Cysteine carboxyl O-methylation of human placental 23 kDa protein

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Abbreviations: AdoMet, S-adenosyl-L-methionine; AFC, S-farnesyl-N-acetyl-L-cysteine;

Abstract

C-Terminal carboxyl methylation of a human placental 23 kDa protein catalyzed by membrane-associated methyltransferase has been investigated. The 23 kDa protein substrate methylated was partially purified by DEAE-Sephacel, hydroxyapatite and Sephadex G-100 gel filtration chromatographies. The substrate protein was eluted on Sephadex G-100 gel filtration chromatography as a protein of about 29 kDa. In the absence of Mg²⁺, the methylation was stimulated by guanine nucleotides (GTP, GDP and GTP γ S), but in the presence of Mg²⁺, only GTP γ S stimulated the methylation which was similar to the effect on the G25K/rhoGDI complex. AFC, an inhibitor of C-terminal carboxyl methylation, inhibited the methylation of human placental 23 kDa protein. These results suggest that the substrate is a small G protein different from the G25K and is methylated on C-terminal isoprenylated cysteine residue. This was also confirmed by vapor phase analysis. The methylated substrate protein was redistributed to membrane after *in vitro* methylation, suggesting that the methylation of this protein is important for the redistribution of the 23 kDa small G protein for its putative role in intracellular signaling.

Keywords: cysteine carboxyl O-methylation, human placenta

Introduction

It has been recognized that a number of membrane proteins possessing a C-terminal -Cys-Aaa-Aaa-Xaa (-CAAX) sequence (where Aaa is an aliphatic amino acid and Xaa is any amino acid) are modified by a series of reactions at the C terminus (Clarke, 1992). These modifications include the addition of an isoprenyl moiety to the sulfur of the cysteine, cleavage of the C-terminal tripeptide, and carboxyl methylation of the resulting α -carboxyl group of the cysteine.

The methyltransferase activity involving the last step of the C-terminal modification was first detected *in vitro* in rod outer segment membranes using an assay which isolated a derivatized cysteine methyl ester residue (Ota and Clarke, 1989; Ong *et al.*, 1989). A rapid assay using a synthetic peptide as a methyl-accepting substrate was also developed. The isoprenylated hexapeptide S-farnesyl-Leu-Ala-Arg-Tyr-Lys-Cys was found to be an effective substrate (Km 2.2 μ M) while the unmodified peptide was not recognized by the enzyme (Stephenson and Clarke, 1990). Subsequently, it has been established that S-farnesyl-N-acetyl-L-cysteine (AFC) is also a substrate for the enzymes from rat and mouse tissues (Volker *et al.*, 1991) and bovine retinal rods (Perez-Sala *et al.*, 1991; Tan *et al.*, 1991), although it has approximately a 10-fold lower binding affinity. In yeast, the STE14 gene product has been shown to represent a similar C-terminal methyl-transferase that can methylate both the RAS proteins and the mating pheromone, α -factor, in intact cells (Hrycyna and Clarke, 1990; Hrycyna *et al.*, 1991).

A number of signaling molecules, including Ras and G-proteins, are targeted to the inner leaflet of the plasma membrane by the modifications. In some cases, palmitoylation of an upstream cysteine is also required (Hancock *et al.*, 1990). These modifications render otherwise hydrophilic proteins hydrophobic, promoting association with membranes. Methyl esterification at the C terminus might increase the hydrophobicity and membrane affinity by blocking the negative charge on the carboxylic acid (Clarke *et al.*, 1988; Ong *et al.*, 1989; Deschenes *et al.*, 1989; Yamane and Fung, 1989). For example, methylation of the mammalian p21K-ras(B) protein is needed for efficient membrane association (Hancock *et al.*, 1991). Alternatively, the methyl ester may be a part of the recognition site for a receptor protein as in the case for methyl esterified mating peptides from the yeasts *Saccharomyces cerevisiae* (Hrycyna *et al.*, 1991; Marcus *et al.*, 1991). Interestingly, loss of the STE14 gene encoding the C-terminal methyltransferase in *S. cerevisiae* does not have a large effect on the function of its substrate RAS proteins, although it does result in sterility based on the loss of α -mating factor activity

(Hrycyna *et al.*, 1991). In mammalian cells, methylation of lamin B, a structural protein of the nucleus, is cell cycle-dependent (Chelsky *et al.*, 1989). Partial inhibition of lamin B methylation in cells treated with 5'-methylthioadenosine slows the assembly of lamin B into the nuclear envelope following mitosis (Chelsky *et al.*, 1989). An additional possible role for methylation in mammalian cells is suggested by the inhibition of mouse peritoneal macrophage chemotaxis by *S*-farnesyl-*N*-acetyl-L-cysteine (AFC), a competitive inhibitor of the methyltransferase (Volker *et al.*, 1991).

A GTP-dependent carboxyl methylation of a number of membrane proteins having molecular weight of 20-23 kDa was first observed by Backlund and Aksamit (1988) in homogenates of mammalian cells. Guanine nucleotide dependency of the methylation suggested that these proteins were members of the small G-protein family, and it was proposed that carboxyl methylation may regulate the function of specific members of this family. It has also been reported that the methylation of the *rap1* proteins in platelets is stimulated by GTP γ S (Huzoor-Akbar *et al.*, 1991).

Small G-proteins have been implicated in a number of functions, including cell proliferation, vesicular transport, secretion and cytoskeletal organization (Barbacid, 1987; Bourne *et al.*, 1990; Valencia *et al.*, 1991). A common feature of these proteins is the role of GTP binding and hydrolysis in a cycle of activation and inactivation of the protein (Bourne *et al.*, 1990). Several proteins belonging to the *ras* and *rho* subfamilies of small G-proteins have a modified C-terminal CAAX sequence, including p21 Ha-ras, *rap1/smg21* and G25K. In addition, the transducin and G-protein γ -subunits, lamin B and cGMP phosphodiesterase also contain the modified C-terminal sequence.

We recently found a heavily methylated 23 kDa protein in human placental cytoplasmic fraction, by using a membrane fraction as an enzyme. The substrate protein was purified from human placenta and the characteristics and a possible role of the protein methylation was investigated.

Materials and Methods

S-Adenosyl-L-[methyl-³H] methionine (Ado[methyl-³H]Met; specific activity, 50 Ci/mmol) was purchased from Amersham International (England). Acrylamide and glycine were obtained from Bio-Rad (America, CA). Guanosine-5'-O-3-thiotriphosphate (GTP γ S) from Boehringer Mannheim (Germany), DEAE-Sephacel and Sephadex G-75 from Pharmacia Co. (Sweden), and (NH₄)₂SO₄, MgCl₂, CaCl₂, ZnCl₂, CoCl₂, CuCl₂, 2,5-diphenyloxazole (PPO) and dithiothreitol (DTT) were from Sigma Chemical Co (U.S.A.). *N*-Acetyl-farnesyl cysteine (AFC) was kindly provided by Dr. W. K. Paik (Department of Biochemistry, Ajou University College of Medicine, Suwon, Korea). The rest

of chemicals were obtained from various commercial sources and were of the highest grade available.

Preparation of methyl acceptor substrate protein from human placenta

One hundred g of human placental tissue was homogenized in 200 ml of 5 mM phosphate buffer (pH 7.2; buffer A) containing 0.5 mM EDTA, 0.5 mM PMSF (phenylmethyl-sulfonyl fluoride), 1 mM DTT and 10% glycerol with poly-tron homogenizer on ice-bath for 3 min, the homogenate was sonicated for 15 sec, and was centrifuged at 39,000 *g* for 30 min to obtain supernatant. The supernatant was treated with finely powered (NH₄)₂SO₄ at 60-70% ammonium sulfate saturation. After 30 min standing, it was centrifuged at 39,000 *g* for 15 min. The precipitate was then dissolved in 10 ml of 10 mM Tris buffer, pH 8.0 (buffer B) containing 0.5 mM EDTA, 0.5 mM PMSF, 1 mM DTT and 10% glycerol, and the suspension was centrifuged at 39,000 *g* for 30 min. Fifteen ml of the supernatant was obtained and it was dialysed against buffer B and was centrifuged at 100,000 *g* for 60 min.

The dialysate was loaded onto DEAE-Sephacel column (1.5×9 cm) which had been equilibrated with buffer B, and the column was washed with buffer B until no A280 absorbing material was eluted. Subsequently, the column was eluted with a linear gradient formed from 0 mM to 0.5 M NaCl in buffer B (pH 8.0), and the flow rate was 0.2 ml/min, and 2 ml of each fractions were collected. Those fractions showing methyl accepting activity were pooled, and the pooled sample was then loaded onto hydroxyapatite column (1.0×5 cm) previously equilibrated with 5 mM phosphate buffer (pH 8.0). The column was eluted with a linear gradient formed from 5 to 300 mM phosphate buffer with a fraction of 1 ml. The fractions showing methyl accepting activity were pooled and concentrated with 75% ammonium sulfate saturation.

After centrifugation, the supernatant was loaded onto Sephadex G-100 gel filtration chromatography column (1.5×65 cm). The flow rate was 0.02 ml/min and the fraction volume was 1 ml. The fraction with peak hemoglobin concentration was considered to be in the range of molecular weight of about 63 kDa. Before the Sephadex G-100 gel filtration chromatography, molecular weight standards (alcohol dehydrogenase, carbonic anhydrase, cytochrome c) were chromatographed and the fractions which shows peak protein concentration were determined.

Preparation of microsomal fraction

Human placental tissue was cut into small pieces and was suspended in 3 volumes of 0.25 M sucrose solution containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. It was then homogenized by Polytron homogenizer, and the homogenate was passed through 3 layers of

cheese-cloth. The homogenate was then centrifuged at 600 *g* for 10 min. The supernatant was centrifuged again at 100,000 *g* for 60 min and the precipitate was resuspended in the same buffer (18 mg/ml) and used as microsomal fraction.

Analysis of [methyl-³H]-labeled proteins by sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) and autofluorography

The chromatographic fractions (5 - 15 μ l) of methyl acceptor substrate protein were incubated with placental microsomal fractions (1 μ l), Ado[methyl-³H]Met (5 nmole, 0.125 Ci) and 1 μ l of 1 M Tris-HCl (pH 8.0) in a total 20 μ l of incubation mixture for 1 h at 37°C. For the study of the effects of guanine nucleotides or AFC, they were added to the reaction mixture with a concentration of 0.1 mM. After the reaction, the incubation mixture was mixed with 3 times modified Laemmli sample buffer and was electro-phoresed on 15 % polyacrylamide gel. The gels were stained with Coomassie brilliant blue. For fluorography, the destained gels were treated in dimethylsulfoxide (DMSO) for 1 h followed by treatment of DMSO containing 20% PPO (w/v) for 1 hr. The gels were dried and exposed to X-ray films at -70°C for 1-7 days.

Results

Purification of the 23 kDa methyl acceptor protein

In order to purify the 23 kDa methyl acceptor protein,

the human placental homogenate was fractionated by ammonium sulfate at 30 - 55% saturation, and the 23 kDa protein was purified by DEAE chromatography (Figure 1) and hydroxyapatite chromatography (Figure 2). After the two chromatographic procedures, the purified sample was loaded onto Sephadex G-100 gel filtration chromatography to estimate molecular weight. The estimated molecular weight of the preparation was about 29 kDa as shown in Figure 3.

Characterization of the methylation of the 23 kDa protein in human placenta

Methylation of the 23 kDa protein required the presence of placental microsome as a source of the methyltransferase, and Ado[methyl-³H]Met as the methyl donor. The methylation occurred in the absence of a soluble protein prenyl transferase, and no isoprenyl substrate such as geranylgeranyl pyrophosphate or mevalonate were added to the reaction mixture.

Although the 23 kDa protein was purified as a soluble protein in the absence of detergents, the ³H-methylated 23 kDa protein associated exclusively with the membrane pellet and not with the soluble proteins (Figure 4B). After the partial purification, the methylation of the 23 kDa protein was stimulated by the addition of GTP γ S (Figure 4A, 4B), suggesting that the 23 kDa protein could be a low molecular weight GTP-binding protein. AFC inhibited the methylation of the protein. This suggests that the methylation is C-terminal prenyl cysteine carboxyl methyl-ation (Figure 4A). The fact was also confirmed by vapor phase analysis (data not shown).

Figure 1. DEAE-Sephacel chromatography of human placental 23 kDa protein. Ammonium sulfate precipitate at 60-70% saturation was applied onto DEAE-Sephacel column (1.5X9 cm) previously equilibrated with 10 mM Tris buffer, pH 8.0 (buffer B) containing 0.5 mM EDTA, 0.5 mM PMSF, 1 mM DTT and 10% glycerol. Elution was carried out with a linear gradient formed from 0 to 0.5 M NaCl in buffer B (pH 8.0), and the flow rate was 0.2 ml/min, and 2 ml of each fractions were collected. The fractions were incubated with microsomal fraction ('Material and Methods') and Ado[methyl-³H]Met followed by SDS-PAGE and autofluorography to analyze the [methyl-³H]-labeled 23 kDa protein.

Figure 2. Hydroxyapatite chromatography of human placental 23 kDa. DEAE-Sephacel chromatographic fractions showing methyl accepting activity was pooled and applied onto hydroxyapatite column (1.0X5 cm) previously equilibrated with 5 mM phosphate buffer (pH 8.0) containing 1 mM DTT, 0.5 mM EDTA, 0.5 mM PMSF and 10% glycerol. Elution was carried out with a linear gradient formed with 5 - 300 mM phosphate buffer (pH 8.0). Assay was performed as described under 'Materials and Methods'.

Figure 3. Migration of the 23 kDa substrate protein on gel filtration. Partially purified substrate protein was applied onto Sephadex G-100 column (1.5 x 65 cm). The activity of the eluted fraction was determined and compared to the elution fractions for proteins of known molecular weight. The molecular weight markers were 1. cytochrome c, 2. carbonic anhydrase, 3. hemoglobin, 4. alcoholic dehydrogenase.

Effects of guanine nucleotides on the 23 kDa methylation

Mg^{2+} inhibited the methylation of the 23 kDa protein (Figure. 4A). GDP or GTP cannot stimulate the methylation of the 23 kDa protein in the presence of Mg^{2+} , but the methylation was stimulated by $GTP\gamma S$ even in the presence of Mg^{2+} . In the absence of Mg^{2+} , the methylation of the 23 kDa protein was stimulated by all the guanine nucleotides employed such as GDP, GTP

and $GTP\gamma S$ (Figure. 4A).

Discussion

A 23 kDa protein methylated by membrane-associated methyltransferase was partially purified from human placenta by DEAE chromatography, hydroxyapatite chromatography and gel filtration chromatography. The methylation of the 23 kDa was shown to be on C-terminal isoprenylated cysteine residue, and there is a significant fraction of the 23 kDa protein in the cytosol of human placenta which have previously underwent isoprenylation and proteolysis.

The 23 kDa protein methylated *in vitro* was almost exclusively associated with the membrane pellet (Figure 4B), suggesting that the methylated form of the 23 kDa protein has higher affinity for the membrane than the unmethylated form. This result is consistent with the other carboxylmethylated proteins, G25K or Ras. A report on G25K isolated from brain membranes indicated that the membrane-bound G25K was mostly methylated (Yamane and Fung, 1989). The *in vitro* methylation of G25K also enhanced the affinity for the membrane (Backlund, 1992). Evidence for the importance of methylation in membrane association has also been reported for Ras, where methylation increases the binding of p21 RasK to the membrane (Hancock *et al.*, 1990).

There were two reports to purify the carboxyl methylated proteins in rabbit brain and rat kidney. In those two studies, the carboxyl methylated protein was identified as G25K. In rabbit brain homogenates, the soluble G25K was isolated as a heterodimer of G25K and an 28 kDa protein (Backlund, 1992). The 28 kDa protein was iden-

Figure 4.A. Effect of Nucleotides and AFC on the 23 kDa protein methylation. Partially purified substrate protein was methylated by microsomal fraction in the absence or presence of 0.1 mM AFC, GDP, GTP, GTPγS as described under 'Materials and Methods'. **B.** After the methylation reaction of the partially purified substrate protein with or without GTPγS as described under 'Materials and Methods', the soluble fraction (S) was separated from the membrane fraction (P) by centrifugation at 12,000×g and the fractions were electrophoresed.

tified later as guanine nucleotide dissociation inhibitor protein, rhoGDI (Backlund, 1993). The G25K protein and rhoGDI co-migrated on gel filtration with molecular weight of 56 kDa. The interaction between G25K and the rhoGDI was also evident from the effect of the rhoGDI on G25K methylation. With free G25K, the guanine nucleotides, GDP, GTP and GTPγS, all stimulated methylation, indicating that the methylation reaction required a guanine nucleotide-bound form of G25K. In contrast, only GTPγS stimulated the methylation in case of the G25K/rhoGDI complex (Backlund, 1992).

In rat kidney cortex, there are two distinct peaks of carboxyl methylated 21-23 kDa proteins on Q-Sepharose anion exchange chromatography. Immunoblotting of these partially purified soluble substrates with a rabbit polyclonal antibody directed against CDC42 (a homolog of G25K) showed the presence of this protein in one of the peaks (Gingras and Beliveau, 1994). Both peaks eluted as proteins of apparent molecular sizes of 40 kDa upon Superose 6 gel-filtration chromatography, suggesting that the carboxyl methylated proteins are complexed with other soluble proteins.

Methylation of the partially purified 23 kDa substrate protein in human placenta is stimulated by GTPγS similar to the previously reported methylation of G25K. But the molecular size of the partially purified protein observed in this study on gel filtration was about 29 kDa, much less than those observed for both the G25K/rho GDI complex (56 kDa) in rabbit brain and 21-23 kDa substrate protein (40 kDa) in rat kidney. This suggests that the 23 kDa protein is either different from 21-23 kDa methyl acceptor molecules reported previously or do not complex with rhoGDI. But the 23 kDa protein could be associated with small molecules.

The effect of guanine nucleotides on the 23 kDa protein methylation in human placenta was different from those

reported previously. The G25K methylation was stimulated by GTPγS, GDP and GTP (Backlund, 1992). But the methylation of the 23 kDa protein which was purified without using detergent was stimulated only by GTPγS in the presence of Mg²⁺ and not by GTP or GDP. In the absence of Mg²⁺, the methylation of the 23 kDa protein was stimulated by all of the guanine nucleotides, GDP, GTP and GTPγS, similar to the effect on the G25K/rhoGDI complex (Backlund, 1992). These data suggest that the purified 23 kDa protein in human placenta is not associated with other protein such as rhoGDI, and the 23 kDa protein itself was not identical with G25K. The effect of Mg²⁺ on the other small G proteins such as Ras was not yet assessed, but the regulation of small G protein methylation could be changed by the intracellular concentration of the ions like Mg²⁺.

In the present study, a carboxyl methylated 23 kDa protein whose methylation was stimulated by GTPγS was partially purified and characterized. The effects of guanine nucleotides on the methylation of 23 kDa was different from those on G25K. In the absence of Mg²⁺, all of the guanine nucleotides GTP, GDP and GTPγS stimulated the methylation of the 23 kDa protein, but in the presence of Mg²⁺, only GTPγS stimulated the methylation, suggesting the role of Mg²⁺ in the modulation of the enzyme activity. The molecular size on gel filtration was different from those of carboxyl-methylation substrate proteins purified from rabbit brain or rat kidney together with the different effect of guanine nucleotides, thus the 23 kDa protein is suggested to be an another protein which was methylated on its C-terminal cysteine residue and a significant fraction of which had previously been isoprenylated and proteolytically degraded. The methylated substrate protein was redistributed to membrane after *in vitro* methylation, suggesting that the methylation of this protein is important in intracellular signaling by redistribution.

Recently mammalian prenylcysteine carboxyl methyltransferase gene was cloned (Dai *et al.*, 1998), so the role of these carboxyl methylated small G protein could be further elucidated.

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