Transformation Activity of Cdc42 Requires a Region Unique to Rho-related Proteins

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Wen Jin Wu, Rui Lin, Richard A. Cerione, and Danny Manor‡

From the Department of Pharmacology, Veterinary Medical Center, Cornell University, Ithaca, New York 14853

The Rho subfamily GTP-binding protein Cdc42 mediates actin cytoskeletal rearrangements and cell cycle progression and is essential for Ras transformation. Expression of a Cdc42 mutant (Cdc42(F28L)) that undergoes spontaneous activation (guanine nucleotide exchange) results in transformation of NIH3T3 fibroblasts. In this report, we show that deletion of residues 120–139 from Cdc42(F28L), which comprise an insert region unique to Rho subfamily proteins but is missing in other GTP-binding proteins, yields a Cdc42 molecule that still undergoes spontaneous GTP-GDP exchange and stimulates both actin cytoskeletal changes and the activation of the cellular targets p21-activated kinase and the c-Jun kinase (JNK1). However, this Cdc42 mutant is unable to transform cells. These findings indicate that the Rho subfamily insert region is dispensable for many of the known signaling pathways initiated by activated Cdc42 but is essential for its regulation of cell growth.

Members of the Rho subfamily of GTP-binding proteins act as molecular switches in a variety of biological response pathways including cell polarity, motility, and cell cycle progression (1, 2). Although it was originally thought that the actions of these GTP-binding proteins were confined to changes in the actin cytoskeletal architecture, it has recently become appreciated that they also stimulate cell cycle progression (3). Dominant-active (GTPase-defective) mutants of Rho subfamily proteins have been reported to have varying degrees of transforming activity, and dominant-negative (guanine nucleotide exchange-defective) mutants were shown to block Ras transformation (4–9).

Previous studies showed that the interactions of Cdc42 or Rac with one of their primary targets, PAK, was not essential for the ability of these GTP-binding proteins to stimulate cell cycle progression, as assayed by DNA synthesis (3). An important step toward ultimately delineating the signaling cascades that mediate cell cycle progression and cellular transformation by Cdc42 or other Rho subfamily proteins will be to identify specific transformation-defective mutants. Toward this end, we have taken advantage of the recent finding that a Cdc42-point mutant (Cdc42(F28L)), which is able to undergo the spontaneous exchange of GTP for GDP without compromising its GTP hydrolytic activity, transforms NIH3T3 cells (4). This “fast cycling” Cdc42(F28L) mutant more closely mimics the transformation of NIH3T3 cells by the Dbl oncoprotein, a guanine nucleotide exchange factor for Cdc42, than do GTPase-defective Cdc42 mutants. Thus, we have used the Cdc42(F28L) molecule as a background to search for transformation-defective mutants.

Of particular interest was a region of 13 amino acids in Cdc42 (residues 122–134), designated the Rho insert region and was missing in Ras and other small G proteins (10, 11). We have recently shown that a Cdc42/Ras chimera (abbreviated ΔL8) in which loop 8 from Ha-Ras (residues 121–127) was substituted for amino acids 120–139 in Cdc42 that includes the insert region, was capable of full GTP binding and GTP hydrolytic activities and was able to functionally couple to various Cdc42 regulators (e.g. Dbl, Cdc42-GTP-activating protein) and targets (12).

Although the Cdc42(ΔL8) mutant was also able to bind to the GDP dissociation inhibitor (GDI), and could be extracted from membranes by the GDI as earlier reported (13, 14), the Cdc42(ΔL8) mutant was no longer susceptible to a GDI-mediated inhibition of GDP dissociation and GTP hydrolysis. However, the Cdc42(ΔL8) mutant did not transform NIH3T3 cells, arguing against the idea that the GDI normally functions as a growth inhibitor/tumor suppressor.

Here, we present evidence for the direct involvement of the insert region in the transformation activity of Cdc42. The data indicate the existence of a distinct, novel signaling pathway that is initiated by Cdc42 activation and impacts on cell growth regulation. The immediate target, downstream from Cdc42, apparently utilizes the insert region as a part of its binding interface with Cdc42.

EXPERIMENTAL PROCEDURES

Cdc42(F28L/ΔL8) gene was generated using polymerase chain reaction with Cdc42(F28L) as a template, in a manner identical to that used for generating Cdc42(ΔL8) (12). The hexahistidine-tagged protein was purified from overexpressing Escherichia coli as described in Ref. 15. For expression in mammalian cells, the Cdc42(F28L/ΔL8) gene was subcloned into the BamHI and EcoRI sites of pcDNA3 and pc4H vectors for transient and stable expression, respectively. Cell culture, immunoprecipitations, and kinase assays were done as described in Ref. 16. Stable cell line selection was achieved using G418 selection as described in Ref. 4. GTP binding activities were measured in a [35S]GTP-γ-S nitrocellulose filtration assay as described in Ref. 17.

DNA synthesis was assayed by measuring [3H]thymidine Incorporation as in Ref. 18. Cells were seeded in 24-well plates at 1 × 10⁵ cells/well in DMEM containing 5% calf serum and grown for 24 h. Cells were then washed in serum-free media and M phase arrest induced by the addition of 80 ng/ml nocodazole. After 4 h cells were washed with serum-free media twice and were pulsed with 5 μCi/ml of the [3H]thymidine for 2 h at 37 °C in DMEM containing 0.5% calf serum. Cells were washed and harvested, and [3H]thymidine incorporation was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

We first set out to examine the biochemical consequences of deleting the insert region from the transforming Cdc42(F28L)
Deletion of the insert region from Cdc42(F28L) does not alter its biochemical properties. A. 

Fig. 1. Deletion of the insert region from Cdc42(F28L) does not alter its biochemical properties. A. 

By immunoblotting with HA antibodies (top panel), expression levels of recombinant Cdc42 were measured in the total lysates of NIH3T3 cells. Fig. 1A shows that like the Cdc42(F28L) point mutant, the Cdc42(F28L/L8) mutant is constitutively active in its ability to bind [35S]GTPγS, and unlike wild-type Cdc42, it does not require excess EDTA to catalyze the exchange of GTPγS for GDP. We then examined whether the constitutively active Cdc42(F28L/L8) mutant was capable of stimulating various signaling activities in vitro. Previous studies have shown that activated versions of Cdc42 directly stimulate members of the PKC family of serine/threonine kinases (19–21). We therefore compared the abilities of the Cdc42(F28L) and Cdc42(F28L/L8) mutants to stimulate PAK activity. Each of these Cdc42 mutants were co-expressed with Myc-tagged PAK in NIH3T3 cells. Fig. 1B shows the results of an experiment where essentially equivalent amounts of Myc-tagged PAK were immunoprecipitated from each lysate and assayed for their ability to phosphorylate the substrate myelin basic protein (MBP). The expression of the Cdc42(F28L) point mutant caused a marked stimulation of PAK activity, compared with cells expressing vector alone or wild-type Cdc42, consistent with previous results (4). Likewise, the Cdc42(F28L/L8) deletion mutant caused a strong stimulation of MBP phosphorylation, equivalent to that observed with Cdc42(F28L).

The stress-responsive nuclear MAP kinase, c-Jun kinase (JNK1), is also activated by Cdc42 (16, 22–24) through its initiation of a signaling cascade. Fig. 1C shows that expression of either Cdc42(F28L) or Cdc42(F28L/L8) in NIH3T3 cells stimulated JNK1 activity as assayed by the phosphorylation of the transcription factor, c-Jun. The overexpression of wild-type Cdc42 also leads to a stimulation of JNK1 while not causing a significant stimulation of PAK (Fig. 1B). This indicates that in NIH3T3 cells, Cdc42 must use another target kinase(s) aside from PAK to initiate signaling pathways that culminate in JNK1 activation, similar to what was observed in the case of Rac1 (9, 25). However, together, these findings demonstrate that the removal of the Rho subfamily insert region from an otherwise activated Cdc42 molecule does not have deleterious effects on the ability of the G protein to stimulate one of its primary cellular targets, PAK, nor its well established nuclear end point, JNK1.

We next examined whether removal of the insert region from Cdc42 altered its ability to stimulate the actin cytoskeletal changes that have been previously associated with the activation of this GTP-binding protein. One of the hallmarks of Cdc42 activation is the induction of filopodia or actin microspikes. This was originally identified upon micro-injecting GTPase-defective forms of Cdc42 into Swiss 3T3 cells (26–28), and more recently was also observed to result from stable expression of the Cdc42(F28L) mutant in NIH3T3 cells (Ref. 4; also see Fig. 2, top panels). We find that expression of the Cdc42(F28L/L8) mutant gives rise to actin stress fiber formation and filopodia extensions in fibroblasts (Fig. 2), such that the cells are essentially indistinguishable from those expressing the Cdc42(F28L) point mutant. The results presented in Fig. 2 (bottom panels) also show that stable expression of either the activated Cdc42(F28L) point mutant or the Cdc42(F28L/L8) mutant in NIH3T3 cells causes a dramatic increase in the formation of focal complexes, as indicated by immunostaining for vinculin, compared with that observed in control fibroblasts.

Although the removal of the Rho subfamily insert region from Cdc42 did not alter its ability to stimulate protein kinase targets nor to induce actin cytoskeletal-associated changes, it had dramatic effects on the ability of Cdc42 to transform fibroblasts. This was initially observed when comparing the growth of NIH3T3 cells in low serum. The data in Fig. 3A show that control NIH3T3 fibroblasts do not proliferate in low serum (1% fetal bovine serum). As previously reported (4), stable expression of the Cdc42(F28L) mutant strongly stimulates the growth of these fibroblasts under conditions of low serum. However, this serum-independent growth is completely eliminated upon removal of the insert region and thus is not observed with the Cdc42(F28L/L8) mutant.

Similar effects were observed when comparing the saturation density of NIH3T3 cells under high serum conditions. Fig. 3B shows that the stable expression of the Cdc42(F28L) protein is accompanied by a marked increase in cell density, consistent
with the ability of this Cdc42 point mutant to undergo a spontaneous activation, thus mimicking the phenotype of Dbl transformed cells (4, 29). However, despite the fact that the Cdc42(F28L/DL8) deletion mutant is also able to undergo spontaneous GTP-GDP exchange, its overexpression is not accompanied by an increased saturation density relative to what is observed in mock-transfected NIH3T3 cells.

The effect of the F28L and F28L/DL8 mutations in Cdc42 on cell cycle progression is directly addressed in the experiments depicted in Fig. 3C. The different stable cell lines were synchronized at G1 phase by nocodazole treatment, and the cellular nucleotide pool was labeled with a brief pulse of [3H]thymidine. Progression through S phase of the cell cycle was then monitored by quantitating the incorporation of the labeled nucleotide into DNA, showing that DNA synthesis in the transformed Cdc42(F28L) cells exceeded that in both parental and Cdc42(F28L/DL8)-expressing cells by ≈10-fold.

The final and perhaps most important indicator for transformation activity involves assays of the growth of NIH3T3 cells in soft agar. Fig. 4 shows that the activated Cdc42(F28L) point mutant strongly promotes the ability of fibroblasts to grow in the absence of a substratum (anchorage-independent growth). However, cells expressing the Cdc42(F28L/DL8) deletion mutant are essentially indistinguishable from control NIH3T3 cells and are unable to induce significant colony formation in soft agar.

It is possible that the transformation signal of Cdc42 is mediated through cross-talk with the Ras-signaling pathway. Such a connection is suggested from the ability of the dominant-negative form of Cdc42 to inhibit Ras transformation (7).

Fig. 5. Transformation by Cdc42(F28L) does not occur through stimulation of Erk. Transient co-transfection of COS-7 cells was carried out with the indicated HA-tagged Cdc42 constructs in pCDNA3 or HA-tagged Ras(G12V) in pDCR together with HA-tagged Erk1 in pCDNA3. Anti-HA antibodies were used to immunoprecipitate and visualize G protein and Erk1 expression levels (top two panels). Immunoprecipitated Erk1 was assayed in vitro for its ability to phosphorylate MBP using [γ-32P]ATP (bottom panel). To obtain similar expression levels of all G proteins, the amount of transfected pDCR-Ras DNA was 500-fold less than that of the Cdc42 constructs.
decrease of Ras-mediated MAP kinase activation and focus in formation in Rat1 fibroblasts (30, 31). We have therefore asked whether Cdc42/F28L and Cdc42/F28L/ΔDL8 show any activation of the Ras pathway by transiently co-transfecting the appropriate Cdc42 construct together with MAP kinase (Erk1) into COS-7 cells and measuring Erk activity in an immune complex kinase assay. As can be seen in Fig. 5, both serum treatment and Ras(G12V) expression lead to significant activation of ectopically expressed Erk1, whereas the expression of wild-type or activated forms of Cdc42 did not result in a significant stimulation of Erk1 activity. Thus, we conclude that transformation by Cdc42(F28L) does not occur via stimulation of Erk activity.

Taken together, these data indicate that the Rho subfamily insert region plays a highly specific role in the ability of activated Cdc42 to transform fibroblasts. This has a number of important structure-function and biological implications. The finding that the Db1 oncprotein, which possesses potent transforming activity, is also an upstream activator of Cdc42 (32–34) first raised the possibility that this GTP-binding protein directly participates in cell growth regulation. However, it has only recently become appreciated that activated versions of Rho subfamily proteins can significantly influence cell cycle progression (3, 35) and in particular that activating mutations of Cdc42 result in cellular transformation (4, 7). The identification of a specific region within the Cdc42 molecule whose removal eliminates its transforming activity provides an important first step toward delineating the signaling pathway(s) involved. The fact that removal of the insert region does not perturb a number of Cdc42-mediated signaling outcomes including the activation of the stress-responsive MAP kinase, JNK1, nor the generation of filopodia or vinculin-rich focal complexes indicates that a very specific and as yet unidentified signaling pathway is essential for the transformation signal. An important effort in the future will be directed toward identifying target molecules that are specifically prohibited from binding to the Cdc42(F28L/ΔDL8) deletion mutant.

Both NMR (36) and x-ray crystallographic (37–39) studies indicate that the Rho subfamily insert region does not function as a “switch domain” upon GTP-GDP exchange. However, it does contribute, together with the switch I and switch II domains, a possible surface for target-binding interactions, such that primary target interactions are likely to occur within the classical effector loop provided by switch I, but secondary interactions could occur within the insert region. It has in fact been reported that mutations within the insert region of Rac prevent it from binding and stimulating one of its more specialized targets, the NADPH oxidase complex (10). Thus, it is now attractive to consider that the insert regions on Cdc42, Rac, and possibly other Rho proteins serve as specific docking sites for binding targets that initiate highly specialized signaling pathways and that one such pathway is essential for cell growth control and when overactivated leads to cellular transformation.
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