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STATHMIN EXPRESSION IN THE PLACENTA AND EMBRYONIC BRAIN

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ABSTRACT. Stathmin is a member of a family of proteins believed to play important roles in neural development. In the current study, expression of stathmin forms in rat brain was examined from day 15 of gestation through the neonatal period and into adulthood. Placental stathmin also was analyzed. Unphosphorylated and phosphorylated forms of stathmin in the brain are highest in the late fetal and early neonatal period. Placenta expresses some larger stathmin-like proteins, but none of the 19 kilodalton forms of stathmin. The results show that stathmin is greatly elevated during a period of rapid brain growth and development, suggesting that stathmin plays a regulatory role in development. The functional significance of the unique pattern of stathmin-like proteins expressed by the placenta is unclear at this time.

Keywords: Stathmin, rat, brain, development, placenta

Stathmin is a cytosolic phosphoprotein believed to be involved in intracellular signaling (Sobel 1991). Though found in almost all tissues, stathmin is expressed most highly in the nervous system (Amat et al. 1991; Doye et al. 1992; Koppel et al. 1990; Maucuer et al. 1993; Schubart 1988; Sugiura & Mora 1995). It is a member of a family of proteins that in mammals includes SCG10 (Anderson & Axel 1985; Stein et al. 1988), SCG10-like protein (SCLIP) (Ozon et al. 1998) and RB3 (Ozon et al. 1997). These latter family members are almost exclusively expressed in neural tissue (Ozon et al. 1997; Ozon et al. 1998). The expression of stathmin and family members in neural tissue supports a regulatory role for these proteins in development of the nervous system. Though it is likely that members of the stathmin family have overlapping functions, they probably play distinct regulatory roles in neural development, given their differences in distribution within the nervous system (Ozon et al. 1997; Ozon et al. 1998), developmental expression (Amat et al. 1991; Anderson & Axel 1985; Doye et al. 1992; Koppel et al. 1990; Maucuer et al. 1993; Ozon et al. 1997; Ozon et al. 1998; Schubart 1988; Sugiura & Mora 1995) and cytosolic vs. membrane localization (Anderson & Axel 1985; Di Paolo et al. 1997; Ozon et al. 1997; Ozon et al. 1998; Sobel 1991).

Several reports (Amat et al. 1991; Doye et al. 1992; Koppel et al. 1990; Maucuer et al. 1993; Schubart 1988; Sugiura & Mora 1995) have suggested that stathmin expression in the brain is high in the late embryonic and early neonatal period, and then declines into adulthood. Ozon et al. (1998) have demonstrated that expression of stathmin mRNA increases in the rat brain from day 16 of gestation to a peak in the early neonatal period and then decreases toward adulthood. The study also showed that expression of SCG10 mRNA followed a similar developmental pattern in the brain. In contrast, SCLIP and RB3 mRNAs did not decline after the neonatal period.

In the current study, we have examined the forms of stathmin expressed in the brains of embryonic, neonatal and pregnant-adult rats and in rat placenta. The data confirm that stathmin is highest in the late embryonic and early neonatal period. Furthermore, the data suggest that the placenta expresses larger stathmin-like proteins, but few if any 19 kilodalton (kDa) forms of stathmin.

METHODS

Animals/tissues.—Tissues were obtained from Sprague-Dawley rats. Adult animals weighed between 180–300 g. Brains from a minimum of five separate animals were pooled for studies of fetal and neonatal stathmin. The 10-day preparation was prepared from multiple placenta-fetus samples taken from two different pregnant rats. All preparations from adult animals involved tissues obtained from at least two separate animals.

Anti-stathmin antibody.—The anti-stathmin antiserum was generated in rabbits against amino-acid residues 32–44 (VPEFPL SPPKKKD) of rat stathmin (Flurkey et al. 1993; Meyer et. al. 1992). This sequence is highly conserved among mammalian stathmins, but it is less conserved among the stathmin family members SCG10 (EAPRT-LASPKKKD), SLIP (PESPVLSSPPKRKD) and RB3 (VPEFNASLPRRRD). The carboxy-terminal five amino acids of the fragment used to generate the antibody are shared by stathmin and SCG10, so some cross-reactivity is possible. In the immunostaining procedure, the antibody was used at a concentration of 1:5000.

Stathmin extraction.—Whole rat brains were suspended in 3 volumes of an ice-cold extraction buffer consisting of 100 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and 0.02% sodium azide at pH 7.0. The suspension was sonicated using three 5-sec bursts at a setting of 2.3 on a Branson Sonifier (Branson Ultrasonic Corp., Danbury, Connecticut). The suspension was centrifuged at 800 × g at 4° C for 4 min, and the supernatant fraction was transferred to a 1.5 mL microfuge tube. Sodium chloride (1 M, in 100 mM Tris-HCl, pH 7.0) was added to a final concentration of 100 mM and the solution was heated to 100° C for 10 min. The tubes were placed on ice for 5 min and centrifuged at 12,000 × g for 5 min, and the stathmin-enriched supernatant fraction was transferred to a clean 1.5 mL microfuge tube. Protein was measured using a colorimetric assay (BCA Protein Assay, Pierce, Rockford, Illinois).

Electrophoresis.—Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli, 1970) on 12% polyacrylamide gels (Bio-Rad Laboratories). Samples (35–70 μg protein) were combined with an equal volume of 2× SDS sample buffer (0.125 M Tris-HCl, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.05% bromophenol blue, pH 6.8) and heated to 100° C for 10 min. Electrophoresis was run at a constant current of 55 mA for approximately 4 h. Proteins in the gels were electrophoretically transferred (60 V, 1 h) to nitrocellulose paper (0.22 μm) using a Trans-Blot Cell (Bio-Rad Laboratories) (Towbin et al. 1979). Samples frozen at −70° C were reanalyzed within 2–3 weeks to confirm results.

Immunostaining.—Open sites on the nitrocellulose paper were blocked by incubating the paper in binding buffer (100 mM Tris-HCl, 0.15 M NaCl, 0.1% BSA, pH 8.0) containing 5% non-fat dry milk. After three 10-min washes in binding buffer, the nitrocellulose was incubated for 22 h in binding buffer containing the anti-stathmin antiserum (1:5000) (Flurkey et al. 1993; Meyer et. al. 1992). Unbound antibody was removed by three 10-min washes in binding buffer, and the nitrocellulose was incubated for 1 h with a goat anti-rabbit gamma globulin coupled to alkaline phosphatase (Sigma Chemical Company, St. Louis, Missouri). Unbound antibody was removed by three 10-min washes in binding buffer, and the alkaline phosphatase was detected using the BCIP/NBT (Pierce Chemical Co., Rockford Illinois).

Density analysis.—Immunostained bands were scanned using an Arcus II scanner and were quantified using the ONE-Dscan program (Scanalytics). Relative stathmin units were calculated by comparing the optical densities of the samples to those of aliquots (5 μl, 7.5 μl and 15 μl) of purified stathmin.

RESULTS

The results show that brain stathmin levels increase from late gestation to the early neonatal period and then decline in older animals (Fig. 1). High levels were present in brains obtained from rats at 2, 5 and 10 days after parturition. Most of the stathmin migrated with a molecular weight of approximately 19,000 (Fig. 1), consistent with unphosphorylated and less-phosphorylated forms of stathmin. However, higher molecular-weight forms (23–24 kDa), consistent with highly phosphorylated stathmin, were observed in brains taken from all fetal and neonatal rats. Lower stathmin levels were found in extracts prepared using brains obtained from nulliparous adult rats and higher molecular-weight forms were not detected (Fig. 1).

No 19 kDa forms of stathmin were detected in placental/fetal tissue from a 10-day pregnant rat. A single 23 kDa protein was detected using the antibody. Fetal and placental tissues could not be reliably separated at this stage of gestation, so the preparation contained elements of both. However, the high molecular

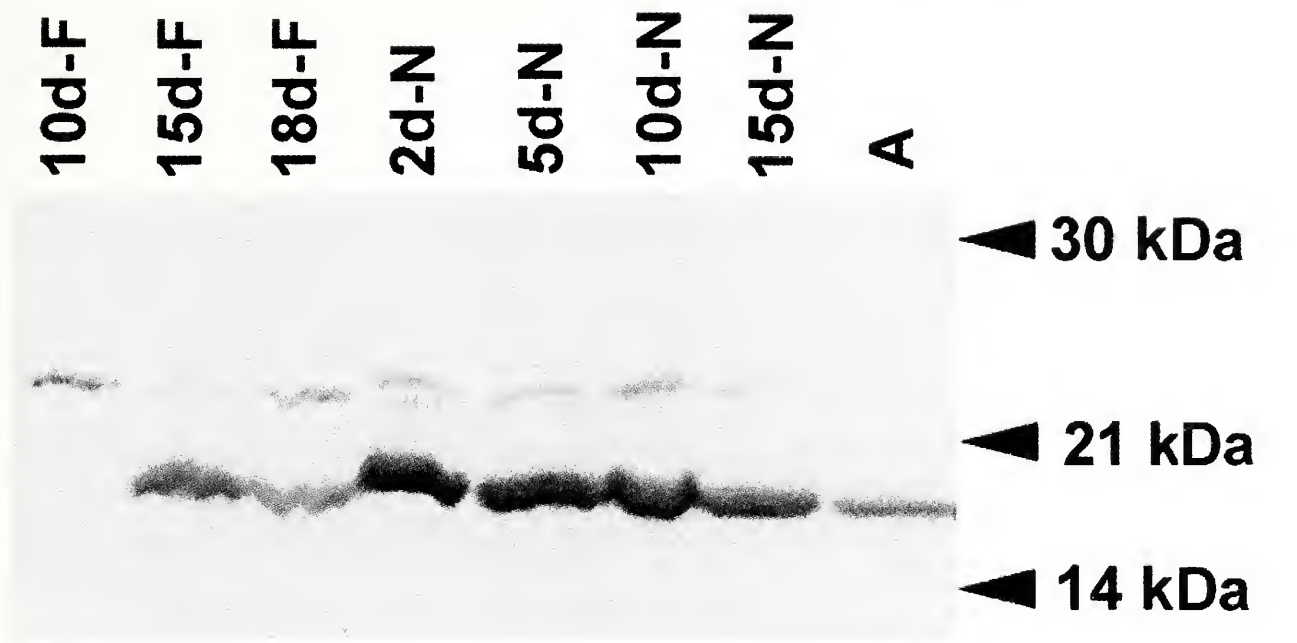

Figure 1.—Extracts (70 μg protein) from rat brain and placenta were analyzed by SDS-PAGE and immunostaining. Samples analyzed were placenta and fetus at 10-days gestation (10d-F)., brains from fetuses at 15 (15d-F) and 18 (18d-F) days of gestation, brains from neonates at 2 (2d-F), 5 (5d-F), 10 (10d-F) and 15 (15d-F) days after parturition, and brain from an adult female (A). Each fetal and neonatal sample represents a minimum of five brains. The numbers at right show the positions of MW standards.

weight protein is likely of placental origin, because placenta accounted for most of the tissue at this stage.

Stathmin was present at a high concentration in brains obtained from fetal rats at 20-days gestation, the expected day of parturition (Fig. 2). Immunostaining of proteins from the 20-day fetus was considerably greater than that of proteins from the 10-day placental/fetal preparation, a preparation that also is presented in Fig. 1 for comparison. Based on its staining relative to the 10-day placental/fetal preparation, the 20-day fetal brain (Fig. 2) contained stathmin at levels comparable to those found in the 2-day neonatal animal (Fig. 1). Higher molecular-weight forms of stathmin in the 20-day fetal brain also were similar to those detected in the brain of the 2 day neonate.

Brain from a 20-day pregnant rat showed almost equal amounts of immunostaining at 19 and 23 kDa (Fig. 2). The appearance of a 23 kDa band is interesting, because no comparable protein was detected in the adult nulliparous animals. The 23 kDa protein appeared to correspond with one of the higher molecular-weight proteins immunostained in extracts from the 20-day fetal brain, 20-day placenta and the 10-day placenta/fetus. The extract from the 20-day placenta did not show evidence of 19 kDa forms of stathmin, in agreement with results obtained with the 10-day placenta/fetus. A 30 kDa protein also was identified in the 20-day placenta.

The immunostained bands in Fig. 2 were analyzed using the ONE-Dscan program (Scanalytics) to obtain an estimate of integrated optical density. The optical densities were then compared to those of different amounts (5 μl, 7.5 μl and 15 μl) of a purified stathmin (Fig. 2), so that staining could be expressed as arbitrary stathmin units. As shown in Table 1, brain tissue from the 20-day fetus contained approximately 10 times as much stathmin-like substance, most of which migrated as a 19 kDa protein(s).

DISCUSSION

The data show that expression of stathmin protein in the rat brain is highest in the late fetal and early neonatal period and then declines into adulthood. In fact, brain stathmin peaks in the period encompassed by day 20 of gestation to day 2 after parturition. This developmental pattern closely parallels that reported (Ozon et al. 1998) for expression of stathmin mRNA in rat brain. The results also are consistent with data from earlier studies (Amat et al. 1991; Doye et al. 1992; Koppel et al. 1990; Maucuer et al. 1993; Schubart

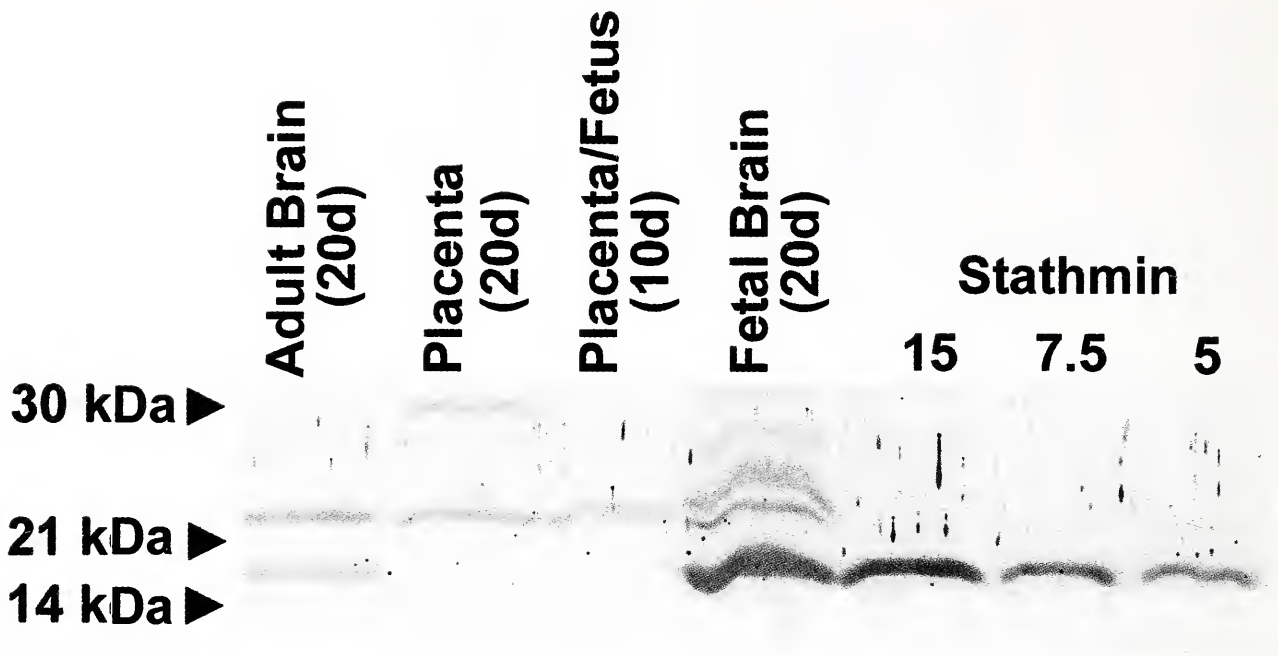

Figure 2.—Extracts (35 μg protein) from rat brain and placenta were analyzed by SDS-PAGE and immunostaining. Samples analyzed were brain (Adult Brain 20d) and placenta (Placenta 20d) from a 20-day pregnant rat, placenta and fetus at 10-days gestation (Placenta/Fetus 10d), brains from fetuses at 20-days gestation (Fetal Brain 20d), and 15 μl, 7.5 μl and 5 μl of a purified stathmin preparation. The numbers at left show the positions of MW standards.

1988; Sugiura & Mora 1995) showing higher levels of stathmin in brains from neonatal animals.

Larger (> 19 kDa) stathmin-like proteins were identified in nearly all brain extracts. Most of these forms probably represent phosphorylated stathmin, because it has been demonstrated previously (Beretta et al. 1993; Cardinaux et al. 1997; Chneiweiss et al. 1989; Chneiweiss et al. 1992; Doye et al. 1992) that brain contains phosphorylated forms of stathmin that migrate with apparent molecular weights of 21,000–25,000. Though larger forms were not detected in a nulliparous adult female rat, the phosphorylated forms likely were present, but at low levels. A 30 kDa form(s) identified in placenta from a 20-day pregnant rat is larger than would be expected for phosphorylated stathmin. This form might represent one of the stathmin-related proteins such as SCG10, SLIP or RB3. The most likely candidate would be SCG10 since this protein shares some homology with stathmin in the region of the molecule used to generate the anti-stathmin antibody (please see Methods), and SCG10 is present at a relatively high concentration in brain. However, SCG10 usually migrates with an apparent molecular weight less than 30,000 (Ozon et al. 1997). Using an anti-RB3 antiserum, Ozon et. al. (1997) reported unidentified "X" proteins that migrate with an apparent molecular weight of 30,000. Hence, the 30 kDa protein could be a yet unidentified stathmin-like protein, perhaps related to RB3.

No 19 kDa stathmin forms were detected in

Table 1.—Stathmin in brain and placenta.

Tissue	Stathmin forms (MW, kDa)	Stathmin amount (relative units)[1]
Brain		
20 Day Pregnant	19	1.8
	23	1.4
	19	17.6
	23	1.4
	24	0.9
20 Day Fetus	27	0.0[2]
Placenta		
	23	0.8
20 Day	30	1.3
10 Day	23	0.8

[1] Units expressed relative to immunostaining of a purified stathmin (see Methods).

[2] Band could not be accurately quantified by the ONE-Dscan program (Scanalytics).

placenta. The smallest form detected in placental tissue obtained at 10 and 20 days of gestation was approximately 23 kDa. Interestingly, a similar-sized protein was prominent in the brain obtained from a 20-day pregnant animal. The dearth of 19 kDa forms of stathmin in placenta may suggest that placental enzymes actively phosphorylate stathmin. Alternatively, the protein immunostained at 23 kDa may represent SCG10 or other stathmin-related protein. In any case, it is remarkable that a tissue undergoing growth and development contains so little unphosphorylated stathmin.

In summary, the results show that expression of stathmin in brain is highest close to the time of parturition. The data also show that the pattern of "stathmin" forms expressed by placental tissue differs from that in brain.

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