Post-translational Processing of RhoA

CARBOXYL METHYLATION OF THE CARBOXYL-TERMINAL PRENYLCYSTEINE INCREASES THE HALF-LIFE OF RhoA

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RhoA and related GTP-binding proteins are modified post-translationally at their carboxyl terminus to form a prenylcysteine methyl ester. The synthesis and post-translational modification of RhoA and Cdc42 were examined in the RAW264 macrophage cell line, and the effect of carboxyl methylation on protein turnover was determined. Cells were labeled with [35S]cysteine, and RhoA or Cdc42 was immunoprecipitated with specific antibodies. Both RhoA and Cdc42 were methylated rapidly in control cells, with little accumulation of unmethylated protein. Carboxyl methylation of RhoA was inhibited by incubation of cells with a carbocyclic adenosine analog, 3-deazaristeromycin, resulting in the accumulation of unmethylated RhoA. Under these conditions, Cdc42 methylation was inhibited only partially. When methylation was inhibited, the RhoA half-life decreased from 31 to 12 h, and the Cdc42 half-life decreased from 15 to 11 h. The increased degradation of unmethylated RhoA demonstrates a novel function for carboxyl-terminal prenylcysteine carboxyl methylation in protecting RhoA and related proteins from degradation.

The Rho family of GTP-binding proteins is a family of important signal-transducing proteins which includes the Rho, Rac, and Cdc42 proteins (1, 2). These proteins are integral components in the signaling pathways for many cellular functions, including the organization of cytoskeletal proteins required for cell motility, adhesion, and proliferation (2, 3). The Rho proteins are also involved in signaling pathways for the activation of specific transcription factors (4). Different members of the Rho family appear to activate distinct signaling pathways. In fibroblasts, activation of Cdc42 is required for cytoskeletal changes involved in filopodia formation (5), whereas the activation of Rac is involved in lamellipodia formation and membrane ruffling (6), and Rho activation results in the formation of actin stress fibers and focal adhesions (7). These cytoskeletal changes are believed to be mediated through specific protein kinases that are activated by the GTP-bound form of the Rho family proteins (8).

All members of the Rho protein family are modified post-translationally at their carboxyl terminus. These proteins are synthesized initially with a carboxyl-terminal Cys-Axx-Axx-Xxx sequence (where Axx is usually an aliphatic amino acid, and Xxx is any amino acid), which is a signal for post-translational processing by the three sequential reactions of prenylation, truncation, and methylation (9). For RhoA and Cdc42, the carboxyl terminus is modified to form a geranylgeranylcysteine methyl ester (10, 11). Lipid modification of the carboxyl terminus is believed to be important for the interaction of Rho with both guanine nucleotide exchange proteins and with downstream effector targets (2).

Carboxyl methylation of proteins containing a carboxyl-terminal prenylcysteine is catalyzed by a membrane-bound prenylcysteine methyltransferase (12). In yeast, the STE14 gene has been shown to encode a membrane-bound prenylcysteine methyltransferase that methylates both yeast RAS and the mating a-factor (13). Both the STE14 methyltransferase and the mammalian prenylcysteine methyltransferase activity can methylate peptide substrates that contain either a carboxyl-terminal farnesyl- or geranylgeranylcysteine (14, 15). In mammalian cells, much of the methyltransferase activity is localized to microsomal membranes (16), but some activity has been reported in neutrophils to be associated with the plasma membrane (17). Because the mammalian methyltransferase has not been purified, it is not certain if multiple forms of the enzyme are present in the membranes.

Carboxyl methylation of some Rho family proteins can be stimulated in vitro by activation of the protein with GTPγS1 (18, 19) or by activation of Rho-dependent signaling pathways in intact cells by the addition of chemoattractants (20). In their inactive form, the Rho proteins can form heterodimers with the Rho guanine nucleotide dissociation inhibitor protein (RhoGDI) (21). Unmethylated Cdc42 from brain (the brain form of Cdc42 is also known as G25K) purifies as a soluble complex with RhoGDI, and RhoGDI blocks methylation until Cdc42 is in the active GTPγS-bound conformation (22). Upon activation, the conformational change brought about by guanine nucleotide binding and the subsequent change in interaction between the GTPase and RhoGDI result in translocation of the protein to the membrane, where methylation by the membrane-bound methyltransferase can occur (22).

The role of carboxyl methylation in Rho protein function has not been defined clearly. It has been reported that methylation may increase membrane attachment (23, 24) and interactions with specific effector proteins (25). Whereas the prenylation and truncation steps are irreversible, carboxyl methylation can be reversed by hydrolysis of the methyl ester to form methanol. A number of esterase (26, 27) and protease activities (27) in cell...

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1 The abbreviations used are: GTPγS, guanosine 5′-O-(3-thiotriphosphate); RhoGDI, Rho guanine nucleotide dissociation inhibitor protein; (His)6-Cdc42, hexahistidine-tagged Cdc42; PBS, phosphate-buffered saline; calcium- and magnesium-free; MEM, modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; DZAri, 3-deazaaristeromycin; AdoHcy, S-adenosyl-l-homocysteine.
extracts have been reported to hydrolyze prenylcysteine methyl esters in vitro, but it has not been demonstrated which of these activities can act in vivo to hydrolyze methyl esters from protein of the Rho family. Several prenylcysteine analogs that inhibit carboxyl methylation also inhibit chemoattractant-stimulated signal transduction, suggesting that methylation may have a direct role in these signal transduction pathways (28). However, these compounds may disrupt chemoattractant signaling pathways by a mechanism that is independent of their inhibition of the methyltransferase (29–31).

To investigate carboxyl methylation of the Rho protein family in vivo, the carboxyl-terminal processing of RhoA and Cdc42 was examined in the RAW264 macrophage cell line. The data reported here show that at least 90% of RhoA and Cdc42 becomes methylated in exponentially growing RAW264 cells. Inhibiting RhoA methylation decreases half-life of the protein significantly, indicating a novel role for carboxyl methylation in protecting RhoA from degradation.

**EXPERIMENTAL PROCEDURES**

**Materials**—A mouse monoclonal antibody, RhoA (26C4), directed against amino acid residues 120–150 of human RhoA (Santa Cruz Biotechnology), was used for immunoprecipitation of RhoA. An anti-Cdc42 antibody was prepared from the serum of rabbits immunized with hexahistidine-tagged Cdc42 (His<sub>6</sub>-Cdc42). The cDNA for the human form of Cdc42Hs (also known as G25K) was obtained from Susan Munemitsu. The His<sub>6</sub>-Cdc42 cDNA was subcloned into the pQE plasmid, and the protein was expressed in Escherichia coli and purified using the QiAexpress system (Qiagen). The (His)<sub>6</sub>-Cdc42 was purified further by chromatography using a Mono Q column (Pharmacia BioTech). The (His)<sub>6</sub>-Cdc42 was dialyzed and applied to a Mono Q column (Pharmacia Biotech), was used for immunoprecipitation of RhoA and Cdc42 from cell extracts. The gels were dried and exposed to X-Omat film (Kodak) at 70 °C for 2 nights at 500 V.

**RESULTS**

**Synthesis and Carboxyl Methylation of RhoA and Cdc42**—The synthesis and post-translational processing of RhoA and Cdc42 were examined by labeling RAW264 cells with [35S]cysteine followed by immunoprecipitation of RhoA and Cdc42 from [35S]-labeled proteins in the cell extracts. Methylation of RhoA and Cdc42 occurs only after the carboxyl terminus is first modified by prenylation and cleavage of the carboxyl-terminal tripeptide. Because carboxyl methylation neutralizes the negative charge of the carboxyl-terminal α-carboxyl group, the methylated protein has a more basic isoelectric point (pI) than the unmethylated protein, and the two forms may be resolved by isoelectric focusing.

Two pI forms of Cdc42 (pI values of approximately 6.4 and 6.9) were also observed in immunoprecipitates of RhoA from [35S]cysteine-labeled cell extracts were separated by two-dimensional PAGE, two labeled proteins with pI values of approximately 5.9 and 6.35 were observed migrating with the 23 kDa of RhoA (Fig. 1). Two pI forms of Cdc42 (pI values of approximately 6.4 and 6.9) were also observed in immunoprecipitates of Cdc42, after separation by two-dimensional PAGE. These two forms of RhoA and Cdc42 were also observed with immunoblots of cell extracts (Fig. 2). As shown below, when methylation was inhibited both RhoA and Cdc42 migrated with the more acidic pI form of the protein. Identification of the methylated proteins on the two-dimensional gel was confirmed by the presence of radiolabeled methyl esters in the proteins when cells were grown with [methyl-<sup>3</sup>H]methylamine. These results indicated that both RhoA and Cdc42, the more basic pI protein was carboxyl methylated, and the more acidic form was unmethylated.

The kinetics of RhoA synthesis and methylation were determined from immunoprecipitates of RhoA after cells were labeled with [35S]cysteine for increasing times. After 1 h of labeling, 76% of the newly synthesized RhoA was already methylated; with increasing labeling times, the radioactivity in

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FIG. 1. Kinetics of RhoA synthesis and methylation. RAW264 cells were incubated with [35S]cysteine for the indicated times, and RhoA was immunoprecipitated as described under “Experimental Procedures.” For the chase period indicated, radioactive [35S]cysteine was replaced with unlabeled cysteine, and the incubation was continued. Immunoprecipitates of RhoA were separated by two-dimensional PAGE, and the radioactivity was detected by autoradiography. Panel A, quantitation of radioactivity in RhoA by scanning densitometry of the autoradiograms. ■ unmethylated RhoA; ●, methylated RhoA; ▲, total methylated and unmethylated RhoA. Panel B, autoradiograms of two-dimensional gels. Gels are orientated with the acidic end to the left and higher molecular weight at the top. Methylated and unmethylated proteins are indicated with arrows.

FIG. 2. Immunoblots of RhoA and Cdc42 separated by two-dimensional PAGE. Extracts of RAW264 cells were solubilized and separated by two-dimensional PAGE. The proteins were transferred to polyvinylidene difluoride membranes, and RhoA and Cdc42 were detected using either anti-Cdc42- or anti-RhoA-specific antibodies. The blots are orientated with the isoelectric focusing dimension horizontally, the acidic end to the left, and the basic end to the right. The arrows indicate the location of unmethylated (u) and methylated (m) forms of the proteins. Panel A, detection of Cdc42 in cell extracts, using anti-Cdc42 antibody at a dilution of 1:500. Panel B, detection of RhoA in cell extracts, using anti-RhoA antibody at a dilution of 1:500.

unmethylated RhoA reached a plateau, while radioactivity in the methylated RhoA continued to increase (Fig. 1). The kinetics indicate that most of RhoA becomes methylated a short time after the protein is synthesized. When further incorporation of radioactivity was blocked by a chase with unlabeled cysteine for 18 h, the unmethylated RhoA decreased significantly, but a detectable amount still remained (Fig. 1). In contrast, the methylated RhoA decreased only slightly during this time. As a result, after a chase of 18 h with unlabeled amino acid, unmethylated RhoA decreased from 16 to 6% of the total labeled RhoA.

In addition to the two spots identified as methylated and unmethylated RhoA, two minor protein spots were also observed in the RhoA immunoprecipitates. The first spot migrated directly above the methylated RhoA, and the second spot migrated between the methylated and unmethylated RhoA spots (Figs. 1B and 3). The identities of these proteins were not determined, and they may represent either further modifications of RhoA or related members of the Rho protein family which were recognized by the anti-RhoA antibody. The protein directly above the methylated RhoA also appears to be methylated because its migration shifts to the unmethylated pi when methylation was inhibited with 3-deazaaristeromycin (Fig. 3). The unprenylated RhoA precursor was not identified in these samples, but it would be expected to migrate just above the prenylated, unmethylated form of RhoA.

Similar kinetics for synthesis and methylation were observed with Cdc42 (data not shown). After 1 h of labeling, only 11% of the radioactivity was in the unmethylated Cdc42; at longer times the label in unmethylated Cdc42 reached a plateau, whereas the radioactivity in methylated Cdc42 continued to increase. After an 18-h chase, the radioactivity in both unmethylated and methylated Cdc42 decreased, such that 9% of the remaining Cdc42 was unmethylated. The total decrease in labeled Cdc42 after the chase indicates that the turnover of Cdc42 was more rapid than RhoA turnover. These results indicate that RhoA and Cdc42 methylation occurs rapidly after protein synthesis, resulting in less than 10% of either protein remaining unmethylated after 18 h.

The steady-state levels of methylated and unmethylated RhoA and Cdc42 were determined from immunoblots of RAW264 cell extracts after separation by two-dimensional PAGE. After transfer to the membranes, Cdc42 or RhoA was
TABLE I

Inhibition of RhoA and Cdc42 carboxyl methylation by 3-deazaaristeromycin

|            | Control (3-h label) | +DZAri (3-h label) | Control (18-h chase) | +DZAri (18-h chase) |
|------------|---------------------|--------------------|----------------------|---------------------|
| **RhoA**   |                     |                    |                      |                     |
| Unmethylated | 194                  | 930                | 17                   | 30                  |
| Methylated  | 1,710               | 695                | 695                  | 438                 |
| Total       | 1,904               | 1,555              | 712                  | 438                 |
| % Unmethylated | 10                  | 69                 | 2                    | 7                   |
| **CDC42**  |                     |                    |                      |                     |
| Unmethylated | 50                   | 334                | 16                   | 26                  |
| Methylated  | 763                  | 673                | 329                  | 265                 |
| Total       | 813                 | 1,047              | 345                  | 291                 |
| % Unmethylated | 6                   | 33                 | 5                    | 9                   |

a Radioactivity was determined from autofluorograms of immunoprecipitates separated on two-dimensional gels, shown in Figs. 3 (RhoA) and 4 (Cdc42).

detected with either anti-Cdc42- or anti-RhoA-specific antibodies (Fig. 2). Similar to the results obtained with immunoprecipitates, each antibody detected two immunoreactive proteins that comigrated with the radiolabeled forms of Cdc42 and RhoA in the immunoprecipitates. Again, the major Cdc42 immunoreactive spot detected on the blots corresponded to the more acidic protein being the unmethylated form of RhoA. A similar shift in labeling of Cdc42 was also observed after treatment with DZAri (Fig. 4 and Table I). After 3 h of labeling in the presence of DZAri, 69% of the newly synthesized RhoA was unmethylated compared with 10% of the total labeled Cdc42 in control cells. After a chase period in the presence of DZAri, unmethylated Cdc42 decreased to 9% of the total labeled Cdc42. Therefore, the increase in cellular AdoHcy inhibited RhoA methylation to a greater extent than the inhibition of Cdc42 methylation.

Effect of RhoA and Cdc42 Methylation on Protein Turnover—The effect of carboxyl methylation on protein turnover was examined for RhoA and Cdc42 by measuring their degradation under conditions where the proteins were either methylated or unmethylated. RAW264 cells were labeled with [35S]cysteine followed by a chase period with unlabeled cysteine to block further labeling of newly synthesized protein, and the radioactivity in RhoA or Cdc42 was determined over time (Fig. 5).

Under control conditions, approximately 90% of the labeled RhoA and Cdc42 was methylated at the start of the chase period (Table I). Because these proteins were more than 90% methylated, the observed protein turnover in control cells is approximately equal to the turnover for the methylated forms of RhoA and Cdc42. The apparent half-life of RhoA in control cells was approximately 31 h (Fig. 5A). Treatment of cells with DZAri inhibited methylation such that 69% of radiolabeled RhoA was unmethylated after 3 h of labeling (Table I). Inhibiting methylation increased RhoA turnover significantly, causing the apparent half-life to decrease from 31 to 12 h (Fig. 5A). The turnover of total cell protein was determined from precipitation of total proteins with cold 5% trichloroacetic acid from cell extracts of control and DZAri-treated cells, and no significant change in total protein turnover was observed with DZAri treatment (data not shown).

Like RhoA, Cdc42 was methylated rapidly under control conditions (Table I), and the half-life of methylated Cdc42 was approximately 15 h under these conditions (Fig. 5B). The half-life of methylated Cdc42 was significantly shorter than the 31-h half-life of methylated RhoA. Treatment of cells with DZAri resulted in only 33% of the radiolabeled Cdc42 remaining unmethylated after 3 h (Table I), and only a small increase in Cdc42 turnover was observed, decreasing the apparent half-life from approximately 15 to 11 h (Fig. 5B). However, 67% of the labeled Cdc42 was still methylated under these conditions, so the observed Cdc42 turnover was equal to the sum of the rates for both methylated and unmethylated Cdc42. The results indicated that incubation of cells with DZAri increased the turnover of RhoA and Cdc42, and the increased turnover was correlated with the inhibition of methylation.
FIG. 5. Effect of carboxyl methyla-

tion on RhoA and Cdc42 turnover.

RAW 264 cells were labeled with [35S]cys-

teine for 3 h followed by a chase with un-

labeled cysteine for the indicated times, as described under “Experimental

Procedures.” RhoA or Cdc42 were then

immunoprecipitated, separated by SDS-

PAGE, and the radioactivity was detected

by autofluorography. The labeled bands

corresponding to RhoA or Cdc42 were

quantitated by scanning densitometry.

The radioactivity remaining in RhoA or

Cdc42 is expressed as a percent of the

radioactivity in each protein at the start

of the chase. Each data point is the average

of duplicate determinations. Panel A, ra-

dioactivity in RhoA immunoprecipitated

from ○, control cells; ■, +0.1 mM 3-dea-

zaaristeromycin. Panel B, radioactivity in

Cdc42 immunoprecipitated from ○, control

cells; □, +0.1 mM 3-deazaazaristeromycin.

DISCUSSION

This study examined the synthesis and methylation of RhoA

and Cdc42 in RAW264 cells, and the results demonstrated an

increased turnover of RhoA when methylation of the carboxyl-

terminal prenylcysteine was blocked. Under the normal growth

conditions, both RhoA and Cdc42 were approximately 90–95%

methylated (Fig. 2), and the kinetics of methylation indicated that

both proteins are methylated a short time after their

synthesis (Fig. 1). The lag between synthesis and methylation

appeared to be a slightly longer for RhoA than for Cdc42, since

at early labeling times unmethylated RhoA accumulated to a

greater extent than the unmethylated Cdc42 (24% unmethyl-

ated RhoA versus 11% unmethylated Cdc42 after 1 h). How-

ever, after an 18-h chase period, approximately equal propor-

tions of RhoA and Cdc42 were methylated.

Raising the cellular AdoHcy concentration inhibited methy-

lation of RhoA and Cdc42, but the inhibition was greater for

RhoA than for Cdc42 (Figs. 3 and 4). The reason for the differ-

ence in inhibition is not clear. A previous report on the mech-

anism of prenylcysteine methyltransferase in mammalian

membranes reported that the enzyme has an ordered Bi Bi

mechanism, where AdoHcy is a mixed type inhibitor with re-

spect to the methylation peptide substrate (38), so AdoHcy may

be a better inhibitor with RhoA as the methylation substrate.

The preferential inhibition of RhoA methylation could also

indicate the presence of two distinct methyltransferases in the

membranes, with each one having a different susceptibility to

inhibition by AdoHcy. Regardless of the detailed mechanism,

the results indicate that raising AdoHcy levels in vivo can preferen-

tially inhibit the methylation of specific protein substrates.

Although methylation of RhoA and Cdc42 occurred rapidly

after synthesis, a small fraction of labeled RhoA and Cdc42

remained unmethylated even after an 18-h chase. The persist-

ence of radioactivity in the unmethylated protein suggests the

presence of a small pool of unmethylated RhoA and Cdc42

which is either methylated very slowly or is never methylated.

This pool of unmethylated protein could be caused by localiza-

tion of the protein to a separate compartment that is inacces-

sible to the methyltransferase. Alternatively, unmethylated

protein may be generated by the slow removal of methyl esters

from a previously methylated protein.

The turnover of Cdc42 was significantly faster than RhoA

turnover. In control cells, the half-life of Cdc42 was approxi-

mately 15 h, whereas the half-life of RhoA was approximately

31 h. The half-life for mammalian proteins can range from

minutes to hundreds of hours (39), so the half-lives of both

RhoA and Cdc42 are within an intermediate range for mam-

alian proteins. Protein degradation is a complex process, and

many structural features can influence protein turnover. Pro-

tein turnover can be regulated by covalent modifications, and

several covalent modifications have been shown to increase
degradation of specific proteins (40). For RhoA, ADP-ribosyla-

tion of the protein by the Clostridium difficile

Toxin A has been

shown to lower the level of RhoA dramatically after an over-
night incubation (41), causing the loss of polymerized actin

fibers and rounding up of the cells.

The data presented here demonstrate significantly faster
degradation of RhoA when the protein is unmethylated, indi-
cating a role for carboxyl methylation in protecting the protein

from degradation. For Cdc42, protein turnover was increased

only slightly by raising the level of AdoHcy; however, a large

fraction of labeled Cdc42 was still methylated under these

conditions (Fig. 4 and Table I). The decrease in half-life was

specific for proteins that were carboxyl methylated because

total protein degradation did not change. In addition to the Rho

proteins, a number of other signal-transducing GTP-binding

proteins contain a carboxyl-terminal prenylcysteine methyl

terine residues may be a signal for degradation which is recog-

nized in a conformation that is more resistant to proteolysis.

Alternatively, the unmethylated carboxyl-terminal prenyl-
cysteine residues may be a signal for degradation which is recog-

nized by specific proteases, or it may direct transport of the

protein to a site for degradation, such as the lysosomes or pro-

tosomes.

Because much of the prenylcysteine methyltransferase

activity is localized to the microsomal fraction (16), methylation

may be required for normal transport and localization of the

newly synthesized Rho proteins. A precedent for methylation
altering peptide transport is the yeast a-factor, where methylation of the a-factor by the STE14 methyltransferase is required for efficient transport of the peptide out of the cell by the STE6 transporter protein (43).

The effect of methylation on RhoA turnover suggests that RhoA levels in vivo may be influenced by competition between methyltransfer and the methylesterase activities. Methionine depletion would decrease the level of AdoMet and result in decreased protein methylation, which would increase degradation of the Rho proteins. In this manner, the level of RhoA and related proteins could be regulated by the cellular methionine pool. Unmethylated RhoA could also be generated by removal of the methyl ester by an esterase from a previously methylated protein. Therefore, activation of a methylesterase could also increase the amount of unmethylated protein, leading to increased protein degradation.

Previous studies have proposed that carboxyl methylation of Rho family of GTP-binding proteins is important for their function in signal transduction. The results presented here provide evidence that carboxyl methylation has a novel role in increasing the half-life RhoA and related proteins. In this role, methylation may not be directly involved in the signal transduction pathway, but methylation may influence signaling by changing the level of RhoA. Studies of additional proteins will be required to determine if the carboxyl methylation of carboxy-terminal prenylketone is a general mechanism for altering the degradation of these signal-transducing proteins.

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