5×10^{-5} M), aminopterin (8×10^{-7} M), thymidine (1.6×10^{-5} M), penicillin/streptomycin, and glutamine. Cultures were screened by immunofluorescence microscopy as described by Burke et al. (1982). Positive cultures were taken through three cycles of cloning by limiting dilution employing thymocytes as a feeder layer. Useful hybridomas were adapted to growth first in DME containing 10% FCS and subsequently into DME supplemented only with 10% Serum Plus™ (Hazelton Systems, Inc.). Purified antibody was obtained from spent culture supernatant by two cycles of ammonium sulphate precipitation (at 45% saturation) and subsequently stored in PBS at 4°C in the presence of sodium azide. The antibody 53FC3 against a 135-kD Golgi membrane protein is described in Burke et al. (1982). A monoclonal antibody (E3) against lamin B_2 was a gift from Erich Nigg (Lausanne, Switzerland).

Labeling of Cells with [^{35}S]Met and $^{32}PO_4$

Cells grown in 35-mm petri dishes were labeled overnight with [^{35}S]met (Amersham Corp., Arlington Heights, IL) or with trans label ([^{35}S]met and [^{35}S]cys, ICN Radiochemicals, Irvine, CA) at 50 μCi/ml, in met free DME containing 5% dialyzed (against PBS) FCS and 10% normal DME (providing 1/10 normal concentration of unlabeled met). For labeling with $^{32}PO_4$, cells were first washed with phosphate free DME and then incubated for the specified time (see Results) in phosphate free DME containing 5% FCS (dialyzed against Hepes-buffered saline) and 1-5 mCi/ml $^{32}PO_4$.

Immunoprecipitation

After labeling, the cells were washed once in PBS and then lysed in a buffer containing 50 mM triethanolamine (TEA), 100 mM NaCl, 0.4% SDS, 1 mM DTT, 1 mM PMSF and 1:1,000 CLAP (10 mg/ml in DMSO of chymostatin, leupeptin, antipain, and pepstatin). After adding Triton X-100 to a final concentration of 2%, the lysate was centrifuged for 5 min in an Eppendorf centrifuge (made by Brinkmann Instruments, Westbury, NY) at 4°C. To the supernatant was added 1-5 μg of monoclonal antibody, 3 μl of rabbit anti-mouse IgG serum and 20 μl of a 50% suspension of protein A Sepharose in PBS. The mixture was then rotated overnight at 4°C. The following morning the protein A Sepharose was washed 5 times in the same buffer containing 0.1% SDS and 0.5% Triton X-100. After two final washes in 50 mM Tris, pH 7.4, the Sepharose pellets were suspended in either one- or two-dimensional gel sample buffer as appropriate.

Phosphoaminoacid Analysis

NRK cells in a 35-mm tissue culture petri dish were labeled with 5 mCi of $^{32}PO_4$ for 4 h in the presence of 600 ng/ml nocodazole. p57 was then immunoprecipitated as described above and the immunoprecipitate fractionated by SDS-PAGE. The gel was subsequently dried, and the labeled p57 band excised, having been first localized by autoradiography (1-h exposure). The gel band was then rehydrated and subjected first to trypsin digestion and then to acid hydrolysis as described by Hunter and Sefton (1980). Phosphoamino acids were resolved by thin layer electrophoresis (Hunter and Sefton, 1980) and localized by autoradiography.

Gel Electrophoresis

One-dimensional SDS-PAGE was performed as described by Laemmli (1970). Two-dimensional gels (nonequilibrium pH gradient as the first dimension) were performed according to the method of O'Farrell et al. (1977). On completion of electrophoresis, gels containing proteins labeled with ^{35}S were fixed in 10% TCA, impregnated with EN^{3}HANCE (New England Nuclear, Boston, MA), dried and exposed to x-ray film at −70°C. Gels containing only ^{32}P-labeled proteins were stained with Coomassie blue, dried, and exposed at −70°C with an intensifying screen (Dupont Cronex Lightning Plus, Wilmington, DE) (Laskey and Mills, 1977).

Immunofluorescence Microscopy

Cells grown on glass coverslips were fixed with formaldehyde and labeled with antibodies according to the general procedures described by Ash et al. (1977). Rhodamine- and fluorescein-conjugated secondary antibodies were obtained from Tago Inc. (Burlingame, CA). Some samples were also stained with Höechst dye 33258 to reveal the chromosomes. Specimens were observed and photographed with a Zeiss Axiophot equipped with an ×63 NA and 1.4 PlanApo objective lens. For confocal microscopy, observations, and photography were performed using a second Axiophot equipped with an MRC Lasersharp confocal imaging system and optical disc storage (Bio-Rad Laboratories, Cambridge, MA).

Protease Protection Analysis

NRK cells labeled with [^{35}S]met/cys were homogenized in a buffer con-

1. *Abbreviations used in this paper*: DxRh, rhodamine-conjugated dextrans; NRK, normal rat kidney; TGN, trans-Golgi network.

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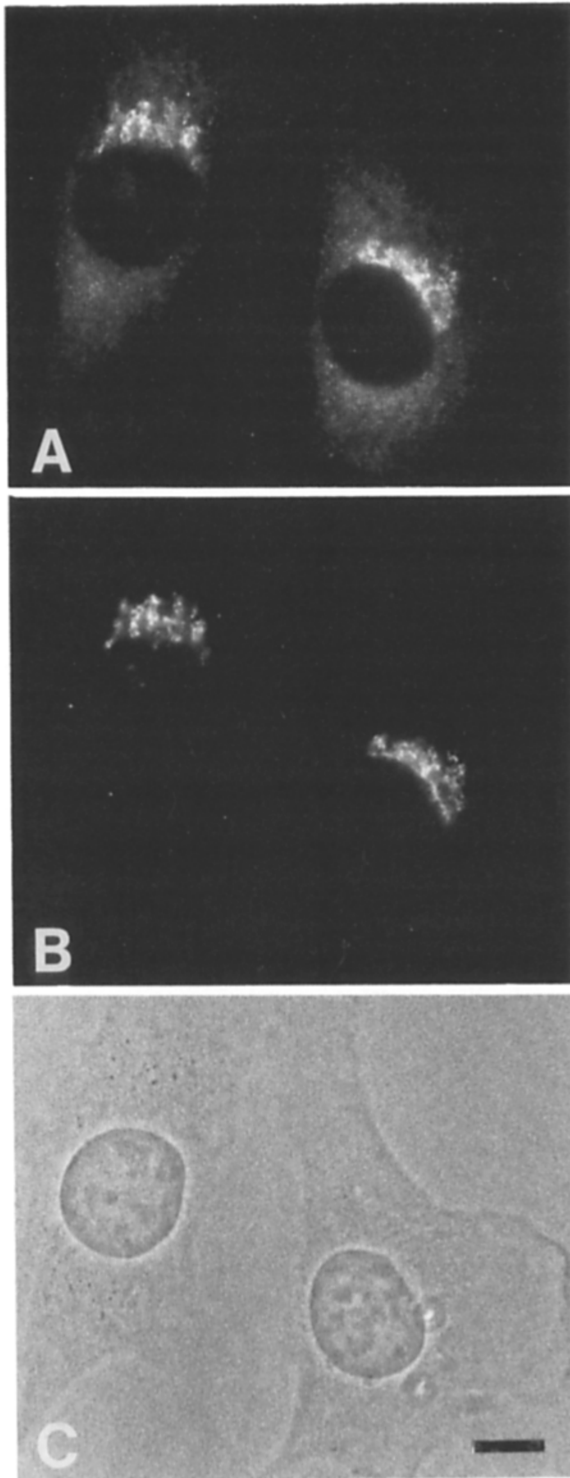

Figure 1. Double immunofluorescence labeling of interphase NRK cells employing the monoclonal antibodies BH3 (*A*) and 53FC3 (*C*, directed against a 135-kD integral Golgi membrane protein). Secondary antibodies were rhodamine-conjugated goat anti-mouse γ heavy chain and fluorescein-conjugated goat anti-mouse μ heavy chain. All of the structures labeled by 53FC3 are labeled by BH3, but not vice versa. Notably, BH3 uniquely labels vesicular structures in the perinuclear region as well as giving a low level of diffuse labeling throughout the cytoplasm. Corresponding phase-contrast images are shown in *B* and *D*. Bar, 10 μm.

taining 10 mM TEA (pH 7.4), 10 mM KCl, 1 mM DTT, 10 μg/ml cytochalasin B. The homogenate was then incubated at 37°C for 1 h with 100 μg/ml proteinase K either in the presence or absence of Triton X-100 as described by Fleischer (1981) and Burke et al. (1982). After the addition of PMSF to 1 mM, the samples were processed for p57 immunoprecipitation. Duplicate samples were assayed for galactosyl transferase activity as described by Bretz and Stäubli (1977), Bretz et al. (1980), and Burke et al. (1982).

Bead Loading

A fixable rhodamine-conjugated dextran (70 kD, catalogue number D-1818; Molecular Probes Inc., Junction City, OR) was introduced into the cytoplasm of NRK cells grown on glass coverslips using the "bead loading" technique described by McNeil and Warder (1988) and employing 75–150 μm glass beads (Sigma Chemical Co., St. Louis, MO). After a 1-h incubation at 37°C the cells were fixed in formaldehyde for 10 min and labeled with BH3 and a fluorescein-conjugated secondary antibody.

Results

Hybridoma BH3, secreting an IgM, was obtained from a fusion involving spleen cells from a mouse that had initially been immunized with a preparation of Triton X-114–solubilized rat liver nuclear membrane proteins. It was identified during a screen employing immunofluorescence microscopy of NRK cells. The staining pattern observed with this antibody is shown in Fig. 1. Clearly the labeling of interphase NRK cells is restricted largely to the perinuclear region and bears a striking resemblance to the pattern observed when these cells are labeled with a monoclonal antibody 53FC3 (Burke et al., 1982), specific for a 135-kD integral membrane protein of the Golgi complex (Fig. 1). However, in contrast to the staining pattern with 53FC3, we always ob-

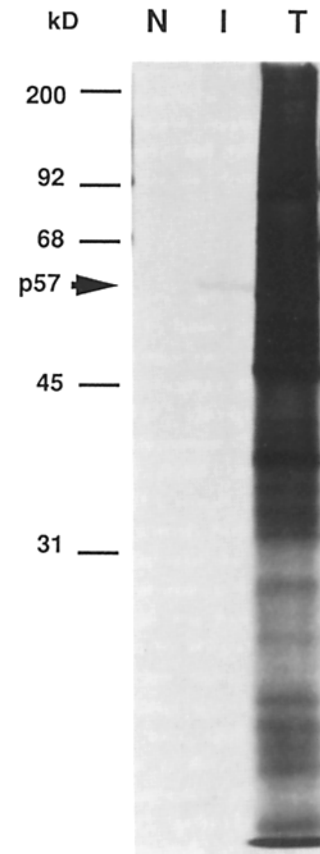

Figure 2. Immunoprecipitation analysis of [^{35}S]met labeled NRK cells employing BH3. (*T*) Total SDS lysate of labeled cells. 50 times this amount was used in each immunoprecipitation. (*I*) Immune sample containing BH3. (*NI*) Nonimmune sample. BH3 clearly immunoprecipitates a single polypeptide of 57 kD.

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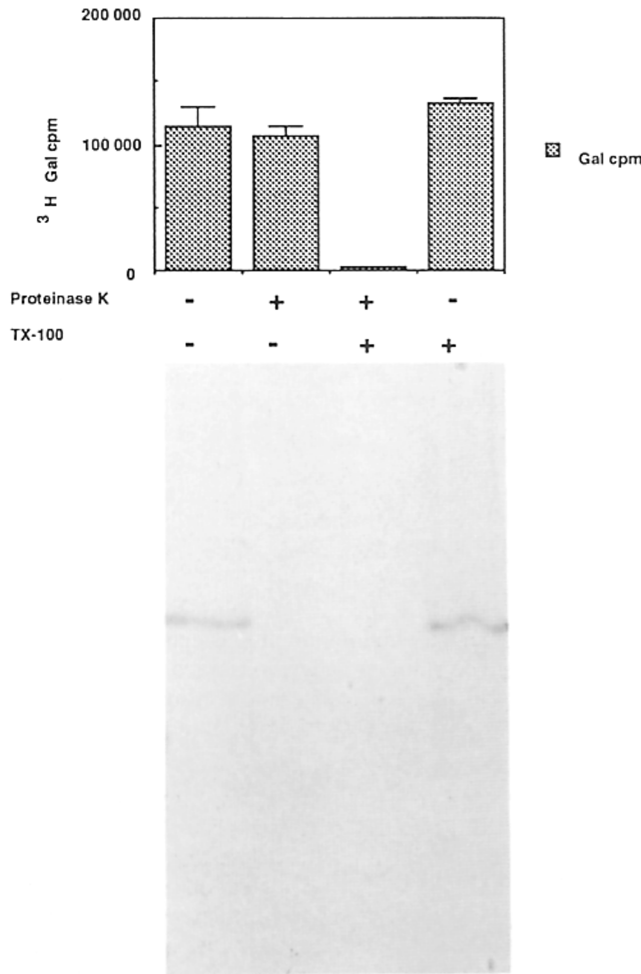

Figure 3. The effects of proteinase K on p57 in homogenates of ^{35}S-labeled NRK cells. Homogenates were digested with 100 $\mu g/ml$ proteinase K either in the presence or absence of Triton X-100 as described in Materials and Methods. Each sample was then processed for immunoprecipitation with BH3 (*bottom*). Samples were also monitored for galactosyl transferase activity (*top*) to demonstrate latency of membranes.

serve a low level of labeling throughout the cytoplasm with BH3 that cannot be attributed to background or to nonspecific binding (Fig. 1). In addition, BH3 also appears to label vesicular structures in the region of the Golgi complex that are not labeled by the Golgi specific antibody 53FC3. A similar pattern of labeling is observed in all mammalian cells so far tested, including rat (NRK), hamster (BHK, CHO), human (SCJO, HeLa), dog (MDCK), and mouse (3T3, PYS-2, P19, P19MES), both embryonic and adult. Immunogold labeling of ultra thin frozen sections indicate that labeling with BH3 (albeit at a low level) is not restricted only to the stacks of cisternae of the Golgi complex but can also be seen over other membranous structures in this region of the cell (B. Burke and G. Griffiths, unpublished observations). Thus, this antibody cannot be considered a Golgi-specific probe.

Immunoprecipitation analysis of NRK cells labeled to equilibrium with either [^{35}S]met or with a mixture of [^{35}S]met and [^{35}S]cys, indicates that BH3 recognizes a single polypeptide of molecular mass 57 kD (Fig. 2). The long exposure times (>2 wk) required for the detection of this protein (p57) suggest that it may be present in only low abundance or that it is a long lived protein that turns over only relatively slowly. To further define the location of p57 with respect to intracellular compartments, homogenates of ^{35}S-labeled NRK cells were digested with proteinase K either in the presence or absence of Triton X-100. After digestion, the samples were analyzed by immunoprecipitation with BH3. As shown in Fig. 3, p57 cannot be detected in proteolysed homogenates even in the absence of detergent when latent galactosyl transferase activity indicates that the membranes of the Golgi complex are impermeable to protease. These results suggest that the epitope on p57 recognized by BH3 is exposed to the cytoplasm. When ^{35}S-labeled NRK homogenates were extracted with 100 mM Na_2CO_3 all of the p57 could be recovered in the supernatant after pelleting of the membranes by ultracentrifugation (Fig. 4). Similar homogenates were also extracted with the detergent Triton X-114. When these extracts were warmed to 33°C, the cloud

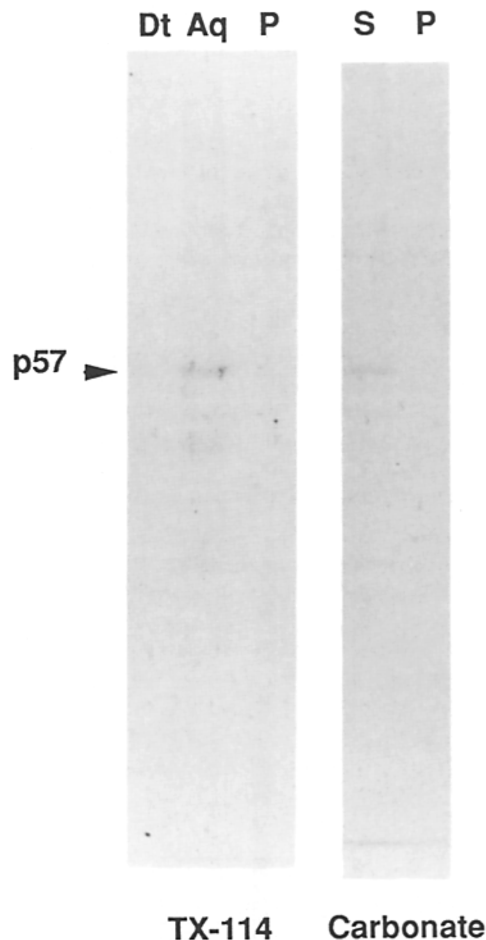

Figure 4. Labeled NRK cells were extracted either in Triton X-114 (*TX-114*) or in Na_2CO_3 (*Carbonate*). The Triton X-114–extracted cells generated two fractions, a detergent insoluble pellet (*P*) and a soluble supernatant. The latter yielded two more fractions upon warming to 37°C, an aqueous phase (*Aq*) and a detergent phase (*Dt*). All three were processed for immunoprecipitation with BH3. For extraction in Na_2CO_3, the cells were homogenized in a 100-mM solution and then airfuged for 15 min at 20 psi and at 4°C to yield a supernatant fraction (*S*) and a membrane pellet (*P*). These were subsequently processed for immunoprecipitation with BH3.

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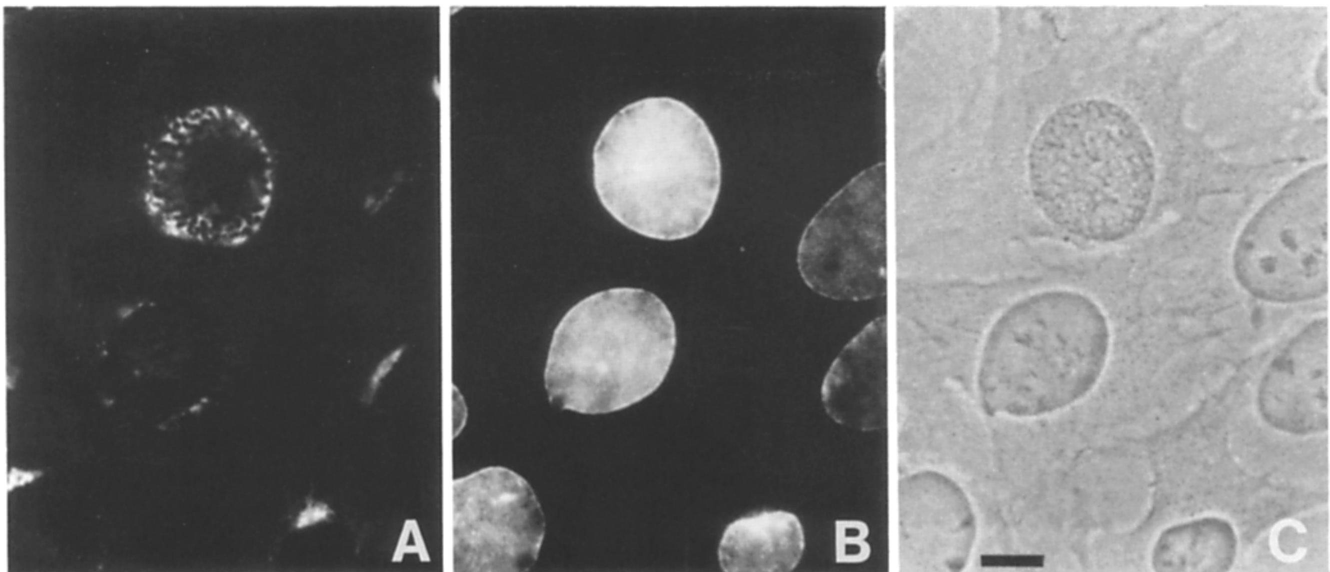

Figure 5. Double label immunofluorescence microscopy of a prophase NRK cell employing BH3 (*A*) and a monoclonal antibody (E3) against lamin B_2 (*B*). Secondary antibodies were rhodamine-conjugated goat anti-mouse γ heavy chain and fluorescein-conjugated goat anti-mouse μ heavy chain. The same field photographed using phase-contrast optics is shown in *C*. Bar, 10 μm.

point of the detergent, p57 could only be detected, by immunoprecipitation, in the aqueous phase of the extract. Little could be detected in the detergent phase (Fig. 4). Taken together, these results suggest that p57 is not an integral membrane protein, a conclusion supported by the further observations described below.

Late in G2 and in early prophase, p57 undergoes a dramatic and unusual redistribution. From its interphase location in the perinuclear region of the cell it rapidly migrates into the cell nucleus. This redistribution is clearly shown in Fig. 5 where the condensing prophase chromosomes appear to be outlined by p57 immunolabeling. Observations with the confocal laser scanning microscope, however, demonstrate that p57 is not found throughout the prophase nucleus but is restricted to the nuclear periphery (Fig. 6). Double label immunofluorescence microscopy employing an antibody against lamin B, indicates that p57 accumulation in the prophase nucleus occurs at a time before disassembly of the nuclear lamina (Fig. 5). That the nuclear envelope is still intact and sealed to large cytoplasmic macromolecules during accumulation of p57 is evident from studies employing fixable rhodamine-conjugated dextrans (DxRh) introduced into NRK cells using the "glass bead" method of McNeill and Warder (1988). When DxRh is loaded into the cytoplasm of G2 NRK cells, immunofluorescence microscopy shows that at a time when p57 has access to the nuclear interior, DxRh is still largely excluded (Fig. 7). This result suggests that nuclear uptake of p57 may represent a signal-mediated process. After nuclear envelope breakdown in prometaphase, p57 becomes uniformly distributed throughout the mitotic cytoplasm (Fig. 8, *A–C*) and remains so during metaphase (Fig. 8, *D–F*) and anaphase (Fig. 8, *G–I*). In telophase and early G1 cells when the nuclear envelope has reformed, p57 is once again excluded from the nuclear interior and returns to the perinuclear region of the cell. This later event appears to occur relatively slowly when compared with the reformation of the Golgi complex (Fig. 8, *J–L*).

Figure 6. Confocal immunofluorescence micrograph of the nuclear region of a prophase NRK cell labeled with BH3 and a fluorescein-conjugated secondary antibody. p57 appears concentrated towards the periphery of the nucleus. Bar, 2.5 μm.

When either NRK or CHO cells were incubated in medium containing $^{32}PO_4$, immunoprecipitation analysis indicated that label was incorporated into p57, albeit at a low level, suggesting that this protein might be phosphorylated. This conclusion was confirmed by the phosphoamino acid analysis described below (Fig. 10). In a first step to determine whether this phosphorylation could be related to the mitotic redistribution of p57, CHO cells were synchronized in pseudometaphase using nocodazole after an overnight thymidine block.

The mitotic cells were subsequently collected by "shake-off." In one experiment, the mitotic cells were replated in medium containing $^{32}PO_4$, either in the presence or absence

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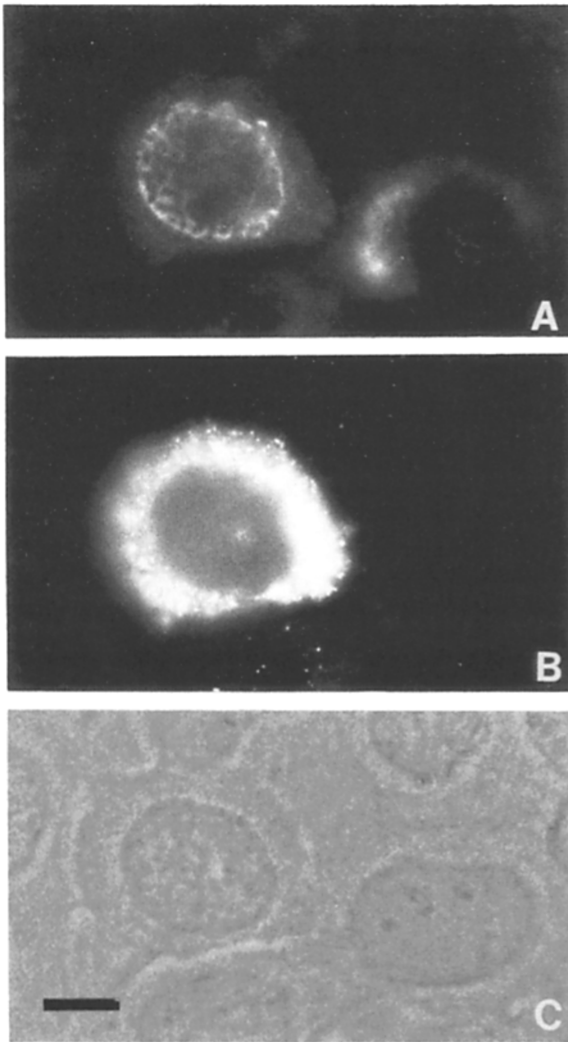

Figure 7. Immunofluorescence microscopy of a prophase NRK cell microinjected with fixable DxRh. NRK cells loaded with DxRh were incubated for 2 h at 37°C and then fixed and labeled with BH3 followed by a rhodamine-conjugated secondary antibody. DxRh distribution is shown in *B* while BH3 labeling can be seen in *A*. The prophase cell shows little DxRh entry into the nucleus whereas p57 has undergone considerable nuclear accumulation. The corresponding phase-contrast image is shown in *C* where several uninjected cells are also visible. Bar, 10 μm.

of nocodazole and incubated at 37°C for 1 h. During this period, the cells incubated in the absence of nocodazole-completed mitosis and entered G1. Subsequent immunoprecipitation (Fig. 9 *A*) indicated that ^{32}P-labeled p57 was only present in the mitotic cells and not the G1 cells, although equivalent amounts of protein could be detected in either immunoprecipitate by Western blotting (Fig. 9 *B*). To exclude the possibility that the nocodazole was inducing the phosphorylation of p57 in some unknown manner, CHO cells were incubated in the presence of nocodazole and $^{32}PO_4$ for 2.5 h after which the mitotic cells were collected by shake-off leaving behind interphase cells on the dish. Both populations of cells were then processed for immunoprecipitation. As before, labeled p57 was only detectable in the mitotic immunoprecipitate (Fig. 9 *C*) despite the fact that far fewer mitotic versus interphase cells were obtained.

These results indicate that p57 becomes hyperphosphorylated during mitosis. Phosphoamino acid analysis of p57 that was immunoprecipitated from NRK cells labeled with 5 mCi of $^{32}PO_4$ in the presence of nocodazole demonstrated that p57 was phosphorylated on both serine and threonine residues (Fig. 10) but not on tyrosine. Analysis by two-dimensional gel electrophoresis of either ^{35}S- or ^{32}P-labeled p57 immunoprecipitates indicates a substantial shift to a more acidic isoelectric point during mitosis (Fig. 11). A comparison of the position of the spots on the mitotic versus interphase gels suggests that p57 may be maximally phosphorylated with ∼4 mol of phosphate per mole of protein during mitosis, assuming that phosphorylation is the only charge altering modification detectable using this gel system. Such heavy phosphorylation would be consistent with the shift up in apparent molecular mass of mitotic versus interphase p57 evident on the two-dimensional gel shown in Fig. 11 *D* and on one-dimensional gels (not shown). Also evident from Fig. 11, (*C* and *D*) is that >50% of the p57 undergoes the shift to a more acidic isoelectric point, presumably because of phosphorylation, in mitotic cells.

Discussion

In this paper, we have described the identification and characterization of a novel 57-kD protein that is localized to the interphase Golgi apparatus and nearby membranes. Chemical fractionation and protease protection studies indicate that it is only peripherally associated with these membranous organelles, and is exposed to the cytoplasm. This protein, p57, is unique in that it undergoes a dramatic redistribution to the nucleus at the end of G2, probably in response to its hyperphosphorylation. Immunofluorescence microscopy of cells loaded with large DxRhs indicate that p57 accumulates in the nucleus before the commencement of nuclear envelope and lamina breakdown at a time when the nucleus is still sealed to cytoplasmic macromolecules. This suggests that the uptake of p57 into the nucleus may be a signal-mediated process, since if the protein is not grossly asymmetric, it would be too large to have access to the nuclear interior by free diffusion through the nuclear pore complexes (for review, see Dingwall and Laskey, 1986).

Redistribution to the nucleus has previously been described for another Golgi-associated protein, the catalytic (C) subunit of the type II cAMP dependent protein kinase. The regulatory (R) subunit of this enzyme is anchored on the cytoplasmic face of membranes of the Golgi apparatus (Nigg et al., 1985*b*). In the presence of elevated cAMP the C subunit dissociates from the R subunit and is rapidly translocated into the nucleus (Nigg et al., 1985*a*). Once cAMP levels fall, the C subunit once again associates with the R subunit that itself undergoes no redistribution. Agents such as forskolin that cause an increase in cAMP resulting in C subunit nuclear translocation, have no discernible effect upon p57 localization (not shown), indicating that these are two quite distinct proteins. They also differ significantly in molecular mass (Nigg et al., 1985*b*).

An intriguing feature of the redistribution of the C subunit in response to cAMP is that it is only the R subunit that actually binds cAMP. This suggests that the C subunit must be in equilibrium between nuclear and cytoplasmic pools and that it is the affinity of the R subunit for C subunits, regulated

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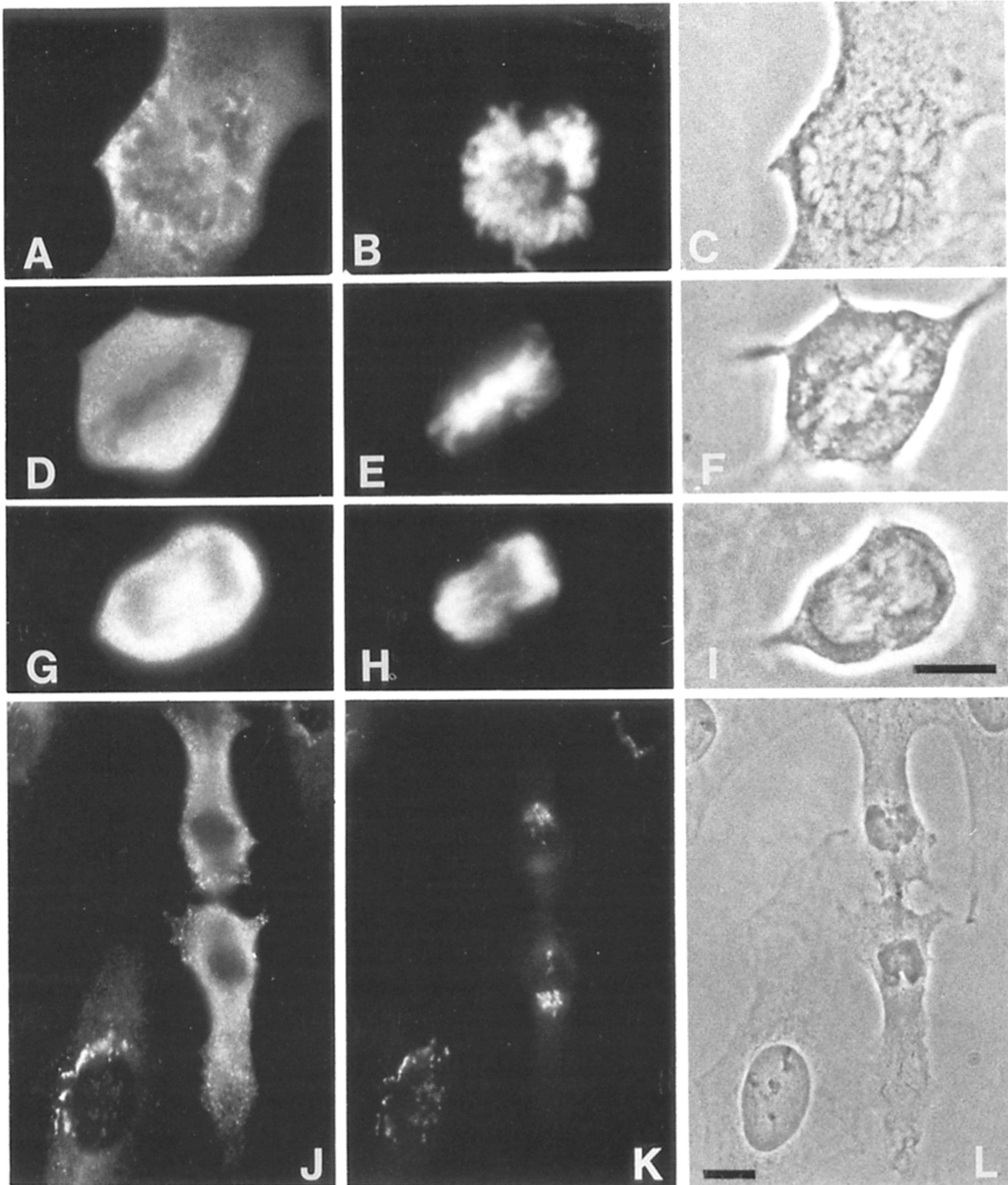

Figure 8. Immunofluorescence labeling of mitotic NRK cells with BH3. (*A*) Prometaphase, (*D*) metaphase, (*G*) anaphase, and (*J*) telophase. In *B*, *E*, and *H*, the corresponding fields are shown stained with Hoechst dye 33258 to reveal the chromosomes, whereas in *K* the cells were double labeled with the anti-Golgi antibody 53FC3 to reveal the state of assembly of the Golgi apparatus. The corresponding phase-contrast images are displayed in the right-hand column. The magnification of *J–L* is somewhat lower than that of *A–I*. Bars, 10 μm.

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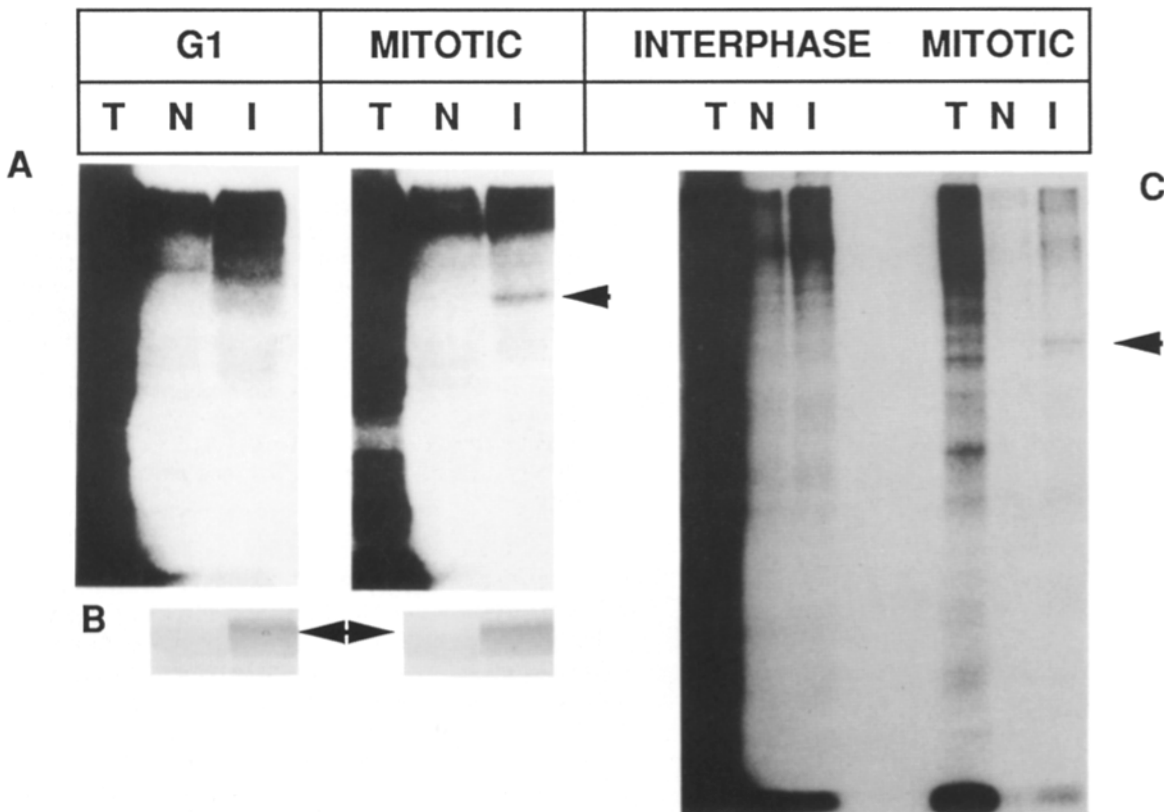

Figure 9. CHO cells were synchronized in pseudometaphase using nocodazole after an overnight thymidine block. The mitotic cells were subsequently collected by shake-off. In *A*, the mitotic cells were replated in medium containing $^{32}PO_4$, either in the presence or absence of nocodazole and incubated at 37°C for 1 h, to yield labeled mitotic or G1 cells, respectively. Both populations of cells were then processed for immunoprecipitation with BH3 (*I*) or a nonimmune control (*N*). The total labeled cell lysate is indicated by *T*. 50 times this amount was used for each immunoprecipitation. An immunoblot of the precipitated samples is shown in *B* indicating that comparable amounts of p57 can be found in both mitotic and G1 cells. In *C*, the CHO cells were incubated in the presence of nocodazole and $^{32}PO_4$ for 2.5 h before shake-off of the mitotic cells. Both the mitotic cells and the interphase cells left behind on the dish were processed for immunoprecipitation with BH3. Approximately five to ten times as many interphase as mitotic cells were used; however, labeled p57 is only detected in the mitotic immunoprecipitate (*arrowhead*).

by cAMP, which controls gross C subunit distribution. Such an equilibration, implying bidirectional movement through the nuclear pore complexes, need not apply to p57 since it only returns to its perinuclear location after dissolution of the nuclear envelope, when it has free access to the cytoplasm. It is probable that p57 contains one or more nuclear localization signals, but at the same time must contain sequences that direct it to the locality of the Golgi complex in interphase cells. One of the functions of the M-phase dependent phosphorylation may be either to inactivate the Golgi targeting sequence or to uncover a cryptic nuclear localization signal, or perhaps both. Since we do not know what molecules p57 binds to on the Golgi complex and nearby membranes, the possibility must remain open that its binding site also undergoes some form of M-phase dependent modification.

Apart from p57, only one other Golgi-associated protein has been suggested to undergo M-phase dependent phosphorylation. This is an 110-kD protein, initially identified in mammalian cells, which is related to the microtubule-associated protein MAP-2 (Allen and Kreis, 1986). It is thought to be involved in the interaction between the Golgi apparatus and the interphase microtubule network. Experiments employing *Xenopus* oocytes and eggs indicate that the amphibian homologue of this protein is hyperphosphorylated during meiotic metaphase. It is possible that this M-phase dependent modification is involved in the modulation of Golgi–microtubule interactions during cell division, although its occurrence in mammalian somatic cells has yet to be demonstrated.

Several Golgi-associated proteins have molecular masses similar to that of p57. Willison et al. (1989) have recently found that a 57-kD protein, TCP-1, encoded by a gene of the mouse T complex, is associated with the cytoplasmic face of interphase Golgi membranes, specifically the trans-Golgi network (TGN). It is suggested that TCP-1 may be involved in acrosome formation in sperm, possibly the budding or targeting of transport or secretory vesicles exiting the TGN. TCP-1 and p57 appear not to be related, however, since TCP-1 apparently remains associated with Golgi-derived vesicles during mitosis while p57 clearly does not. In addition, p57, in contrast to TCP-1, is not restricted to the TGN. A similar argument applies to the one other known peripheral protein of the Golgi apparatus with a molecular mass in the 50–60 kD range GC1 (Chicheportiche et al., 1984; Chicheportiche and Tartakoff, 1987). This protein of 54 kD

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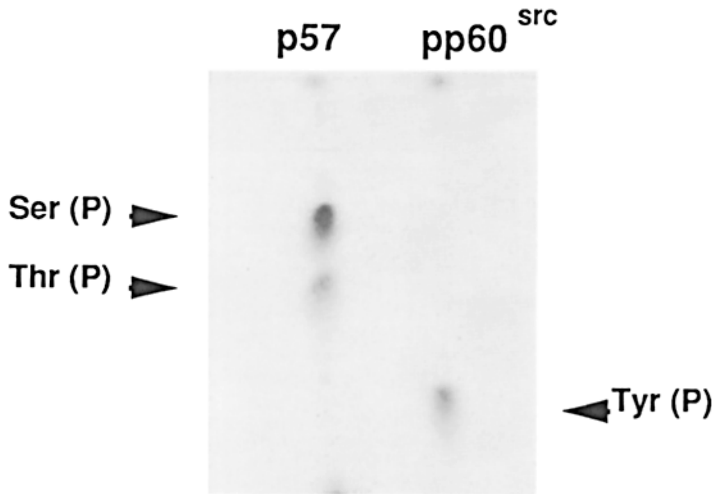

Figure 10. NRK cells were labeled overnight with 5 mCi of $^{32}PO_4$ in the presence of nocodazole. p57 was subsequently immunoprecipitated from these cells and the immunoprecipitate fractionated on an SDS gel. After autoradiography, p57 was excised from the gel, digested with trypsin, and then subjected to acid hydrolysis. Phosphoamino acids were then separated by thin layer electrophoresis and detected by autoradiography. The positions of nonradioactive standards were determined using ninhydrin. $pp60^{src}$ was employed as a source of labeled phosphotyrosine, *Tyr(P)*. It is clear that p57 contains only phosphoserine, *Ser(P)*, and phosphothreonine, *Thr(P)*.

and a second immunologically related polypeptide of 86 kD are localized exclusively to two or three medial cisternae of the Golgi apparatus. There is no indication that either the 54- or the 86-kD protein ever undergo any M-phase dependent redistribution independent of the Golgi apparatus.

What then might be the function of p57? One possibility would be that it is somehow involved in the normal interphase functions of the Golgi apparatus. Since, however, it does not appear to be associated with just a single compartment it would have to be argued that its function was common to several biochemically distinct compartments. One obvious possibility would be that it is involved in either vesicle budding or targeting and that its M-phase dependent modification could be involved in the cessation of intra-Golgi transport during mitosis. Such an explanation, however, does not account for the movement of this protein into the nucleus at the end of G2. In addition, as can be seen in Fig. 8 (*J–L*), p57 appears to return to its interphase location relatively slowly, at a time when it is known that normal interphase vesicular traffic has already resumed (Warren et al.,

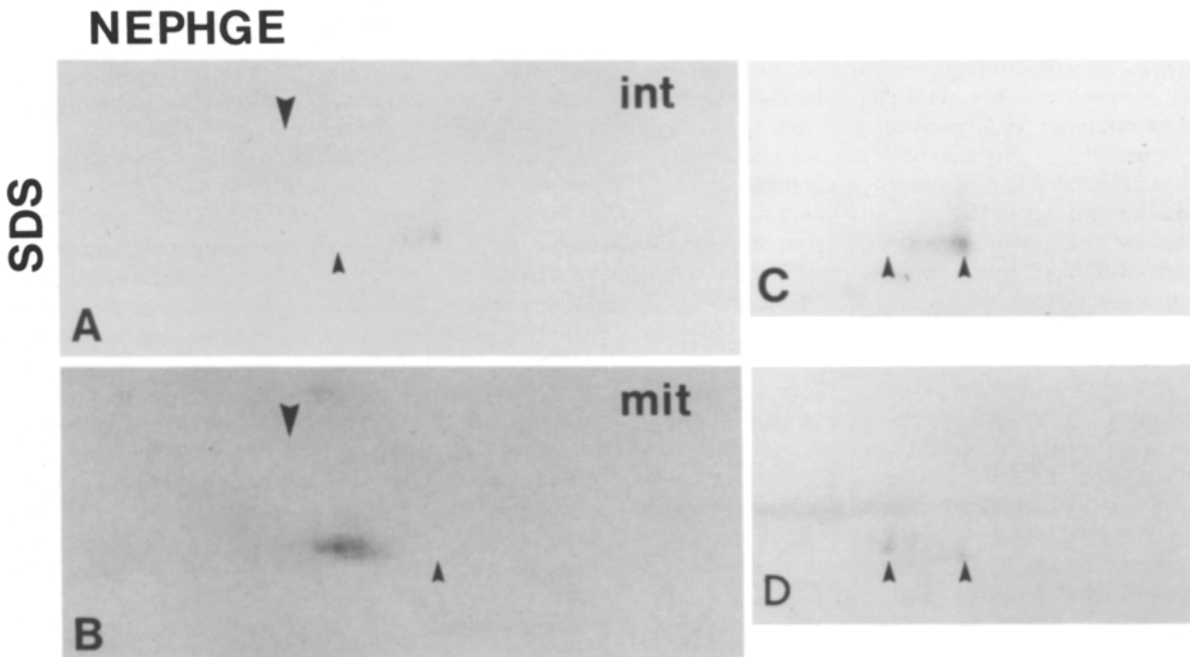

Figure 11. p57 was immunoprecipitated from interphase NRK cells labeled with [^{35}S]met (*A*) and from mitotic NRK cells labeled with $^{32}PO_4$ (*B*). The immunoprecipitates were subsequently fractionated on identical two-dimensional gels both of which were stained with Coomassie blue, processed for fluorography, and dried in parallel. The position and total number of spots on each autoradiograph indicates that p57 possesses 5–6 charge isoforms. The small arrowheads indicate the positions of the major charge isoforms on either autoradiograph. The large arrowheads show the position of a nonradioactive protein in the immunoprecipitate (detected by Coomassie blue staining; *not shown*) on each gel, which provides an internal standard for the position of p57. *C* and *D* show a pair of interphase and mitotic immunoprecipitates both labeled with [^{35}S]met. This indicates that at least 50% of the total p57 undergoes a shift in isoelectric point during mitosis. Note also that p57 has undergone a slight shift up in apparent molecular mass in *B* and *D*.

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1983). Another potential role for p57 may be as an effector of mitotic progression, possibly involved in some aspect of prophase nuclear restructuring such as chromatin condensation or nuclear envelope breakdown. The latter might be consistent with the apparent concentration of p57 towards the periphery of the prophase nuclei. It could also in principle, play the role of a reporter molecule involved in the synchronization of cytoplasmic and nuclear events. In this way, it is conceivable that the association of p57 with the interphase Golgi complex and nearby membranes could be misleading in terms of p57 function, just as it is for the cAMP dependent protein kinase described in detail above.

p57 appears not to correspond to any of the other known regulatory proteins implicated in mitotic progression. Its molecular mass is very close to what has been described for the cyclins, which are involved in the regulation of p34 activity (for review, see Cross et al., 1989). However, we have failed to obtain any immunological data which would confirm identity with the cyclins (Burke, B., and K. Swenson, unpublished observations). Furthermore, in contrast to the cyclins, p57 does not appear to undergo any large scale degradation after metaphase. In *Drosophila* early embryos for instance, cyclins become undetectable by immunofluorescence microscopy by early anaphase (Lehner and O'Farrell, 1989). Because protein kinases play a key role in mitotic regulation, we have tested p57 immunoprecipitates for kinase activity; however, to date none has been convincingly found.

If p57 is required for chromatin condensation or for some aspect of nuclear envelope breakdown, it should be possible to demonstrate this in vitro employing mitotic extracts such as has been described by Suprynowicz and Gerace (1986). After preadsorption of a CHO mitotic cytosol fraction with the antibody against p57, the ability of the cytosol to promote breakdown of interphase nuclei may then be assessed microscopically. Similarly, if p57 is required for normal interphase Golgi function this may also be determined in vitro employing the cell free intra-Golgi transport assay developed by Rothman and his colleagues (Balch et al., 1984), or perhaps in vivo by microinjection (Burke and Warren, 1984). Such experiments will hopefully shed more light on the function of p57.

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Note added in proof: We have recently obtained a second independent antibody (TC11) against p57 that gives identical results to those described in this paper.

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