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Identification and Developmental Regulation of a Neuron-specific Subunit of Cytoplasmic Dynein

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Cytoplasmic dynein is the microtubule minus-end–directed motor for the retrograde axonal transport of membranous organelles. Because of its similarity to the intermediate chains of flagellar dynein, the 74-kDa intermediate chain (IC74) subunit of dynein is thought to be involved in binding dynein to its membranous organelle cargo. Previously, we identified six isoforms of the IC74 cytoplasmic dynein subunit in the brain. We further demonstrated that cultured glia and neurons expressed different dynein IC74 isoforms and phospho-isoforms. Two isoforms were observed when dynein from glia was analyzed. When dynein from cultured neurons was analyzed, six IC74 isoforms were observed, although the relative amounts of the dynein isoforms from cultured neurons differed from those found in dynein from brain. To better understand the role of the neuronal IC74 isoforms and identify neuron-specific IC74 dynein subunits, the expression of the IC74 protein isoforms and mRNAs of various tissues were compared. As a result of this comparison, the identity of each of the isoform spots observed on two-dimensional gels was correlated with the products of each of the IC74 mRNAs. We also found that between the fifteenth day of gestation (E15) and the fifth day after birth (P5), the relative expression of the IC74 protein isoforms changes, demonstrating that the expression of IC74 isoforms is developmentally regulated in brain. During this time period, there is relatively little change in the abundance of the various IC74 mRNAs. The E15 to P5 time period is one of rapid process extension and initial pattern formation in the rat brain. This result indicates that the changes in neuronal IC74 isoforms coincide with neuronal differentiation, in particular the extension of processes. This suggests a role for the neuronal IC74 isoforms in the establishment or regulation of retrograde axonal transport.

INTRODUCTION

The intracellular movement of membranous organelles toward the minus ends of microtubules is driven by the motor protein cytoplasmic dynein (Brady, 1991; Schroer and Sheetz, 1991; Holzbaur and Vallee, 1994). In neurons, dynein is responsible for retrograde axonal transport of membranous organelles from the synapse to the cell body (Brady, 1991; Vallee and Bloom, 1991). Retrograde axonal transport is the mechanism by which viruses, neurotrophins, and material to be degraded or recycled are moved from the synapse along the process to the cell body (Hammerschlag and Brady, 1989). The cytoplasmic dynein protein complex is made up of two ~530-kDa heavy chains, two or three ~74-kDa intermediate chains (IC74), and four 50- to 60-kDa polypeptides (Paschal *et al.*, 1987; Collins and Vallee, 1989; Vallee *et al.* 1989; Neely *et al.* 1990). Cytoplasmic dynein has two globular heads that contain the heavy chains and the motor domains of the molecule (Vallee *et al.*, 1988; Mikami *et al.*, 1993). The heads are connected by stalks

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to a common base, which binds the membranous organelles. The IC74 subunits of cytoplasmic dynein are related to the IC78 subunit of *Chlaymdomonas* flagellar outer arm dynein (King *et al.*, 1991; Mitchell and Kang, 1991; Paschal *et al.*, 1992; Ogawa *et al.*, 1995; Vaughan and Vallee, 1995; Wilkerson *et al.*, 1995), a subunit that participates in the binding of flagellar dynein to the A microtubule (King *et al.*, 1991, 1995). The similarity of the IC subunits suggests that the cytoplasmic IC74 subunits are also located at the base of the molecule and that they are involved in binding cytoplasmic dynein to membranous organelles (King and Witman, 1990; Paschal *et al.*, 1992; Ogawa *et al.*, 1995).

At least six IC74 isoforms are identified in cytoplasmic dynein from the adult rat brain, most of which are phosphorylated in vivo (Dillman and Pfister, 1994). Moreover, two distinct but closely related IC74 genes, IC74–1 and IC74–2, which are spliced to produce five mRNAs (IC74–1A and -1B and IC74–2A, -2B, and -2C) have been identified (Vaughan and Vallee, 1995). Because there are just two to three IC74 subunits per dynein molecule, these results indicate that the pool of brain cytoplasmic dynein is not homogeneous, but rather that heterogeneous cytoplasmic dynein molecules are produced by the assembly of different IC74 subunits into the dynein complex.

Brain tissue contains various specialized cell types. Thus, to investigate the different pools of cytoplasmic dynein, we first examined the IC74 subunit pool and its phosphorylation state at the cellular level by characterizing cytoplasmic dynein isolated from cultured neurons and glia (Pfister *et al.*, 1996). We found that the IC74 subunit pools of dynein from glia and neurons are different. Cytoplasmic dynein from glia is made up exclusively of the products of the IC74–2C mRNA: the IC74–2C isoform and its post-translationally modified phospho-IC74–2C isoform. Because glia express only the IC74–2C subunit, the pool of cytoplasmic dynein is homogeneous in these cells. Furthermore, the presence of this IC74 subunit in the dynein complex is sufficient for constitutive cytoplasmic dynein function and regulation in cells.

In contrast, the expression of IC74 isoforms in cultured neurons is not as well understood. The IC74 isoform pattern of dynein from neurons was similar, but not identical, to that of dynein from adult brain (Pfister *et al.*, 1996). When we compared dynein from brain and cultured cortical neurons, the same six isoforms were found in both samples, but the relative amounts of the isoforms differed. Moreover, when dynein from brain or neurons was treated with phosphatase, two of the six IC74 spots resolved on two-dimensional gels were eliminated, while four IC74 spots remained. Furthermore, while all five known IC74 mRNAs were identified in brain, the IC74–2A mRNA was not found in neurons. Neurons, with the products of three additional mRNAs, utilize a wider range of IC74 subunits than glia. As it is thought that the dynein molecule has two to three subunits of IC74, the cytoplasmic dynein molecules from neurons must differ in their IC74 polypeptide composition.

Therefore, to determine whether the IC74 isoform diversity was a unique characteristic of nerve cells and to more completely understand the IC74 isoform composition of neuronal cytoplasmic dynein, additional investigations were undertaken. In particular, given the presence of IC74 isoforms in neurons but not glia, we wanted to determine whether there were neuron-specific IC74 isoforms. We also sought to understand the basis for the observation that some of the IC74 isoforms are less abundant in cultured neurons than in the adult brain. Finally, we wanted to complete the correlation of the IC74 mRNAs with the IC74 protein isoforms resolved by two-dimensional gel electrophoresis.

By comparing the protein and mRNA of the IC74 isoforms in different tissues, we have matched all but one of the IC74 mRNAs with the IC74 spots seen when brain dynein is analyzed by two-dimensional gel electrophoresis. We find that while all tissues have the IC74–2C subunit and its phospho-isoform first identified in glia, neither the glial nor the neuronal IC74 isoform pattern is typical. Many tissues have both the IC74–2C and the IC74–2B isoforms, although the relative amounts of the two isoforms vary in different tissues. The IC74–1A isoform is neuron specific. We also find that the relative abundance of the neuronal IC74 isoforms changes during brain development, in particular from the 15th day of gestation (E15) to the fifth day after birth (P5). However, the relative amounts of the IC74 mRNAs change very little during the same developmental period. The timing of the changes in the relative amounts of neuronal IC74 isoforms coincides with the initial burst of process extension and pattern formation in the brain. These results have important implications for the structure, function, and regulation of cytoplasmic dynein in cells. In particular, they suggest an important role for specific IC74 isoforms in brain function and development.

Nomenclature

Whenever possible, we propose that the dynein IC74 isoform spots observed on two-dimensional gels be identified with the designation of their mRNA and, when appropriate, the inclusion of the prefix phospho-. The arbitrary alphanumeric spot designations should be reserved for situations when the spots are not conclusively identified.

MATERIALS AND METHODS

Immunoprecipitation, Electrophoresis, and Immunoblotting

Immunoprecipitation and electrophoresis were performed as described previously (Dillman and Pfister, 1994; Pfister *et al.*, 1996), except that the rat kidney, testis, and spleen were suspended in two volumes of lysis buffer and homogenized with two 10-s pulses of a Tissumizer (Tekmar, Inc., Cincinnati, OH) set at 25% power. To immunoprecipitate cytoplasmic dynein from specific portions of the brain, the cerebral cortex, cerebellum, and medulla/pons regions were removed from an adult rat brain by dissection and homogenized in lysis buffer. To obtain adult meninges, five adult rat brains were removed, placed in ice cold phosphate-buffered saline, and the meninges covering the brain were gently teased away from the rest of the brain tissue. Embryonic brain and meninges were collected from fetuses obtained from timed, pregnant female Sprague-Dawley rats (Taconic, Germantown, NY). Immuno-blotting was as described previously, except polyvinylidene difluoride membrane or nitrocellulose were used and the Renaissance ECL (DuPont, Wilmington, DE) was used to detect the bound antibody (Dillman and Pfister, 1994).

Phosphatase Treatment

Dynein immunoprecipitated from rat tissue was treated with 200 U of lambda phosphatase (New England Biolabs, Beverly, MA) while bound to Protein A beads for 40 min at 30°C. The Protein A beads were then washed twice with Immunoprecipitation Wash Buffer I and the protein was eluted from the beads with IEF sample buffer.

Reverse transcriptase-polymerase chain reaction (RT-PCR) of IC74 mRNA

RNA from rat tissue was isolated using the Qiagen RNeasy kit (Chatsworth, CA), according to the manufacturer's directions, as described previously. The first strand cDNA was made using the SuperScript Preamplification System (Life Technologies, Gaithersburg, MD) according to the manufacturer's directions, with oligo-dT as the primer. The first strand synthesis was followed with an RNase H treatment. The first strand cDNA was diluted 1:5 with dH_2O and 1 μl of cDNA per 20 μl reaction was used in the PCR reaction. The PCR reaction conditions and reagent concentrations were as per the Life Technologies' directions for PCR amplification of first strand cDNA using a hot start protocol as follows: 94°C for 5 min, then 30 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 60 s. Six oligonucleotides designed to distinguish the two genes and five alternative splice variants of IC74 (Vaughan and Vallee, 1995) were synthesized by Life Technologies as follows:

G1/S = 5′- GAAGCTGGAAGCCAAGACGATC -3′,
G1/AS = 5′- ACCTGGGTAACCTTTGACACGC -3′,
G2/S1 = 5′- CGAAGCCTTGCTTCAGAGCATG -3′,
G2/AS1 = 5′- TCTTCCTCCTCCTCATCTTCTTTGG -3′,
G2/S2 = 5′-ACGCCAAGTGAAGCTGGAAGC -3′,
G2/AS2 = 5′- TGGCTTCCAGCTTCACTTGGC -3′.

The oligonucleotides were used in the following four combinations: G1/S and G1/AS, G2/S1 and G2/AS1, G2/S1 and G2/AS2, and G2/S2 and G2/AS1. To distinguish the different PCR products generated by these primers, the products of each PCR were separated on 8% acrylamide gels, 1× TBE buffer. A *Hae*III digest of pBR322 DNA (Marker V, Boehringer Mannheim, Indianapolis, IN) was used to determine the sizes of the PCR products.

RESULTS

Analysis of the IC74 Cytoplasmic Dynein Isoforms from Brain and Other Tissues

To identify the cytoplasmic dynein IC74 isoforms unique to brain tissue, we analyzed cytoplasmic dynein from adult rat brain and other tissues (Figure 1). Clear differences in the spot patterns of the IC74 region were observed when dynein from the various tissues was analyzed by two-dimensional gel electrophoresis. Three general IC74 patterns were identified from an analysis of six tissues. A summary diagram of the relative positions of the IC74 spots is drawn in Figure 1F. By far the most complex IC74 isoform pattern was observed in cytoplasmic dynein from adult brain (Figure 1A). As previously observed, in brain there are six spots arranged in two arcs (Dillman and Pfister, 1994). The spots of the acidic arc are referred to as the A spots, while the spots of the basic arc are the B spots. The A1 spot is the major spot, and the B and B1 spots are present in roughly equal amounts. From previous work, it is known that the product of the IC74–2C mRNA, the IC74–2C isoform, is resolved at the B spot and that the phospho-IC74–2C isoform is one of the polypeptides that co-migrates with the B1 spot (Pfister *et al.*, 1996). The A spot migrates slightly faster than the B1 spot in this gel system. The A spot is not always resolved from the A1 spot, and usually overlaps the A1 spot. Two poorly resolved spots extend diagonally and upward from the A1 and B1 spots. They are referred to as the A2 and B2 spots or "tails." We have previously demonstrated that these spots are generated by phosphorylation of other IC74 polypeptides (Pfister *et al.*, 1996).

The IC74 isoform pattern of cytoplasmic dynein from testis is simpler than that of dynein from brain (Figure 1B). The B and B1 spots are the major spots, as the A1 spot is not observed. In the absence of the A1 spot, the A spot is resolved more clearly. A diagnostic feature of the A spot is that the position of its most basic end is directly under the B1 spot. The discrete A2 and B2 tails seen in brain are not observed in testis.

Dynein from the three other tissues, kidney, liver, and spleen (Figure 1, C–E) demonstrate the third IC74 isoform pattern. In these tissues, the B spot (IC74–2C isoform) predominates. There is relatively less of the B1 spot in these tissues than in brain or testis. All three of these tissues have a spot just to the left of the B1 spot. Although it lies along the arc of the A spots, we conclude from numerous analyses that this spot was not identified in the previous analysis of brain cytoplasmic dynein. We will refer to this as the X spot. The X spot is a minor one in the IC74 region of dynein from liver or kidney. However, the staining of the X spot in dynein from spleen is comparable to that of the B spots. The X spot seen in these three tissues has a slower mobility on gels than the brain-specific A1

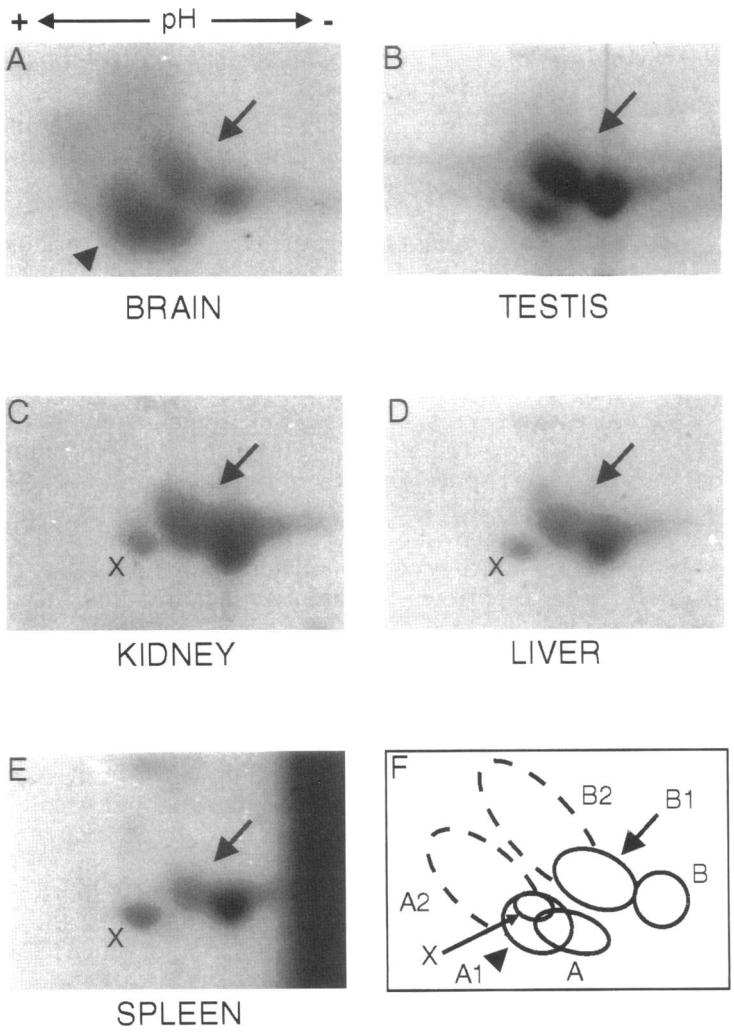

Figure 1. Differences in the IC74 isoforms of cytoplasmic dynein from adult rat brain and other tissues. Adult rat brain and other tissues were homogenized in lysis buffer, the dynein was immunoprecipitated from the lysates with antibody 74.1, and analyzed by two-dimensional gel electrophoresis as described in MATERIALS AND METHODS. Only the IC74 region of the gel is shown; the gels were silver stained. (A) Adult brain, (B) testis, (C) kidney, (D) liver, (E) spleen, and (F) interpretive drawing summarizing and identifying the IC74 isoforms seen in the five tissues. The protein loading and silver staining of each gel were adjusted for maximal resolution of all the spots. Six spots are seen when dynein from brain is analyzed: A, A1, A2, B, B1, and B2. Three spots are seen when dynein from testis is analyzed: B, B1, and A. Three spots are seen when dynein from kidney, liver, and spleen is analyzed: B, B1, and X. The arrow points to the B1 spot, and the arrowhead points to the A1 spot. The X spot is identified on the drawing with the long arrow and on the gel panels with an x. The acidic and basic ends of the IEF pH gradient are indicated with a + and − at the top of panel A.

spot. Figure 1F summarizes our interpretation of the complex IC74 spot patterns seen in these rat tissues. Note that the A and X spots overlap the A1 spot. Furthermore, the A2 and B2 spots or tails, which we previously have shown were phosphorylated in vivo, are found only in brain tissue.

By comparing the two-dimensional gel IC74 isoform patterns of control and phosphatase-treated dynein from brain, neurons, and glia, we previously have shown that two IC74 isoforms, one of which is the phospho-IC74–2C isoform, co-migrate at the B1 spot (Pfister *et al.*, 1996). When phosphate is removed from the phospho-IC74–2C isoform, it migrates at the B spot, not the B1 spot. Therefore the presence of a B1 spot after phosphatase treatment is diagnostic of the presence of a distinct unphosphorylated IC74 isoform (see also Figure 5B and Table 1.

To characterize the B1 spot of cytoplasmic dynein from testis and liver, immunoprecipitated dynein from those tissues was treated with the general protein phosphatase, lambda phosphatase. No major change in the relative amounts of the B and B1 spots was observed when cytoplasmic dynein from testis was treated with phosphatase (Figure 2A). In this regard cytoplasmic dynein from testis is similar to that from brain. Dynein from both tissues retains a major B1 spot after phosphatase treatment, which indicates the presence of the unphosphorylated IC74 isoform. There was also no change in the appearance of the A spot. When cytoplasmic dynein from liver was treated with phosphatase, three spots were still observed in the IC74 region, however, the relative amount of the B1 spot had diminished considerably, while that of the B spot (IC74–2C isoform) was larger (compare Figure 2B to Figure 1D). This suggested that most, but not all, of the protein contributing to the B1 spot in liver was the phospho-IC74–2C isoform and that after removal of the phosphate by lambda phosphatase, the resulting IC74–2C isoform migrated at the B spot. The position of the X spot was unaltered, indicating that it

Table 1. Correlation of the polypeptide products of the IC74 mRNAs with IC74 isoform spots resolved by two-dimensional gel electrophoresis

			Cells and tissues observed to express:	
mRNA	Protein product	Two-dimensional gel spot	mRNA	Protein
IC74-1A	IC74-1A	A1	Neurons, brain	Neurons, brain
	Phospho-IC74-1A	A2		Neurons, brain
IC74-1B	IC74-1B	A	Neurons, brain, testis	Neurons, brain, testis
	Phospho-IC74-1B	A1		Neurons, brain
IC74-2A	IC74-2A	Unknown (Predicted to overlap the B1 spot)	P20 and older brain	NA
IC74-2B	IC74-2B	B1	Neurons, brain, testis, liver	Neurons, brain, testis, liver
	Phospho-IC74-2B	B2		Neurons, brain
IC74-2C	IC74-2C	B	All cells and tissues	All cells and tissues
	Phospho-IC74-2C	B1		All cells and tissues

The analysis of the product of IC74-2C mRNA is from Pfister *et al.* (1996). The remainder of the analysis is described in the text.

was not phosphorylated. When dynein from kidney was treated with phosphatase, the results were similar to that of dynein from liver (our unpublished observations). These two experiments thus provided further evidence that two isoforms co-migrate with the B1 spot.

Identification of IC74 Gene Products and mRNA in Testis and Liver

To determine which of the two IC74 genes are responsible for the A spot observed in the testis and to further analyze the X spot seen in liver, kidney, and spleen, we probed blots of two-dimensional gels with an antibody specific for the IC74–2 isoforms. The IC74–2 antibody is specific for a 15-amino acid sequence found only at the carboxyl terminus of IC74 gene 2. Figure 3, A and C, shows that the IC74–2 antibody detects both of the B spots in cytoplasmic dynein from testis and liver, indicating that they are products of the IC74–2 gene. We also probed blots of dynein from testis and liver with the 74.1 antibody, which reacts with products of both the IC74–2 and IC74–1 genes. The A spot in testis is detected only by the 74.1 antibody. This result suggests that the A spot is likely to be a product of the IC74–1 gene. Neither antibody recognized the X spot. When blots of dynein from spleen and kidney were probed, the results were similar to those with dynein from liver (our unpublished observations).

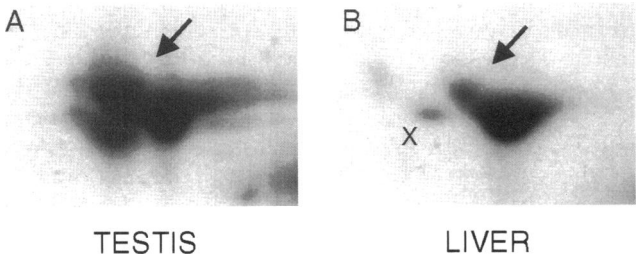

Figure 2. Dephosphorylation modifies the IC74 isoform distributions of cytoplasmic dynein from liver. Cytoplasmic dynein was immunoprecipitated from testis and liver, treated with lambda phosphatase, and analyzed by two-dimensional gel electrophoresis as described in MATERIALS AND METHODS. The gels were silver stained. (A) IC74 region of a gel showing phosphatase-treated dynein from testis. (B) IC74 region of a gel showing phosphatase treated dynein from liver. The arrows point to the B1 spots, and the X spot is indicated with an x. Phosphatase treatment of dynein from liver, but not testis, reduces the relative staining of the B1 spot. Phosphatase treatment did not alter the A spot in testis or the X spot in liver.

To determine which of the five IC74 mRNAs are expressed in testis and liver, we used RT-PCR with primers specific for each of the alternatively spliced regions of the two genes. When testis mRNA from testis was analyzed with probes that distinguish between the two IC74–1 messages, a product of the size diagnostic for the IC74–1B mRNA was observed (Figure 4B). There was no evidence of IC74–1A mRNA in testis. A similar analysis of liver mRNA found no evidence of either IC74–1 message (Figure 4C). When testis and liver mRNAs were analyzed with probes specific for the IC74–2 messages, the IC74–2B and IC74–2C mRNAs were identified in both tissues (Figure 4, B and C). Interestingly, no evidence of IC74–2A mRNA was seen in either tissue.

Correlation of the Products of the IC74 mRNAs with the Two-dimensional Gel Protein Spots

To correlate the two-dimensional gel IC74 isoform spots with the products of the IC74 mRNAs, we first

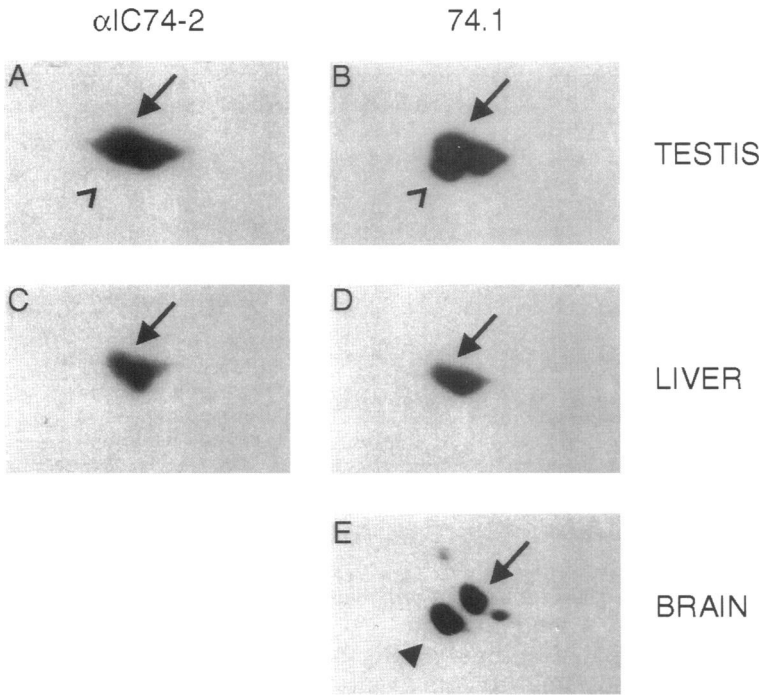

Figure 3. Identification of the IC74 gene 2 products in cytoplasmic dynein from liver and testis. Cytoplasmic dynein from the indicated tissues was immunoprecipitated, resolved by two-dimensional gel electrophoresis, transferred to polyvinylidene difluoride, and probed with the antibodies 74.1 and anti-IC74–2 as described in MATERIALS AND METHODS. Only the IC74 region of the blots is shown. (A and B) Testis dynein, (C and D) liver dynein, and (E) brain dynein. (A and C) The IC74–2 specific antibody was used as probe; (B, D, and E) antibody 74.1 was used as probe. Note that neither antibody recognized the X spot in liver. The IC74–2 specific antibody recognized only the B and B1 spots in testis and liver. Therefore, the B spots found in dynein from testis and liver are a product of gene IC74–2. The antibody 74.1 recognized the A spot in dynein from testis, and the A, A1, B, B1, and B2 spots in dynein from brain. Therefore the A spots found in testis and brain are likely to be products of gene IC74–1. The arrows point to the B1 spot. The open arrowhead points to the position of the A spot of testis dynein. The filled arrowhead points to the A1 spot.

used the Expasy Server (University of Geneva) to calculate the molecular mass and isolectric point of each of the predicted IC74 mRNA products. To determine whether the calculated data predicted the observed two-dimensional gel IC74 spot pattern, the data for each mRNA product was drawn as a circle of arbitrary size on a model two-dimensional gel at the position of its calculated pI and molecular mass (Figure 5A). The predicted results did resemble the electrophoretic data. However, the two-dimensional gel IC74 spot pattern was better approximated when the presentation of the calculated data was modified in two ways (Figure 5B). First, the estimated molecular masses of the three IC74–2 mRNA products were adjusted simultaneously so that each member of the group had an apparent molecular mass ~3 kDa higher than calculated. This modification assumes that the IC74–2 gene products migrate slightly anomalously in our gel system. Second, the spots were redrawn as ovals whose relative sizes were taken from the IC74 spots in equivalent positions on the two-dimensional gel analysis of brain dynein (Figure 5B). After these manipulations, comparisons of the expression of IC74 mRNA and the two-dimensional gel IC74 spot patterns of various tissues and cells were used to verify the positions of all but the IC74–2A mRNA gene products (Table 1).

From our studies of glial dynein IC74 isoforms, we know that the polypeptide migrating at the B spot is the product of mRNA IC74–2C, and that phospho-IC74–2C is one of the isoforms that co-migrates with the B1 spot (Pfister *et al.*, 1996). Because a B1 spot is observed after lambda phosphatase treatment of cytoplasmic dynein from liver, testis, brain, and cortical neurons, it is likely that the phosphatase-insensitive protein co-migrating with B1 is a separate product of an IC74 mRNA. The B1 spot is recognized by the IC74–2 specific antibody. Therefore, it is likely to be a product of the IC74–2 gene. Aside from IC74–2C, liver, testis, cortical neurons, and brain have only one IC74–2 mRNA in common, the IC74–2B mRNA. Therefore, the second isoform co-migrating with the B1 spot is the product of IC74–2B. The A spot is found in dynein from brain, cortical neurons, and testis. As this spot is not recognized by the IC74–2 specific antibody, it is likely to be a product of the IC74–1 gene. The only IC74–1 mRNA found in testis is IC74–1B; its protein product is therefore assigned to the A spot. The A1 spot is observed only in dynein from brain and cortical neurons. It is also not recognized by the IC74–2 specific antibody. Therefore, it is also likely to be a product of the IC74–1 gene. The IC74–1A mRNA is the only IC74 mRNA expressed in brain and cortical neurons. Therefore, the A1 spot is the product of the IC74–1A mRNA. As brain is currently the only source for IC74–2A mRNA, no unique protein isoform can be assigned as a product of that message. However, its calculated molecular mass and isoelectric point suggest that it may not be resolved from the IC74–2B product. If this is the case, it too would co-migrate with the B1 spot (Figure 5B). This analysis is also unable

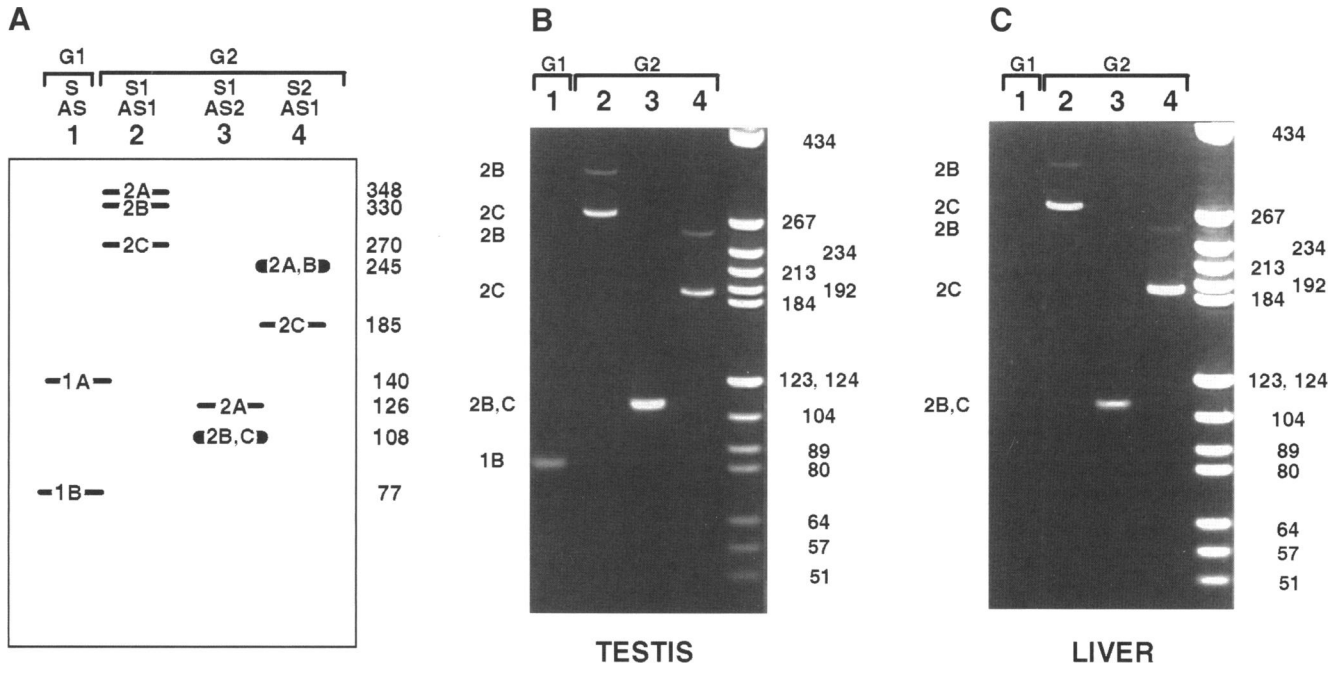

Figure 4. Identification of the IC74 mRNAs in liver and testis. A) Diagram, drawn as a model gel, of relative position and sizes (in bp) of the PCR products predicted to result from combinations of primers specific for the alternatively spliced regions of the two IC74 genes. On the right side of the gel diagram are the predicted sizes of the individual PCR products diagramed on lanes of the gel. Overlaid onto the gel lanes of the drawing are the specific mRNAs that would generate the diagramed products. Lane 1, the primer set for gene IC74–1 (G1) will produce a 140-bp product if the full length mRNA, IC74–1A, is present, and a smaller 77 bp product if IC74–1B is present. Lanes 2–4, for gene IC74–2 three combinations were used. Lane 2, with primers G2/S1 and G2/AS1 each mRNA will produce a product of a different size: a product of 348 bp, diagnostic of the full length mRNA IC74–2A; 330 bp diagnostic of the excision of the first alternatively spliced region, mRNA IC74–2B; and a product of 270 bp diagnostic of the excision of both alternatively spliced regions, mRNA IC74–2C. Lane 3, the G2/S1 and G2/AS2 primers are diagnostic for excision of the first alternatively spliced region. mRNA IC74–2A generates a product of 126 bp; mRNAs IC74–2B and IC74–2C, generate products of 108 bp. Lane 4, when primers diagnostic for splicing of the second alternatively spliced region, G2/S2 and G2/AS1, are used, mRNAs IC74–2A and IC74–2B generate products of 245 bp, while IC74–2C generates a product of 185 bp. B) Ethidium stained gel analysis of IC74 mRNA isolated from testis. mRNA was isolated from adult rat testis as described in the MATERIALS AND METHODS. Lane 1, analysis of IC74–1 mRNA, a product of 77 bp was identified, diagnostic of mRNA IC74–1B. Lane 2, analysis of IC74–2 mRNA, products of 330 and 270 bp, diagnostic of IC74–2B, and IC74–2C, were identified. Lane 3, analysis of mRNA based on the excision of the first alternatively spliced region. A product of 108 bp diagnostic of either IC74–2B or IC74–2C was identified. Lane 4, analysis of mRNA based on excision of the second alternatively spliced region. Products of 245 bp, diagnostic of IC74–2A or IC74–2B, and 185 bp, diagnostic of IC74–2C, were observed. Therefore, products of the sizes diagnostic for mRNAs IC74–1B, IC74–2B, and IC74–2C were found in mRNA from testis. No IC74–1A or IC74–2A mRNA was detected. C) Ethidium stained gel analysis of IC74 mRNA isolated from liver. mRNA was isolated from adult rat liver as described in MATERIALS AND METHODS. Lane 1, analysis of IC74–1 mRNA, no products were identified. Lane 2, analysis of IC74–2 mRNA, products of 330 and 270 bp, diagnostic of IC74–2B and IC74–2C, were identified. Lane 3, analysis of mRNA based on the excision of the first alternatively spliced region. A product of 108 bp, diagnostic of either IC74–2B or IC74–2C, was identified. Lane 4, analysis of mRNA based on excision of the second alternatively spliced region. Products of 245 bp, diagnostic of IC74–2A or IC74–2B, and 185 bp, diagnostic of IC74–2C, were observed. Therefore, mRNA from liver produces products of the sizes diagnostic for mRNA IC74–2B and IC74–2C. No IC74–1A, IC74–1B or IC74–2A mRNA was detected.

to assign a known IC74 mRNA to the protein that runs at the X spot. The pI predicted for the IC74–2C polypeptide (pH 5.16) agrees within 0.26 U with the pI observed by isoelectric focusing (pH 4.9) (Dillman and Pfister, 1994).

Developmental Regulation of Brain IC74 Cytoplasmic Dynein Isoforms

The IC74–1A mRNA and its product, the IC74–1A isoform (A1 spot), were observed only in brain tissue and cultured neurons (Pfister *et al.*, 1996). Interestingly, we found that the relative amount of the IC74–1A isoform (A1 spot) in cultured cortical neurons was less than that observed in adult brain. We explored several reasons that might account for the differences seen when the isoforms from cultured cells and brain were compared. Because we analyzed dynein from cortical neurons, we considered the possibility that the expression of IC74 isoforms is different in different regions of the brain. However, we found that dynein from different regions of the brain (cerebral cortex, cerebellum, and medulla-pons) give the

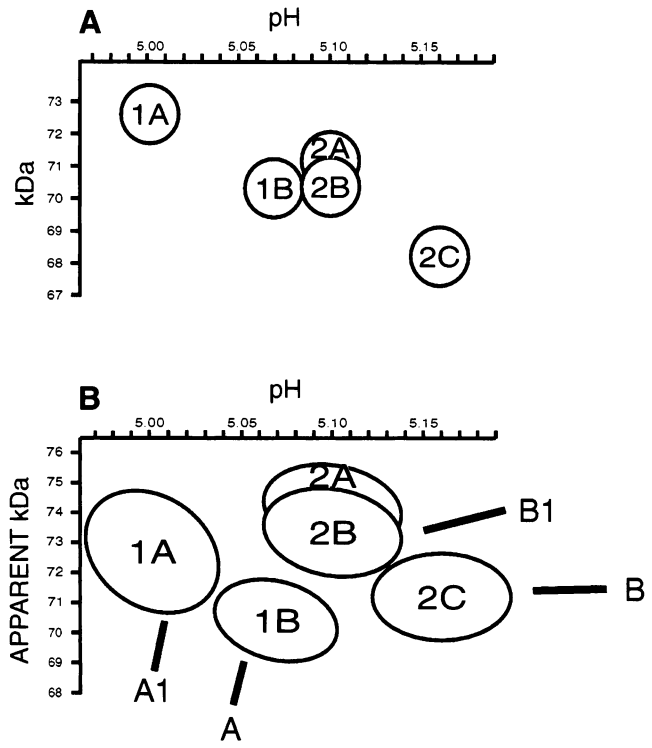

Figure 5. Correlation of the mRNA and protein expression patterns of the IC74 cytoplasmic dynein isoforms. (A) Computer-generated prediction (Expasy Server, University of Geneva) of the two-dimensional gel positions of the products of the IC74 mRNAs. The amino acid sequences predicted by the mRNA nucleotide sequences were used to calculate the isolectric points and molecular masses of each polypeptide. (B) Approximation of the observed gel positions of the IC74 protein isoforms produced by the indicated IC74 mRNAs. This panel was obtained by taking the image in panel A and increasing the apparent molecular masses of the three IC74–2 gene products simultaneously, by ~3 kDa. The shape of each mRNA product was drawn to conform to those observed in Figure 1A. Written inside each oval is the IC74 mRNA whose protein product is predicted to generate a spot on two-dimensional gels. The letters outside each shape indicate the two-dimensional gel spot labels used in Dillman and Pfister (1994) and Figure 1F.

same isoform pattern as dynein from whole adult brain (our unpublished observations). We therefore concluded that the pattern was not related to a specific functional region of the brain. We then realized that the cortical neuron cultures were derived from brains taken at embryonic day 18 (E18). We therefore investigated whether the different amounts of the IC74 isoforms, in particular the IC74–1A isoform (A1 spot), might be the result of changes in the expression of specific IC74 isoforms during brain development. To test this we examined the developmental expression of the IC74 isoforms in cytoplasmic dynein from rat brains or brain regions obtained from individuals at ages E15 to P5. Several changes in the relative amounts of IC74 isoforms were found (Figures 6 and 7).

Two major IC74 spots, B and B1, were observed in dynein immunoprecipitated from an E15 cerebral cortex (Figure 6A). This is a markedly different pattern than that observed when adult brain dynein is analyzed (Figure 6F). A similar IC74 isoform pattern is observed when dynein from an E15 brain was analyzed (our unpublished observations). By E18 (Figure 6B), the A spots were clearly seen. Although the relative amounts of the B and B1 spots were similar to that observed on E15, a greater amount of the B2 spot was observed. As might be expected, given the relative amounts of the A1 and B1 spots, the A2 spot was not as extensive as the B2 spot. At P0 (Figure 6C), the relative amount of the B1 spot is now greater than that of the B spot and the amount of the A spots continues to increase. The B2 spot remains more intensely stained than the A2 spot. Similar results are seen at P2 (Figure 6D). By P5 (Figure 6E), the IC74 isoform pattern is similar to the adult pattern (Figure 6F), except perhaps for the relative staining of the A2 and B2 spots. Interestingly, we consistently observe that both the A2 and the B2 spots seem more extensive in dynein from P0-P5 than dynein from adult brain.

To distinguish between the polypeptide product of mRNA IC74–2B and the phospho-IC74–2C isoform, which co-migrate at the B1 spot, cytoplasmic dynein isolated from brains at different developmental stages was treated with lambda phosphatase and analyzed by two-dimensional gel electrophoresis. With dynein from an E15 brain there was only a minor change in the relative amounts of the B and B1 spots (Figure 7A, compare with Figure 6A). With dynein from an E18 brain, however, we found that there was much more of the B1 spot than the B spot (compare Figures 6B and 7B). This indicates that the relative increase in amount of the B1 spot seen in Figure 6B is due to a relative increase in the amount of IC74–2B isoform, not the phospho-IC74–2C isoform. As was observed with dynein from adult brain (Figure 7D), phosphatase treatment of dynein from E18 and P1 brains (Figure 7C) eliminated the A2 and B2 spots. This is evidence that when the phosphate is removed, the protein spread out in the B2 spot migrates at the B1 spot. Furthermore, because the IC74–2B isoform is the only embryonic isoform that migrates at the B1 spot after dynein is treated with phosphatase, this suggests that the B2 spot is the phospho-form of IC74–2B and not IC74–2C. Phosphatase treatment of these dynein samples also enhances the resolution of the A1 and A spots.

To obtain evidence that the changes in isoform expression are restricted to neurons, we analyzed the IC74 isoform pattern of the meninges covering the brain. There was no difference in the IC74 isoform pattern when meningeal cytoplasmic dynein from E18 and adult brains were compared (our unpublished observations).

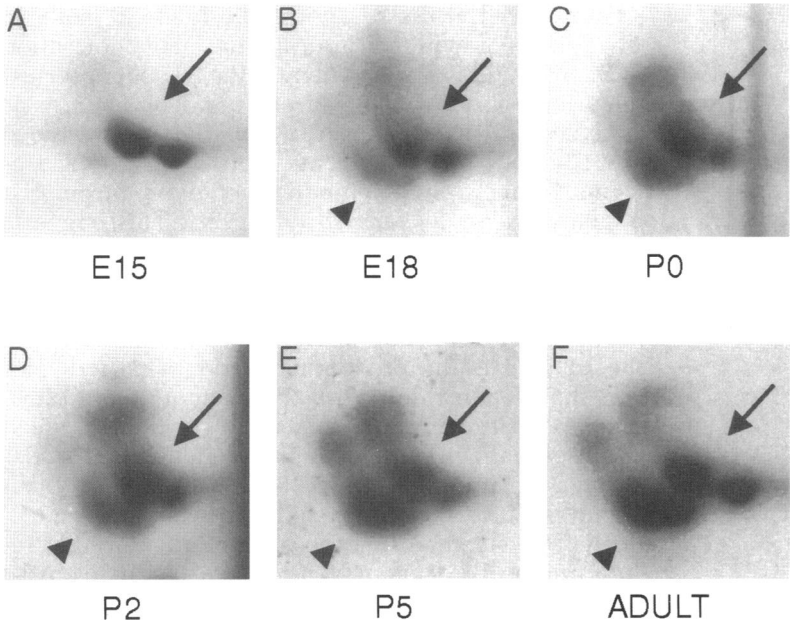

Figure 6. Changes in the expression of the cytoplasmic dynein IC74 isoforms during rat brain development. Rat brains or cerebral cortices were harvested at the indicated day of gestation (E), or postnatal day (P). The tissue was homogenized and the dynein was immunoprecipitated and then analyzed by two-dimensional gel electrophoresis as described above. (A) E15, (B) E18, (C) P0, (D) P2, (E) P5, and (F) adult. (A and B) Dynein from cerebral cortex; (C–F) Dynein from whole brain. At E15, the major spots are B and B1. By E18, the B2 spot is clearly visible, as are the A spots. Dynein from E18 cortex is very similar to that found in cultured cortical neurons (Pfister *et al.*, 1996). At P0, the amount of the B1 spot appears greater than that of the B spot. By P5, an IC74 isoform pattern very similar to that of the adult is observed. Dynein immunoprecipitated from E15 and E18 whole brains had relative IC74 isoform amounts indistinguishable from those in panels A and B, respectively, and adult cortex has the same IC74 pattern as adult brain (our unpublished observations). Arrows point to the B1 spot, arrowheads to the A1 spot. For each sample, the protein loading and staining were adjusted for maximal resolution of all the spots.

Expression of IC74 mRNAs during Brain Development

We next examined the expression of the IC74 mRNAs during brain development, using the RT-PCR assay. At E13 only the IC74–2C message is detected (Figure 8B). By E14, a small amount of IC74–2B is seen in addition to the IC74–2C message (Figure 8C). On E15, the IC74–1A, IC74–1B, IC74–2B, and IC74–2C mRNAs are seen (Figure 8D). By E18 the relative amount of the mRNA for IC74–1B dropped slightly, while that of mRNA IC74–2C decreased considerably (Figure 8E). These levels stay relatively constant until at least P5 (Figure 8H). Interestingly, while the IC74–2A message is found in adult brain (Pfister *et al.*, 1996), it is not detected at any of these embryonic or early postnatal time points. When the expression of IC74 mRNA at later times was analyzed, a very small amount of the IC74–2A mRNA was detected at P20 (our unpublished observations), and by P26 (Figure 8I) the level of IC74–2A was comparable to that seen in the adult (Figure 7C in Pfister *et al.*, 1996).

DISCUSSION

The IC78 subunit of flagellar outer arm dynein is known to bind the dynein to its cargo, the A microtubule (King *et al.*, 1991, 1995). Because the IC74 subunit of the retrograde motor protein, cytoplasmic dynein, is similar to the IC78 flagellar subunit, it is thought to be involved in binding the dynein complex to membranous organelles (Paschal *et al.*, 1992; King *et al.*, 1995; Vaughan and Vallee, 1995; Wilkerson *et al.*, 1995). We previously identified six isoforms of the IC74 subunit when dynein from brain was analyzed by two-dimensional gel electrophoresis, and we found that most of the IC74 isoforms could be labeled in vivo with ^{32}P (Dillman and Pfister, 1994). We have been analyzing the relationships of the different IC74 isoforms. In a study of IC74 at the cellular level, we discovered that the IC74 isoforms found in dynein from cultured glia and neurons were considerably different (Pfister *et al.*, 1996). When dynein from glia was analyzed by two-dimensional gel electrophoresis, two IC74 protein isoforms were resolved. Further study showed that they were the IC74–2C isoform and the phospho-IC74–2C isoform. When the IC74 isoforms of dynein from cultured neurons were analyzed, the pattern of spots on two-dimensional gels was found to be similar to that of adult brain. However, we observed that the relative amounts of the neuronal IC74 isoforms and the brain IC74 isoforms were different. We therefore sought in this study to establish the basis for the differences in the relative amounts of IC74 isoforms in dynein from neurons and brain. We also sought to determine if there were neuron-specific isoforms, and to correlate the products of the four IC74 mRNAs expressed in neurons with the remaining IC74 isoforms resolved on two-dimensional gels.

To identify the products of the IC74 mRNAs on two-dimensional gels, we compared the expression of IC74 isoforms and IC74 mRNA from different tissues (Figure 5B and Table 1). The product of the IC74–2C mRNA, which we refer to as the IC74–2C isoform, is the most basic isoform and resolves at the B spot on two-dimensional gels. It has been found in all cells

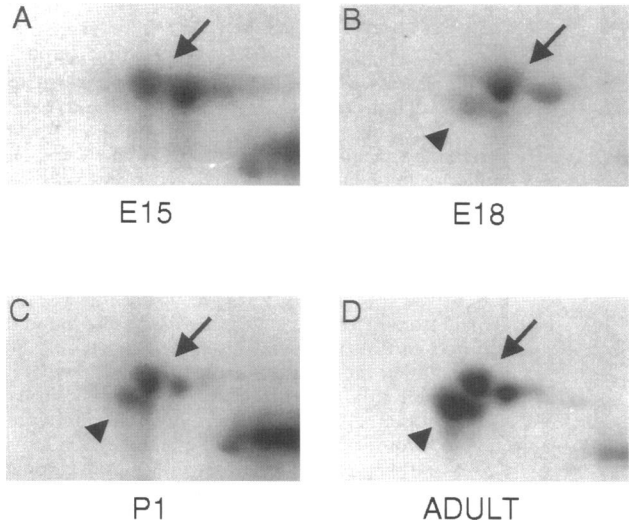

Figure 7. Analysis of the basal expression of IC74 isoforms during brain development by treatment of immunoprecipitated cytoplasmic dynein with phosphatase. Rat brains or cortices were harvested at the indicated day of gestation (E), or postnatal day (P). The tissue was homogenized, the dynein was immunoprecipitated, treated with lambda phosphatase, and then analyzed by two-dimensional gel electrophoresis as described above. The gels were silver stained. (A) E15, (B) E18 cortex (same sample as in Figure 6B), (C) P1, and (D) adult. The B and B1 spots are approximately equal on E15, while the B1 spot predominates on E18. As was observed with dynein from cortical neurons, phosphatase treatment enhances resolution of the A spots (Pfister *et al.*, 1996). Arrows point to the B1 spot, arrowheads to the A1 spot. For each sample, the protein loading and staining were adjusted for maximal resolution of all the spots.

and tissues examined so far. Although it is the only IC74 gene product found in glia, it is a relatively minor isoform in neurons (Pfister *et al.*, 1996). The IC74–2B isoform is one of the isoforms that migrates at the B1 spot; in brain and testis, it is a major isoform. In most other tissues it is a minor isoform. The IC74–1B isoform is resolved at the A spot, is found only in testis and brain, and is a relatively minor isoform in both tissues. The IC74–1A isoform is found only in brain, where it is presumably the major isoform in neurons. The IC74–2A mRNA is found only in adult brain, and we have been unable to identify its protein-product as a unique spot on two-dimensional gels. The calculated molecular weight and isolectric point of the IC74–2A product predict that it would not be resolved from the B1 spot on our two-dimensional gels. If this is the case, that would mean three different isoforms co-migrate at the B1 spot: the IC74–2A, IC74–2B, and phospho-IC74–2C isoforms.

The phosphorylation relationships of the IC74–2 isoforms (B spots) observed on two-dimensional gels are now well understood. The IC74–2C isoform resolves at the B spot, and metabolic labeling with ^{32}P indicates that its phospho-isoform is one of the isoforms co-migrating at the B1 spot (Pfister *et al.*, 1996). Also resolved at the B1 spot is the IC74–2B isoform. Two pieces of evidence indicate that phosphorylation of the IC74–2B isoform generates the B2 spot observed when cytoplasmic dynein from brain or neurons is analyzed on two-dimensional gels. First, only the B and B1 spots, the IC74–2C and phospho-IC74–2C isoforms, respectively, are found when dynein from glia is analyzed on two-dimensional gels. This suggests that the B2 spot is not the product of hyper-phosphorylated IC74–2C. Second, cytoplasmic dynein from embryonic brain has a relatively large B2 spot, and after phosphatase treatment, which eliminates the B2 spots, the B1 spot is the predominant spot. This indicates that the protein resolved in the B2 spot before phosphatase treatment migrates at the B1 spot after phosphatase treatment. Interestingly, while there is a relatively large amount of the IC74–2B isoform in dynein from testis, no B2 spot or tail has been observed in testis. This suggests that the phosphorylation that results in the B2 spot may be the result of a kinase specific to neurons.

The phosphorylation relationships of the IC74–1 isoforms, the A spots, are not as well defined as those of the IC74–2 isoforms. Because the two A spots are more distinctly resolved after phosphatase treatment of neuronal and embryonic brain cytoplasmic dynein, it appears likely that both IC74–1A and -1B have phospho-isoforms. By analogy with the IC74–2 isoforms it appears probable that the phospho-IC74–1B isoform migrates with the A1 spot, and that the phospho-IC74–1A isoform migrates with the A2 spot. It also seems likely that the very faint ^{32}P labeling observed over the A spot (IC74–1B isoform) (Dillman and Pfister, 1994; Pfister *et al.*, 1996) is the result of its overlapping distribution with the IC74–1A isoform (A1 spot) on two-dimensional gels. Phosphatase treatment does not change the appearance of the IC74–1B isoform (A spot) of dynein isolated from testis. This suggests either that 1C74-1B is not phosphorylated in testis, or that phosphorylation does not alter its migration.

The IC74 isoforms of brain cytoplasmic dynein isolated from mammals, including sheep, cow, and mouse appear similar to those reported for rat brain (our unpublished observations). When cytoplasmic dynein isolated from embryonic chicken brain was analyzed on two-dimensional gels, three IC74 spots were observed (Toyoshima *et al.*, 1992; Gill *et al.*, 1994). Two appear equivalent to the B and B1 spots we observe in rat brain. The third spot is more difficult to correlate with the dynein spots from rat brain. As embryonic brain dynein was analyzed in both these studies, this may not represent the full complement of the IC74 isoforms found in adult chicken brain.

The relationship of the X spot polypeptide to the IC74 isoforms is ambiguous. It migrates near the IC74–1 isoforms on two-dimensional gels and is

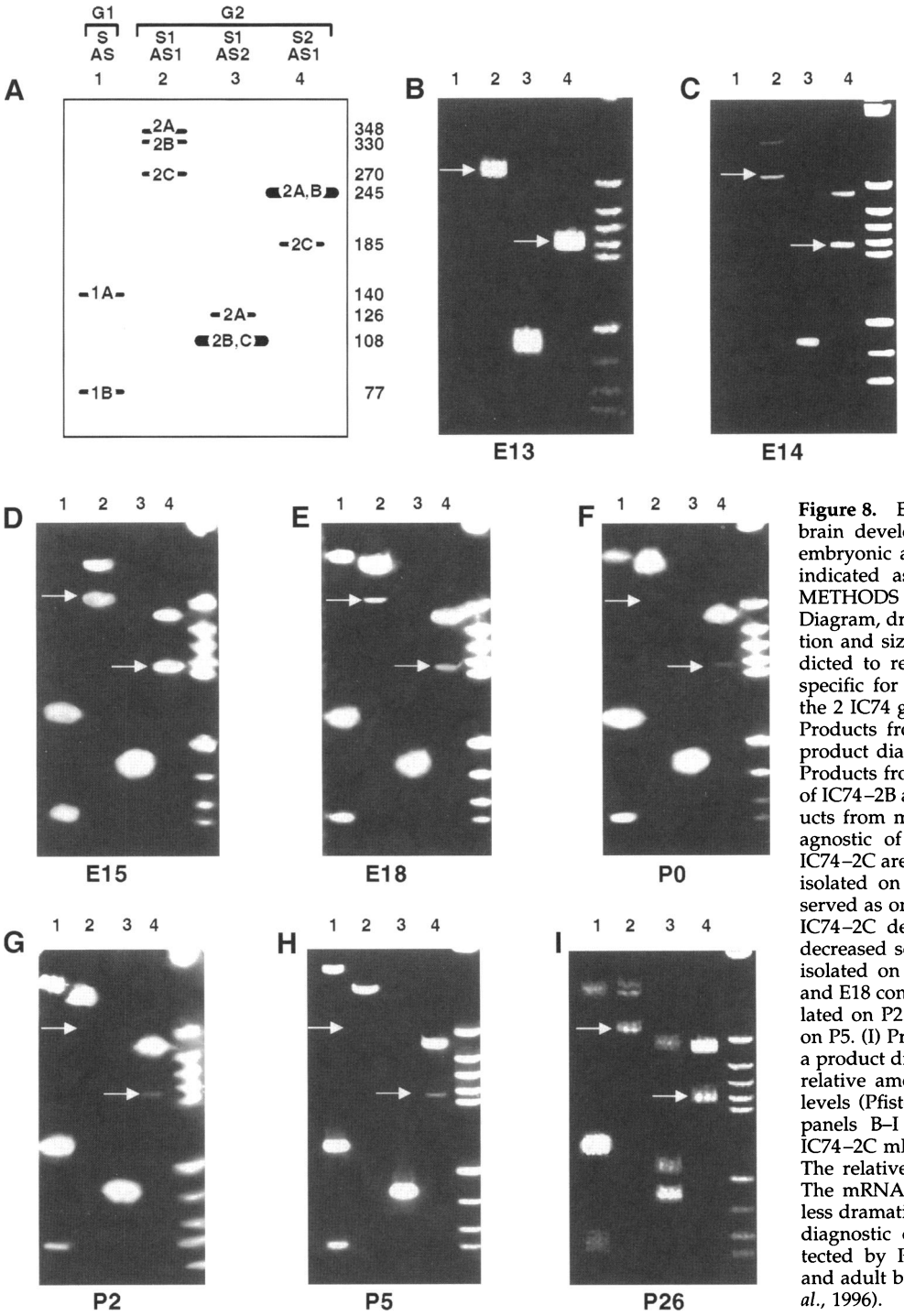

Figure 8. Expression of IC74 mRNA during rat brain development. mRNA was isolated from embryonic and postnatal rat brain on the days indicated as described in MATERIALS AND METHODS and was analyzed by RT-PCR. (A) Diagram, drawn as a model gel, of relative position and sizes (in bp) of the PCR products predicted to result from combinations of primers specific for the alternatively spliced regions of the 2 IC74 genes, as described in Figure 4A. (B) Products from mRNA isolated on E13, only a product diagnostic of IC74–2C is observed. (C) Products from mRNA isolated on E14, products of IC74–2B and IC74–2C are observed. (D) Products from mRNA isolated on E15, products diagnostic of IC74–1A, IC74–1B, IC74–2B, and IC74–2C are observed. (E) Products from mRNA isolated on E18. The same products were observed as on E15, though the relative amount of IC74–2C decreased considerably and IC74–1B decreased somewhat. (F) Products from mRNA isolated on P0. The trends observed from E15 and E18 continue. (G) Products from mRNA isolated on P2. (H) Products from mRNA isolated on P5. (I) Products from mRNA isolated on P26, a product diagnostic of IC74–2A is seen, and the relative amount of IC74–2C has reached adult levels (Pfister *et al.*, 1996). The white arrows in panels B–I point to bands diagnostic of the IC74–2C mRNA in the second and fourth lanes. The relative amount of IC74–2C drops at E15. The mRNA IC74–1B shows a similar although less dramatic trend in relative amount. Products diagnostic of mRNA IC74–2A are faintly detected by P20 (our unpublished observations) and adult brain levels are seen by P26 (Pfister *et al.*, 1996).

found in many tissues and cultured cell lines. Antibody 74.1 immunoprecipitates the X spot along with the entire dynein complex under nondenaturing conditions. However, neither the IC74–2–specific antibody (αIC74–2) nor the general IC74 antibody (74.1) detects it on blots, and the X spot is not immunoprecipitated with antibody 74.1 when the dynein complex is solubilized under denaturing conditions (our unpublished observations). While the A2 spot, phospho-IC74–1A, is also not detected on blots by the 74.1 antibody (Figure 3E), it is immunoprecipitated by this antibody when the dynein complex is denatured

(Dillman and Pfister, 1994). This suggests that X does not have the 74.1 epitope that is located in the amino terminal 60 amino acids, and is conserved in both IC74 gene families (Vaughan and Pfister, unpublished observations). The position of the X spot does not shift after phosphatase treatment and in the N1E-115 neuroblastoma and MDCK cultured cell lines, it is not labeled in vivo with ^{32}P (our unpublished observations). It therefore does not appear to be phosphorylated. Although we cannot rule out the possibility that the X spot is an IC74 subunit modified in a way that renders it nonreactive with antibody 74.1, the bulk of the evidence suggests that it is either not related to the IC74 subunits characterized here, or is at best only distantly related to them.

With the products of at least four different mRNAs from two genes, and only two or three IC74 subunits per dynein molecule, neurons must have a heterogeneous pool of cytoplasmic dynein. Our analysis cannot yet determine whether individual dynein molecules in neurons are made up of the same IC74 gene product, or mixtures of different IC74 gene products. In *Chlamydomonas* flagella, each outer arm dynein has the product of two distinct IC genes (Mitchell and Kang, 1991; Wilkerson *et al.*, 1995). Each IC has a different function. IC69 is thought to be involved in the assembly and regulation of the dynein complex (King and Witman, 1990; King *et al.*, 1991; Mitchell and Kang, 1993). IC78 binds to α tubulin and therefore is involved in binding the outer arm dynein complex to its cargo (King *et al.*, 1991). However, our analysis has demonstrated that one gene product is necessary and sufficient for cytoplasmic dynein assembly and function (Pfister *et al.*, 1996). In glia, general microtubule-minus end-directed intracellular organelle transport, and its regulation (Bomsel *et al.*, 1990; Corthesy-Theulaz *et al.*, 1992; Aniento *et al.*, 1993; Fath *et al.*, 1994; Lafont *et al.*, 1994), must be accomplished with just the IC74–2C isoforms. It is therefore tempting to speculate that the other IC74 gene products allow dynein to bind to specialized cargo, or to allow dynein to be regulated in specific ways. Neurons are specialized for the axonal transport of membranous organelles over long distances, and dynein must be synthesized in the cell body and transported to the synapse before it is utilized in retrograde transport (Hammerschlag and Brady, 1989). The IC74–1A and IC74–2B isoforms, and their respective phospho-isoforms, are the major isoforms in neurons and the IC74–1A isoform, the A1 spot, is unique to neurons; both of these isoforms are found in the axons that make up the optic nerve (Dillman and Pfister, 1994). In addition, nerve growth factor induces differentiation of PC-12 (pheochromocytoma) cells, one characteristic of which is the extension of long neurite processes. We have found that nerve growth factor changes the levels of IC74 messages in PC-12 cells, resulting in increased synthesis of the IC74–2B isoform (our unpublished observations).

Regulation of the expression of the major neuronal IC74 isoforms during rat brain development further supports the hypothesis that they may be involved in a specialized function in the neuron. At E13, only the IC74–2C mRNA is detected, while by E15, all the mRNAs, except IC74–2A, are present. Even though almost all of the IC74 mRNAs are present at E15, the IC74-2B and IC74-2C gene products dominate the IC74 polypeptide pool. Between E15 and E18 however, the relative amounts of the IC74–2B polypeptide and its phospho-isoform increase dramatically. Over the course of the next week, the relative amounts of the IC74–1 proteins slowly increase, reaching adult levels by P5. The relative levels of the phospho-IC74–2B and phospho-IC74–1A isoforms (the B2 and A2 spots) are also greatest during this time period. Coincident with the increased relative expression of the other IC74 isoforms, the relative amount of the IC74–2C protein isoform decreases, a pattern also observed for the IC74–2C mRNA. Interestingly, no IC74–2A mRNA is detected even as late as P5, although it is found in adult brain. Analysis of the expression of mRNA at later times indicates that the IC74-2A mRNA reaches adult levels between P20 and P26. It is therefore synthesized at a much later time in brain development than the other IC74 isoforms.

The relative level of expression of many of the structural microtubule associated proteins varies during brain development. However, the most striking changes in the level of these proteins is reported to occur after birth (reviewed by Tucker, 1990). Although Paschal and associates showed, using SDS-PAGE, that there was little change in the amount of the IC74 subunit during P1- P29 (Paschal *et al.*, 1992), we found changes in the relative amounts of the IC74 isoforms that occur earlier in brain development. The timing of the changes in IC74 isoforms is consistent with the hypothesis that they have a specialized role in neurons. By E15/E18 neurogenesis is almost complete, and the initial stage of process sprouting and pattern formation is underway (Bayer and Altman, 1995). The extension of processes and the maintenance of neuronal connections is dependent on neurotrophic factors and these growth factors must be transported from the synapse or growth cone to the cell body by retrograde transport (Hammerschlag and Brady, 1989). As dynein is the retrograde transport motor, it is noteworthy that the changes observed in dynein isoforms are consistent with the requirement for retrograde transport of developmentally important neurotrophic factors to maintain process growth. These results encourage the speculation that coincident with the extension of processes, the neuron switches from the IC74–2C isoforms used for con-

stitutive membrane trafficking to IC74 isoforms specialized for the requirements of long distance retrograde axonal transport.

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