Communication

Guanine Nucleotide-dependent Carboxyl Methylation of Mammalian Membrane Proteins*

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A guanine nucleotide-dependent protein carboxyl methylation is demonstrated in mammalian cell membranes. The methylation of membrane proteins of M, 20,000–23,000 requires S-adenosylmethionine, GTP or nonhydrolyzable GTP analogs, and a cytoplasmic methyltransferase. The protein methyl groups are stable at neutral pH and under basic conditions hydrolyze to produce methanol. The specific methyl acceptor proteins and methyltransferases varied between tissues and cell types, suggesting that these methylations have cell-specific functions. The guanine nucleotide-dependent carboxyl methylations provide a possible mechanism for regulating the function of GTP-binding membrane proteins in the transduction of receptor-mediated signals of eukaryotic cells.

Protein carboxyl methylation is a post-translational modification found in both prokaryotic and eukaryotic cells (1, 2). Carboxyl methylations are physiologically reversible protein modifications since methyl esters can be removed by hydrolysis to produce methanol. In bacteria, genetic and biochemical evidence demonstrates that the carboxyl methylation of specific glutamyl residues of membrane proteins is involved in regulating chemotaxis (3, 4). In eukaryotic cells, carboxyl methylation has been reported for a variety of proteins (5–11); however, a regulatory function analogous to the γ-glutamyl methylation involved in bacterial chemotaxis has not been demonstrated (1). Unlike the bacterial γ-glutamyl methyltransferase, the eukaryotic protein carboxyl methyltransferases that have been isolated have a broad protein substrate specificity (2, 12), and methylate D-aspartyl (13) and L-isoadaspartyl (13, 14) residues. Because of these properties, it has been proposed that the D-aspartyl/L-isoadaspartyl carboxyl methyltransferase functions in the repair of damaged proteins (1, 15).

In eukaryotic cells, receptor-mediated functions often involve interaction of receptors with guanine nucleotide-binding proteins, which couple the receptors to effector proteins involved in producing the cellular second messengers. Several families of guanine nucleotide-binding proteins have been characterized, including the G-protein (16) and ras-protein (17) families, and other GTP-binding protein families (18–20). These proteins are membrane-associated and are characterized by the specific binding of guanine nucleotides and by a GTPase activity. Clarke et al. (21) have recently demonstrated the carboxyl methylation of Ha-ras in transformed rat embryo fibroblasts. The methylation of Ha-ras was relatively base-stable and near stoichiometric, and the authors propose that the methyl ester may be at the carboxyl-terminal amino acid (21).

Here, we demonstrate that several membrane proteins can be carboxyl methylated by S-adenosylmethionine in an enzymatic reaction that is greatly stimulated by the presence of guanine nucleotides. The guanine nucleotide dependence and the physiologically reversible nature of carboxyl methylations suggest that these methylations may regulate the function of some guanine nucleotide-binding membrane proteins.

EXPERIMENTAL PROCEDURES

RAW264 cells were grown and the membranes isolated as described (22, 23). Rat brain and liver tissues were obtained from Pel-Freez Biologicals. Humanuffy-coat cells were obtained from normal volunteers, and the cell pellets were stored frozen until homogenization. Homogenization was carried out in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% 2-mercaptoethanol, 5 mM MgSO₄, 0.1 mM phenylmethylsulfonyl fluoride, and 10% sucrose. The samples were homogenized using a Potter-Elvehjem tissue grinder, and the homogenate was centrifuged for 10 min at 600 × g. The supernatant was collected and centrifuged for 45 min at 60,000 × g. The resulting supernatant was used as the source of cytosol proteins. The pellet was resuspended in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% 2-mercaptoethanol and used for the membrane proteins. The standard methylation reactions of 0.2 ml contained the following: 0.1 M sodium phosphate (pH 6.8), 1 mM EDTA, 30 μCi/ml [methyl-3H]S-adenosylmethionine (15 Ci/mmol, Du Pont-New England Nuclear), 1.5 mg/ml RAW264 membranes, and 1 mg/ml RAW264 cytosol. The reactions were incubated for 45 min at 37 °C. Membranes were then collected by centrifugation for 5 min at 15,000 × g. The cytosol proteins were precipitated from the supernatant by the addition of an equal volume of ice-cold 10% trichloroacetic acid. For extraction of the methyl acceptor protein from the membranes, the membrane pellets were resuspended in 0.1 M sodium phosphate (pH 6.8) with the addition of either 1% sodium cholate, 1% Nonident P-40, or 2M NaCl. The suspensions were incubated on ice for 20 min, and the residual membranes were collected by centrifugation for 5 min at 15,000 × g. The proteins were solubilized and were separated by SDS-polyacrylamide gel electrophoresis (24) using gels of 12.5% polyacrylamide. The radioactivity was detected by autoradiography.

The total GTPyS-dependent methylation of membrane proteins was determined by methylation of the membranes under the standard conditions except that the reaction time was increased to 90 min and the reactions were done in the absence or presence of 0.1 mM GTPyS. The membranes were then pelleted by centrifugation for 5 min at 15,000 × g, and the supernatant was removed. The membranes were resuspended in 0.5 ml of ice-cold 0.1 M sodium phosphate, pH 6.8, and the membranes were pelleted and resuspended two additional times. The final membrane pellet was solubilized in 50 μl of 0.1 M Tris, pH 6.8, and 2% SDS, and the radioactivity was determined by liquid scintillation spectrometry. The GTPyS-stimulated methylation was calculated as the difference between the incorporation of radioactivity in the presence and absence of GTPyS.

The methylated membrane proteins were eluted from the SDS-polyacrylamide gels, and the hydrolysis of the methyl esters was measured as described (4). The hydrolyzed methyl esters were reacted with 0.1 M HCl in a boiling water bath for 2 hr and were measured by the carboxyl content of the hydrolysate.

The abbreviations used are: SDS, sodium dodecyl sulfate; GTPyS, guanosine 5′-O-(3-thiotriphosphate); GDPβS, guanosine 5′-O-β-thio-diphosphate; p(NH)pG, guanyl-5′-yl imidodiphosphate; p(NH)ppA, adenylyl-5′-yl imidodiphosphate.

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with 3,5-dinitrobenzoyl chloride, and the radioactive product was recrystallized as described (25). Immune precipitation using the anti-
ras monoclonal antibody, Y13-259 (Oncogene Science), was carried out as described (26).

RESULTS AND DISCUSSION

Experiments to examine the methylation of membrane proteins were carried out using the macrophage cell line RAW264. The chemotactic and phagocytic properties of this cell line have been previously reported (22), and the cell line has been used to characterize the inhibition of chemotaxis by methylation inhibitors (27) and by bacterial toxin inhibitors of G-proteins (23, 28). The methylation of RAW264 membranes was determined by incubation of membranes with [methyl-3H]S-adenosylmethionine in the presence of the cytosol fraction from RAW264 cells, and the incorporation of radioactivity was determined after separation of the proteins by SDS-polyacrylamide gel electrophoresis. The addition of GTPγS to the reaction mixture greatly increased the radioactivity determined after separation of the proteins.

The nucleotide specificity for the methylation reaction was examined as shown in Fig. 2. The addition of ATP or the ATP analog, p(NH)ppA, had little effect on the methylation of membrane proteins. The addition of GTP partially stimulated the methylation of proteins of M, 23,000, 22,400, and 20,000, and the nonhydrolyzable GTP analogs, GTPγS and p(NH)ppG, had an even greater effect on the methylation. The addition of GDPβS was relatively ineffective in stimulating the proteinn methylation but did increase slightly the methylation of the M, 22,400 protein. The data indicate that the stimulation of methylation was specific for GTP or GTP analogs.

Methyl groups covalently modify proteins at several different amino acid residues by a variety of linkages (1,2). Protein carboxy methyl esters are characterized by their hydrolysis under basic conditions to produce methanol. Aspartyl-β-methyl esters are rapidly hydrolyzed at slightly basic pH, while glutamyl-γ-methyl esters are relatively more resistant to hydrolysis (29). The guanine nucleotide-dependent methylations observed after separation by SDS-polyacrylamide gel electrophoresis must be stable enough under mildly basic conditions to survive the pH of the polyacrylamide gel buffers. After separation by SDS-polyacrylamide gel electrophoresis, the major methylated proteins of M, 23,000 and 22,400 were extracted from the gel and the base hydrolysis of the methyl group examined. Incubation of the methylated protein overnight at pH 11 converted the radioactivity into a volatile product, which was shown to be methanol by reaction with 3,5-dinitrobenzoyl chloride to form the radioactive 3,5-dinitrobenzoic acid methyl ester (25) and recrystallization three times to a constant specific radioactivity. The rate of methyl ester hydrolysis was determined (Fig. 3), and a rapid rate of hydrolysis (t1/2 = 120 min) was observed only when the pH was increased to pH 11. The pH dependence for hydrolysis of the methylated M, 22,400 and 23,000 proteins indicates that the methyl ester is more stable than typical protein aspartyl-β-methyl esters (29) and the rate of hydrolysis of the methyl group is more consistent with either a glutamyl-γ-methyl ester (4, 29) or an α-carboxyl methyl ester (29).
were determined. The methylated proteins were incubated for gel electrophoresis. The major methylated proteins of the group. indicated times at 37 °C in the following buffers: Experimental Procedures and separated by SDS-polyacrylamide. 23,000 were eluted from the gel, and rates of methyl group hydrolysis acetate (pH 4.3 pmol of methyl groups/mg of membrane protein, with corresponding methyltransferases. An estimate of the abundance of the 23,000 protein. Assuming a stoichiometry of 1 mol of methyl group/mol of protein and a molecular weight of 23,000, about 0.01% (w/w) of the membrane proteins. This is a 20.1K - 14.4K - 67K - 94K - 30K - 12K - dye front -.0.1M sodium borate (pH 11.0). The initial protein bound methyl groups = 380 cpm.

RAW264 macrophage cells appeared to be an abundant source of both the methyl acceptor proteins and the corresponding methyltransferases. An estimate of the abundance of the methyl acceptor proteins in the RAW264 membranes was calculated by quantitation of the GTPγS-stimulated methylation as described under "Experimental Procedures." The GTPγS-stimulated methylation resulted in the addition of 4.3 pmol of methyl groups/mg of membrane protein, with the majority of that increase associated with the methylation of the 23,000 protein. Assuming a stoichiometry of 1 mol of methyl group/mol of protein and a molecular weight of 23,000, the GTPγS-stimulated methyl acceptor proteins make up about 0.01% (w/w) of the membrane proteins. This is a minimum estimate, since the proteins may be partially methylated in vitro and the methyl transfer reaction may not be complete. Several guanine nucleotide-binding proteins have been purified, and there is a large range in the levels present in membranes. In brain, Gα is approximately 1% of the total membrane proteins (30), and the ADP-ribosylation factor is 0.2% of the protein in cholate extracts of the membranes (31). In fibroblasts transformed with viral H-ras, H-ras comprised 0.15% of the membrane protein; however, in nontransformed fibroblasts, the level of cellular ras expression is much lower (32).

The ras proteins are membrane proteins which bind guanine nucleotides and have molecular weights in the range of 20,000–24,000. Since the methylation of ras has been recently reported (21), it was of interest to determine if the guanine nucleotide-dependent methyl acceptor proteins belong to the ras family. The monoclonal antibody Y13-259 known to immune precipitate K-ras, H-ras, and N-ras (26) was used to immune precipitate the methylated membrane proteins. None of the methylated membrane proteins from RAW264 cells were immune precipitated with the Y13-259 antibody. Therefore, the guanine nucleotide-dependent methyl acceptor proteins observed here are distinct from the ras family. Methylation of ras may still occur in RAW264 cells; however, the low level of ras expected in the RAW264 membranes would make it difficult to detect its methylation under these conditions. This is consistent with the report on ras methylation in transformed rat embryo fibroblasts, where in control fibroblasts the level of ras expression was too low to detect any methylation (21).

Different tissues and cell types were examined in order to determine if the methyltransferases were localized to specific cells. The cytosol fractions from several sources were incubated with RAW264 membranes in the presence of [methyl-3H]S-adenosylmethionine, and the methylation of membrane proteins was determined in the absence or presence of GTPγS (Fig. 4). Humanuffy-coat cells were an abundant source of guanine nucleotide-dependent methyltransferases. With theuffy-coat cell cytosol, the methylation of M, 23,000 and 22,400 proteins was observed, along with the methylation of a third protein of M, 22,000. This additional methylated band may be due to the presence of an additional methyltransferase or may be due to modifications of the methyl acceptor proteins that alter their mobility on the SDS-polyacrylamide gels. Purification of the methyltransferases and methyl acceptor proteins is currently in progress to differentiate between these possibilities. Like RAW264 cells, theuffy-coat cells also had methyltransferase activity for a M, 20,000 protein. The cytosol from liver and brain also contained some guanine nucleotide-dependent methyltransferase activity, although the activity was not as great as found in buffy-coat cells or RAW264 cells.
In contrast, HeLa cells lacked the guanine nucleotide-dependent methyltransferase for any of the membrane proteins. Therefore, it appears that there is specificity in the methyltransferase present in different cell types.

The high level of methyltransferase activity in both RAW264 and buffy-coat cells suggests that carboxyl methylation of the M, 22,400 and 23,000 proteins may have a role in myeloid cell functions. The methylation reactions that we report here are distinguished from previously reported methylations by their sensitivity to guanine nucleotide stimulation. The substrates for the methylations are membrane-associated, and different methyltransferases are present in different tissues. The effect of guanine nucleotides on the methylation reaction suggests that activation of a guanine nucleotide-binding membrane protein may stimulate carboxyl methylation of the protein, resulting in an alteration of the signal transduction functions. These properties indicate that the guanine nucleotide-dependent protein carboxyl methylations may play an important role in the regulation of specific cellular receptor systems. In addition, the methylation reaction we describe may provide a method to identify and quantitate a novel family of guanine nucleotide-binding membrane proteins, in a manner analogous with the use of bacterial toxins to characterize the G-protein family by ADP-ribosylation.

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