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Prenylated protein methyltransferase of rat cerebellum is developmentally co-expressed with its substrates

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High levels of prenylated protein methyltransferase are expressed in the developing rat cerebellum and are responsible for methylation of endogenous G-proteins and 50–52 kDa synaptosomal proteins. Enzyme activity in cerebellar synaptosomes of 3 week postnatal rats is 2-fold higher than that found in adult rat cerebellum. A 10-fold rise in activity occurs at the end of the second and during the third postnatal weeks, followed by a subsequent decline. Expression of the enzymes' substrates follows the same pattern. The high methyltransferase activity in 3-week-old cerebellum coincides with the period of granule cell migration and synaptogenesis, suggesting a regulatory role for the enzyme and its substrates in cerebellar ontogenesis.

Isoprenylated protein methylation; G-protein; Cerebellar ontogenesis

1. INTRODUCTION

The C-terminal cysteine of mature ras and ras-related GTP-binding proteins has a thioether-linked prenyl group [1,2] and is modified by the membrane-bound [3–5] prenylated protein carboxyl methyltransferase (PPMTase) [6–8]. Methylation is the only reversible modification [9] in a sequence of biochemical reactions that lead to maturation of these proteins [1,2], thus suggesting a regulatory role for the PPMTase. Recent studies demonstrated that the enzyme recognizes hexapeptides with a C-terminal-farensylated cysteine [3] and simple substrates such as *N*-acetyl-*S*-farnesyl cysteine [7,8]. Use of such substrates enabled quantitative determination of the PPMTase in rat liver [3], brain [8] and in bovine rod outer segments [7]. We have previously characterized the enzyme and its endogenous GTP-binding protein substrates in neuroblastoma N1E-115 and in pheochromocytoma PC-12 cells [5,10–12]. Neurite-like outgrowth in both cell lines was found to coincide with a marked and persistent increase in methylation of GTP-binding proteins, suggesting that development and maintenance of neuronal cells requires substantial expression of the PPMTase. The present study characterizes the PPMTase of rat cerebellum and its substrates and demonstrates their marked coordinated rise during the critical period of cerebellar granule cell migration and synaptogenesis.

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Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AFC, *N*-acetyl-*S*-farnesyl cysteine; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; FTA, *S*-farnesyl thioacetic acid; PPMTase, prenylated protein methyltransferase; SDS, sodium dodecyl sulfate; Tris, Tris[hydroxymethyl]amino methane.

2. MATERIALS AND METHODS

[methyl-^{3}H]*S*-Adenosyl-L-methionine ([methyl-^{3}H]AdoMet, 15 Ci/mmol) and [α-^{32}P]GTP (approximately 3,000 Ci/mmol) were purchased from New England Nucler. *S*-Adenosyl-L-homocysteine and AdoMet were from Sigma Chemical Co. AFC and FTA were prepared, purified and tested for purity by NMR analysis as detailed elsewhere [7]. Purity of the substances used here was >95%. Charles River adult male rats and new borns were grown as detailed previously [13]. Cerebella were removed after decapitation, and homogenized in 0.32 M sucrose containing 50 mM Tris-HCl, pH 7.4, 3 mM EDTA, 1 mM EGTA, 5 U/ml aprotinin and 5 μg/ml pepstatin to yield 10% (w/v) homogenates. Nuclear fractions were obtained by 10 min 600 × *g* centrifugation, synaptosomal fractions by 20 min 14,500 × *g* centrifugation and microsomal fractions by 50 min 100,000 × *g* centrifugation steps. Pellets were resuspended in the homogenization buffer and stored at −70°C. Methyltransferase assays were performed at 37°C in 50 mM Tris HCl buffer, pH 7.4, using 75–125 μg protein, 25 μM [methyl-^{3}H]AdoMet (300,000 cpm/nmol) and 150 μM AFC (prepared as a stock solution in DMSO) in a total volume of 50 μl. DMSO concentration in all assays was 4%. Various protein and AFC, or [methyl-^{3}H]AdoMet concentration were used in several experiments as indicated in the text. Reactions were terminated after 10 min by adding 500 μl chloroform methanol (1:1) and a subsequent addition of 250 μl H_2O, mixing and phase separation. A portion of 125 μl of the chloroform phase was dried down at 40°C and 200 μl of 1 N NaOH/1% SDS solution added. The [^{3}H]methanol thus formed was counted by the vapor phase equilibrium method as detailed previously [5,11]. Typical background counts (no AFC added) were 50–100 cpm, while typical reactions with AFC yielded 500–6,000 cpm. Assays were performed in triplicate and background counts subtracted. Methyla-

tion of endogenous substrates, α-^{32}P blot overlay assays and gel electrophoreses were performed as detailed previously [10–12].

3. RESULTS

Subcellular fractions of adult rat cerebellum were assayed for PPMTase activity with saturating concentrations of AFC (150 μM) and [methyl-^{3}H]AdoMet (25 μM). Comparable enzyme activaties were detected in the nuclear, synaptosomal and microsomal fractions (respectively, 39.5, 28.5 and 30.9 pmol AFC-[methyl-^{3}H]ester formed/min/mg protein). No activity was detected in the cytosol. Methylation of AFC was linear with time for at least 10 min and with protein (up to 125 μg). Substrate and methyl donor curves of the synaptosomal membrane enzyme, yielded typical Michaelis–Menten kinetics with K_m values of 27 μM for AFC and of 2.2 μM for [methyl-^{3}H]AdoMet. Methylation of AFC was strongly inhibited (>90%) by 200 μM *S*-adenosyl-L-homocysteine, a competitive inhibitor with respect to AdoMet [14], and by 200 μM *S*-farnesyl thioacetic acid (FTA) (>80%), a competitive inhibitor with respect to AFC [14]. Inhibition curves with the latter enabled estimation of its inhibition constant (K_i = 2.8 μM). Carboxymethylation of endogenous synaptosomal proteins, with apparent M_r's of 50–52 kDa and 20–30 kDa, was also inhibited by FTA (Fig. 1). Proteins in the first group are unique to the cerebellum [15], while those in the second group are the ubiquitous methylated small GTP-binding proteins [16] (Fig. 1, right panel). Methylation of a third group of proteins (M_r 33–36 kDa) was not blocked by FTA (Fig. 1), suggesting that, unlike the former ones, they are not substrates for the PPMTase.

The developmental pattern of PPMTase activity in the cerebellum of postnatal rats is shown in Fig. 2. Enzyme activity was relatively low on postnatal day 7,

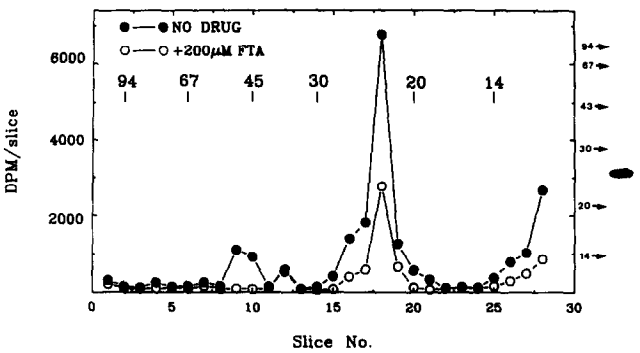

Fig. 1. Carboxyl methylation of proteins in synaptosomes of adult rat cerebellum. Synaptosomal membranes (200 μg) were methylated using 25 μM [methyl-^{3}H]AdoMet (15 Ci/mmol) in the presence (●) or in the absence (○) of 200 μM FTA, and protein-[methyl-^{3}H]esters determined after electrophoretic separation in 12.5% SDS-polyacrylamide gels as detailed previously [11]. Note that methylation of the 50–52 kDa proteins and the GTP-binding proteins, but not of the 33–36 kDa proteins, was inhibited by FTA. (Right panel) Autoradiogram of an [α-^{32}]GTP blot overlay of the separated proteins. Data are of one out of 3 experiments that yielded similar results.

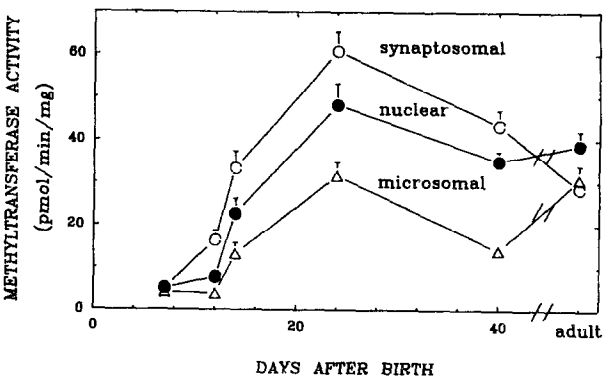

Fig. 2. Prenylated protein methyltransferase activity during the development of the rat cerebellum. Methyltransferase assays were performed as detailed in methods using 75–125 μg of nuclear (●), synaptosomal (○), or microsomal (△) membrane proteins prepared from cerebella of rats of the indicated postnatal days. Data represent the mean values ± S.D. (vertical bars) of three separate experiments (means of 2 separate determinations).

increased significantly during the second postnatal week (3- to 4-fold increase) and continued to rise sharply during the third postnatal week, reaching peak values on day 24, where activities were more then 10-fold higher as compared to those observed on day 7. During these developmental periods PPMTase specific activity was higher in the synaptosomal fractions as compared to that of the nuclear and microsomal fractions. As seen in Fig. 2, on day 24, enzyme specific activities in the nuclear and in the microsomal fractions were either similar to or about 20% higher than those observed in the corresponding fractions of the adult cerebellum. In contrast, specific activity of the PPMTase in the synaptosomal fraction declined sharply after the third postnatal week, resulting in a 50% lower activity in the adult cerebellar synaptosomes.

Alterations in synaptosomal protein substrates of the PPMTase during postnatal cerebellar development (Fig. 3) resembled those of the enzyme (Fig. 2). As shown, levels of methylated GTP-binding proteins and of the 50–52 kDa proteins increased during the second and third weeks after birth and declined thereafter. In contrast, levels of the 33–36 kDa methylated proteins were high on days 7 and 12 and declined during the time period of PPMTase activity increase (Fig. 3).

4. DISCUSSION

The PPMTase of the rat cerebellum described here has properties similar to the enzymes found in bovine rod outer segments [7], whole mouse and rat brains [4,8] and in N1E-115 [5,10] and PC-12 [11,12] cells. Namely, the enzyme is bound to the membranes and methylates the prenylated cysteine analog AFC, as well as endogenous G-proteins (Fig. 1) known to be prenylated [1,2,17]. Consistent with the role of G-proteins in cytoskeletal organization, vesicular transport and signal

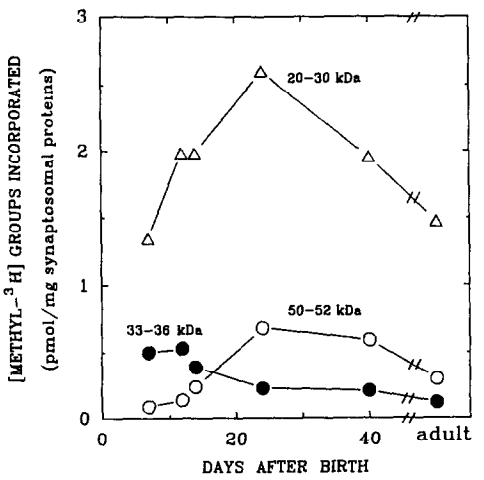

Fig. 3. Methylation of synaptosomal proteins during the development of the rat cerebellum. Synaptosomal membranes of cerebella from rats of the indicated postnatal days were methylated and protein-[methyl-^{3}H]esters determined as detailed in Fig. 1. Data present the amount of [methyl-^{3}H]esters formed in the 50–52 kDa, the 33–36 kDa and 20–30 kDa proteins as a function of age.

transduction [16], cerebellar methyltransferase activity is found in all sub-cellular membrane fractions but not in the cytosol.

Expression of the cerebellar PPMTase is developmentally regulated. At the end of the second and during the third postnatal weeks, enzyme levels rise sharply in all membrane fractions. This is followed by a decline, reaching the activity levels found in the adult cerebellum. This decline is particularly marked in the synaptosomal fraction. Activity of the synaptosomal PPMTase appears to be well coordinated with its specific endogenous substrates (Fig. 2), which are small GTP-binding proteins and the 50–52 kDa proteins (Figs. 1 and 3). Recent studies showed that the latter are uniquely expressed in the cerebellum [15], which is also the brain region with the highest PPMTase activity [18]. The present results suggest that the 50–52 kDa proteins and small GTP-binding proteins may play a significant role in the developing cerebellum and warrants efforts towards their identification. It is important to note that the period in which the highest PPMTase activity and substrates levels are expressed in the rat cerebellum coincides with the period of time characterized by massive granule cell migration and synaptogenesis [19]. This coincidence is similar to that observed in cultured PC-12 and N1E-115 cells [5,10–12], and strengthens the hypothesis that methylation of ras and of other prenyl cysteine-containing proteins may be necessary for cell migration, neurite outgrowth and synapse formation.

The present observations raise several interesting questions. (i) Is the expression of other enzymes involved in G-protein maturation (i.e. protein prenyl transferases) also developmentally regulated? (ii) Is there a cerebellum-specific prenylated protein methylesterase? (iii) Which types of small GTP-binding proteins are expressed at high levels particularly during granule cell migration? (iv) Are the unique synaptosomal 50–52 kDa protein substrates for the methyltransferase involved in granule cell migration and synaptogenesis? (v) Would selective protein methylation inhibitors, such as FTA, interfere with the postnatal development of the rat cerebellum? Since the necessary experimental tools are now available, such questions can be answered quite rapidly.

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