

# Mitosis and Inhibition of Intracellular Transport Stimulate Palmitoylation of a 62-kD Protein

Dorothy I. Mundy and Graham Warren

Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom

**Abstract.** Recent studies suggest that a cycle of acylation/deacylation is involved in the vesicular transport of proteins between intracellular compartments at both the budding and the fusion stage (Glick, B. S., and J. E. Rothman. 1987. *Nature (Lond.)*. 326:309–312). Since a number of cellular processes requiring vesicular transport are inhibited during mitosis, we examined the fatty acylation of proteins in interphase and mitotic cells. We have identified a major palmitoylated protein with an apparent molecular weight of 62,000 (p62), whose level of acylation increases 5–10-fold during mitosis. Acylation was reversible and p62 was no longer palmitoylated in cells that have exited mitosis and entered G1. p62 is tightly bound to the cytoplasmic side of membranes, since it was sensitive to digestion with proteases in the absence of detergent and was not removed by treatment with 1 M KCl. p62 is removed from membranes by nonionic detergents or

concentrations of urea >4 M. The localization of p62 by subcellular fractionation is consistent with it being in the *cis*-Golgi or the *cis*-Golgi network. A palmitoylated protein of the same molecular weight was also observed in interphase cells treated with inhibitors of intracellular transport, such as brefeldin A, monensin, carbonylcyanide *m*-chlorophenylhydrazone, or aluminum fluoride. The protein palmitoylated in the presence of brefeldin A was shown to be the same as that palmitoylated during mitosis using partial proteolysis. Digestion with two enzymes, alkaline protease and endoprotease lys-C, generated the same <sup>3</sup>H-palmitate-labeled peptide fragments from p62 from mitotic or brefeldin A-treated cells. We suggest that the acylation and deacylation of p62 may be important in vesicular transport and that this process may be regulated during mitosis.

A substantial number of proteins of both cellular and viral origin contain long-chain fatty acids covalently bound to the polypeptide backbone (McIlhinney, 1990). However, the functional consequences of this post-translational modification are unknown. There are two major classes of acyl proteins. One class contains the 14-carbon saturated fatty acid, myristate, which is added co-translationally and remains stably bound throughout the lifetime of the protein. The second class of proteins contains the 16-carbon saturated fatty acid, palmitate, and is modified post-translationally. In most cases, palmitic acid appears to be linked via thioester bonds to internal cysteines (Olson, 1988) although *O*-ester linkages may also occur (Jing and Trowbridge, 1990). Palmitoylated proteins fall into two categories depending on whether the palmitoyl group undergoes turnover or not. Viral glycoproteins are the best studied example of irreversibly palmitoylated proteins. The viral glycoproteins are acylated shortly after synthesis in a compartment that resides between the ER and the *cis*-Golgi (Bonatti et al., 1989) and do not appear to undergo reversible acylation because inhibitors of protein synthesis block palmitoylation of these

proteins. However, Olson and Spizz (1986) have demonstrated that <1% of the palmitoylated proteins in BC<sub>3</sub>H1 muscle cells are transmembrane glycoproteins. The majority of the palmitoylated proteins in cells do not contain carbohydrate and are acylated by a mechanism that is independent of protein synthesis. The reversible fatty acylation of several palmitoylated proteins has been demonstrated *in vivo*, including the transferrin receptor, p21<sup>N-ras</sup>, the neuronal growth cone protein GAP-43, several erythrocyte cytoskeletal proteins, and p64, a protein which is deacylated in response to growth factor stimulation (Omary and Trowbridge, 1981; Magee et al., 1987; Staufienbiel, 1988; Skene and Virag, 1989; James and Olson, 1989). Palmitoylation may determine the extent to which a protein is associated with membranes, but many palmitoylated proteins are now known to associate independently of fatty acylation (Hancock et al., 1989). The role of covalently bound fatty acids may therefore be more complex than simply serving as a hydrophobic membrane anchor.

Recent reports have suggested a role for fatty acylation in intracellular transport (Glick and Rothman, 1987). The mechanism of vesicle mediated transport is thought to be the same from the ER to the Golgi complex and through the Golgi stack up to the *trans*-Golgi network (see Warren, 1990).

Dr. Mundy's present address is Department of Neurology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 80309.

Transport from the *cis*-to-medial cisternae has been particularly well characterized (Rothman and Orci, 1990), and palmitoyl-CoA is thought to be required for both the budding of transport vesicles (Glick and Rothman, 1987; Pfanner et al., 1989) and for subsequent fusion with the next cisterna (Pfanner et al., 1990). Membrane fusion requires the recruitment of several proteins from the cytosol to the membrane to form a complex which can catalyze the fusion reaction (Rothman and Orci, 1990). Reversible palmitoylation of one or more of the proteins in such a complex has been proposed as a possible mechanism that would allow rapid recycling of transport components. The palmitoylation target has not, however, been identified.

One approach to identifying possible target proteins is to exploit inhibitors of intracellular transport. In mammalian cells membrane traffic is inhibited during mitosis. This includes endocytosis, exocytosis, and receptor recycling (Warren, 1985). In addition, neither newly synthesized proteins (Featherstone et al., 1985) nor glycolipids (Kobayashi and Pagano, 1989) are transported through the secretory pathway to the cell surface. In fact, all vesicle-mediated traffic appears to be inhibited. By comparing the palmitoylation patterns between interphase and mitotic cells we have identified a major palmitoylated protein (p62) which is only clearly detected during mitosis. The same protein becomes acylated in cells treated with the intracellular transport inhibitors brefeldin A (BFA)<sup>1</sup>, monensin, carbonylcyanide *m*-chlorophenylhydrazide (CCCP), and aluminum fluoride, suggesting that acylation and deacylation of this protein is important in the transport process.

## Materials and Methods

### Materials

Cell culture media were purchased from Northumbria Biological Laboratories (Northumbria, UK). <sup>3</sup>H-palmitate (60 Ci/mmol) was purchased from New England Nuclear (Boston, MA). BFA was purchased from Epicenter Technologies (Madison, WI) and stored according to the manufacturer's instructions. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), BDH Chemicals Ltd. (Dagenham, Essex, England), or Boehringer-Mannheim Diagnostics, Inc. (Houston, TX) unless otherwise stated.

### Cells

The 15B clone of CHO cells (Gottlieb et al., 1974) were maintained as monolayers in  $\alpha$ -MEM containing 10% FCS, 100 U/ml penicillin-streptomycin, 2 mM glutamine, and non essential amino acids. Other cells were maintained as follows: normal rat kidney (Louvard et al., 1982), A431 (Woodman and Warren, 1988), HeLa (Lucocq et al., 1987), and BHK (Green et al., 1981).

### Mitotic Cells

Mitotic 15B cells were prepared essentially as described by Balch et al. (1987) except that cells were grown to confluency in 850-cm<sup>2</sup> roller bottles. After incubation with nocodazole (100 ng/ml) for 3 h, mitotic cells were selectively removed from the monolayer by spinning the roller bottle at 300 rpm for 3 min at 37°C using a modified roller apparatus (Klevecz, 1975). This method yielded  $\sim 5\text{--}8 \times 10^7$  cells/roller bottle with a mitotic index of 94–98% as judged by staining with Hoechst dye 33258. Mitotic cells had to be labeled and used within 5–6 h of exposure to nocodazole or the cells would begin to exit mitosis spontaneously.

1. *Abbreviations used in this paper:* BFA, brefeldin A; CCCP, carbonylcyanide *m*-chlorophenylhydrazide.

### Labeling with <sup>3</sup>H-Palmitate

Interphase cells, grown in 60-mm culture dishes, were washed three times with serum-free growth medium and usually labeled in MEM buffered with 20 mM Hepes, pH 7.4, containing 7.5% FCS and 50  $\mu$ Ci/ml <sup>3</sup>H-palmitate. The FCS was filtered through a 0.2- $\mu$ m nitrocellulose filter before use. Mitotic cells were labeled in suspension and maintained in the mitotic state by including 100 ng/ml of nocodazole. Cells were incubated at 37°C for 60–90 min as indicated in the figure legends. Monolayers were released using either trypsin/EDTA or Tris/salt (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, and 1 mM Na<sub>2</sub>HPO<sub>4</sub>) containing 5 mM EDTA. Cells were pelleted at low speed and washed once with Dulbecco's PBS. The cell pellets were then extracted for at least 1 h on ice with 200  $\mu$ l 1% Triton X-100 in Dulbecco's PBS containing 1 mM EGTA, 1 mM EDTA, 10 mg/ml soy bean trypsin inhibitor, 1 mM iodoacetamide, and 10  $\mu$ g/ml each of chymostatin, leupeptin, antipain, and pepstatin. The nuclei were removed by centrifugation in an Eppendorf microfuge (Brinkman Instruments Co., Westbury, NY) at 14,000 rpm for 30 s at room temperature. Protein was determined by the BCA method (Pierce Chemical Co., Rockford, IL), using BSA as a standard and equal amounts of protein were precipitated by the method of Wessel and Flugge (1984). The precipitates were solubilized in Laemmli buffer and run on a 5–20% SDS-polyacrylamide gradient gel. Gels were treated with Amplify (Amersham Corp., Arlington Heights, IL) and dried for fluorography. Gels were exposed for 7–30 d to film (X-OMAT AR; Eastman Kodak Co., Rochester, NY) at –80°C. Densitometry was performed on a video densitometer from Bio-Rad Laboratories (model 620; Richmond, CA).

### Microsome Preparation and Extraction with Urea, Salt, or Triton X-114

Mitotic or interphase cells were labeled and washed once with homogenization buffer (0.25 M sucrose in 10 mM Tris-HCl, pH 7.4, and 1 mM magnesium acetate). The cell pellet was diluted with 4 vol of homogenization buffer and the cells broken by 10 passes through a ball bearing homogenizer with a clearance of 0.008 in. (Balch and Rothman, 1985). Postnuclear supernatants were prepared by centrifugation at 800 *g* for 5 min at 4°C. These were then diluted with 3–4 vol of 1 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE buffer), and centrifuged in a table-top ultracentrifuge (model TL-100, TLA 100.3 rotor; Beckman Instruments, Inc., Palo Alto, CA) at 100,000 rpm for 30 min at 4°C. The microsomes were either prepared directly for gel electrophoresis or treated as follows. To treat membranes with urea or high salt the microsomes were resuspended in homogenization buffer containing either urea or KCl and incubated for 30 min on ice. The treated membranes were separated into a pellet and supernatant fraction by centrifugation in an SW 50.1 rotor (Beckman Instruments, Inc.) for 1 h at 100,000 *g*. Samples were then prepared for gel electrophoresis as described above. Microsomes were treated with 1% Triton X-114 essentially as described by Bordier (1981) and equal volumes were loaded for electrophoresis.

### Protease Treatment of Membrane Fractions

Post-nuclear supernatants from <sup>3</sup>H-palmitate-labeled cells were incubated for 15 min at 37°C with 1, 5, or 50  $\mu$ g/ml of proteinase K, or 2 or 10  $\mu$ g/ml of trypsin, which had been pretreated with 1 mM PMSF, in the presence or absence of 0.5% Triton X-100. Reactions were stopped by transfer to ice and the addition of PMSF to a final concentration of 4 mM. The proteins were then precipitated as described above and analyzed by SDS-PAGE. Galactosyltransferase activity was measured by the method of Bretz and Staubli (1977) on replicate incubations from unlabeled cells.

### Subcellular Fractionation

Post-nuclear supernatants were prepared, as described above, from <sup>3</sup>H-palmitate-labeled and unlabeled mitotic or interphase cells. 2 ml of the post-nuclear supernatant was layered on a sucrose gradient consisting of 50% (1 ml), 45% (1.5 ml), and 2 ml each of 40, 35, 30, 25, and 15% sucrose (wt/wt) in TE buffer and centrifuged at 25,000 rpm for 15 h in an SW 28.1 rotor at 4°C. Fractions of  $\sim 1.2$  ml were collected from the bottom. To analyze the <sup>3</sup>H-palmitate labeled fractions, 0.5 ml of each fraction was diluted to 3 ml with TE buffer and pelleted at 40,000 rpm in an SW 50.1 rotor for 1 h. The proteins were then precipitated and processed for electrophoresis and fluorography as described above. The unlabeled fractions were analyzed for the following marker activities: galactosyl transferase for the Golgi apparatus (Bretz and Staubli, 1977); choline phosphotransferase for

the ER (Percy et al., 1991); alkaline phosphodiesterase for the plasma membrane (Storrie and Madden, 1990);  $\beta$ -hexosaminidase for lysosomes (Storrie and Madden, 1990); and succinic dehydrogenase for mitochondria (Pennington, 1961). The *cis*-Golgi compartment was assayed by Western blotting with  $^{125}\text{I}$ -protein A using an antibody to p58, a protein which is a marker for this compartment (Saraste et al., 1987). The proteins in each fraction were precipitated and separated on 8% SDS-PAGE and transferred to Immobilon P in 10 mM CAPS, pH 11, containing 10% methanol. Membranes were blocked with 5% nonfat dry milk in TS (20 mM Tris, pH 7.5, 150 mM NaCl) and 1% Tween-20 and then incubated for 2 h at 25°C with a 1:1,000 dilution of an antibody to p58 followed by  $^{125}\text{I}$ -protein A for 1 h.

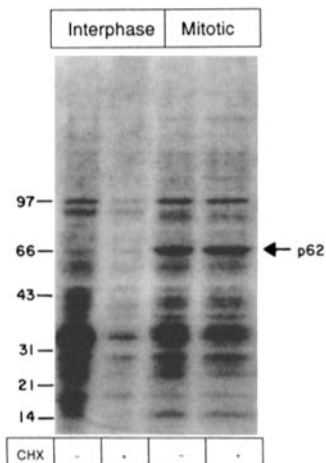
### Cleveland Gels

Partial proteolysis was performed by the method of Cleveland et al. (1977) using a kit from Promega Biotec (Madison, WI) with 0.05  $\mu\text{g}$  alkaline protease and 0.25  $\mu\text{g}$  endoproteinase Lys-C exactly as described by the manufacturer. Alkaline protease cleaves at the carboxylic sides of aromatic and neutral amino acids. Endoproteinase Lys-C cleaves at the carboxylic side of lysine. Purified microsomes from mitotic or BFA-treated cells were first separated by 10% SDS-PAGE. The piece of the gel containing p62 was excised by comparison to a fluorograph of a portion of the gel that had been treated with Amplify, dried, and exposed to film. These gel pieces were then layered on a 10–20% gradient gel, overlaid with the protease solution and electrophoresed. Gels were then treated as above and fluorographed.

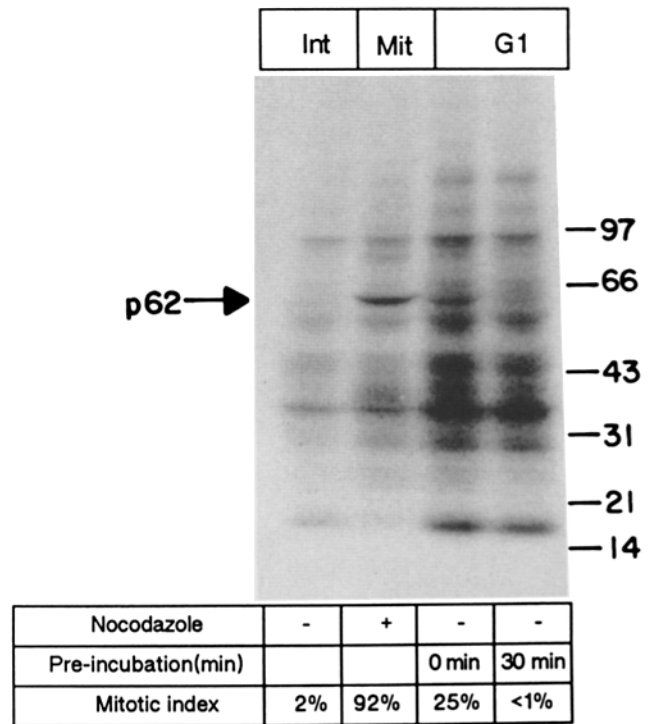
## Results

### p62 Is Acylated in Mitotic Cells

When mitotic and interphase cells were labeled with  $^3\text{H}$ -palmitate the patterns of acylated proteins, resolved by electrophoresis, were different. The most striking difference was the appearance of a 62-kD protein in mitotic cells, which was not apparent in interphase cells (Fig. 1). The acylation of this protein was increased from 5- to 10-fold during mitosis. This was subsequently shown to be a protein (see below) and so will be referred to as p62. Mitotic cells were prepared using the microtubule inhibitor nocodazole, which is also required during the labeling period to ensure that the cells remain mitotic. The appearance of p62 was not caused by the presence of this microtubule inhibitor because treatment of interphase cells for up to 3 h with nocodazole had no effect on acylation. To show that existing and not newly synthesized p62 was being acylated, cells were labeled with  $^3\text{H}$ -palmitate in the



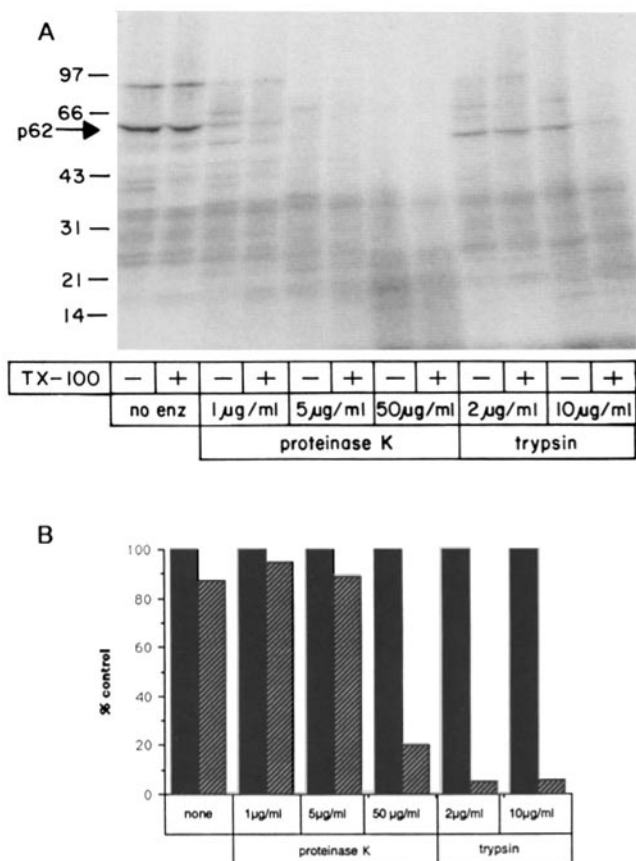
**Figure 1.** p62 is palmitoylated in mitotic but not interphase cells. Interphase and mitotic cells were preincubated for 20 min with or without 10  $\mu\text{g}/\text{ml}$  cycloheximide (CHX) before labeling for 90 min with  $^3\text{H}$ -palmitate. Note the presence of p62 (arrowhead) in mitotic but not interphase cells. The molecular weight marker proteins for this and succeeding figures are: phosphorylase b (97 kD); bovine serum albumin (66 kD); ovalbumin (43 kD); carbonic anhydrase (31 kD); soy bean trypsin inhibitor (21 kD); and lysozyme (14 kD).



**Figure 2.** p62 is no longer palmitoylated in cells that have exited mitosis. Interphase cells (*Int*), mitotic cells (*Mit*), and *G1* cells were labeled with  $^3\text{H}$ -palmitate for 60 min. *G1* cells were generated by incubating mitotic cells in the absence of nocodazole for 0 or 30 min before adding the  $^3\text{H}$ -palmitate. The mitotic index indicated on the figure was determined at the end of the labeling period.

presence of cycloheximide at a concentration that inhibited protein synthesis >98%. The pattern of acylated proteins in mitotic cells was completely unchanged when protein synthesis was inhibited (Fig. 1). The amount of labeling in interphase cells was markedly reduced by treatment with cycloheximide but the pattern remained essentially the same with one exception. A protein at  $\sim 35$  kD disappeared. This protein was also sensitive to digestion with glycopeptidase F (unpublished observations) which indicates that it is a glycoprotein and might therefore be expected to be sensitive to inhibitors of protein synthesis. A similar reduction in the labeling of proteins with  $^3\text{H}$ -palmitate in the presence of protein synthesis inhibitors has been reported by several other investigators (Magee and Courtneidge, 1985; McIlhinney et al., 1985; Olson and Spizz, 1986). In mitotic cells protein synthesis is already inhibited (Buell and Fahey, 1969), which may explain the relative lack of effect of cycloheximide on fatty acylation in those cells.

The acylation of p62 is tightly associated with the cell cycle. To show this, mitotic cells were allowed to exit mitosis and enter *G1* by removing nocodazole and then pulse labeled with  $^3\text{H}$ -palmitate as they did so. As shown in Fig. 2, p62 was not labeled if  $^3\text{H}$ -palmitate was added to cells 30 min after removal of nocodazole (for 1 h), a point at which most of the cells were in late telophase or early *G1*. If cells were labeled (for 1 h) immediately after the nocodazole was removed, the amount of label on p62 was also substantially reduced. The mitotic index for the cells at this intermediate



**Figure 3.** p62 is found on the cytoplasmic side of membranes. (A) Post-nuclear supernatants from  $^3\text{H}$ -palmitate labeled cells were treated with the indicated concentrations of proteinase K or trypsin in the presence or absence of detergent as described in Materials and Methods, analyzed by SDS-PAGE, and fluorographed. (B) Replicate incubations from unlabeled cells were analyzed for galactosyltransferase activity. Note that most of the  $^3\text{H}$ -palmitate labeled proteins, including p62, are digested in the absence of detergent. ■, no detergent; ▨, +Triton X-100.

stage was 25%. In pulse-chase experiments p62 lost label only very slowly (data not shown), much slower than the time needed for the cells to exit mitosis and for p62 to cease being a substrate for acylation.  $^3\text{H}$ -palmitate is incorporated into all cellular lipids and without the ability to block reincorporation of the label into the protein of interest, definitive pulse-chase experiments are not possible. The label on p62 was removed by treatment with hydroxylamine at pH 7.0 indicating that the palmitate is attached to the protein by a thioester linkage (data not shown).

### p62 Is Tightly Associated with Membranes

Post-nuclear supernatants were prepared from  $^3\text{H}$ -palmitate-labeled cells and fractionated to yield membranes and cytosol. All of the palmitoylated proteins, in both interphase and mitotic cells, were found in the membrane fraction and not in the cytosol (data not shown). p62 was sensitive to digestion with proteases (Fig. 3 A) in contrast to the lumenally disposed Golgi marker, galactosyl transferase, which was sensitive only in the presence of Triton X-100 (Fig. 3 B). This shows that at least part of the p62 (that carrying the palmitoyl group) is exposed on the cytoplasmic face of mem-

branes. All of the palmitoylated proteins appear to be sensitive to protease digestion in the absence of detergent, which is consistent with previous reports that the majority of the acylated proteins are cytoplasmically oriented (Wilcox and Olson, 1987). p62 was tightly associated with membranes since it is not removed by KCl up to 1 M (Fig. 4 B). Sodium carbonate treatment at pH 11 removed the  $^3\text{H}$ -palmitate label and therefore could not be tested. p62 was removed from membranes by urea but only by concentrations in excess of 4 M (Fig. 4 A).

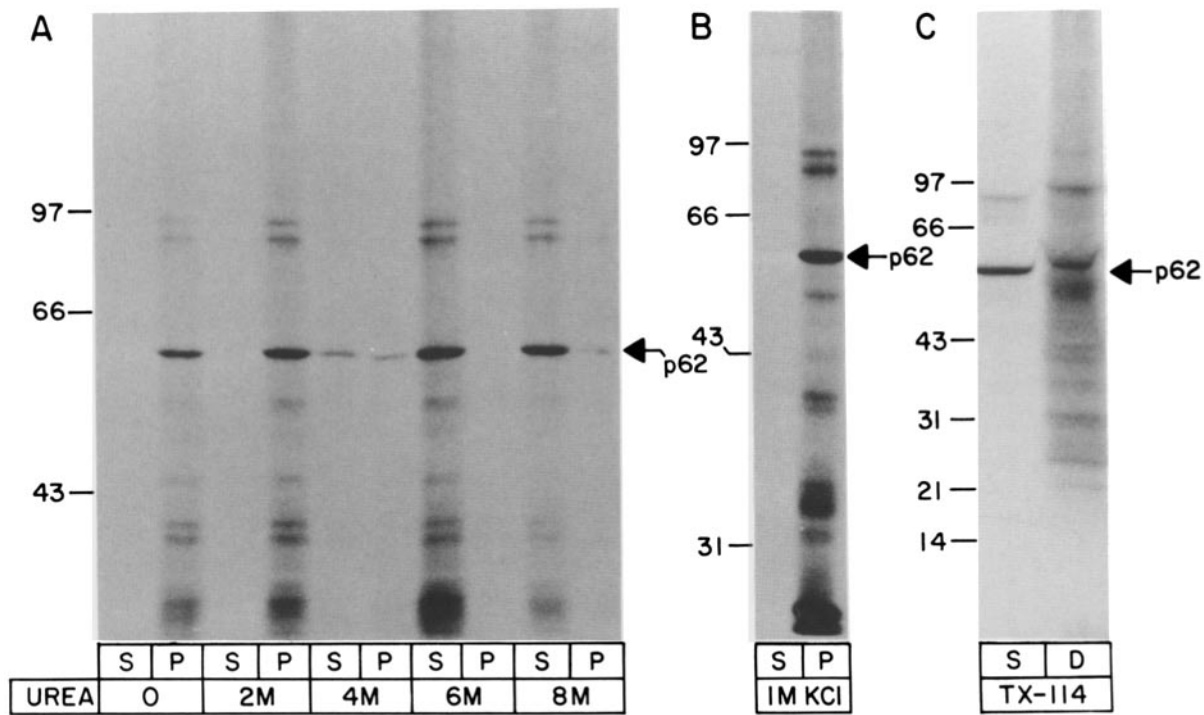
The detergent Triton X-114 can be used to separate integral membrane proteins from soluble proteins by phase separation although this characterization is not always definitive. When membranes are treated with Triton X-114 all the palmitoylated proteins except two, p62 and an ~88-kD protein, remained in the detergent phase (Fig. 4 C). In Triton X-114, p62 appears to separate into two bands. The lower molecular weight band is found in the aqueous phase and a higher molecular weight band remains in the detergent phase. It is not yet known if these are different proteins or if they represent the same protein, perhaps with an additional posttranslational modification. p62 also does not appear to be *N*-glycosylated because treatment with glycopeptidase F had no effect on its molecular weight (data not shown).

### p62 Cofractionates with a Marker for the *cis*-Golgi

To determine the subcellular location of acylated p62, post-nuclear supernatants from interphase and mitotic CHO cells were fractionated on sucrose gradients. p62 was distributed throughout fractions 1-9, but peaked in fractions 4-7 (Fig. 5, A and C). We cannot yet determine whether the nonacylated form has the same distribution. Fractions from the sucrose gradient were also blotted using an antibody to p58, a marker for the *cis*-Golgi (Fig. 5 B). p62 and p58 cofractionate exactly (Fig. 5 C), which is consistent with the interpretation that p62 is contained in this compartment. Fig. 6 shows the distribution of various marker enzymes on the sucrose gradients. The acylated form of p62 does not cofractionate with markers for lysosomes, mitochondria, ER, or the plasma membrane. p62 also does not cofractionate with the *trans*-Golgi marker, galactosyl transferase. Interestingly, whereas the ER marker, choline phosphotransferase, and the Golgi marker, galactosyl transferase, are completely separable in interphase cells (data not shown), in mitotic cells, the ER shifts in density to comigrate with the galactosyl transferase peak (see Fig. 6). This is possibly because of the loss of ribosomes (Zeligs and Wollman, 1979) since protein synthesis is inhibited during mitosis.

### p62 Is Labeled in Interphase Cells Treated with BFA

The fungal metabolite, BFA, rapidly and reversibly blocks transport of proteins out of the ER and also results in the dramatic disassembly of the Golgi apparatus by the resorption of most of the Golgi membranes into the ER (Misumi et al., 1986; Lippincott-Schwartz et al., 1989, 1990). When interphase CHO cells were labeled with  $^3\text{H}$ -palmitate in the presence of BFA there was a striking and selective increase in the labeling of a component with the same molecular weight as p62 (Fig. 7 A). Labeling did not require preincubation with the drug and was not altered by pretreatment of the cells with cycloheximide (Fig. 7 B). BFA treatment does



**Figure 4.** p62 can be removed by urea and Triton X-114 but not by salt. Microsomes, prepared from mitotic cells labeled for 60 min with  $^3\text{H}$ -palmitate were resuspended in the indicated concentrations of urea (A) or KCl (B) and incubated on ice for 30 min before centrifugation at 400,000  $g$  for 30 min to yield a membrane pellet (P) and supernatant (S) fraction. The proteins were precipitated and separated on 10% SDS-polyacrylamide gels. (C) Microsomes were extracted with Triton X-114 and separated into a supernatant (S) and a detergent (D) phase. Equal volumes were precipitated and the proteins separated on a 5–20% SDS-polyacrylamide gel. p62 is marked with an arrowhead.

seem to reduce the labeling of most of the other proteins but only p62 labeling is increased. Partial proteolysis of p62 from mitotic cells and interphase cells treated with BFA confirmed their identity (Fig. 8). Digestion with two different enzymes generated the same  $^3\text{H}$ -palmitate labeled fragments from p62 isolated from either mitotic or BFA-treated cells. Alkaline protease digestion yielded three major fragments with approximate mol wt of 43,000, 34,000, and 21,000 and several minor fragments. Digestion with endoprotease lys-C generated a 21- and a 31-kD fragment although the digestion did not go to completion. The digestion with endoprotease lys-C may be inefficient because of the long destaining period before the band was excised and run in the second dimension. This was necessary because a portion of the gel had to be dried and fluorographed before the appropriate band could be excised (see Materials and Methods). This result also demonstrates that the palmitoylated component is a protein.

BFA had no effect on the level of p62 labeling in mitotic cells (Fig. 7 A) showing that the effects are not additive and suggesting it mimics the mitotic mechanism. The effect of BFA was very rapid, with p62 appearing within 5–10 min of addition of the drug and reaching a maximum at  $\sim 30$  min (Fig. 9). This rapid appearance of label on p62 is consistent with the time course of the effects of BFA on CHO cells, where a morphologically identifiable Golgi apparatus was shown to disappear within 15 min after the addition of the drug (Doms et al., 1989). The effect of BFA on the appearance of p62 was reversible (Fig. 10, A and B). In the presence of BFA labeling of p62 was very prominent, and if cells were

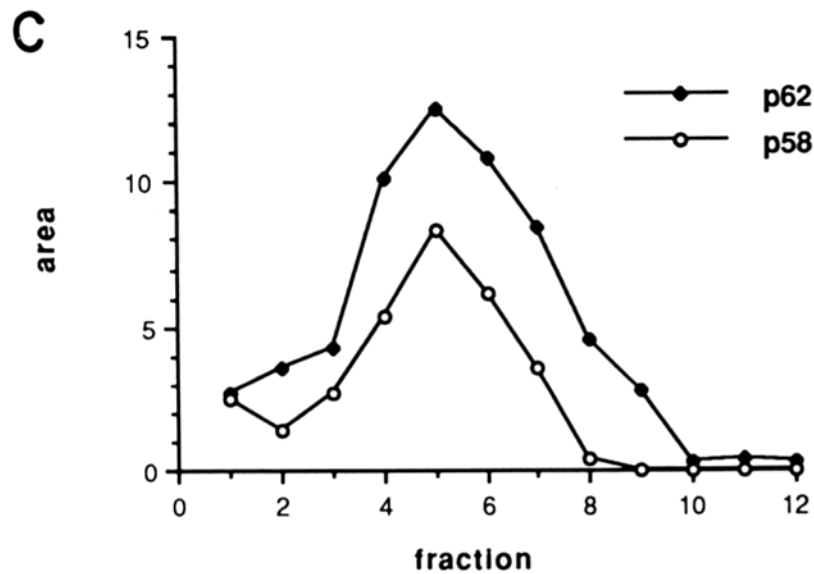
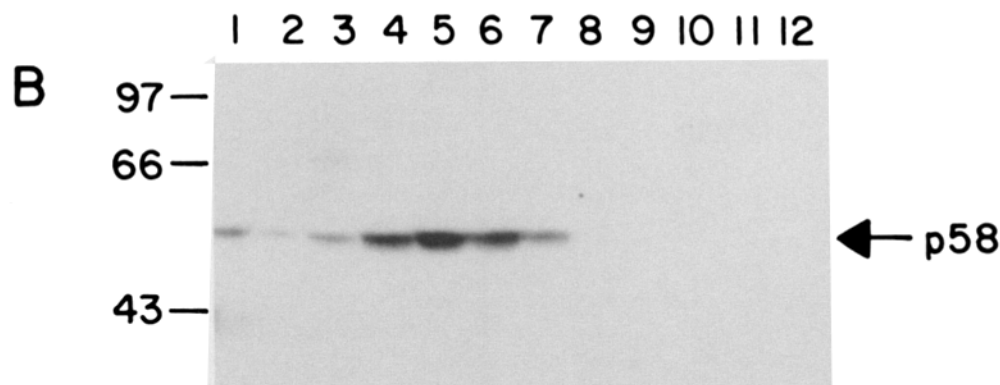
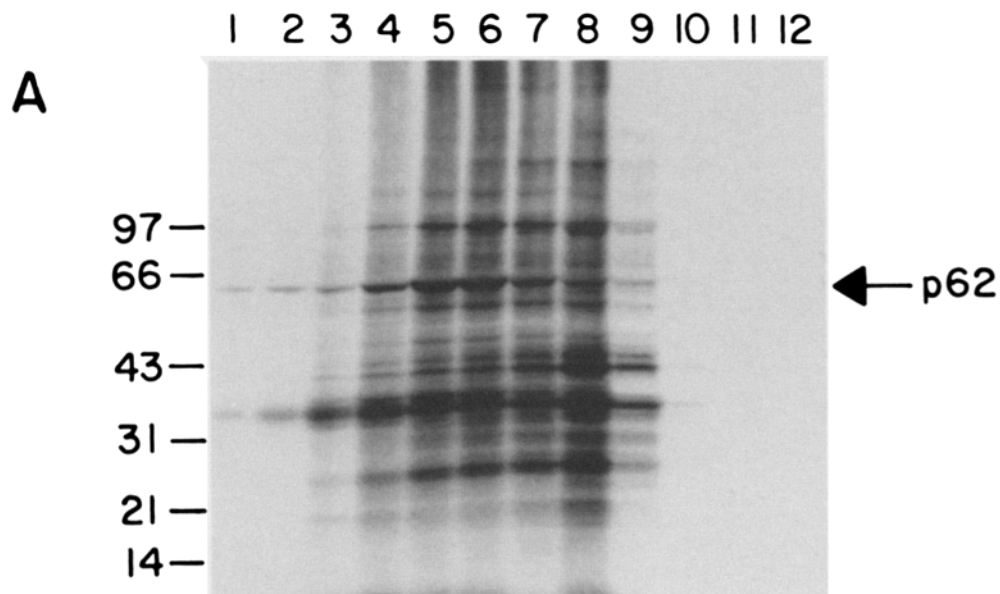
first pretreated with BFA for 15 min but the drug was removed during the labeling period, some labeling of p62 was apparent. If, however, label was added 15 min after the pretreatment then little if any labeled p62 was seen.

The redistribution of Golgi enzymes to the endoplasmic reticulum caused by BFA is prevented by nocodazole (Lippincott-Schwartz et al., 1990). Pretreatment of interphase cells for 1 h with 20  $\mu\text{g}/\text{ml}$  of nocodazole, before labeling in the presence of both BFA and nocodazole, did not prevent the acylation of p62 (data not shown). This would indicate that p62 is not becoming acylated because it has been redistributed to the ER as a result of treatment with BFA. Incubation at 16°C also blocks retrograde transport and, therefore, the disassembly of the Golgi apparatus, but p62 still becomes acylated in the presence of BFA at this temperature. These treatments do not, however, prevent the dissociation of the 110-kD protein, which is the earliest (within 30 s) observable effect of BFA treatment in cells (Donaldson et al., 1991). This would imply that acylation of p62 occurs after the dissociation of the 110-kD protein but before the actual disassembly of the Golgi apparatus.

The effect of BFA on p62 labeling is not limited to CHO 15B cells. Fig. 11 shows that CHO wild-type, normal rat kidney, A431, HeLa, and BHK cells respond in the same way. A protein of the same molecular weight as p62 became heavily labeled with  $^3\text{H}$ -palmitate in these cell types.

#### ***A p62 Is also Labeled in Interphase Cells Treated with other Inhibitors of Intracellular Transport***

BFA inhibits intracellular transport and causes the disassem-



**Figure 5.** p62 cofractionates with p58, a marker for the *cis*-Golgi. (A) Fractions from  $^3\text{H}$ -palmitate labeled cells were diluted and fractionated to obtain membranes as described in Materials and Methods. The proteins were precipitated and separated by 5–20% SDS-PAGE and fluorographed. (B) The same fractions in A were separated on 8% SDS-PAGE and Western blotted with an antibody to p58 as described in Materials and Methods. The membranes were dried and exposed at  $-80^\circ\text{C}$  to Kodak X-OMAT AR film for 24 h. (C) Densitometry of the gels in A and B.

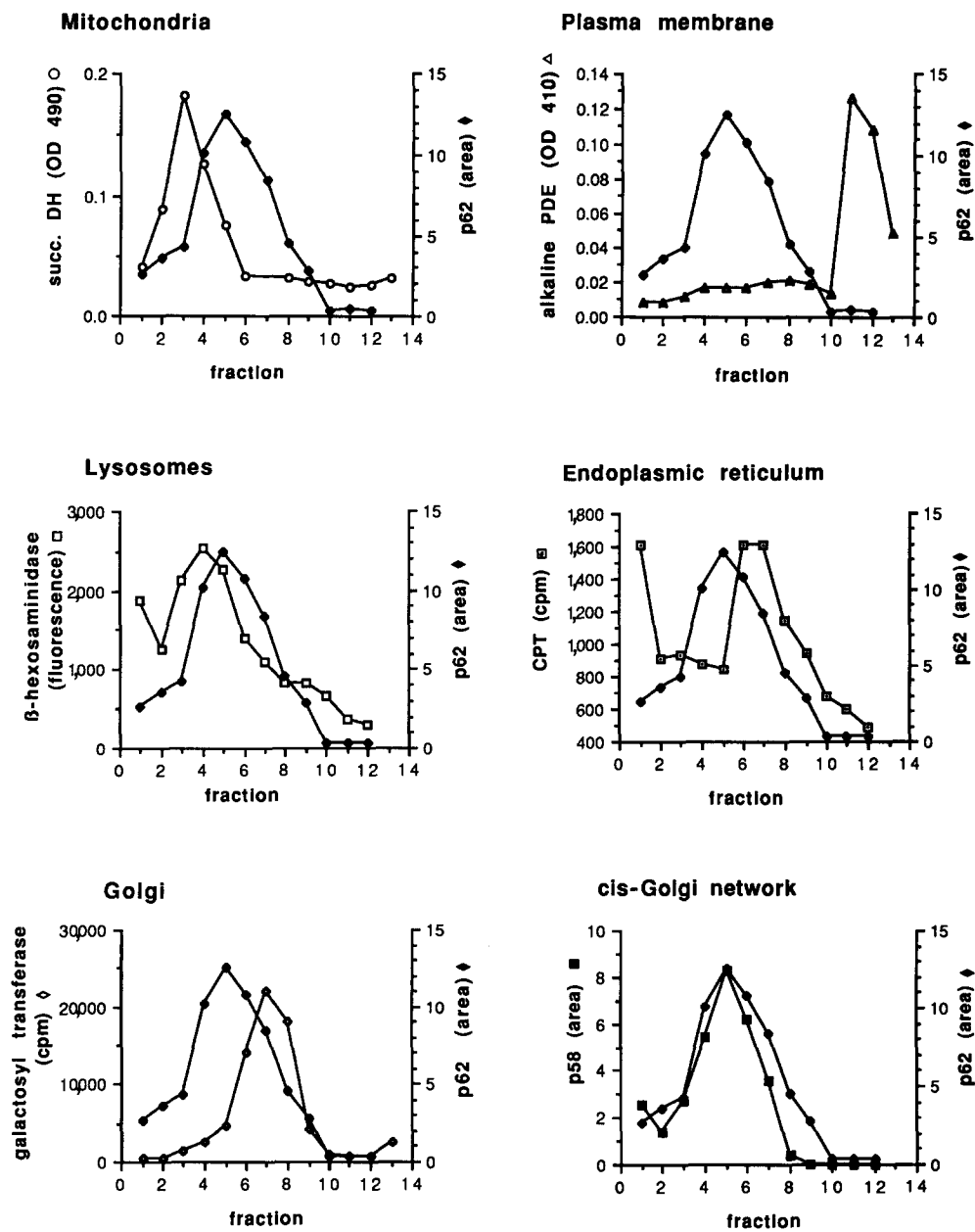
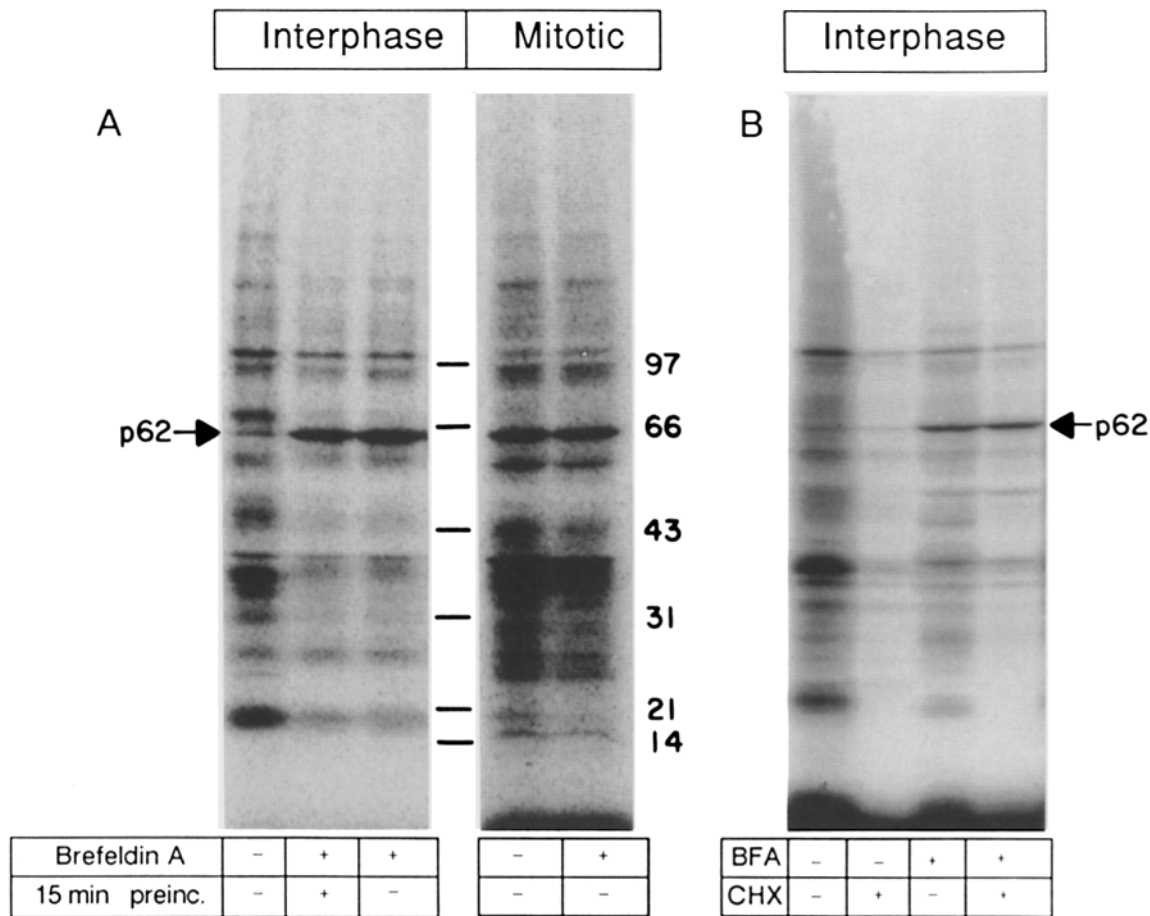


Figure 6. The distribution of marker enzymes and p62 on sucrose gradients. Post-nuclear supernatants were prepared from mitotic cells, which were either labeled for 90 min with <sup>3</sup>H-palmitate or unlabeled, and fractionated on sucrose gradients as described in Materials and Methods. The amount of p62 in each fraction was determined by densitometry and plotted against markers for mitochondria, the plasma membrane, lysosomes, the ER, and the *trans*- and *cis*-Golgi apparatus. p62 cofractionates exactly only with p58, the marker for the *cis*-Golgi apparatus.

bly of the Golgi apparatus and both effects also occur during mitosis (Burke et al., 1982; Hiller and Weber, 1982), although during mitosis there is no evidence of redistribution of Golgi enzymes to the ER (Lucocq et al., 1989). As described above, acylation of p62 occurs even when disassembly of the Golgi apparatus is prevented by pretreatment with nocodazole. To further support the hypothesis that the increase in palmitoylation of p62 is related to an effect on transport rather than due to the disassembly of the Golgi apparatus, several other inhibitors of intracellular transport were tested for their ability to alter the pattern of fatty acylation. CCCP has been used to inhibit transport of proteins at a stage between the ER and the Golgi apparatus (see Burkhardt and Argon, 1989, for references). A 62-kD protein becomes palmitoylated in interphase cells treated with CCCP (Fig. 12 A). Labeling of p62 in the presence of CCCP is

prevented if cells are labeled in medium containing glucose. Preincubation with CCCP is not required and the labeling pattern is the same in the presence or absence of BFA. Monensin also inhibits intracellular transport of many proteins to the cell surface and they accumulate in the Golgi apparatus (Tartakoff, 1983; Griffiths et al., 1983). As shown in Fig. 12 B, a protein with the same molecular weight as p62 becomes acylated in cells treated with monensin. As in BFA-treated and mitotic cells, labeling was not sensitive to cycloheximide (data not shown). Aluminum fluoride has been used both in intact cells and in cell-free systems to block transport at a step which is dependent on a GTP-binding protein (Melançon et al., 1987; Orci et al., 1989). Fig. 12 B compares the labeling of p62 in interphase cells with BFA, monensin, or aluminum fluoride-treated cells. Neither aluminum fluoride nor monensin were as efficient at



**Figure 7.** A p62 protein is palmitoylated in interphase cells treated with BFA. (A) Mitotic or interphase cells were labeled with  $^3\text{H}$ -palmitate for 60 min in the presence or absence of 6  $\mu\text{g}/\text{ml}$  of brefeldin A (BFA). One sample was pre-incubated with the drug for 15 min before labeling. (B) Interphase cells were preincubated in the presence or absence of 10  $\mu\text{g}/\text{ml}$  of cycloheximide for 20 min before labeling for 60 min with  $^3\text{H}$ -palmitate in the presence or absence of 6  $\mu\text{g}/\text{ml}$  of BFA. For cycloheximide-treated cells the drug was present both before and during the labeling period.

increasing the label on p62 as BFA. If, however, cells were preincubated with monensin for 2 h, the amount of label on p62 reached the level in BFA-treated cells.

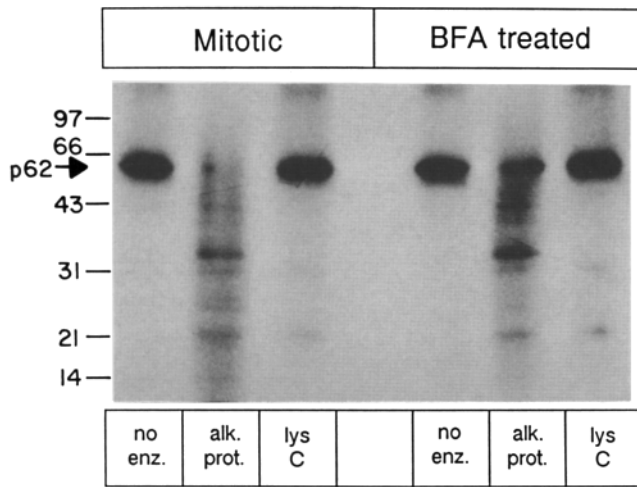
### Discussion

A number of cellular processes requiring vesicular transport are inhibited during mitosis making this a powerful tool for studying the transport process. Since fatty acylation is required during intra-Golgi transport we examined the palmitoylation of proteins in interphase and mitotic cells. In this manner we have identified a protein, p62, as a potential candidate for the palmitoylation target required in transport. Several lines of evidence point to the involvement of p62 in intracellular transport. First, there is a good correlation between the level of p62 and the stage in the cell cycle. p62 can be labeled by  $^3\text{H}$ -palmitate in mitotic cells, a state in which intracellular transport is inhibited, but not in interphase or G1 cells where transport has resumed.

Second, p62 can be labeled in interphase cells treated with inhibitors of intracellular transport. BFA blocks the movement of proteins to the Golgi apparatus probably by disrupting the formation of the putative Golgi vesicle coat (Orci et al., 1991). The retrograde pathway continues, however,

which results in the redistribution of at least some of the Golgi enzymes back to the ER, and this results in the disassembly of the Golgi apparatus (Lippincott-Schwartz et al., 1990). The effect of BFA on the fatty acylation of p62 is striking and very rapid and is consistent with the time required for BFA to exert its effects on the cell. Golgi reassembly after removal of BFA takes  $\sim 30$  min, again similar to the time course for p62 to cease being a major substrate for palmitoylation. Also, the effect of BFA on palmitoylation is not related to the redistribution of p62 or another protein to a different compartment since the increase in the palmitoylation of this protein is seen even when redistribution is inhibited with nocodazole. Several cell types were treated with BFA and in each case there is a dramatic increase in the level of acylation of a  $\sim 62$ -kD protein. Monensin inhibits transport of many proteins at the level of the Golgi stack (Tartakoff, 1983; Griffiths et al., 1983). CCCP inhibits transport primarily between the ER and the Golgi stack (Fries and Rothman, 1980; Kabcenell and Atkinson, 1985; Argon and Milstein, 1984) but may inhibit at a later stage as well (Burkhardt and Argon, 1989), and aluminum fluoride, which is thought to interact with GTP binding proteins required in transport, acts at both the ER to Golgi stage (Beckers and Balch, 1989) and within the Golgi stack itself (Melançon et

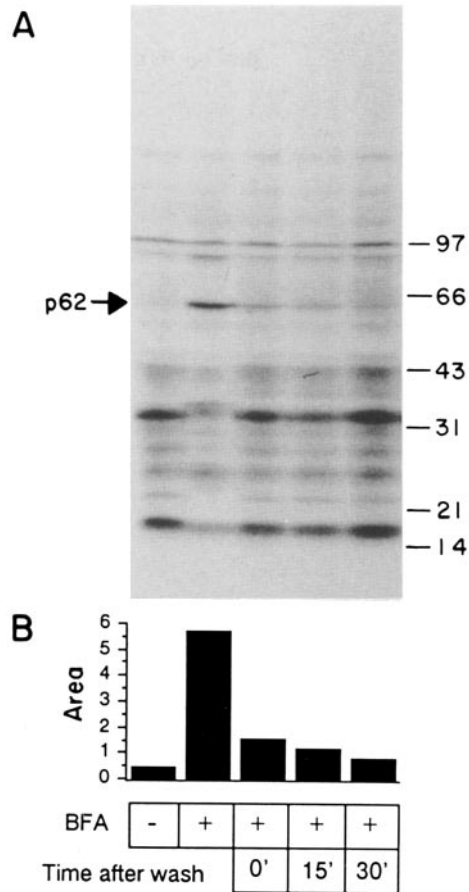




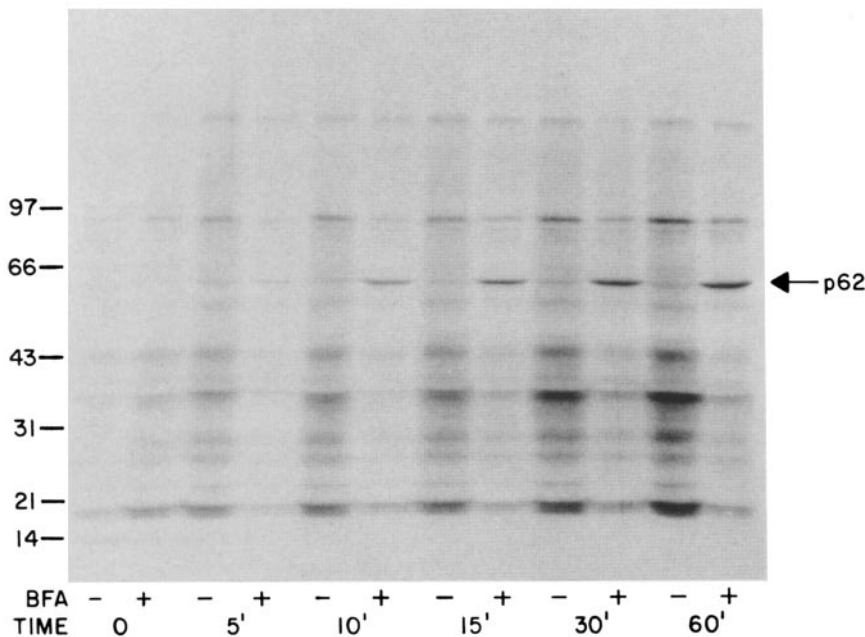
**Figure 8.** p62 from mitotic cells is the same protein as p62 in interphase cells treated with BFA. p62 from mitotic and BFA-treated cells was subjected to Cleveland analysis to determine if it is the same protein. Alkaline protease treatment generated three major  $^3\text{H}$ -palmitate-labeled fragments with a relative molecular mass of  $\sim 43,000$ ,  $34,000$ , and  $21,000$  in both cases. Endoproteinase Lys-C digestion generated 21-kD and 31-kD acylated fragments although the digestion did not go to completion.

al., 1987). In all three cases, the acylation of p62 is increased.

Third, the acylated form of p62 is tightly associated with the cytoplasmic surface of membranes which cofractionate on sucrose step gradients with the *cis*-Golgi. p58 was originally described as a marker for the *cis*-Golgi (Saraste et al., 1987). However, recent evidence suggests that it may be more accurate to refer to this region of the cell as the *cis*-Golgi network, which consists then of the intermediate compartment between the ER and the Golgi and the *cis*-most cisterna of the Golgi apparatus. One of the functions of this compartment appears to be the recycling of proteins



**Figure 10.** The effect of BFA is reversible. Interphase cells were preincubated for 15 min with  $6 \mu\text{g/ml}$  of BFA and then labeled for 60 min with  $^3\text{H}$ -palmitate either in the continued presence of BFA or after its removal by extensive washing. Label was added either 0, 15, or 30 min after the removal of BFA, as indicated in the figure. (A) Fluorogram. (B) Quantitation of the label in the p62 protein in each lane.



**Figure 9.** BFA causes rapid palmitoylation of p62. Interphase cells were prelabeled for 30 min with  $^3\text{H}$ -palmitate and labeling was continued in the presence or absence of  $6 \mu\text{g/ml}$  BFA for up to 60 min.

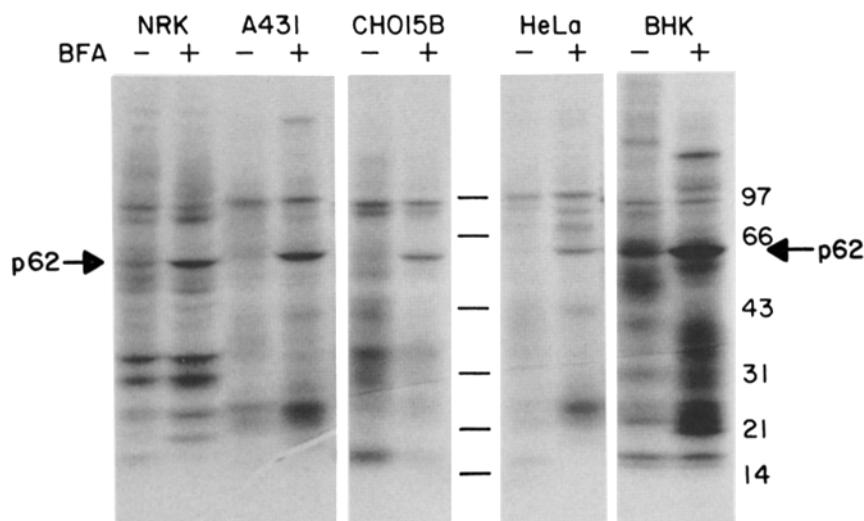


Figure 11. BFA stimulates the acylation of a p62 protein in several types of cells. Each cell type was grown in 60-mm dishes and labeled with  $^3\text{H}$ -palmitate for 60 min in the presence or absence of 6  $\mu\text{g}/\text{ml}$  BFA.

back to the ER since the KDEL receptor is also localized to this region of the cell (Lewis and Pelham, 1990). Lippincott-Schwartz et al. (1990) showed that the intermediate compartment, in contrast to the Golgi apparatus, remains as a distinct organelle in the presence of BFA. The localization of p62 in this intermediate compartment or *cis*-Golgi network is also consistent with our finding that p62 is not redistributed to a different compartment upon BFA treatment. By our method we only detect the acylated form of p62 and do not know the distribution of the deacylated form. There are several possibilities. First, this is the true distribution of p62 and the bulk of the protein is associated with the intermediate compartment. Second, p62 may be associated with all the organelles throughout the transport pathway but the portion

that is in the intermediate compartment becomes acylated because the enzyme is located there. Or third, p62 moves throughout the pathway but when transport is inhibited during mitosis it accumulates in this compartment either because it is acylated or because some downstream event is blocked. The exact subcellular localization of p62 will therefore require the production of an antibody.

p62 is labeled in cells treated with cycloheximide, indicating that the protein exists in an acylated and a deacylated form. The changes in the labeling of p62 can best be explained by an acylation/deacylation cycle. In the steady state, in interphase cells, the deacylated form predominates but inhibition of transport either during mitosis or in the presence of transport inhibitors causes the acylated form to accumu-

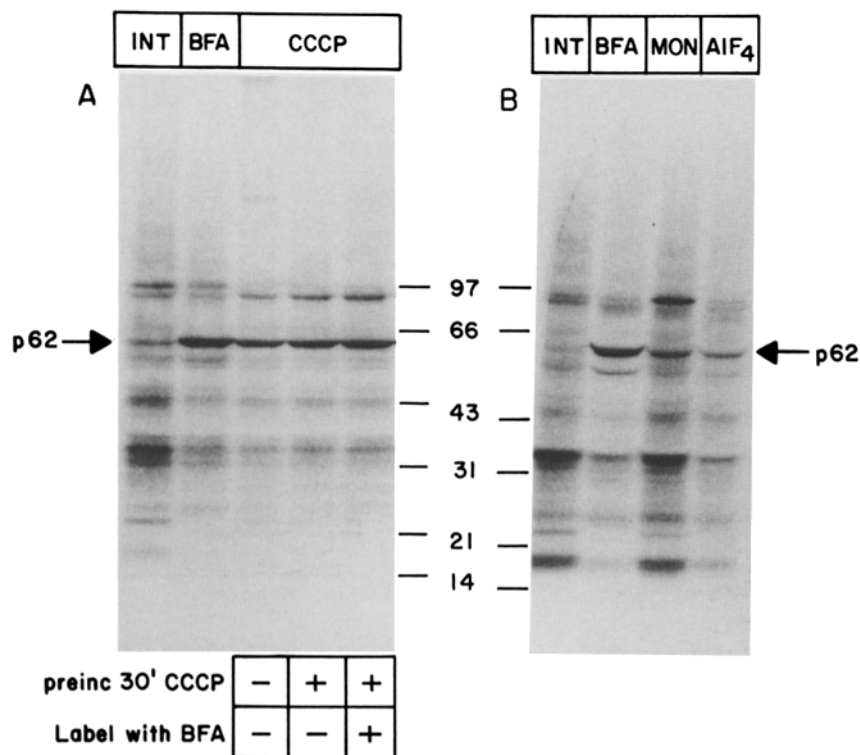


Figure 12. Other inhibitors of intracellular transport also stimulate the acylation of a p62 protein. (A) Interphase cells were labeled for 60 min with  $^3\text{H}$ -palmitate in glucose free DME in the absence (*INT*) or presence of 6  $\mu\text{g}/\text{ml}$  BFA or 10  $\mu\text{M}$  CCCP. Where indicated, the cells were preincubated for 30 min with CCCP and then labeled in the presence or absence of BFA. (B) Cells were labeled as in A with no additions (*INT*) or with 6  $\mu\text{g}/\text{ml}$  BFA, 10  $\mu\text{M}$  monensin or aluminum fluoride (3 mM NaF; 50  $\mu\text{M}$  AlCl<sub>3</sub>) without preincubation.

late. This could be due to a direct effect on the acylase or by inhibiting the deacylase. Either would provide a means of identifying the enzyme(s) involved. p62 may become acylated as a mechanism to inhibit its function or because it is not being consumed. This might be an indirect effect caused by inhibition of a reaction further downstream which in turn prevents p62 from discharging its palmitoyl group in the normal manner. If rapid turnover of the acyl group is occurring, it could be due to the uncoupling of p62 from a regulatory subunit. In the presence of radioactive palmitate, label would accumulate under these conditions. These possibilities cannot yet be distinguished.

The function of p62 is unknown but it could be a component of the transport mechanism which is thought to be shared by all vesicle-mediated steps from the ER to the *trans*-Golgi network. This is supported by the fact that inhibitors that act at different steps of intracellular transport have the common effect of increasing the level of acylation of p62 and the effects of mixing inhibitors are not additive. Transport is thought to be initiated by the formation of a coat complex (Waters et al., 1991), which must bind to Golgi membranes yet cannot do so by binding to the cytoplasmic tails of transported proteins, as does the clathrin coat (Pearse, 1988), because transport is nonselective. One possible solution, which arises from the known dependence of budding on added palmitoyl-CoA, is that the coat could attach by reversible addition of palmitoyl groups to one or more of the coat components. It is therefore of interest that one of the major proteins of the coat complex,  $\delta$ -COP, has almost the same molecular weight as p62. If true, this would mean that this coat protein remains bound to membranes during mitosis and treatment with BFA, a result at variance with the known behavior of one of the other coat proteins,  $\beta$ -COP (Donaldson et al., 1990). It is, however, possible that the coat disassembles under these conditions and the different components behave differently. It has recently been demonstrated that BFA prevents the assembly of the nonclathrin-coated vesicles in a cell-free system (Orci et al., 1991) and the authors speculate that a possible target for BFA is either a subunit of the coat itself or the binding site in the membrane. If p62 were the target, irreversible acylation may explain inhibition of transport.

Both budding and fusion show a dependence for added palmitoyl-CoA and there is no reason to believe that the palmitoyl requirement is different for each step. Another suggestion for the role of p62 might therefore be as an acylating enzyme or the acyl donor providing palmitoyl groups for both steps of vesicle mediated transport. This is consistent with its localization in the *cis*-Golgi network, which is also the site for acylation of viral glycoproteins (Bonatti et al., 1989). The acylated enzyme may accumulate because a block in transport prevents it from discharging its acyl group. If p62 is an acylating enzyme that may explain the general reduction in the acylation of most proteins upon treatment of cells with BFA. Finally, it has been suggested that palmitoylation can alter the interaction of a protein with GTP-binding proteins (for references see McIlhinney, 1990). This is interesting since GTP-binding proteins are thought to be required for transport (Beckers and Balch, 1989; Melançon et al., 1987) and guanine nucleotides can also modulate the effects of BFA on cells (Donaldson et al., 1991). Perhaps the acylation of p62 affects its interaction with a GTP binding

protein which is required in the transport pathway. Suggestive though these arguments might be, the role played by p62 must await its purification and further biochemical and genetic characterization.

The observed increase in palmitoylation of p62 during mitosis is dramatic and readily apparent even in total cell extracts. Ours is the first demonstration that the acylation of a protein can be altered during the cell cycle. Although the functional consequences of protein palmitoylation have not been defined, the data suggest that fatty acylation may play an important role in regulating cellular processes during mitosis.

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