A Single cDNA Encodes Two Isoforms of Stathmin, a Developmentally Regulated Neuron-enriched Phosphoprotein*

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Stathmin, a 19-kDa neuron-enriched soluble phosphoprotein, has been recently proposed as an ubiquitous intracellular relay for the diverse extracellular signals regulating cell proliferation, differentiation, and functions through various second messenger pathways (Sobel, A., Boutterin, M. C., Beretta, L., Chneiweiss, H., Doye, V., and Peyro-Saint-Paul, H. (1989) J. Biol. Chem. 264, 3765-3772). Internal sequences of the protein from rat brain were determined after purification by two-dimensional polyacrylamide gel electrophoresis, electrophoretic transfer onto Immobilon, and in situ proteolysis. Oligonucleotide mixtures based on these sequences were used to clone a cDNA for stathmin from a rat PC12 cell Agt10 library. The deduced amino acid sequence reveals partial homologies with the coiled coil structural regions of several intracellular matrix phosphoproteins. Using this cDNA as a probe, we show that the expression of stathmin mRNA parallels that of the protein during brain ontogenesis, reaching a maximum at the neonatal stage. In vitro translation of the derived cRNA yielded all the known molecular forms of stathmin, namely its α and β isoforms in their unphosphorylated and phosphorylated states. Thus, a single cDNA codes for both biologically relevant isoforms of the protein, indicating that they differ by co- or post-translational modifications.

ExPERIMENTAL PROCEDURES

Polyacrylamide Gel Electrophoresis—One-dimensional electrophoresis (15) was performed on 13% acrylamide-containing gels, and two-dimensional PAGE was run according to Carscallen (16) and previously described (8).

Stathmin Sequence Analysis—Stathmin-enriched S3 fractions were prepared by a 100 °C treatment of the rat brain soluble protein fraction (8). For sequence analysis, proteins (500 μg/gel) were run on semi-preparative two-dimensional PAGE gels (8) and electroblotted onto Immobilon membranes (17). The Amido Black-detected PI spots were cut out from three blots, pooled, and digested with trypsin (18, 19). Peptides eluted from the membrane were purified by high pressure liquid chromatography, and the sequences of the three most abundant ones (peptides I, II, and III, see Fig. 2) were determined by gas-phase sequencing.

Immunological Procedures—A rabbit was immunized with 2 mg of synthetic peptide I (Neosystem Laboratories, Strasbourg, France) with complete Freund’s adjuvant and boosted 6 weeks later. An antibody against the entire stathmin protein was prepared previously (8).

For Western blots, proteins from gels were electroblotted onto nitrocellulose; the membrane was saturated with casein (2.5%) and probed with either antiserum as indicated. Bound antibodies were detected with 125I-protein A and autography.

cDNA Library Screening—Two oligonucleotide mixtures (mix I and mix II) of all the codon combinations corresponding to peptides I and II were synthesized (gene assembler, Pharmacia LKB Biotechnology Inc.) using deoxynucleosine where codon ambiguity involved all four nucleotides. The derived 10-end-labeled probes (specific activity, ≈5 × 10⁸ cpm/μmol) were used to screen 10⁶ clones from a Agt10 PC12 cell cDNA library (gift of Drs. J. Patrick and J. Boulter (20)). The final washes being performed in 2 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7) containing 0.1% SDS at 62 °C (mix I) and 50 °C (mix II). Primary screening with mix I yielded positive clones at a frequency of 1/10,000, and the positivity of 9 out of 100 clones was confirmed by hybridization with mix II.

Protein phosphorylation-dephosphorylation reactions are known to be major intracellular regulatory mechanisms fol-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) J04979.

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†‡ The abbreviations used are: PAGE, two-dimensional gel electrophoresis; SDS, sodium dodecyl sulfate.

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of 10 clones was confirmed with mix II. Clone AS62b that contained the longest positive EcoRI insert among four selected clones was further used for cDNA sequencing and in vitro expression.

**Nucleotide Sequencing**—Clone AS62b insert was subcloned in both orientations into the plasmid vector pBluescript (Stratagene, San Diego, CA). Single-stranded DNA templates were prepared by infection of cultures with M13 helper phage KO7 and were sequenced by the chain termination method (21) using synthetic primers and a modified T7 DNA polymerase (Sequenase, United States Biochemical Corp., Cleveland, OH).

**RNA Blot Analysis**—Total RNAs were isolated from rat brain as described (22). Northern blots were prepared with 25 pg of glyoxalated RNA according to the procedure of Thomas (23) and probed with the 32P-labeled (24) fragment of stathmin cDNA. Final washes were performed at 50 °C in 0.1 SSC, 1.0% SDS.

**In Vitro Transcription and Translation**—Linearized expression plasmids pS62b were transcribed using the T3 RNA polymerase and a cap analog (Stratagene). Approximately 5 µg from the resulting cRNA were translated (2 h, 30 °C) in a rabbit reticulocyte lysate system (Promega) (25). After translation, boiled extracts were treated (1 h, 30 °C) with alkaline phosphatase (0.2 units/µl) at pH 9.5 or directly prepared for two-dimensional PAGE as described (8).

**RESULTS AND DISCUSSION**

The increasingly phosphorylated states of the α and β isoforms of stathmin (α0–α3, β0–β3) are partially resolved by two-dimensional PAGE as a set of unphosphorylated (N1, N2) and phosphorylated (P1, P2, P3) spots (Fig. 1) (8). The amino acid sequences of three tryptic peptides (I, II, III) of the two-dimensional PAGE purified α1 form (spot P1) of rat brain stathmin were determined (Figs. 1 and 2). Polyclonal antibodies raised against synthetic peptide I specifically recognized all the two-dimensional PAGE spots corresponding to both α and β isoforms of stathmin from rat brain (Fig. 1) or from the rat pheochromocytoma-derived PC12 cells (not shown). This result confirmed that the sequence determined is indeed that of stathmin and showed that it is common to its two isoforms.

We therefore used mixtures of synthetic oligonucleotides designed from this sequence and that of peptide II to screen a Agt10 PC12 cell cDNA library (20). Clone AS62b contained the longest positive insert (Fig. 2) and displays an open reading frame with sequences corresponding to those of peptides I, II, and III. The NH2 terminus of the protein was designated by the only ATG start codon existing between peptide I and the closest stop codon located upstream with the same open reading frame. The calculated molecular weight of the corresponding 149-amino acid protein is 17,269, in good agreement with the apparent M, of 19,000 determined for stathmin by SDS-gel electrophoresis. Similarly, the 1080-base pair length of the insert containing a poly(A) tail and a 5'-untranslated sequence is close to the 1.1 kilobase size of the corresponding mRNA determined by Northern blot analysis using clone AS62b as a probe (Fig. 4A). Together, these observations indicate that clone AS62b encompasses at least the nearly complete sequence of the mRNA for stathmin.

The cDNA-deduced amino acid sequence contains a high proportion of charged amino acids (47%) possibly responsible for the characteristic heat stability of stathmin, which remains soluble at 100 °C (8). Furthermore, hydrophyty analysis (26) revealed an increasing hydrophilicity from the N-terminal toward the C-terminal part of the molecule (not shown).

Stathmin is also rich in OH-containing residues (11 serines and 2 threonines); two of them (serines 16 and 63) are located in potential phosphorylation sites (Lys/Arg-Lys-X-Ser-X) for the cAMP-dependent protein kinase (4) for which stathmin is a good substrate (9). However, both isoforms of stathmin can be phosphorylated on at least three sites (13) and by presumably distinct kinases activated by diverse second messengers (5–7, 11, 12). Other phosphorylation sites for the CAMP-dependent and the other kinases phosphorylating stathmin in vivo remain therefore to be determined.

A search for protein sequence homology (27) revealed weak similarities between stathmin and a set of phosphoproteins of the intracellular (cytoplasmic or nuclear) matrix: myosin heavy chains (28, 29), type II cytoketin (30), tropomyosin (31), and lamins A and C (32, 33) (Fig. 3). These homologies reach up to 51% for residues 47–82, within a putative predicted (34, 35) α-helical region of stathmin (residues 47–124). Within this region, the sequence of stathmin displays a heptad repeat structure (abcdefg), where residues a and d are hydrophobic or uncharged and which resembles a similar organization described for the above mentioned proteins (28–33). Such heptad repeats are known to be able to yield coiled coil interacting structures (29, 32, 33, 36, 37) which might thus also contribute to the molecular mechanisms by which stathmin...
min fulfills its proposed functional role as an ubiquitous intracellular relay for extracellular regulations.

During brain development, the expression of stathmin protein increases until birth and then decreases toward adulthood (8, 38); it was shown by immunoprecipitation of in vivo translation products that the postnatal decrease of the protein was accompanied by a similar decline of its mRNA (38). We show by Northern blot analysis that both the increase and decrease of stathmin expression are paralleled by the expression of its mRNA (Fig. 4A). The various phases of the expression of stathmin are therefore at least in part under pretranslational and possibly transcriptional control.

To further characterize the protein encoded by clone AS62b, in vitro-transcribed stathmin cRNA was used as a template for translation in rabbit reticulocyte lysate. As expected for stathmin (8), the translated proteins remained soluble after a 100 °C treatment used for their partial purification. In addition, when analyzed by two-dimensional PAGE, the corresponding [35S]methionine-labeled spots comigrated with the unphosphorylated and phosphorylated forms of the endogenous stathmin originally present in the reticulocyte lysate (Fig. 4B). Labeling experiments with [γ-32P]ATP indeed demonstrated the presence of protein kinase activity in the reticulocyte lysate (not shown). Furthermore, alkaline phosphatase treatment resulted in the conversion of the in vitro-translated proteins toward their most basic forms comigrating with the unphosphorylated states α0 and β0 of the two stathmin isoforms (Fig. 4B). Altogether, these results clearly demonstrate that clone AS62b encodes proteins possessing the known biochemical properties of stathmin and whose phosphorylation takes place readily in the in vitro translation system.

The most important conclusion from the above experiments is, however, that the two α and β isoforms of stathmin are encoded by a single cDNA corresponding to a single cellular mRNA. Indeed, since both isoforms were expressed in the in vitro translation system, they differ only by co-translational or post-translational modifications. The amino acid following the initiator methionine being alanine, it is likely, according to Huaga et al. (39), that, in the intact cell, the methionine is cleaved and that the resulting N terminus is acetylated; these reactions are indeed known to take place also in the reticulocyte system (40). Alternate or complementary modifications on possibly different amino acid residues should, however, also be considered.

Biological regulations of these post-translational reactions might then be responsible for the observed variations in the relative expression of the α and β isoforms in various tissues and cell types (8), in relation to their respective contributions to the role of stathmin as an ubiquitous intracellular relay for the regulations of cells by factors of their extracellular environment.

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