Developmental Tissue Expression and Phylogenetic Conservation of Stathmin, a Phosphoprotein Associated with Cell Regulations*

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Stathmin is a 19-kDa phosphoprotein presumably involved in regulations of cell proliferation, differentiation, and functions as an intracellular relay for extracellular signals activating diverse second messenger pathways. Antisera prepared against the whole protein or against two peptides (residues 15–27 and 134–149) recognized the two isoforms (α and β) of stathmin in their different phosphorylated states on immunoblots. Also, the possible existence of a family of stathmin-related proteins is suggested by the detection with some sera of proteins of 17, 21, and 60 kDa in brain.

Stathmin and its diverse molecular forms were detected in all mouse tissues tested, in varying concentrations. Depending on the tissue, it is 2–100 times more abundant in the neonate than in the adult. It is most abundant in brain at both developmental stages, the protein levels being paralleled by the expression of the corresponding mRNA as detected with a specific cDNA probe. Antibodies directed against the rat protein also reacted with stathmin-like proteins in the brain of other mammals, birds, reptiles, amphibians, and some fish species, and the various isoforms could be recognized on immunoblots. In conclusion, our results suggest that stathmin is most likely involved in two distinct types of regulations: 1) "developmental" regulations, related to cell proliferation, differentiation, and maturation, and 2) "functional" regulations mostly at the adult stage, and typically in the nervous system. In addition, stathmin is also phylogenetically well conserved at least in vertebrates. Together, these observations support the proposed ubiquitous nature and general importance of stathmin in biological regulations.

Protein phosphorylation-dephosphorylation is a major intracellular regulatory mechanism triggered by the generation of second messengers in response to extracellular signals (reviewed in Ref. 1). A set of soluble phosphoproteins (proteins "1–16") was identified in our laboratory in diverse biological systems using two-dimensional polyacrylamide gel electrophoresis (PAGE)2. Among them we recently proposed stathmin (originally described as protein "7–8") to be a ubiquitous intracellular relay, possibly integrating diverse second messenger signals triggered by various extracellular agents (7). Proteins most likely identical to stathmin have been identified by others in various cell types (reviewed in Ref. 7), in particular P19 (8–10), pp17 or prosolin (11, 12), and p18 (13). Phosphorylation of stathmin could be related in various instances to the regulation of cell proliferation (3, 14, 15), differentiation (6), and functions (2, 4, 5, 8, 16).

Stathmin (Mr ≈ 19,000) is highly abundant and a major phosphoprotein substrate in neurons (16), which allowed its purification from bovine and rat brain (7, 9). Its expression in brain is maximal at the neonatal period (10, 16), a developmental profile also described for its mRNA (10, 17).

In the various tissues and cell types, stathmin is present as several isoelectric variants (pI ≈ 6.2–3.6) resolved by two-dimensional PAGE (7, 8, 12). We showed recently that they reflect the existence of two distinct isoforms, α and β, in their basal and increasingly phosphorylated states (18). A single cloned cDNA codes for both isoforms of stathmin, which differ only by posttranslational modifications (17). Its sequence displays a strong homology (19) with the developmentally regulated neuronal protein SCG10 (20, 21).

In this study, we present a detailed analysis of the distribution of stathmin in mouse tissues. We also demonstrate the higher expression of stathmin in all tissues at earlier stages of development. We show finally that stathmin is well conserved throughout the evolution of vertebrates.

EXPERIMENTAL PROCEDURES

Materials

Male Wistar rats were from Iffa-Credo (France), Swiss mice from the local breeding facility, and male Fauve de Bourgogne rabbits from CEBGA (France). Frozen brain samples from postmortem humans was kindly provided by Dr. P. Javoy-Agid (Paris, France), from frog (Rana) by Dr. M. Nicolet (Besançon, France), from Torpedo marmorata by Dr. S. Birman (Geneve-Yvette, France), and from lamprey by Dr. J. Massoulié (Paris, France). Lizards (Pseudacris macus) were kindly provided by Dr. Grenet (École Normale Supérieure, Paris, France). Synthetic peptides were custom-prepared by NeoSystem Laboratories (Strasbourg, France). Other chemicals and their sources were: leupeptin, aprotinin, pepstatin (Sigma), reagents for polyacrylamide gels (Serva), 0.2-μm nitrocellulose (Schleicher and Schuell), and 125I-protein A (Amersham Corp.).

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† The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.

Methods

Tissue Extracts—Animals were killed by cervical dislocation, and their forebrains were dissected, quickly frozen, and kept at -20 °C. Frozen brains were homogenized in 9 volumes of homogenization buffer (10 mM Tris-HCl, pH 7.4, 0.02% NaN3, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM EDTA) in a Teflon-glass Potter-Elvehjem homogenizer and centrifuged at 13,000 rpm for 3 min. The resulting “low-speed supernatant” extract was stored at -20 °C until use. For organ studies, tissues from newborn and adult mice were quickly dissected, homogenized in 9 (brain) or 4 (other tissues) volumes of homogenization buffer, and centrifuged at 100,000 rpm for 6 min in a Beckman TL-100 centrifuge. The supernatant was adjusted at 100 mM NaCl, incubated at 100 °C for 2 min, and centrifuged again at 100,000 rpm. The resulting stathmin-enriched “S3” supernatant (7) was dialyzed against 0.2 mM Tris-HCl buffer, pH 7.4, containing 0.2 mM NaCl, to allow lyophilization and further concentration of samples for gel electrophoresis.

Antisera—The rabbit polyclonal antiserum prepared against the entire stathmin molecule extracted from two-dimensional gels was described previously (7). This serum was further affinity purified according to Olmsted (22) and used for immunoblots at a concentration corresponding to a 1:500 dilution of the original serum.

Two synthetic peptides corresponding to stathmin residues 15-27 and 134-149, with an additional amino-terminal tyrosine, were also used to generate stathmin-specific antisera: peptide I (17), -Tyr-Ala-Ser-Gly-Gln-Ala-Phe-Glu-Leu-Iso-Leu-Ser-Pro-Asn-; and peptide C, -Tyr-Arg-Lys-Ala-Lys-Glu-Ser-Pro-Ala-Asp-Glu-Thr-Glu-Ala-Asp-. Rabbits were immunized with 2 mg of peptide in complete Freund’s adjuvant. The anti-peptide C serum used in this study comes from a bleeding 6 weeks after immunization, whereas the anti-peptide I was obtained 6 weeks after a booster injection at 6 weeks after immunization. The anti-peptide I serum was used at a 1:10,000 dilution for immunoblots, which allowed detection of stathmin in 0.6 μg of total protein from a brain S3 extract (corresponding approximately to 5-10 ng of stathmin). For similar results, the anti-peptide C serum was used at a 1:2,000 dilution.

Polyacrylamide Gel Electrophoresis—Discontinuous polyacrylamide electrophoresis in one dimension was performed according to Laemmli (23) on 13% acrylamide gels. Two-dimensional PAGE was performed as described previously (2). Isoelectric focusing gels contained 2% total ampholines, pH 5-7 and 3-10, in the proportions 4:1. The second dimension was run on 13% acrylamide gel. For protein detection gels were silver-stained.

Immunoblotting—Electrotransfer of one- and two-dimensional gels on nitrocellulose was performed with an LKB semi-dry blotting apparatus (12 V, 90 min). Nitrocellulose blots were stained with Ponceau Red (0.2% in 1% acetic acid), destained with transfer buffer, and blocked with 2.5% casein for 1 h (20 °C). After overnight incubation at 4 °C with antiserum diluted in 1 casein, bound antibodies were detected with [125I]-protein A and overnight to 48 h of autoradiography on Kodak XAR-5 film with a Kodak X-Omat rapid intensifying screen. Quantification of immunoreactive bands on autoradiograms was performed with an Imstar image analysis system.

Protein Assay—Concentrations of proteins were estimated by the method of Bradford (24) using IgG as standard.

RESULTS

The two isoforms of stathmin, in their increasingly phosphorylated states (α0–α3, β0–β3), migrate as a set of isoelectric variant (pI ≈ 6.2-5.6) protein spots on two-dimensional gels: two nonphosphorylated spots, N1 (α0) and N2 (β0), and the phosphorylated spots P1 (α1), P2 (α2), and P3 (β2, α3, β3) (7, 18). In the present work, we used specific rabbit polyclonal antisera to analyze the tissue and developmental expression of stathmin, as well as its evolutionary conservation.

Antisera

A first anti-stathmin serum was obtained against the whole rat brain protein (7). After sequencing part of rat stathmin and the molecular cloning of its cDNA (17), we prepared antisera directed to synthetic peptides. One was made against peptide I, close to the amino-terminal region of the protein (residues 15-27). The other was prepared against peptide C, corresponding to the 16 carboxyl-terminal amino acids of stathmin (residues 134-149), a region mostly divergent from the stathmin homologous protein SCG10.

Fig. 1 shows that the major band recognized at 19 kDa on one-dimensional immunoblots of adult and neonatal rat brain extracts is the only one common to all three types of antisera, including anti-peptide C. Therefore, it corresponds specifically to stathmin. This is further confirmed by two-dimensional immunoblot experiments where the three types of
antigens also recognized all stathmin spots at the 19-kDa level (see also Refs. 7 and 17), as shown here in adult and neonatal brain with anti-peptide I (Fig. 2).

The anti-peptide I and to a lesser extent the anti-protein but not the anti-peptide C serum interacted also with a group of unidentified 60-kDa proteins present specifically in brain and, like stathmin, more abundant at the neonatal stage (Figs. 1 and 2). These results indicate that the 60-kDa proteins contain a domain homologous most likely to the amino-terminal region of stathmin.

In neonatal brain, the anti-protein serum also interacted with a couple of minor 17- and 21-kDa bands visible on onedimensional immunoblots, the 21-kDa band being also recognized by the anti-peptide I serum (Fig. 1). Thus these proteins also share some homology with stathmin, the significance of which remains to be elucidated.

**Tissue Distribution in Adult and Neonate Mouse**

We examined the distribution of stathmin and of its various forms, as well as its expression at the mRNA level, in various tissues of the mouse, together with their variations from the neonatal to the adult stage. For immunoblots of stathmin-enriched S3 extracts the anti-peptide I serum was used, since it yielded the most sensitive detection of the protein in the mouse (see Fig. 6). Qualitatively similar results were obtained also with the anti-peptide C serum (not shown), thus ensuring the specificity of the immunodetection of stathmin.

**Adult**—Stathmin was detected in almost all adult mouse tissues or cell types tested (Fig. 3, Table I). It was most abundant in forebrain and present in lower but similar concentrations in cerebellum and testis. Compared to the brain, some tissues like spleen, lung, and pituitary contained about 5 times less stathmin, whereas other tissues like heart, tongue, skeletal muscle, or liver contained 25–100 times less. Kidney was the only tissue where we were unable to detect stathmin, even after prolonged exposure of the immunoblot autoradiograms. The total blood cell fraction, as well as the separate red cell fraction (not shown), contained relatively high amounts of stathmin, about 30% of the concentration in brain.

**Neonate**—As described previously for adult (10, 16), we found that stathmin is 2–50 times more abundant in all mouse neonatal tissues than in the corresponding adult tissues (Fig. 3; Table I). As a result of this generally higher expression of stathmin, there is only a 3-fold range in the concentrations of stathmin among all neonate tissues tested but brain, as opposed to a 30-fold range in the adult.

Finally, no stathmin was detected in blood serum, confirming the exclusively intracellular location of this protein.

Northern blot analysis with a specific cDNA probe revealed a great abundance of stathmin mRNA in brain, traces in heart and tongue, and virtually no detectable levels in skeletal muscle and liver (Fig. 4). The expression of the mRNA for stathmin thus qualitatively parallels the expression of the protein itself.

Two-dimensional immunoblots of heart, spleen, lung, and blood cells (Fig. 5) revealed the presence of the same diverse forms of stathmin, although in variable relative amounts, as in brain. The corresponding proteins were also detected by silver staining (not shown). In the lung and mostly in the heart, however, the lower concentration of total stathmin did not allow the detection of all forms.

**Neonate**—As described previously for brain (10, 16), we found that stathmin is 2–50 times more abundant in all mouse neonatal tissues than in the corresponding adult tissues (Fig. 3; Table I). As a result of this generally higher expression of stathmin, there is only a 3-fold range in the concentrations of stathmin among all neonate tissues tested but brain, as opposed to a 30-fold range in the adult.

The higher abundance of stathmin in the neonate is also qualitatively paralleled by the expression of its mRNA, which was easily detected on Northern blots (Fig. 4) in all neonatal tissues tested.

The two-dimensional patterns of stathmin forms from neonatal tissues recognized on silver-stained gels (not shown) or immunoblots were also globally similar to the patterns of adult tissues (Fig. 5).
Stathmin: Tissue Expression, Development, and Conservation

Tissue Expression and Development

Stathmin-enriched S3 boiled extracts (adult 500 μg, neonatal 100 μg of protein/sample) from various tissues were submitted to two-dimensional PAGE immunoblotting with the anti-peptide I serum. The areas corresponding to the nonphosphorylated (N1, N2) and phosphorylated (P1, P2, P3) forms of stathmin are shown.

Phylogenetic Conservation and Expression in Vertebrate Brain

Since stathmin is most abundant in brain, we used this tissue to examine its conservation and expression through evolution of vertebrates.

Conservation—On one-dimensional immunoblots, the anti-peptide I serum recognized a 19-kDa stathmin-like protein in all vertebrate classes with similar intensity, slightly less in birds (quail) and fish (Torpedo) (Fig. 6). When the same samples were examined on two-dimensional gels, all species displayed similar amounts of silver-stained protein at the same locations as those of the immunoreactive proteins (140 μg/sample) from forebrains of the indicated vertebrate species. Low-speed supernatant proteins (700 μg/sample) from forebrains of the indicated vertebrate species were analyzed by two-dimensional immunoblotting with the anti-peptide I serum. The areas of the 19-kDa immunoreactive proteins are shown. N1, N2, P1, P2, and P3 indicate the locations of the corresponding spots in the mouse. Proteins migrating at the same intensity were present in the human, lizard, and frog, whereas the immunoreactive stathmin-like proteins in quail and Torpedo are more basic.

Two-dimensional Patterns of Immunoreactive Stathmin-like Proteins—To further characterize the immunoreactive 19-kDa stathmin-like proteins through evolution, we performed two-dimensional immunoblots of brain extracts (low-speed supernatant) with the anti-peptide I serum (Fig. 7). Several isoelectric variants of the immunoreactive proteins were detected, migrating in most cases exactly like the mouse or rat stathmin forms N1, N2, P1, P2, and P3, as it was also checked by direct comigration of the human, lizard, and frog samples with 32P-labeled PC12 cell extracts (not shown). These 19-kDa immunoreactive proteins are therefore indeed homologous to stathmin and may be designated by the same name.

In quail and Torpedo the immunoreactive spots migrated with more basic isoelectric points, not comigrating with the PC12 32P-labeled stathmin spots. However, their pattern in quail is very similar to the mouse pattern. In addition, we checked with Torpedo that the immunoreactive proteins remain, like stathmin, in the heat-soluble fraction. Therefore, the proteins detected in quail and Torpedo most likely also correspond to the various forms of stathmin in these species.

DISCUSSION

The phosphorylation of stathmin could be related, in diverse biological systems, to the intracellular mechanisms involved in the regulations of cell proliferation, differentiation, and functions by extracellular agents activating various second messenger pathways (reviewed in Ref. 7). In order to get further clues on the general importance of this protein in biological regulations, we studied here its tissue distribution, its expression through development, and its evolutionary conservation.

In spite of the homology (19) between stathmin and the developmentally regulated superior cervical ganglion protein SCG10 (20), all three of our antisera were specific for stathmin in the 19-kDa range. In addition, none of them recognized a protein migrating as SCG10, as assessed by direct comparison with an anti-SCG10 antisera (20).3

Other proteins of approximately 17, 21, and 60 kDa displayed some immunological homology with stathmin, with the exception of its COOH-terminal sequence. In addition,
the two-dimensional pattern of the 60-kDa proteins suggests that they might be actually isoelectric variants of a single protein.

Together, these observations suggest the existence of a family of proteins containing domains homologous to some regions of stathmin. Such a family was first identified on the basis of Northern blot (20) and direct sequence homologies between stathmin and SCG10 (19). All these proteins also share with stathmin its higher expression at the neonatal than at the adult stage. However, unlike stathmin, they seem to be restricted to the nervous tissue. It will thus be interesting to test if proteins of the same family can be identified in other biological systems.

The previously proposed ubiquity of stathmin (7) is demonstrated here by its detection in virtually all mouse tissues tested. The lack of stathmin ("P19") immunoreactivity in all adult tissues but brain and testis reported by Schubart (10) is thus most likely due to a lower sensitivity of the antibodies and/or of the detection system used.

In all tissues tested, the expression of stathmin was higher in the neonate than in the adult, both at the protein and mRNA levels. This generalizes previous observations with brain where the expression of stathmin reaches a peak at the neonatal stage (10, 16, 17). The high expression and wide distribution of stathmin suggest that it plays a general role at this stage of development, when mostly the proliferation, differentiation, and maturation of cells are taking place in the diverse tissues.

The overall decrease of the expression of stathmin toward adulthood is very variable among tissues. As reported previously (7, 9), brain is the richest tissue also at the adult stage. The high abundance of stathmin in the testis (see also Ref. 10) probably reflects the "developmental" nature of this organ, composed mostly of cells in their proliferation, differentiation, and maturation phases. On the other hand, the presence of stathmin in the other adult tissues, including the nucleus-free, nonproliferating red blood cells, is in good agreement with its previously proposed involvement in regulations of fully differentiated cell functions (7).

In summary, these observations suggest the existence of two relatively independent types of roles for stathmin, possibly carried by the same or by very similar molecules: 1) a "developmental" role, mostly expressed during the embryonic and perinatal periods, would be related to the regulations of cell proliferation, differentiation, and maturation during tissue development; 2) a "functional" role, particularly emphasized in brain, would then be rather related to regulations of differentiated cell functions.

A stathmin-like 19-kDa protein was immunodetected in most vertebrate species tested, with the exception of some fish. Stathmin is thus quite well conserved among all vertebrate classes. Recently, a very high homology was also found between the amino acid sequences of rat and bovine (19), and an even higher one between the rat and human stathmin sequences. Such a high phylogenetic conservation is in good agreement with the presumably ubiquitous and general role of stathmin in biological regulations.

The sequence of the peptide I region is particularly well conserved. The immunoreactive signal was however slightly weaker with quail and Torpedo, which also display more basic stathmin-like forms on two-dimensional immunoblots than mammals. Also, in view of the very broad existence of a stathmin-like molecule in most species including Torpedo, the absence of immunoreaction with carp and lamprey, which belong to phylae having diverged early in evolution, is most likely due to a divergence in this region of the molecule rather than to the lack of stathmin.

The serum prepared against the whole protein displayed a continuously decreasing immunoreaction from mammals (with the exception of the mouse) to fish. On the other hand, the COOH-terminal region was recognized only in mammals (with the antiserum prepared against the corresponding rat sequence. Interestingly, this is also a region of divergence with SCG10 as well as with the other (17, 21, and 60 kDa) stathmin-related proteins discussed above.

Altogether, these results indicate that some domains of stathmin have been better conserved than others. They include in particular the area of peptide I, which interestingly also contains one or two of the predicted phosphorylation sites of stathmin (17, 19). It is very likely that these most conserved regions correspond to domains of the molecule which are important for its function.

In conclusion, the wide tissue distribution, the developmental regulation, and the phylogenetic conservation of stathmin are further indications of its importance as a ubiquitous and general intracellular relay for the diverse extracellular agents regulating the development and the differentiated functions of animal tissues.

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