Mammalian Cdc42 Is a Brefeldin A-sensitive Component of the Golgi Apparatus*

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In this study, we have used immunocytochemical and fractionation approaches to provide a description of the localization of the mammalian Cdc42 protein (designated Cdc42Hs) in vivo. A specific anti-peptide antibody was generated against the C-terminal region of Cdc42Hs. Using affinity-purified preparations of this antibody in indirect immunofluorescence experiments, Cdc42Hs was found to be localized to the Golgi apparatus. Similar to the well-characterized non-clathrin coat proteins ADP-ribosylation factor (ARF) and β-COP, the perinuclear clustering of Cdc42Hs is rapidly dispersed upon exposure of the cells to the drug brefeldin A, suggesting that it too may play a role in the processes of intracellular lipid and protein transport. Employing cell lines possessing inducible forms of ARF, we demonstrate here a tight coupling of the nucleotide-bound state of ARF and the subcellular localization of Cdc42Hs. Specifically, the expression of wild-type ARF had no effect on the brefeldin A sensitivity of Cdc42Hs while, as is the case for ARF and β-COP, expression of a GTPase-deficient form of ARF (ARF(Q71L)) renders these Golgi-localized proteins resistant to brefeldin A treatment (Teal et al., 1994; Zhang et al., 1994). Moreover, the induced expression of a mutant form of ARF with a low affinity for nucleotide resulted in constitutive redistribution of Cdc42Hs in the absence of brefeldin A treatment. These results suggest that Cdc42Hs may play a role in cell morphogenesis by acting on targets in the Golgi that direct polarized growth at the plasma membrane.

The Ras superfamily of GTP-binding proteins plays critical regulatory roles in a variety of cellular functions including growth control, intracellular traffic, and cytoskeletal organization (for a review, see Hall, 1994). A general role in cell morphogenesis for the Rho subfamily proteins has recently been demonstrated by results obtained with microinjection of mutants or modified Rho and Rac proteins (Kozma et al., 1995; Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995). Cdc42Hs belongs to the Rho subfamily of low molecular weight GTPases and is the mammalian homolog of the Saccharomyces cerevisiae cell-division-cycle protein Cdc42Sc, previously shown to function in the process of polarized growth in yeast (Johnson and Pringle, 1990). The human and yeast proteins are 80% identical, whereas the most closely related proteins in the mammalian Ras superfamily, Rac 1,2 and RhoA, share roughly 70 and 50% homologies with the Cdc42Hs sequence, respectively (Shinjo et al., 1990). Several regulatory activities specific for Cdc42Hs or related Rho family members have been identified in vitro. These include the dbl oncogene product, which accelerates GDP-GTP exchange (Hart et al., 1991), the Cdc42Hs-GAP (Hart et al., 1991; Barford et al., 1993), and Rho-GDI (Leonard et al., 1992). Moreover, a number of putative targets have recently been identified and characterized including the Wiskott-Aldrich Syndrome Protein (WASP) (Symons et al., 1996), the p21-activated serine/threonine kinases (PAKs) (Manser et al., 1994; Martin et al., 1995; Bagrodia et al., 1995a), and the p70 S6 kinase (Chou and Blenis, 1996). All of these suggest an important function for Cdc42Hs in processes critical for normal cell growth and differentiation. However, little is known regarding the specific biochemical mechanisms by which Cdc42Hs is regulated or how its various effector activities are manifested in vivo.

The high degree of sequence conservation between the yeast and human Cdc42 proteins results in full complementation of CDC42-deficient yeast strains by the human cdc42 gene (Shinjo et al., 1990). Thus, the underlying function of the yeast and mammalian Cdc42 proteins might be similarly conserved along with the regulatory proteins that modulate their nucleotide-bound state. This view has been supported by the finding that the dbl oncogene encodes a guanine nucleotide exchange factor with in vitro specificity for the Cdc42Hs protein (Hart et al., 1994), whereas deletion analysis in S. cerevisiae has demonstrated that the CDC24 gene product, which contains a region with sequence similarity to Dbl, interacts with the Cdc42Sc protein (Ziman and Johnson, 1994). The region of similarity between Cdc24 and Dbl consists of ~250 amino acids and has been shown to be necessary for both the transformation and nucleotide exchange activity of oncogenic Dbl (Hart et al., 1994). Moreover, Zheng et al. (1994) have demonstrated that the recombinant Cdc24 protein can act as a potent exchange factor for either the mammalian or yeast Cdc42 in vitro.

Consistent with its proposed role in cell polarity in yeast, Cdc42Sc has been shown to localize to the site of bud emergence at the plasma membrane (Ziman et al., 1993). Recent progress in purifying and characterizing regulatory and target proteins for Cdc42Hs has prompted similar interest in its subcellular location with the expectation that this knowledge will provide clues to its in vivo function. The evidence reported in this study indicates that a significant fraction of the Cdc42Hs in mammalian cells is localized to the Golgi apparatus. This membrane-bound Cdc42Hs pool in resting cells is immediately shifted from the perinuclear Golgi complex to the cytosol in BFA1-treated cells. This responsiveness of the Golgi-localized Cdc42Hs to brief BFA treatment is similar to that of proteins

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1 The abbreviations used are: BFA, brefeldin A; ARF, ADP-ribosylation factor; TBS, Tris-buffered saline.
that have been demonstrated to be components essential for intracellular trafficking. In this regard, the observed properties of cellular Cdc42Hs, both in distribution and responsiveness to BFA and the GTP-binding protein ARF, are remarkable in their biochemical similarities to the non-clathrin coated vesicle component β-COP. Taken together with microinjection studies implicating Cdc42Hs in cell filopodia genesis (Kozma et al., 1995; Nobes and Hall, 1995), the results presented here, which describe a close coupling of Cdc42Hs to the ARF GTPase cycle, suggest a role for mammalian Cdc42 in the processes leading to the formation and targeted delivery of Golgi-derived vesicles in cells.

MATERIALS AND METHODS

Reagents—BFA was purchased from Epicenter Technologies (Madison, WI) and stored as a 5 mg/ml stock solution in ethanol at –20°C. An antibody for Cdc42Hs was raised in rabbits immunized with a synthetic peptide corresponding to the C-terminal 22 amino acids of Cdc42Hs, conjugated to keyhole limpet hemocyanin (Shinjo et al., 1990). Affinity purification of the Cdc42Hs antibodies was achieved using peptides written on a 48-well plate coated with bicarbonate activated beads. This antibody was found to be highly specific for Cdc42Hs and did not react with its closest homologs (Rac1, Rac2, or RhoA; data not shown). Monoclonal anti-β-COP M3A5 (Allan and Kreis, 1986) was a gift of T. Kreis (University of Geneva, Switzerland) and was used at a 1:50 dilution for immunolocalization and at a 1:1000 dilution for immunoblotting. The monoclonal antibody 10B6 (Wood et al., 1991), which recognizes a resident Golgi protein, was a gift of W. Brown (Cornell University, Ithaca, NY) and was used at a 1:100 dilution in PBS (120 mM NaCl and 20 mM potassium phosphate, pH 7.4) for immunolocalization. Mouse monoclonal antibody specific for mannosidase II was obtained from Babco (Richmond, CA) and used at a 1:100 dilution for immunoblotting.

Bodipy® and rhodamine-labeled secondary antibodies were obtained from Molecular Probes (Portland, OR) and used at a 1:200 dilution in PBS. Immunoblots were developed with enhanced chemiluminescence as per manufacturer’s instructions after sequential incubations of primary antibody and a secondary anti-rabbit or anti-mouse horseradish peroxidase conjugate purchased from Amersham diluted 1:5000 in TBS-T.

RESULTS

Colocalization of Cdc42Hs and Markers for the Golgi Apparatus—The subcellular distribution of Cdc42Hs was determined by performing a total cellular fractionation and immunoblotting equivalent amounts of protein from each of the fractions of homogenized rabbit liver. As shown in Fig. 1, 40 µg of each fraction revealed a significant amount of Cdc42Hs in the crude “GH” Golgi fraction collected from the 1.22/1.15 M sucrose interface (see “Materials and Methods”). Identification of the membranes in this fraction being significantly enriched in the Golgi apparatus was confirmed by probing the upper half of the transfer membrane with the monoclonal antibody 53PC3, which recognizes a resident enzyme of the Golgi complex, mannosidase II (Burke et al., 1982; Baron and Garoff, 1990). Preparations were also examined by scanning electron microscopy and found to be substantially enriched with the highly fenestrated membrane structures characteristic of the Golgi (data not shown).

In all cell types examined, the anti C-terminal Cdc42Hs antibody revealed a prominent perinuclear staining pattern, as illustrated for untreated NR-6 cells in Fig. 2A. The perinuclear distribution observed with the anti-Cdc42 antibody overlapped to a significant extent in all cells with markers for the Golgi complex, including the 110-kDa subunit of the coatomer complex, β-COP (Fig. 2C; Robinson and Kreis, 1992). Components of the coatomer complex, including the ARF GTase, have been shown to be essential for the proper formation of transport vesicles in the Golgi cisternae (for a recent review, see Schekman and Orci, 1996).

Coatomer proteins are redistributed to the cytosol of treated cells within minutes after treatment with BFA, which results...
in a cessation of vesicle formation at the Golgi membrane, halting the constitutive secretory process. BFA has been shown to affect transport from the endoplasmic reticulum to the Golgi complex by blocking the process of vesicle coating at the cytoplasmic face of donor membranes (Orci et al., 1991). The short term (<1 min) consequence of BFA treatment, namely, the redistribution of select transport proteins from the Golgi to the cytosol, is attributed to the inhibition by BFA of the guanine nucleotide exchange activity of the ARF GTP-binding protein that functions in isolated Golgi membranes.

Fig. 2, B and D, show that BFA treatment caused a rapid change in the cellular localization of both Cdc42 and β-COP in that neither protein showed its characteristic perinuclear distribution after a 1-min treatment. Some nuclear staining by both the anti-Cdc42 and anti-β-COP antibodies was observed in cells that had been treated with BFA, although we failed to detect either protein by immunoblotting highly purified nuclei isolated from BFA-treated NR6 cells (data not shown). Under conditions that resulted in this rapid redistribution of Cdc42 and β-COP, the structure of the Golgi complex remained intact as shown in E and F, in which the pattern of immunofluorescence was obtained with a monoclonal antibody directed against a protein marker of the cis and medial cisternae of the Golgi complex (Wood et al., 1991).

The finding that Cdc42Hs is localized to the Golgi complex in mammalian cells led us to further characterize the effect of BFA treatment on its cellular distribution. Fig. 3, A and B, again illustrate the extensive overlap between Cdc42Hs (Fig. 3A) and the coat protein β-COP (Fig. 3B), as visualized using the anti-Cdc42Hs antibody and a monoclonal antibody that recognizes this coatomer subunit (Allan and Kreis, 1986). Treatment of the cells with BFA causes a redistribution of both proteins (Fig. 3, C and D) with kinetics that are essentially identical, and, significantly, the addition of fresh culture medium to cells exposed to BFA for 1 min resulted in the rebinding of Cdc42Hs to Golgi membranes (t1/2 ~ 30 min; data not shown). This reestablishment of Golgi membrane binding by Cdc42Hs in cells washed free of BFA was concomitant with the rebinding observed for β-COP.

Likewise, conditions that block the effects of BFA treatment on the redistribution of β-COP similarly affected Cdc42Hs. For example, pretreatment of cells with AlF4− has previously been shown to render cells insensitive to the effects of BFA on coatomer binding (Donaldson et al., 1991). When cells normally responsive to BFA were preincubated with AlF4− before the addition of BFA, Cdc42Hs as well as β-COP remained bound to Golgi membranes (Fig. 3, E and F). This AlF4−-induced block of BFA effects has been interpreted as evidence for a heterotrimeric G protein α subunit acting upstream of coat proteins and contributing to the regulation of their membrane binding (Ktistakis et al., 1992).

Additional support for the view that Cdc42Hs may be regulated by the same cellular factors that determine the distribution of coatomer proteins is provided by the finding that in cell lines such as PtK1 that are resistant to the action of BFA on coatomer proteins, the subcellular location of Cdc42Hs is also unaffected by BFA treatment. Fig. 4 compares the effect of BFA on the distribution of Cdc42Hs (A and B) and β-COP (C and D) in PtK1 cells. The ineffectiveness of BFA in certain cell lines seems to be due to the absence of a factor in the membrane fraction that can be reconstituted in heterokaryons (Ktistakis et al., 1991). It is not known whether this membrane component represents the BFA target that can block normal ARF guanine nucleotide exchange activity, but this proposed BFA-sensitive factor seems to determine the BFA effects on Cdc42Hs as well.

Given the similarities observed for Cdc42 and proteins previously linked to the GTPase cycle of ARF, we wanted to examine whether Cdc42Hs and ARF participate in a common biochemical pathway at the Golgi membrane. Previously, several laboratories have shown that transfection of mutant forms of ARF possessing distinct functional properties (e.g. GTPase-deficient) result in dramatic changes in the distribution of coatomer proteins and their sensitivity to BFA (Zhang et al.,...
1994; Dascher and Balch, 1994; Teal et al., 1994). To investigate whether the expression of mutant forms of ARF resulted in changes of CDC42 localization or an altered sensitivity to BFA treatment, cell lines possessing inducible forms of ARF3 (Zhang et al., 1994) were used in Cdc42 immunolocalization experiments. The expression of a GTPase-deficient form of ARF3 that possesses a mutation at codon 71 (designated ARF(Q71L)) blocks the action of BFA on the localization of β-COP in normal rat kidney cells. This observation further supports ARF as a critical component of the coatamer-binding cycle because the induction of the ARF(Q71L) mutant resulted in a loss of constitutive secretory activity in these cells (Zhang et al., 1994). Fig. 5 demonstrates that the presence of the GTPase-deficient form of ARF3 also blocks the action of BFA on the Golgi population of Cdc42Hs. In A, control normal rat kidney cells, after the induction of the wild-type form of ARF3, were stained for the presence of Cdc42Hs using the anti-C-terminal antibody. As is the case for the NR6 cells, Cdc42Hs is localized to the Golgi membranes of normal rat kidney cells (Fig. 5A) and is rapidly redistributed upon exposure to 5 μg/ml BFA (Fig. 5B). In contrast, the expression of the ARF3(Q71L) mutant made Cdc42Hs insensitive to the addition of BFA (Fig. 5D). The distribution of Cdc42Hs after the induction of ARF(Q71L) but before the addition of BFA is shown in Fig. 5C. The failure of BFA to redistribute the Golgi-bound Cdc42Hs was absolutely dependent on the addition of interferon, which was necessary to induce the expression of the ARF3(Q71L) mutant.

The expression of a second ARF3 mutant, in which an isoleucine was substituted for an asparagine at position 126 (ARF(N126I)), was found to cause a significant redistribution of the Golgi-bound population of Cdc42Hs. Fig. 6A shows that when normal rat kidney cells transfected with an inducible form of ARF(N126I) were stained for the presence of Cdc42Hs, the typical perinuclear localization of Cdc42 revealed by staining with the C-terminal antibody was observed. Remarkably, however, after a 9-h induction period, the perinuclear localization of Cdc42Hs was no longer detected. Given that the ARF(N126I) mutant binds both GDP and GTP poorly and is defective for guanine nucleotide exchange, these results are consistent with the idea that disruption of the normal nucleotide exchange activity of ARF prevents the association of Cdc42Hs with Golgi membranes (see below).

**DISCUSSION**

Several recent observations suggest that Cdc42 plays a key role in signaling cascades that affect cell morphogenesis. The demonstration that Swiss-3T3 fibroblasts microinjected with Cdc42Hs can induce the outgrowth of plasma membrane filopodia (Kozma et al., 1995; Nobes and Hall, 1995) is consistent in a broad topological sense with the earlier observations in *S. cerevisiae* that assigned Cdc42Sc a critical role in the establishment of cell polarity (Adams et al., 1990). Our present results, which indicate that the Golgi membranes are the predominant location for Cdc42 in mammalian cells, suggest that the regulation of filopodia formation by Cdc42Hs may be coupled to a transport activity.

The role of the Golgi apparatus in orchestrating the biochemical events that control the formation of transport vesicles and the incorporation of biomolecules that determine vesicle targeting has been well established, and several proteins have been identified that play critical roles in Golgi-mediated transport, including the coatamer subunits, e.g., β-COP (Serafini et al., 1991), and the low molecular weight GTP-binding protein ARF. The high molecular weight coatamer complex cycles between a soluble form when ARF is in the inactive or GDP-
bound state and a membrane-attached form when ARF is active or GTP-bound. Agents that block the delivery of vesicles to the plasma membrane such as GTPγS and BFA have been used to disrupt the normal cycling of this complex, thereby demonstrating the critical importance of coatsmier membranes to cytosol translocation in the proper maintenance of the constitutive secretory machinery. Our indirect immunofluorescence experiments show a similar (Golgi) localization for Cdc42Hs and the non-clathrin coated vesicle (coatomer) protein β-COP and that the localization of both proteins is similarly affected by BFA and different mutants of ARF.

The mechanism by which expression of the ARF(Q126I) mutant results in the constitutive redistribution of Cdc42Hs is most likely a consequence of the nucleotide-bound state of the ARF protein. In vitro binding assays with ARF(Q126I) indicate that this mutant binds guanine nucleotides very weakly,\(^2\) indicating that in vivo, this ARF mutant is largely nucleotide-free. Thus, the nucleotide-free form of ARF most likely acts as a dominant negative suppressor of normal ARF function by binding to and blocking the action of the ARF guanine nucleotide exchange factor present in the Golgi membranes, as is the case for related mutants of other Ras-like GTP-binding proteins (Hart et al., 1994). The observation that the induction of the ARF(Q126I) mutant has the same effect as BFA treatment (i.e. eliminating the perinuclear distribution of Cdc42Hs) suggests that ARF acts upstream or in coordination with Cdc42Hs in the Golgi membrane. One possible biochemical connection between these two GTPases has been recently suggested from data characterizing a synergistic effect of ARF and Cdc42Hs toward phospholipase D activity (Singer et al., 1995), raising the possibility that they both may bind to distinct activation sites on a common target enzyme. Significantly, the phospholipase D activity in this study was Golgi-localized and, consistent with a role for Cdc42Hs and ARF in its activation, was blocked by treatment of the Golgi membranes with BFA (Ktistakis et al., 1995).

Taken together, our findings coupled with the observation that BFA treatment blocks the phorbol 12-myristate 13-acetate-induced formation of filopodia and retards the wound-healing response (Bershadsky and Futerman, 1994) make it tempting to speculate that Cdc42Hs contributes to the mechanism by which new lipid and protein are delivered to a chosen point control through its ability to stimulate the 70-kDa S6 kinase (Chou and Blenis, 1996). This, a picture seems to be emerging in which Cdc42 directly influences a variety of cellular activities that bear on the cytoskeleton and various cellular cycle-cell cycle events. It is difficult to imagine that these different activities would occur randomly; rather, it seems more likely that they would need to be coordinated. Perhaps such coordination is mediated at the Golgi membranes, where Cdc42Hs may initiate actin-based trafficking events that are critical and/or coupled to normal cell cycle progression. Through the use of epitope-tagged mutants of Cdc42Hs, we hope to be able to identify additional participants in each of the effector activities for Cdc42Hs and thus begin to obtain a clearer picture of its overall biological function as well as better understand how its different effector activities are coordinated.

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