Identification and Molecular Characterization of Unc-33-Like Phosphoprotein (Ulip), a Putative Mammalian Homolog of the Axonal Guidance-Associated *unc-33* Gene Product

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The control of neuritic extension and guidance is critical for the development, maturation, and regeneration of functional neuronal circuits. We identified a neuronal 64–85 kDa phosphoprotein, the expression of which in mouse brain is regulated during development, reaching a peak at ~5 d postnatal, when maturation of neurons and synaptic connections is highly active. The amino acid sequence of the mouse protein deduced from its cloned cDNA reveals similarities with that of the neuritic outgrowth- and guidance-related product of the *unc-33* gene in *Caenorhabditis elegans*. The regulation of

its phosphorylation in response to nerve growth factor, as well as its localization in neurites and growth cones and at the neuromuscular junction, further indicates that Ulip (for Unc-33-like phosphoprotein) is not only a structural but likely is also a functional mammalian homolog of Unc-33, potentially involved in the control of neuritic outgrowth and axonal guidance.

Key words: neuronal development; axonal guidance; neuritic elongation; Ulip; *unc-33*; hydantoinases; toad-64; *crmp-62*; phosphoprotein; PC12 cells; neuromuscular junction

The development of the nervous system requires, after proliferation and migration of neuronal precursor cells, the growth of neuritic processes and their guidance toward the appropriate targets with which they will establish synaptic contacts. Several intracellular proteins, the expression or regulation of which could be related closely to neuritic growth and its control, have been characterized (for review, see Skene, 1989). Genetic screening of mutants impaired in the proper targeting of neuronal circuits in invertebrates such as *Caenorhabditis elegans* (White et al., 1986) yielded a large set of mutants exhibiting abnormal, uncoordinated (*unc* mutation) movements (Brenner, 1974; Swanson et al., 1984). Aside from mutations in genes coding for muscle proteins, proteins implicated in migration of precursor cells, neurotransmitter release, and synapse formation, several mutations resulting in axonal outgrowth defects were identified (Hedgecock et al., 1985; Desai et al., 1988; Siddiqui and Culotti, 1991; McIntire et al., 1994). Some of these correspond to extracellular guidance molecules such as *unc-6* (Desai et al., 1988; McIntire et al., 1994) for which netrins have been identified recently as their mammalian homologs (Serafini et al., 1994), whereas others like *unc-33*

(Li et al., 1992) or *unc-44* correspond to intracellular, often cytoskeleton-associated or related proteins (Otsuka et al., 1995).

In our own search for proteins involved in the relay and integration of intracellular signals triggered by extracellular regulatory factors, we identified stathmin (Sobel, 1991), a small, ubiquitous, and cytosolic phosphoprotein that is highly expressed in neurons of the developing brain (Chneiweiss et al., 1989; Koppel et al., 1990) and is phosphorylated in response to growth and differentiation factors such as nerve growth factor (NGF) (Doye et al., 1990). It is the generic member of a highly conserved protein family (Maucuer et al., 1993) that includes the neuronal differentiation-related protein SCG10 (Anderson and Axel, 1985; Stein et al., 1988).

We have observed previously that a rabbit polyclonal antibody originally directed against an internal peptide of stathmin also recognized a 64 kDa brain-specific phosphoprotein (Koppel et al., 1990). This protein is highly expressed in the neonatal mouse and is downregulated in the adult, therefore appearing to be implicated potentially in the development of the nervous system. We report in this study the further molecular purification and characterization of this phosphoprotein, as well as the molecular cloning of its cDNA. The corresponding amino acid sequence reveals similarities with that of a bacterial hydantoinase and with the neuronal development-related gene product of *unc-33* in *C. elegans*; we therefore designate the mouse protein as Ulip (for Unc-33-like phosphoprotein). Mutations in the *unc-33* gene were shown to result in neuritic outgrowth and guidance defects (Hedgecock et al., 1985; Hedgecock et al., 1987; Desai et al., 1988). Ulip appears also to be part of a Unc-33-related protein family, another member of which is represented by the toad-64 protein identified recently in rat (Minturn et al., 1995) and the chick *crmp-62* protein (Goshima et al., 1995), which was published after submission of this paper and was proposed to be involved in the intracellular response to collapsin. Together with the molecular properties of the mouse Ulip protein, our present characterization of its biological expression and regulation in neuronal cells

Received Aug. 3, 1995; revised Oct. 10, 1995; accepted Oct. 16, 1995.

This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Association Française contre les Myopathies, the Association pour la Recherche contre le Cancer, and the Ligue Nationale Française contre le Cancer. T.B. was the recipient of postdoctoral fellowships from the Fondation pour la Recherche Médicale and FEBS. C.C.-D. was supported partially by Grant 92-167 from Direction de la Recherche et des Etudes Techniques to Dr. F. Rieger. We thank Dr. M. Fardeau for his constant support and Dr. F. Rieger for discussions and technical help for the neuromuscular junction experiments. We also thank the following people: Dr. R. Hen for the gift of the Uni-Zap neonatal mouse brain cDNA library; Dr. H. Chneiweiss for the cultured striatal neurons; J. D'Alayer for his major contribution to peptide sequencing; Dr. G. Géraud for his help with confocal microscopy; S. Ozon and V. Manceau for their help in antisera preparation and characterization; and Drs. S. Lawler and A. Maucuer for critical reading of this manuscript.

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suggests that it is most likely a functional mammalian homolog of the *unc-33* gene product, which suggests further that it participates in the control of neuritic outgrowth and axonal guidance during the development and regeneration of the mammalian nervous system.

MATERIALS AND METHODS

Materials

Swiss mice were from the local breeding facility. Frozen brain samples from postmortem humans were provided by Dr. F. Javoy-Agid (INSERM U289, Paris, France). DEAE–Sephacrose Cl-6B, phenyl–Sephacrose Cl-4B, nucleic acid purification (NAP)-25 columns, an electrophoresis molecular weight kit, an isoelectric focusing calibration kit, and ampholines were from Pharmacia (Uppsala, Sweden). The dT-primed Uni-Zap cDNA library from whole neonatal mouse brain and the helper phage kit were from Stratagene (La Jolla, CA). The prokaryotic pET19 vector expression kit was from Novagen (Madison, WI). The Sequenase Version 2.0 DNA-sequencing kit was from United States Biochemical (Cleveland, OH). PC12 cells were purchased from the American Type Culture Collection (Rockville, MD), culture media and fetal calf serum were from Gibco (Epagny, France), and horse serum was from Seroviol (Vogelgrun, France). Other materials were obtained as follows. Aprotinin, pepstatin, leupeptin, urea, dithiothreitol, Trizma, agarose, isopropylthiogalactoside (IPTG), and phenol were from Sigma (Deisenhofen, Germany). Restriction enzymes, T4-DNA ligase, and polynucleotide kinase were from New England Biolabs (Beverly, MA). Calf intestine alkaline phosphatase and NGF were from Boehringer Mannheim (Mannheim, Germany). Reagents for polyacrylamide gels, serum albumin, and casein were from Serva (Heidelberg, Germany). Ammonium sulfate, silver nitrate, and other standard reagents were from Prolabo (Paris, France). SDS was from Fluka (St. Quentin-Fallavier, France). [γ - 32 P]ATP, 125 I-labeled protein A, the Megaprime DNA-labeling system, and electro-generated chemiluminescence (ECL) Western blotting reagents were from Amersham (Buckinghamshire, UK).

Methods

Partial purification of Ulip. Partially purified Ulip was obtained from neonatal mouse brains through three purification steps. Neonatal mouse brains (800; 70 gm) were homogenized with a Polytron homogenizer in 4 vol of homogenization buffer (25 mM Na phosphate, pH 7.8, 1 mM EGTA, 10 μ g/ml leupeptin, 25 μ g/ml aprotinin, and 10 μ g/ml pepstatin). Homogenates were centrifuged for 10 min at 4000 $\times$ g. Pellets were resuspended in 2 vol of homogenization buffer, homogenized, and centrifuged as before. The supernatants of the two centrifugations were assembled, sonicated, and centrifuged for 1 hr at 100,000 $\times$ g. The supernatant (S2) was loaded on a DEAE–Sephacrose Cl-6B column (1.75 cm² $\times$ 26 cm) equilibrated with 100 ml of buffer A (25 mM Na phosphate, pH 7.8, 1 mM EGTA) at a flow rate of 30 ml/hr. Proteins were eluted in 300 ml of a linear gradient of 0–250 mM NaCl in buffer A, and 5 ml samples were collected. The Ulip-containing fractions were pooled, and solid ammonium sulfate was added up to 20% saturation. This pool was loaded on a phenyl–Sephacrose Cl-4B column (1.75 cm² $\times$ 22 cm) that was equilibrated previously with 100 ml of buffer B (10 mM Na phosphate, pH 7.8, 1 mM EGTA) containing 20% saturated ammonium sulfate. The proteins were eluted in a decreasing linear gradient from 20 to 0% saturated ammonium sulfate in buffer B. The Ulip-containing fractions were pooled and dialyzed twice against 20 vol of buffer A. The proteins were concentrated on a small (10 ml) DEAE–Sephacrose Cl-6B column and eluted with 400 mM NaCl in buffer A. The eluate was desalted on a Sephadex G-25 (NAP-10) column and concentrated further to a final volume of 0.5 ml by evaporation. In the final purification step, the concentrated fraction was chromatographed in three successive runs on two serially connected Superose 12 fast protein liquid chromatography (FPLC) columns in buffer C (50 mM Na phosphate, pH 7.2, 150 mM NaCl) at a flow rate of 0.3 ml/min. Fractions (0.6 ml) were collected, and Ulip-enriched fractions were pooled and used for further analysis. The presence of Ulip at the successive purification stages was tested by one-dimensional Western blot using the cross-reactive anti-stathmin antibody. Proteins were quantified using the method of Bradford (1976).

PAGE. One-dimensional electrophoresis was performed on 13% polyacrylamide gels according to Laemmli (1970). Two-dimensional PAGE was performed as described previously (Sobel and Tashjian, Jr., 1983). The isoelectric focusing gels contained 2% total ampholines, pH 6–8 and

3–10 at a ratio of 4:1. The second dimension was run on 10% acrylamide gels. Proteins were either immunoblotted (see below) or silver-stained as described previously (Sobel et al., 1989).

Western blot analysis. Proteins were transferred from gels onto nitrocellulose in a semidry electroblotting apparatus; maximal recovery of Ulip was achieved in 48 mM Tris and 39 mM glycine buffer containing 5% methanol, instead of 20%, for optimal stathmin transfer. The membrane was saturated with casein (2.5%) in immunoblot solution (12 mM Tris-HCl, pH 7.4, 160 mM NaCl, 0.1% Triton X-100) and probed with either an antiserum against peptide I of rat stathmin (1:10,000) (Koppel et al., 1990) or an antiserum against recombinant Ulip (1:20,000) diluted in immunoblot solution containing 1% casein. Bound antibodies were detected either with 125 I-labeled protein A and autoradiography or with peroxidase-bound anti-rabbit antibodies and the ECL kit (Amersham).

Protein sequence analysis. Ulip-enriched fractions were separated on two-dimensional polyacrylamide gels. Gels were fixed for 30 min in 25% ethanol and 10% acetic acid and stained for 3 min in 0.1% amidoblack in 1% acetic acid and 40% methanol. Gels were destained in 1% acetic acid, and the spots corresponding to the major form of Ulip were cut out from three gels, pooled, and digested with 2 mg/ml endoprotease Lys C. The peptides eluted from the gel then were separated by HPLC on a DEAE–C18 column with a gradient of 0–55% acetonitrile in 0.1% trifluoroacetic acid. Five clearly separated peptides (I–V) were microsequenced by automatic Edman degradation.

cDNA library screening. Molecular genetic procedures were performed according to standard protocols (Sambrook et al., 1989). Two degenerate oligonucleotides corresponding respectively to peptides V and IV were synthesized (Genset, Paris, France): 5'-TC(T/C)TCIACICC(A/G)TTIG-TICC(T/C)TCIGGIATIGCIGT-3' (Oli-1); and 5'-TTICC(A/G)TCIG-CCCCA(T/C)TCIC(G/T)CCA(T/C)TT-3' (Oli-2). Phages (7.5×10^5) from a Uni-Zap neonatal mouse brain cDNA library were screened with the two 32 P-end-labeled oligonucleotides ($\sim 2.5 \times 10^6$ cpm/pmol). The final washes were performed in 2 $\times$ SSC (1 $\times$ SSC: 150 mM NaCl, 15 mM Na citrate, pH 7) containing 0.1% SDS at 60°C (Oli-1) and 55°C (Oli-2). Primary screening with Oli-1 identified no clearly positive clone, whereas 14 positive clones were identified with Oli-2 (frequency, $\sim 1/5000$ clones).

Nucleotide sequencing. The Bluescript plasmid vector containing the *EcoRI* and *XhoI* inserts was excised from the phage genome using the ExAssist helper phage (Stratagene) and was introduced into the *SolR* strain of *Escherichia coli* as described by the manufacturer (Stratagene). Twelve clones with inserts from 1300 to 2100 bp were analyzed further. Restriction analysis and sequencing from the two ends showed that these clones differed only in the length of their 5' ends. *AluI* fragments of the longest insert were subcloned into Bluescript and were sequenced fully by the Sanger dideoxy-termination method (Sanger et al., 1977) using T7 and T3 complementary primers and a modified T7 DNA polymerase (Sequenase). Eight additional Ulip-specific oligonucleotides were used to verify the order and the orientation of the subcloned fragments in the entire cDNA insert.

Bacterial expression of recombinant Ulip. An *NdeI* restriction site was introduced into the cDNA coding for Ulip by PCR at the putative ATG translation start site: this was carried out using an oligonucleotide containing the mutated sequence (AATCGCCTATGTCTACCAGGG-CAAGAAG) and an oligonucleotide 3' to the *AatII* site (TAAG-GCATCTGGAAGTGGGT). The mutated PCR product and the original *AatII*–*XhoI* fragment of the Ulip cDNA were ligated into the *NdeI*–*XhoI*-digested pET19 vector. Nova-Blue bacteria (Novagen) were transformed, and ampicillin-resistant strains were selected for Ulip cDNA inserts and sequenced to verify ligation sites and the PCR product. A BL-21 *E. coli* strain then was transformed with Ulip cDNA-containing vectors, and expression was induced with 5 mM IPTG. Almost all of the His-tagged Ulip protein produced was found in nonsoluble inclusion bodies. The protein was dissolved in a buffer containing 6 M urea, and the protein was purified further on a His-Bind column as described by the manufacturer (Novagen).

Northern blot analysis. Total RNAs were isolated from several tissues as described previously (Taylor et al., 1986). RNAs were separated on an agarose gel containing formaldehyde (Sambrook et al., 1989), transferred to a Hybond N membrane (Amersham) in 20 $\times$ SSC, and stained with 2% methylene blue. The blots were probed with a multi-prime-labeled ($\sim 1.5 \times 10^6$ cpm/ μ g DNA) *EcoRV*–*XhoI* fragment of the Ulip cDNA. Final washes were performed in 0.1 $\times$ SSPE (1 $\times$ SSPE: 150 mM NaCl, 9 mM Na phosphate, 1 mM EDTA) and 0.1% SDS at 60°C.

Antiserum production. Rabbits were immunized with 1 mg of synthetic peptide V (Neosystem, Strasbourg, France) coupled to keyhole limpet hemocyanin or with 0.1 mg of recombinant Ulip in complete Freund's

adjuvant and boosted 4 weeks later with half the amount of peptide (protein) in incomplete Freund's adjuvant.

Cell culture. PC12 cells were grown in RPMI 1640 supplemented with 10% horse serum, 5% fetal calf serum, 2 mM glutamine, and 5 μ g/ml gentamycin. Cells were passaged using 0.025% trypsin and 1 mM EDTA in phosphate-buffered saline (PBS). For induction of differentiation, 20 ng/ml 2.5S NGF was added to the cultures. Differentiation usually was induced during 3 d. For two-dimensional PAGE analysis, cells were scraped from the culture dishes in PBS. They were centrifuged for 10 min at 1500 $\times$ g and sonicated in 3 vol of homogenization buffer containing 50 mM Tris-HCl, pH 7.2, instead of Na phosphate.

Immunofluorescence procedures. PC12 cells were fixed in methanol at -20°C (6 min) and treated with blocking solution containing 3% bovine serum albumin (BSA) in PBS (3 times, 10 min each). The cells were incubated (1 hr) with the antibodies diluted in the blocking buffer and washed with PBS containing 0.1% Tween 20. Fluorescein-conjugated goat anti-rabbit antibodies (Tago, Burlingame, CA) were used to reveal anti-Ulip antibodies, and rhodamine-conjugated antibodies (Bioss, Compiègne, France) were used to reveal anti-neurofilament (68 kDa) or anti-tubulin antibodies (Sigma).

Striatal neurons from 20 d gestational rat embryos were cultured for 8 d and then fixed in 2% *p*-formaldehyde in PBS containing 1 mM Ca^{2+} for 30 min at 4°C . This was followed by another incubation in 0.1% glycine and 0.1% Triton X-100 in PBS for 30 min at room temperature. Incubations with antibodies were performed as for PC12 cells.

Control immunolabeling experiments were performed using either preimmune serum or serum preincubated with the recombinant Ulip antigen (10 μ g protein/ μ l serum).

Immunostaining procedures on muscle tissue were performed as described previously (Cifuentes-Diaz et al., 1994). Briefly, frozen adult muscles were cut at -28°C . Cryostat sections (6 μ m) were incubated directly for 1 hr with the anti-Ulip antiserum diluted 1:500 in blocking solution, washed (1 hr) in PBS containing 0.1% Tween 20, and incubated with fluorescein-conjugated anti-rabbit antibodies (dilution 1:100). After washing, preparations were treated with rhodamine-conjugated α -bungarotoxin (1:1000, Molecular Probes, Eugene, OR).

For immunostaining of whole-mount-teased fibers, small bundles of fixed (1% *p*-formaldehyde) muscle fibers were incubated first with 0.1 M glycine in PBS (30 min) and then with 3% BSA and 0.5% Triton X-100 in PBS (30 min). They were incubated next with anti-Ulip antiserum (1:500) overnight at 4°C , washed, and incubated with secondary antibody. The specificity of the staining was assessed by the omission of the primary antibody or by the use of preimmune serum. Preparations were observed with a conventional fluorescence microscope or with a confocal laser scanning microscope (MRC-600, Bio Rad, Hercules, CA) mounted on an Optiphot II Nikon microscope as described previously (Mège et al., 1992).

RESULTS

Ulip is a phosphoprotein present in mouse brain as at least two different isoforms

Two-dimensional Western blot analysis of the soluble fraction of newborn mouse brains showed that an antiserum raised against a peptide corresponding to stathmin residues 15–27 recognized, in addition to the characteristic stathmin spots in the region of 19–20 kDa, three spots corresponding to a more basic protein with an M_r of ~ 64 kDa (Fig. 1A) (Koppel et al., 1990). This protein, originally designated P60 and which we propose to name Ulip (see introductory remarks), was found in the high-speed supernatant of a mouse brain extract but, unlike stathmin, it was precipitated after boiling.

The molecular characterization of Ulip based on its identification with the anti-stathmin antiserum allowed the production of two rabbit polyclonal sera specific for this protein: (1) an antiserum raised against synthetic peptide V corresponding to the sequence of an internal peptide of Ulip; and (2) an antiserum raised against a recombinant protein produced in bacteria. As shown in Figure 1B, both antisera recognized the Ulip protein in the brain and showed no cross-reactivity with stathmin. Because of its higher sensitivity and specificity, the anti-recombinant Ulip antiserum was used primarily for further investigations. Neverthe-

less, the results obtained with this antiserum always could be reproduced qualitatively with the antiserum raised against the synthetic peptide.

All three available antisera recognized several spots corresponding to various molecular forms of Ulip on two-dimensional immunoblots of a neonatal mouse brain-soluble extract (Fig. 1C). Two groups of spots were recognized by the various antisera in brain extracts: group A, with an M_r of ~ 64 kDa, and group B, with an M_r of ~ 70 kDa. The anti-stathmin antiserum detected three increasingly acidic forms within group A, with isoelectric pH (pI) values of 7.1, 6.9, and 6.8 (spots 3, 4b, and 5, respectively). In addition, the anti-Ulip antiserum allowed the detection of additional spots in group A (spots 1, 2, 4a, and 6), as well as spots in group B. The protein forms of both groups migrated with a similar pI pattern, differing only in spots 1 and 6, which could not be detected in group B, and in spot 4, which appeared as a doublet (a and b) in group A, whereas only spot 4a could be detected in group B.

Spots 1–6 migrated at isoelectric points differing by ~ 0.1 pH unit from each other, corresponding approximately to the charge brought by the addition of a phosphate group. The difference between the isoelectric points of A4a and A4b is too small to be explained by an additional phosphate group and, therefore, corresponds more likely to another type of post-translational modification.

The acidic forms 1–6 of both groups A and B could be converted mostly to two more basic forms (pI ~ 7.4), designated A_N and B_N, respectively, after treatment with alkaline phosphatase for 10 min at 37°C (Fig. 1C). Furthermore, the *in vitro* phosphorylation of the partially purified fraction of Ulip with protein kinase A (PKA), casein kinase II, or cdc2 kinase produced radioactive spots corresponding to forms A1–A5 (data not shown). The acidic forms 1–6, therefore, correspond to increasingly phosphorylated states of the corresponding nonphosphorylated forms N. Together, these observations indicate that groups A and B correspond most likely to the various unphosphorylated (N) and phosphorylated (1–6) states of two isoforms of Ulip. Of interest, a third isoform (C) with an M_r of ~ 85 kDa was recognized by all three antisera in the rat PC12 pheochromocytoma cell line, as described below (see Fig. 6).

Because the B isoform is not recognized by the anti-stathmin antibody, whereas it is detected with both the anti-peptide V and the anti-recombinant Ulip antisera, it lacks the stathmin-related epitope. This could be the result of a post-translational modification of alternative splicing, or of the expression of a second, closely related gene. Because the lower M_r form is recognized by the anti-stathmin antiserum, one can exclude the possibility that isoform A is a result of a proteolytic degradation of isoform B. In agreement with this, the recombinant Ulip protein, which contained an additional 20 amino acid "His" tag (see Materials and Methods), migrated only slightly above the A isoform and clearly below the B isoform on SDS gels (data not shown).

Partial purification of Ulip and cloning and sequencing of a corresponding cDNA

Partial purification of Ulip was achieved from neonatal mouse brains, where it is most abundant. Brains were homogenized, and a high-speed supernatant was prepared and chromatographed on a DEAE-Sepharose column (see Materials and Methods). Ulip eluted in a single peak between 110 and 220 mM NaCl. It then was chromatographed further on a phenyl-Sepharose column in a decreasing linear gradient from 12 to 2% ammonium sulfate

Figure 1. *A*, Two-dimensional PAGE and immunoblot analysis of a mouse-brain-soluble fraction. A soluble protein extract from neonatal mouse brains (40 μ g of protein) was electrophoresed on two-dimensional gels (pH 6–8, 13% polyacrylamide) and either silver-stained (*left*) or blotted onto nitrocellulose and subjected to immunodetection with an anti-stathmin antiserum (1:10,000) (*right*). *Stathmin* and *Ulip* spots are indicated. *B*, Characterization of specific anti-Ulip antisera. Antisera were raised specifically against Ulip by injecting rabbits either with peptide V (see Fig. 2) or with the recombinant Ulip protein. The soluble fraction from neonatal mouse brains (15 μ g of protein) was electrophoresed on a 13% polyacrylamide gel and immunoblotted with anti-stathmin (1:10,000 dilution), anti-recombinant Ulip (1:20,000), or anti-peptide V (1:2,000) antiserum. Note that the antiserum directed against the recombinant Ulip is the most specific (see *Ulip*); *St.*, stathmin. *C*, Multiple forms and *in vitro* dephosphorylation of Ulip. Protein (40 μ g) from the soluble fraction of a neonatal mouse brain homogenate was separated by two-dimensional PAGE (pH 6–8, 10% acrylamide), and the Ulip protein was revealed with the indicated antisera (same dilutions as in *B*). Two groups of forms with respective M_r of ~64 kDa (group *A*) and ~70 kDa (group *B*) are indicated, as are the corresponding nonphosphorylated (*N*) and phosphorylated (*P*) forms. The phosphorylated forms are numbered, most likely according to their degree of phosphorylation. The form number 4 seems to be subdivided further in *a* and *b*, differing probably by some other post-translational modification (see text). All of the forms recognized by the anti-recombinant Ulip also were recognized by the anti-peptide V antiserum (data not shown). *Control*, untreated brain homogenate; *phosphatase*, 20 U of alkaline phosphatase was added to the protein fraction and incubated at 37°C for 10 min, resulting in the conversion of most phosphorylated forms of Ulip toward their unphosphorylated states N_A and N_B .

saturation. After desalting and concentration, this peak was purified further by molecular sieve, FPLC chromatography on Superose 12HR. The resulting Ulip fraction was enriched ~100-fold compared with the original soluble fraction of the neonatal mouse brain (data not shown).

The enriched Ulip-fraction proteins were separated by two-dimensional PAGE, the A3 spot of Ulip was cut out, and the sequences of five internal peptides were determined (Fig. 2). Two degenerate oligonucleotides (Oli-1 and Oli-2) based on the respective sequences of peptides V and IV were used to screen

750,000 clones of a neonatal mouse brain Uni-Zap cDNA library at high stringency. Oli-1 yielded no clearly positive clones, whereas Oli-2 detected 14 positive clones, all of which contained 1500- to 2000-bp-long inserts that differed only by the length of their 5' ends.

The longest sequence identified is presented in Figure 2. It displays an open reading frame (ORF) with a translation initiation consensus sequence (Kozak, 1987) upstream of an initial ATG. The ORF codes for a protein of 570 amino acids, which contains the sequences of all five peptides that were sequenced earlier

gaattcggcagcagcaaaagaaaaaagaaaaaaataacaaaaaagaacccccagccggcgctgaacgccagtgaggagggtgagtgaggcg 94
 gggggcgggtcggggcgaagggggaactggggagatttactattgtctcgcagccgcgcggggagcccgaggggggcgaggcgagaggaggcg 190
 gcacgaccggcgccggcgaccattcgcctccacctgatctggggcgctgtgcgctgagccaggcgcgagcagcagcagtagcagcgaggcagacat 286

cccctgcagccagaatcgccacc ATG TCC TAC CAG GGC AAG AAG AAC ATT CCG CGG ATC ACG AGT GAC CGT CTT CTA 363
 M S Y Q G K K N I P R I T S D R L L 18

ATC AAG GGA GGG AGA ATC GTC AAT GAT GAT CAG TCC TTT TAT GCT GAT ATT TAC ATG GAG GAT GGC TTA ATA 435
 I K G G R I V N D D Q S F Y A D I Y M E D G L I 42

AAG CAA ATT GGA GAC AAT CTG ATT GTC CCC GGA GGT GTG AAG ACC ATC GAG GCC AAC GGG AAG ATG GTG ATC 507
 K Q I G D N L I V P G G V K T I E A N G K M V I 66

CCT GGA GGC ATT GAC GTC CAT ACC CAC TTC CAG ATG CCT TAC AAG GGA ATG ACC ACA GTG GAC GAT TTC TTC 579
 P G I D V H T H F Q M P Y K G M T T V D E F F 90

CAA GGG ACA AAG GCT GCC TTA GCG GGA GGC ACC ACC ATG ATC ATT GAC CAT GTG GTA CCT GAA CCT GAG TCT 651
 Q G T K A A L A G G T M I D H V V P E P E S 114

AGC CTG ACA GAG GCC TAT GAA AAG TGG CGT GAG TGG GCC GAT GGG AAG AGC TGC TGT GAC TAT GCT TTG CAT 723
 S L T E A Y E K W R E W A D G K S C C D Y A L H 138

GTG GAC ATC ACC CAC TGG AAT GAC AGC GTC AAG CAG GAG GTA CAG AGC CTC AGC AAG GAA AAA GGC GTT AAC 795
 V D I T H W N D S V K Q E V Q S L S K E K G V N 162

TCC TTC ATG GTT TAC ATG GCC TAC AAG GAT TTA TAT CAA GTG TCC AAC ACA GAG CTC TAT GAG ATC TTC ACC 867
 S F M V Y M A Y K D L Y Q V S N T E L Y E I F T 186

TGC CTG GGA GAG CTG GGG GCC ATT GCT CAA GTT CAT GCC GAG AAT GGA GAT ATC ATT GCC CAG GAG CAG GCA 939
 C L G E L G A I A Q V H A E N G D I I A Q E Q A 210

CGG ATG CTG GAA ATG GGG ATA ACC GGC CCA GAA GGC CAC GTT CTG AGC AGA CCG GAA GAG CTG GAA GCT GAG 1011
 R M L E M G I T G P E G H V L S R P E E L E A E 234

GCT GTG TTC CGT GCC ATC ACC GTC GCC AGC CAG ACC AAC TGC CCC CTC TAT GTC ACC AAG GTC ATG AGC AAG 1083
 A V F R A I T V A S Q T N C P L Y V T K V M S K 258

AGC GCG GCT GAT CTC ATC TCA CAA GCC AGG AAG AAA GGA AAT GTG GTC TTT GGC GAG CCC ATC ACT GCC AGC 1155
 S A A D L I S Q A R K K G N V V F G E P I T A S 282

CTG GGA ATA GAT GGA ACT CAT TAC TGG AGT AAG AAC TGG GCC AAA GCA GCT GCG TTT GTG ACA TCC CCA CCT 1227
 L G I D G T H Y W S K N W A K A A A F V T S P P 306

CTG AGC CCT GAC CCT ACC ACA CCT GAC TAC ATC AAC TCC TTG CTG GCC AGC GGG GAT CTG CAG CTC TCT GGA 1299
 S P D P T T D Y I N S L L A S G D L Q L S G 330

AGT GCC CAC TGT ACC TTT AGC ACT GCC CAG AAA GCC ATT GGG AAG GAC AAC TTC ACA GCC ATC CCT GAA GGC 1371
 S A H C T F S T A Q K A I G K D N F T A I P E G 354

ACC AAT GGC GTG GAG GAG CGT ATG TCT GTC ATC TGG GAC AAG GCT GTG GCC ACG GGG AAG ATG GAT GAA AAC 1443
 T N G V E E R M S V I W D K A V A T G K M D E N 378

CAG TTT GTG GCC GTG ACA AGC ACC AAT GCT GCC AAG ATA TTC AAC CTG TAC CCT CGA AAG GGG AGA ATA GCT 1515
 Q F V A V T S T N A A K I F N L Y P R K G R I A 402

GTG GGC TCT GAC AGC GAC CTT GTC ATC TGG GAT CCA GAT GCC TTG AAG ATT GTC TCT GCC AAG AAC CAC CAG 1587
 V G S D S D L V I W D P D A L K I V S A K N H Q 426

TCG GTT GCC GAA TAC AAC ATC TTT GAA GGG ATG GAG CTG CGT GGT GCA CCT CTG GTG GTT ATC TGC CAG GGC 1659
 S V A E Y N I F E G M E L R G A P L V V I C Q G 450

AAG ATC ATG CTG GAA GAT GGC AAC CTG CAC GTG ACC CAG GGG GCT GGC CGC TTC ATT CCC TGC AGC CCA TTC 1731
 K I M L E D G N L H V T Q G A G R F I P C S P F 474

TCT GAC TAT GTC TAT AAG CGC ATT AAA GCA AGG AGG AAG ATG GCA GAC CTG CAT GCA GTC CCA AGA GGC ATG 1803
 S D Y V Y K R I K A R R K M A D L H A V P R G M 498

TAT GAT GGA CCA GTG TTT GAC TTG ACC ACC ACC CCC AAG GGG GGC ACC CCA GCT GGC TCT ACT CCG GGC TCT 1875
 Y D G P V F D L T T T P K G G T F A G S T R G S 522

CCC ACT CGG CCA AAC CCG CCA GTG AGG AAC CTC CAT CAG TCA GGA TTT AGC CTG TCA GGC ACC CAA GTG GAT 1947
 P T R P N P P V R N L H Q S G F S L S G T Q V D 546

GAG GGT GTC CGC TCA GCT AGC AAA CGC ATT GTG GCA CCC CCT GGA GGC CGT TCT AAC ATC ACA TCC CTG AGT 2019
 E G V R S A S K R I V A P P G G R S N I T S L S 570

taagccctcccaagaggaggcagaagcaaaaaaaaaaaaaaaaaaactcgagggggg 2076

from purified Ulip, indicating that it indeed encodes this protein. There is no significant homology between the sequences of Ulip and stathmin, suggesting that the cross-reactivity with the anti-stathmin antiserum affected only a restricted epitope and, thus, was fortuitous. The calculated molecular mass of the protein corresponding to the identified sequence is 61,940 Da, and its theoretical pI is 6.3. It is hydrophilic and contains several predicted α helices and β sheets, but none of them of extensive length.

In agreement with the observation that Ulip is a phosphoprotein *in vivo*, its sequence displays consensus sites for several protein kinases (Fig. 2, *shaded boxes*) such as PKA (S363, S522, and S553), casein kinase II (T84, S226, and T543), and “proline-directed” kinases such as cdk or mitogen-activated protein kinase (MAPK) (S308, T313, and S472). This is also in agreement with *in vitro* phosphorylation results indicating that the partially purified Ulip protein is a good substrate for casein kinase II, cdc2, and PKA (data not shown).

Molecular conservation of Ulip and sequence homologies with a D-hydantoinase and Unc-33

A search for protein sequence homology in the GenBank database revealed that the Ulip amino acid sequence is 77% identical to that of the rat neuronal protein designated toad-64 (accession number Z46882) (Minturn et al., 1995). This protein, therefore, likely is a member of the same protein family as Ulip (see also Discussion).

Databank screening also revealed several short, Ulip-related human fetal brain-expressed-sequence tags (ESTs) (Adams et al., 1993), which cover three domains (*EST I*, *EST II*, and *EST III* in Fig. 3) that together represent more than half of the Ulip sequence. There is a high degree of similarity between the corresponding regions of the mouse Ulip and the human EST-derived amino acid sequences (81, 76, and 96% identity within EST domains I, II, and III, respectively). Furthermore, the anti-Ulip antiserum recognized a protein with an M_r of 64 kDa on a Western blot of a human brain extract, as in the mouse (data not shown). Therefore, it is likely that the EST sequences correspond to one or more human proteins of the Ulip protein family (see also Discussion).

GenBank database screening also revealed homologies with sequences from more distant species: a bacterial D-hydantoinase (Jacob et al., 1987), and the product of the *unc-33* gene from the nematode *C. elegans* (Li et al., 1992) (Fig. 4).

The amino acid sequence of the *Pseudomonas putida* D-hydantoinase covers approximately three quarters of the Ulip sequence (residues 14–469), with nearly 40% identity. D-Hydantoinases catalyze the hydrolysis of D-5-monosubstituted hydantoins and D-dihydropyrimidines to yield the corresponding N-carbamyl-D-amino acid (Syldatk et al., 1990). In preliminary experiments, no D-hydantoinase activity was detected with recombinant Ulip produced in bacteria.

The amino acid sequence of the *C. elegans unc-33* gene product covers nearly the entire length of Ulip with 33.5% identity. It has been proposed that Unc-33 plays a role in

axonal guidance and outgrowth in *C. elegans* (Desai et al., 1988; McIntire et al., 1994). Animals bearing the mutated gene are severely uncoordinated, almost paralyzed, and are partially egg-laying-defective. The gene product of *unc-33* exists in three alternatively spliced forms (of 854, 676, and 523 amino acids), which differ only in the length of their N-terminal extension (Li et al., 1992). The sequenced form of Ulip corresponds to the shortest of the three Unc-33 forms.

Of interest, the triple alignment of Ulip with hydantoinase and Unc-33 reveals a highly conserved region of 34 amino acids (Fig. 4, see region b: 380–413 in Ulip) with 59% overall identity among the three sequences (84% between Unc-33 and Ulip, 65% between hydantoinase and Ulip). The similarity between Ulip and Unc-33, a protein of almost the same length, is dispersed throughout the sequence with a lower degree of similarity in the C-terminal quarter (d) of the protein. The shorter D-hydantoinase sequence reveals a slightly higher (40%) similarity with Ulip than Unc-33 (36%) in the N-terminal three quarters (a–c) of the Ulip sequence, but it lacks the C-terminal d region.

Brain specificity and developmentally regulated expression of Ulip

The striking molecular similarity of Ulip with the Unc-33 protein, which is implicated in the regulation of axonal guidance and outgrowth in *C. elegans*, led us to investigate their potential biological similarities.

Northern blot analysis of total RNA extracts from various tissues (Fig. 5A) revealed a 5.5 kb Ulip mRNA in newborn mice, mostly expressed in the brain and more weakly expressed in the heart and muscle. In the adult, there was no detectable 5.5 kb signal in any of the tissues tested. However, a 2 kb mRNA not observed in the brain was detected in the testis even at high stringency (65°C in 0.1× SSC, 0.1% SDS).

Western blot analysis of the expression of Ulip in various tissues from the neonatal mouse showed that the protein also is expressed almost exclusively in the brain (Fig. 5B). In agreement with the Northern blot results, a much weaker but clear expression was detected in the heart, as was a very weak but still detectable expression in the lung and muscle, possibly attributable, at least in part, to the presence of neuronal cells in the corresponding tissue preparations. In the adult animal, the expression of the Ulip protein could be detected only in the brain, where its expression was at least 20-fold lower than in the neonate, because even a 20-fold longer autoradiographic exposure of the Western blot revealed a weaker signal (Fig. 5B). Also correlating well with the Northern blot results, a very weak band corresponding to the Ulip protein was detected on the Western blot of the adult testis.

In light of the results showing mostly a neonatal brain-specific expression of Ulip, we further examined its brain-expression pattern during the neonatal period of development (Fig. 5C). The protein becomes detectable in the cortex and the striatum at embryonic day 16, reaching a peak at approximately postnatal day 5, when neuronal differentiation and synapse formation are very

Figure 2. Complete nucleotide sequence of the cloned cDNA and deduced amino acid sequence of Ulip from mouse brain. The nucleotide sequence is numbered starting from the first nucleotide at the *EcoRI* insertion site. Amino acids are numbered from the ATG start codon after the boxed consensus Kozak sequence. The sequences of peptides I–V determined by protein sequencing are underlined, and the regions corresponding to the two oligonucleotides (*Oli-1* and *Oli-2*) used for the library screening are overlined. Consensus phosphorylation site sequences are indicated in *shaded boxes* (with the presumed phosphorylation residue in a *black box*) for PKA, casein kinase II (*dashed underline*), and “proline-directed” kinases (*solid underline*).

1									
EST	I-MSYQGGKNIP	RITSDRILIK	GGKIVNDDQS	FYADIYMEDG	LIKQIGENLI	VPGGVKTIEA	NGRMVIPGGI	DVNTYLQKPS	QGMTAANDFF
Ulip	MSYQGGKNIP	RITSDRILIK	GGKIVNDDQS	FYADIYMEDG	LIKQIGENLI	VPGGVKTIEA	NGRMVIPGGI	DVHTHFQMPY	KGMTTVDDFF 90
toad	MSYQGGKNIP	RITSDRILIK	GGKIVNDDQS	FYADIYMEDG	LIKQIGENLI	VPGGVKTIEA	HSRMVIPGGI	DVHTRFQMPD	QGMTSADDF
	*****	*****	***	*****	*****	*****	*****	***	***
143									
EST	QGTAAALVGG	TTMIIDHVVP	EPGSSLLTSF	EKWHEAADTK	SCCNYSLHVD	ITS-I			
Ulip	QGTAAALVGG	TTMIIDHVVP	EPSSSLTEAY	EKWREWADGK	SCCDYALHVD	ITWVNDVSKQ	EVQSLSKKEG	VNSFMVYMAV	KDLYQVSNTE 180
toad	QGTAAALVGG	TTMIIDHVVP	EPGTSLLAAF	DQWREWADSK	SCCDYSLHVD	ITEWHKGIQE	EMEALVKDHG	VNSFLVYMAF	KDRFLQLTDSQ
	***	***	***	***	***	***	***	***	***
270									
Ulip	LYEIFTCLGE	LGAIQVHAE	NGDIIAQEQA	RMLEMGITGP	EGHVLSRPEE	LEAEAVFRAI	TVASQTNCPL	YVTKVMSKSA	ADLISQARKK 270
toad	IYEVLVSIRD	IGAIQVHAE	NGDIIAEEQQ	RILDLGITGP	EGHVLSRPEE	VEAEAVNRSI	TIANQTNCPL	YVTKVMSKSA	AEVIAQARKK
360									
Ulip	GNVVFGEPI	ASLGIDGTHY	WSKNWAKAAA	FVTSPLSPD	PTTPDYINSL	LASGDLQLSG	SAHCTFSTAQ	KAIGKDNFTA	IPEGTNGVEE 360
toad	GTVVYGEPI	ASLGTDGSHY	WSKNWAKAAA	FVTSPLSPD	PTTPDFLNSL	LSCGDLQVTG	SAHCTFNATAQ	KAVGKDNFTL	IPEGTNGTEE
370									
EST	II-V	ATGKMDENQF	VAVTSTNAAK	IFNLYPRKGR	IAGVSDADV	IWDPKLTKTI	TAKSHKSAVE	YNIFEGMECH	GSPLVVISQG
Ulip	RMSVIWDKAV	ATGKMDENQF	VAVTSTNAAK	IFNLYPRKGR	IAGVSDSDLV	IWDPDALKIV	SAKNHQSVAE	YNIFEGMELR	GAPLVVICQG 450
toad	RMSVIWDKAV	VIGKMDENQF	VAVTSTNAAK	VFNLYPRKGR	ISVGSDADLV	IWDPDVSKTI	SAKTHNSALE	YNIFEGMECR	GSPLVVISQG
		*	*****	*****	*****	*****	*****	*****	*****
476									
EST	KIVFEDGNIN	VNKGMRFP	RKAFPE-II						
Ulip	KIMLEDGNLH	VTQAGRFIP	CSFFSDYVYK	RIKARRKMD	LHAVPRGMYD	GPVFDLTTTP	KGGTPAGSTR	GSPTRPN-PP	VRNLHQSGFI 539
toad	KIVLEDGTLH	VTEGSGRYIP	RKFFPDFVYK	RIKARSRLAE	LRGVPRGLYD	GPVCEVSVTP	KTVPASSAK	TSPAKQQAPP	VRNLHQSGFS
	**	***	*	***	*			**	*****
522									
EST	FSGTQVDEGV	-RSASKRIVA	PPGGRSNITS	LS-III					
Ulip	LSGTQVDEGV	-RSASKRIVA	PPGGRSNITS	LS 570					
toad	LSGAQIDDNI	PRRTTQRIVA	PPGGRANITS	LG 572					
	***	*	*****	*****	*				

Figure 3. Amino acid alignment of Ulip with human ESTs and rat toad-64. Three human fetal brain EST-derived amino acid sequences obtained by combining overlapping shorter fragments from GenBank (I, combination of ESTs 05414 from Ulip residues 1–60 and EST 04167 from Ulip residues 61–143; II, EST 06020; III, EST 03046; respective GenBank accession numbers are T07524, T06278, T08129, and T05158) and the toad-64 sequence (GenBank accession number Z46882) (toad) were aligned with Ulip. Alignment with the Clustal V software reveals an overall 81% identity between Ulip and the EST fragments (I, 81%; II, 76%; III, 96%) and 76% identity between Ulip and the toad-64 protein. Double identities between sequences are indicated by *colons*, and triple identities are indicated by *stars*.

active. It then decreases to very low levels as soon as postnatal day 20.

Regulation of Ulip phosphorylation by NGF in PC12 cells

The PC12 cell line often is used as an *in vitro* model for neuron-like differentiation. Indeed, NGF induces the flattening of cells and the outgrowth of neurites as well as the appearance of molecular markers of neurons (Greene and Tischler, 1982).

Two-dimensional Western blots with antisera directed against the mouse protein showed that Ulip is detected easily in rat PC12 cells (Fig. 6*B*), where it also can be identified clearly on gels by silver-staining (Fig. 6*A,B*). The rat PC12 protein actually comigrated with the purified mouse brain protein on two-dimensional PAGE gels (data not shown). These results are in good agreement with the apparent evolutionary conservation of the Ulip protein. As in the brain, only the specific anti-Ulip antisera recognized its B isoform. As mentioned above, all antisera also recognized a third likely isoform (C) of the Ulip protein in PC12 cells, with an M_r of ~85 kDa (Fig. 6*B*), which is expressed at a level comparable with that of the A isoform. This isoform was also detected by silver-staining.

The presumably phosphorylated forms of the C isoform, like the B isoform, have identical isoelectric points to those of the A isoform. It is unlikely that the interaction with the C isoform represents a fortuitous cross-reactivity of three distinct antisera. This isoform, therefore, probably corresponds to another alternatively spliced form of Ulip, including additional exons that might account for its larger size, or to a closely related protein of the same family.

It was of great interest to see whether the phosphorylation pattern of Ulip was changed after induction of the differentiation of these cells toward a neuron-like phenotype. PC12 cells were labeled with [32 P](PO $_4$) $^{-3}$ *in vivo*, and the radioactive phosphoproteins from control or NGF-induced cells were analyzed by two-dimensional PAGE and autoradiography. As shown in Figure 6*C*, at least two forms, A3 and A4, were radiolabeled in control cells. These phosphoproteins coincided clearly with the known Ulip spots on silver-stained gels as well as with the immunostained spots on comigrations with the soluble fraction of PC12 cells (data not shown).

After NGF treatment for 30 min, the A4 form became undetectable. This might be attributable either to the phos-

A

hyd.		MTKIIK	NGTIVTATDT	YEADLLIKDG	KIAMIGQHLE	EKGA-EVIDA	KGCYVFPGGI	DSHTHLDMPF	GGTVTKDDFE	75
Ulip	MSYQGGKNIP	RITSRLLIK	GGRIVNDQDS	FYADIYMEDG	LIKQIGDNLI	VPGGVKTIEA	NGKMVIPGGI	DVHTHPQMPY	KGMTTVDDFF	90
unc33		MSILLVK	NAQIVNDDAI	FVADILIEDG	IIQNVAPNLE	APEGAEVLDA	AGKLALPAGI	DVYTQVT---	--DSSVDDLS	72
		*	**	**	*	*	*	**	**	
hyd.	SGTIAAAGG	TTTIIDFCLT	NKGEPLKKA	ET-WHNKAG	KAVIDYGFHL	MISEITDDVL	EELPKVIAEE	GITSFKVFMA	YKNVPQADDG	164
Ulip	QOTKAALAGG	TTMIIDHVVP	EPESLSLEY	EK-WREWADG	KSCCDYALHV	DITHWNSVK	QEVQSLSKEK	GVNSPFMVMA	YKDLYQVSNT	179
unc33	TGCKSAIAGG	TGTIVEVVRP	RGAESVVS	KRVKNQLEKS	GISCHVALSV	AITDFCE---	QEMSELVKNE	GINSFVL---	--DGVSLTDD	154
	*	*	*	*	*	*	*	*	*	
hyd.	TLYRTLVA	ELGALVMVHA	ENGVDIDYLT	KKALAEGNTE	PIYHALTRPP	EVEGEATGRA	CQLTELAGSQ	LYVVHVTCQA	AVEKIAQARN	254
Ulip	ELYEIFTCLG	ELGALIAQVHA	ENGDIIAQEQ	ARMLEMGITG	PEGHVLSRPE	ELEAEAVFRA	ITVASQTNCP	LYVTKVMSKS	AADLISQARK	269
unc33	KLLELFEHVK	RLGALIRVVP	ENKSIVAMLE	KKMLKLGVTG	PEGFPQSRPE	SLEADRVSGV	CVLGNLASC	ISIVQVSSAD	SLAAIEKARA	244
	*	***	*	**	*	*	*	*	*	
hyd.	KGLDVWGETC	PQYLVLDDQS-	YLEKPDFEGA	KYVWSPPLR-	EKWHQEVLEW	ALKNGQLQTL	GSDQCSPDFK	GQKELGRGDF	TKIPNGGPMV	342
Ulip	KGNVVFGEPI	TASLGIDGTH	YWSKNWAKAA	APVTSPPPLSP	DPTTPDYINS	LLASGDLQLS	GSAHCTFS-T	AQKAIGKDNF	TAIPEGTNGV	358
unc33	SGALAHABIA	SAAVTADGSA	LFSQDLRFAS	AHLTDVPLRR	G--APDRMIG	ALSTQPLVVC	TSGHRPVN--	SATRVAAKDF	AIAQKGSTGA	330
	*	*	*	**	*	*	*	*	*	
hyd.	EDRVLSILFSE	GVKKGRITLN	QFVDIMSTRI	AKLFLGLPRK	GTIAVGSAD	LVIFDPDIER	VISAETHHMA	VDYNAFEGMK	VTGEPVSVRA	432
Ulip	EERMSVINDK	AVATGKMDEN	QFVAVTSTNA	AKIFNLYPRK	GRIAVGSDSD	LVIWDPDALK	IVSAKNHQS	AEYNIPEGME	LRGAPLVVIC	448
unc33	EERMAVVWER	AVRSGRIDAM	RFVAVTSTNA	AKMFNMPKK	GRIAVGADAD	LVIWDASGKR	VLESSRAQSS	QENSMYDGLT	VHSVVTATIV	420
	*	*	*	**	*	*	*	*	*	
hyd.	EANLLSVINN	ISENQGTANI	452							
Ulip	QGKIMLEDGN	LHVTQAGGRF	IPCSPFSDYV	YKRIKARRKM	ADLHAVPRGM	YDGPVFDLTT	TPKGGTPAGS	TRGSPTRPNP	PVRNLHQSGF	538
unc33	GGKIAYQNGE	VREAPVAGGF	LRLSPNSPYL	FSMVGQRDKF	ANVERVERE-	---ASSQQQK	PQQNGHHKNS	GDFDRNRT--	---KVMESSI	501
	*	*	*	*	*	*	*	*	*	
hyd.										
Ulip	SLSGTQVDEG	VRSASKRIVA	PPGGRSNITSLS	570						
	*	*	*	*	*	*	*	*	*	
unc33	DFGG-----	--SAANRPRN	PPGGRT--TGFW	523						

B

Figure 4. Alignment of Ulip with *unc-33* and a D-hydantoinase. *A*, Triple alignment of the amino acid sequences of Ulip with those of the *C. elegans* *unc-33* gene product and of the D-hydantoinase (*hyd.*) from *P. putida* is shown. Double identities between sequences are indicated by colons, and triple identities are indicated by stars. *B*, Regions are delimited according to their degree of similarity (percentages refer to identities with the sequence of Ulip within each region or at right within their whole length). Note the well preserved *b* region, presenting 59% overall identity among the three proteins.

phorylation of A4 to a highly phosphorylated form or to the dephosphorylation of A4. Based on their migration pattern, the three spots indicated by stars above A3 and A4 on Figure 6C most likely correspond to forms B4a, B5, and B6. They were heavily phosphorylated under control conditions, whereas the addition of NGF induced a decrease in the intensity of the two more acidic spots.

It appears, therefore, that the phosphorylation of Ulip forms in PC12 cells is modified in response to NGF, possibly in

relation to the control of neuronal differentiation and neurite outgrowth.

Intracellular distribution of Ulip in PC12 cells and in primary cultured neurons

To attain deeper insight into the possible biological role of Ulip, its intracellular distribution in PC12 cells and in cultured rat striatal neurons was examined by immunofluorescence with the anti-recombinant Ulip antiserum (Fig. 7). When the same cultured neurons were labeled

Figure 5. Tissue specificity and developmental regulation of the expression of Ulip in the mouse. *A*, Northern blot. Five micrograms of total RNA of the indicated tissues from newborn or adult mouse were separated on a 1% agarose gel containing formaldehyde, transferred to nylon membranes, and hybridized with the ^{32}P -labeled *EcoRV*-*XhoI* fragment of the Ulip cDNA (see Materials and Methods). The integrity and the quantitative equivalence of the RNAs were checked before hybridization by staining with methylene blue (data not shown). The right panel displays the entire length of the first and the last lanes of the left panel (newborn brain and adult testis), showing the weak 2 kb signal observed only in adult testis. *B*, *C*, Immunoblots. *B*, Sixty micrograms of protein homogenates from the indicated tissues were separated by one-dimensional PAGE on a 10% polyacrylamide gel, transferred onto nitrocellulose membranes, and probed with the anti-recombinant Ulip antiserum (1:50,000) and detected with ^{125}I -labeled protein A. As indicated, all of the tissues examined in the newborn and adult animals were the same except for the thymus in the newborn and the testis in the adult mouse. The immunoblots were exposed either without ($\times 1$) or with ($\times 20$) a Kodak Quanta III screen for the same duration, the latter yielding an ~ 20 -fold enhanced signal. Stars refer to the minor, higher M_r bands visible in the newborn brain sample, most likely corresponding to the B and C Ulip isoforms (see Figs. 1, 6). *C*, Fifty micrograms of protein from mouse brain cortex or striatum homogenates at the indicated stages of development were separated by one-dimensional PAGE, transferred onto nitrocellulose membranes, and revealed with cross-reactive anti-stathmin antibody (1:10,000) and ^{125}I -labeled protein A, showing a peak of Ulip expression at ~ 5 d postnatal.

either with immune serum preincubated with the recombinant Ulip protein or with preimmune serum at the same dilution, the fluorescence was reduced strongly, indicating that the labeling with the anti-Ulip serum was highly specific.

In PC12 cells, the protein was cytoplasmic and was absent from the nuclei. After induction of differentiation with NGF, Ulip was also detectable in all of the neurites, where the antiserum clearly labeled varicosities (arrowheads; the lower intensity of neurite labeling in Fig. 7*B* is attributable to the focus on the cell body and nucleus). In agreement with results from immunoblot experiments (data not shown), no difference was observed in the expression level of Ulip between NGF-treated and nontreated cells. No clear colocalization of the Ulip protein with cytoskeletal elements could be observed by double-staining PC12 cells with antibodies recognizing cytoskeletal proteins such as tubulin, actin, and the M_r 68 kDa neurofilament isoform (data not shown).

Primary neurons from E20 rat striatum were examined after 8 d in culture (Fig. 7). Like PC12 cells, all of the neurons displayed immunoreactivity in the cytoplasm, whereas very little if any signal could be detected in the nuclei. The neurites and axons were labeled along their entire length, with a strong staining at the varicosities (arrowheads) and at the growth cones (arrows).

Expression of Ulip at the neuromuscular junction

The fact that Ulip was associated with neuritic processes grown in culture prompted us to examine its presence at peripheral motor nerve endings at the neuromuscular junction. Double-labeling experiments were performed using the anti-Ulip antiserum and the rhodamine-conjugated α -bungarotoxin, which binds to acetylcholine receptors (Fig. 8). Synaptic sites, which were identified by their α -bungarotoxin labeling on cross-sections of adult gastrocnemius muscle, displayed a moderate but clearly positive Ulip

Figure 6. Ulip expression and phosphorylation in PC12 cells. Proteins from the soluble fraction of PC12 cells were separated by two-dimensional PAGE. The entire silver-stained two-dimensional gel is shown with the area of Ulip boxed (*A*). In *B*, the same area enlarged is displayed together with the corresponding areas of Western blots probed with sera directed against stathmin, peptide V, or recombinant Ulip. Numbers refer to the various forms of each of the A (64 kDa), B (70 kDa), and C (85 kDa) isoforms of Ulip, as defined in Figure 1. Note that the A and C isoforms are recognized by all the three antisera, whereas the B isoform is not recognized by the anti-stathmin antiserum. For this experiment, the anti-peptide V antibody was used after affinity purification against peptide V. *C*, *In vivo* phosphorylation in PC12 cells. PC12 cells were incubated for 4 hr with ^{32}P -labeled *o*-phosphate without (*control*) or with (+NGF) the addition of NGF (200 ng/ml) for the final 30 min. Proteins were separated by two-dimensional PAGE and detected by autoradiography. Numbers refer to the corresponding phosphorylated forms of the A isoform of Ulip, whereas the asterisks indicate most likely increasingly phosphorylated B forms.

staining. Triple labeling with anti-Ulip, anti-neurofilament antibody, and α -bungarotoxin showed that Ulip immunoreactivity was associated with intramuscular nerve branches located near the neuromuscular junction and was recognized by the positive staining with the anti-neurofilament antibody (data not shown).

To confirm further the location of Ulip at the neuromuscular junction, we performed whole-mount preparations of teased sternocleidomastoid muscle fibers (Fig. 8). Ulip immunoreactivity examined by confocal fluorescence microscopy appeared at two different levels at motor nerve endings: as a staining pattern that closely matched the α -bungarotoxin labeling, and as a positive immunoreaction delineating the preterminal region associated with the neuromuscular junction.

DISCUSSION

The present characterization of the developmentally regulated neuronal Ulip phosphoprotein led us to identify both molecular and biological similarities with the product of the *C. elegans* gene *unc-33*. Ulip thus is likely to be its functional mammalian homolog, potentially related to the molecular processes underlying axonal guidance and neuritic elongation that take place during development and regeneration within the nervous system.

Biological and molecular properties of Ulip

The expression of Ulip appeared preferentially in the nervous system, where it is highly dependent on development; these findings support a role of Ulip related to the molecular processes allowing and/or controlling specific stages of neuronal development.

In PC12 cells and primary neurons in culture, Ulip could be detected in the cell body, but not in the nucleus, and in all of the neuritic processes, showing a marked labeling of varicosities and growth cones. These observations clearly demonstrate the neuronal expression of Ulip and its likely involvement in neuronal growth and/or differentiation. Ulip was detected in peripheral nerve endings at the adult neuromuscular junction, where it was coincident with the nicotinic acetylcholine receptor labeling, but Ulip was associated also with presynaptic regions. This latter observation reflects the presence of Ulip in neuronal processes and, possibly, in associated terminal Schwann cells.

In the mouse brain, at least two Ulip isoforms, A and B, are present, with respective M_r values of 64 and 70 kDa, whereas a third, additional M_r 85 kDa C isoform is expressed in rat PC12 cells. In the mouse brain, a single 5.5 kb mRNA was detected, which likely codes for the most abundant A isoform. The various forms of Ulip might be products of the same gene and translated from alternatively spliced mRNAs, as was shown also for *Unc-33* (Li et al., 1992). Another possibility is that the diverse forms recognized by the various antisera correspond to closely related proteins of a common, conserved family, as suggested by the comparison of all of the related sequences available so far (see below). Interestingly, an additional mRNA of ~ 2.0 kb was found in the testis, where traces of the protein also were detected.

Ulip is a phosphoprotein, because it is sensitive to alkaline phosphatase and incorporates radioactive phosphate, which is in good agreement with the presence of consensus phosphorylation sites for several types of protein kinases within its amino acid sequence. *In vivo*, the phosphorylation state of Ulip in PC12 cells was modified in response to the neuronal growth, differentiation, and survival factor NGF. This is a clear indication of a possible regulation of the activity of this protein, and of its potential implication in intracellular cascades involved in the regulation of neuronal differentiation and maturation.

Molecular and functional similarities

The databank search with the Ulip sequence revealed similarities with mammalian sequences, toad-64, several brain ESTs

Figure 7. Immunofluorescence labeling of Ulip in PC12 cells and rat striatal primary neurons. Control (*A*) and NGF-induced (*B*) PC12 cells were fixed with methanol, and E20 neurons from rat striatum (*C–F*) were fixed after 8 d in culture with 2% *p*-formaldehyde. Cells were labeled with the anti-recombinant Ulip antiserum (1:500; *A–D*), with anti-Ulip antiserum preincubated with 10 $\mu\text{g}/\mu\text{l}$ recombinant Ulip protein (*E*), or with preimmune rabbit serum at the same dilution as in *A–D*. Primary antibodies were revealed with rhodamine-conjugated anti-rabbit secondary antibodies. Note neurites with strongly immunoreactive varicosities (arrowheads) and growth cones (arrows). In *B* the focus is mainly on the cell body, resulting in lower apparent fluorescence intensity in neurites; in *D*, only the axonal extension of a neuron can be seen, the cell body being outside the field. Scale bars, 25 μm ; magnification in *B*, *C*, *E*, and *F* as in *A*.

and, more provocatively, a *P. putida* bacterial hydantoinase and the *C. elegans unc-33* gene product. After submission of this paper, two additional Ulip-related sequences were reported, crmp-62 in chick (Goshima et al., 1995) (accession number U17277) and munc in mouse (S. Tontsch, unpublished data; accession number X87242).

Ulip exhibits puzzling sequence similarities with several bacterial hydantoinases (Yang et al., 1993), the most striking of them

with an enzyme from *P. putida* (Jacob et al., 1987), the sequence of which covers the N-terminal three quarters of Ulip. The only mammalian hydantoinase-like protein that displays a certain similarity with Ulip is a hamster dihydro-orotase (Simmer et al., 1990; Williams et al., 1990), which has a high degree of similarity (60% identity) with Ulip only in a 37-amino-acid stretch (Ulip residues 67–103). This region is well preserved in all dihydro-orotases known so far, but the probable critical amino acids for Zn^{2+}

Figure 8. Ulip expression at the neuromuscular junction. Transverse sections of adult mouse gastrocnemius muscle (*left*) or whole-mount-teased fibers from sternocleidomastoid muscle (*right*) were double-stained with the anti-recombinant Ulip antibody (revealed with a fluorescein-conjugated secondary antibody) and rhodamine-conjugated α -bungarotoxin. On the muscle sections examined by conventional fluorescence microscopy, immunoreactivity to Ulip was present at the synaptic sites (*arrows*) identified by α -bungarotoxin binding as well as on interstitial nerve fibers (*arrowheads*). Confocal microscopic analysis of teased muscle fibers revealed that Ulip immunoreactivity entirely recovered the distribution of acetylcholine receptors (*filled curved arrows*), thus appearing yellow on the superposed image. It was present additionally in scarce extrasynaptic regions revealed by the green staining on the superposed image (*open curved arrows*). Scale bars, 25 μ m.

binding (Williams et al., 1990) are not fully conserved in Ulip. Because the rest of the dihydro-oxotase sequence shows almost no homology with Ulip, it is not likely that Ulip corresponds to this enzyme. The fact that no hydantoinase activity could be detected in preliminary experiments with recombinant Ulip also does not support a direct functional homology with this class of enzymes. However, the observed similarities might correspond to the conservation of a functional, enzymatic, or regulatory domain related to the involvement of Ulip in a step of neuron-specific metabolism important for neurite outgrowth and axonal guidance during neuronal differentiation.

In view of the neuronal and developmental expression of Ulip, its sequence similarity with Unc-33 seems to be more directly relevant at the functional level. Three forms of Unc-33 are expressed in *C. elegans*, which are the products of differentially spliced mRNAs with M_r values of 55.5, 72.1, and 90.8 kDa, differing only in the length of their N-terminal extensions (Li et al., 1992) and corresponding to the expression of three mRNAs of 2.8, 3.3, and 3.8 kb, respectively. The amino acid sequence of the shortest Unc-33 form covers the entire sequence of the presumably shortest, A form of Ulip, with an overall identity of 33.5%. Although there are clearly several genes for Ulip-related proteins, it is possible that some of the Ulip forms with molecular sizes in the mouse or rat comparable with those found for *unc-33* isoforms in *C. elegans* are derived also by alternative splicing of a given gene.

In *unc-33 C. elegans* mutants, nearly all neurons inspected, including sensory, motor, and interneurons, show neuritic outgrowth defects (Hedgecock et al., 1985; Desai et al., 1988): some neuritic processes follow abnormal pathways and have swollen endings and prematurely terminated axons, which altogether underline the developmental importance of the Unc-33 protein. The developmental regulation of Ulip expression, mostly at a stage at which neuronal differentiation and maturation take place, indicates that its molecular similarity is indicative of a functional similarity with Unc-33 and, therefore, that it also might play a role in the control of neurite elongation and/or axonal guidance. The relatively low-level presence of Ulip in the adult brain might reflect its presence in areas where neurogenesis remains active or its involvement in maintaining neuronal stability, for example, at the junctional level. This latter possibility is supported by the presence of Ulip in adult nerve endings, as at the neuromuscular junction.

Interestingly, the two other Unc-33-related sequences reported in the literature, toad-64 in the rat (Minturn et al., 1995) and *crmp-62* in chick (Goshima et al., 1995), are expressed like Ulip mostly during development in the nervous system but, in contrast with Ulip, they were not detected in any other tissue even at low levels. Comparison of their amino acid sequences reveals that toad-64, *crmp-62*, and *munc*, a mouse sequence that recently became available in the sequence databanks, display ~97% amino acid identities, whereas Ulip is only ~76% identical to any of the other three sequences. This observation reveals, as suspected on the basis of the protein expression profiles discussed above, that Ulip is a member of a protein family: it should be designated, therefore, as Ulip1, the other group of Unc-33-related sequences then being Ulip2—highly conserved between birds (*crmp-62*) and mammals (toad-64 and *munc*). Several human ESTs are at least 96% identical either with Ulip1 or with the various known Ulip2 sequences, further demonstrating the high evolutionary conservation of each of these two members of the Ulip protein family. Additional human EST sequences display 72–76% identity with

either Ulip1 or Ulip2: they belong to the partial sequence designated *Hcrmp-1* by Strittmatter and coworkers (Goshima et al., 1995), which covers the Ulip1 and -2 sequences from their residue 59 to their C-terminal extremity. The corresponding protein could be designated, therefore, as Ulip3, to indicate its clear inclusion in the same protein family. The recognition of these three members of the Ulip family allows us to understand further the correspondence of the three EST domains defined in Results and shown in Figure 3: EST I (1–60) corresponds to Ulip2; EST I (61–143) and EST II correspond to Ulip 3; and EST III corresponds to Ulip1. Finally, some ESTs display lower degrees of similarity (50–60% identity) with the above-described Ulip sequences, caused by sequencing errors or by reflecting the existence of more members of the Ulip family. This overall analysis of available sequences also might explain some of our results concerning the expression of Ulip mRNA or protein, particularly in non-neuronal tissues and potentially in non-neuronal cells within the nervous system. It is likely that, although various members of the Ulip family are expressed most highly in neuronal cells, they also have distinct expression patterns, with respect both to the cell type considered and to differentiation stages during development.

We characterized Ulip as highly regulated during developmental stages in the mouse brain corresponding to active neurogenesis and synapse formation; its regulated phosphorylation suggests further its potential implication in the intracellular cascades responsible for the relay and integration of signals controlling neuronal differentiation. The present molecular and biological characterization of Ulip (Ulip1) strongly indicates that it is a structural and functional mammalian homolog of Unc-33. It appears, therefore, to be a good candidate for participating in neuronal differentiation and maturation, presumably through the control of neurite elongation and/or of axonal guidance during the development of the nervous system. Our data suggest further that Ulip plays a role during development in other tissues and cell types, and in the adult, where it might participate, for example, in the normal or post-traumatic replacement and regeneration of neurons; Ulip also is involved potentially in the promotion or maintenance of the stability of neuronal connections.

Toad-64 was recognized to be upregulated very early after division of neuronal cells and was considered to be an early marker of cells committed to a neuronal phenotype (Minturn et al., 1995). *Crmp-62* was suggested to be involved in the intracellular cascade initiated in response to collapsin (Goshima et al., 1995). Most of the ESTs corresponding to Ulip3 were identified from a human embryonic brain library (Adams et al., 1993), suggesting an expression pattern and a developmental regulation at least partially similar to those of Ulip1 (Ulip) and Ulip2 (toad-64 and *crmp-62*). However, the actual function(s) of each of the vertebrate proteins of the Ulip family is likely to be of broader significance than those originally proposed for toad-64 and *crmp-62*. Because all Ulip-related vertebrate proteins display functional and molecular similarities with the *C. elegans unc-33* gene product (32–35% sequence identity), we believe that their collective designation as “Ulip” proteins (1, 2, 3, ...) is the most appropriate because it refers to their proven resemblance to Unc-33. Their identification will allow the further characterization of their common and differential biological functions.

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