Differential Expression and Phosphorylation of the 74-kDa Intermediate Chains of Cytoplasmic Dynein in Cultured Neurons and Glia*

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The 74-kDa intermediate chains (IC74) of the cytoplasmic dynein complex are believed to be involved in the association of dynein with membranous organelles. While each dynein molecule is thought to have two or three IC74 subunits, at least six different IC74 protein isoforms were found in dynein from brain. Therefore we investigated the relationships of the brain cytoplasmic dynein IC74 isoforms and their association in the dynein complex at the cellular level. We found that cultured cortical neurons and glia express distinct IC74 isoforms. The IC74 isoform pattern observed in dynein from cortical neurons was generally similar to that found in dynein from adult brain, indicating that there are different populations of cytoplasmic dynein in neurons. Two IC74 isoforms were observed on two-dimensional gels of dynein from glia, while a single glial IC74 mRNA was detected. Metabolic labeling of glial dynein with $^{32}$P followed by treatment of the isolated dynein with phosphatase in vitro demonstrated that one of the glial IC74 isoforms is the product of the single glial IC74 mRNA and that the other is its phosphoisoform. A single mRNA product and its phosphoisoform are therefore sufficient for constitutive dynein function and regulation in glial cells.

Cytoplasmic dynein is a ubiquitous minus end-directed microtubule-based motor protein (1, 2). In neurons, it is believed to be the motor for retrograde transport of membranous organelles from the synapse to the cell body (3, 4). Retrograde transport is essential for the movement of neurotrophic factors from the synapse to the cell body and for the recycling and degradation of cellular components (5, 6). The cytoplasmic dynein complex is composed of two 530-kDa heavy chains, which make up two globular heads, as well as intermediate chains of −74 kDa (IC74) and 53–59 kDa (7). Molecular analyses indicate that portions of the cytoplasmic dynein IC74 are related to the ICs from the outer arm dynein of flagella (8–11). In addition, the antibody 74.1 directed against the IC74 subunit of cytoplasmic dynein cross-reacts with a polypeptide of similar molecular mass in mammalian glia and flagella (8). It is known that the two IC subunits from the outer dynein arm of Chlamydomonas flagella are located at the base of the molecule (12, 13) and that one, IC78, participates in binding the outer arm to its cargo, the A tubule of the axoneme outer doublet microtubules (12). The similarity of flagellar and cytoplasmic dynein ICs makes it likely that the cytoplasmic dynein IC74 is also located at the base of the two stalks and is involved in binding to cargo, such as membranous organelles.

While quantitative analyses of the polypeptide composition of the cytoplasmic dynein complex indicate that it contains only two or three IC74 subunits per molecule (4, 7, 14, 15), we recently identified at least six different IC74 isoforms in dynein immunoprecipitated from adult rat brain (16). Therefore, there must be populations of cytoplasmic dynein in brain that differ in the composition of their IC74 subunits. Since many of the brain IC74 isoforms were labeled in vivo with $^{32}$P, some of the IC74 diversity is presumably generated by posttranslational modification of a more limited number of polypeptides, and the role of phosphorylation in the regulation of cytoplasmic dynein is under active investigation (16–18). Recently two cytoplasmic dynein ic74 genes, and five alternative splice mRNA variants were identified in brain (19) which suggested that at least five distinct IC74 polypeptides can be synthesized. Therefore, we sought to understand the circumstances which generate the multiple IC74 isoforms and to determine if different populations of cytoplasmic dynein could be identified.

As brain tissue is made up of many specialized cell types, the IC74 isoforms of two brain-specific cell types, cultured cortical neurons and glia, were investigated at the protein and mRNA levels. In addition, the role of phosphorylation in generating the IC74 isoforms in brain and the cultured cells was investigated. We report that neurons and glia express different IC74 isoforms. Two IC74 isoforms were found when cytoplasmic dynein from glia was analyzed by two-dimensional gel electrophoresis. One isoform is the product of the single dynein IC74 mRNA transcribed in glia, and the other is its phosphoisoform. Three additional ic74 gene products and their phosphoisoforms are found in neurons. These results demonstrate that there are cell specific differences in the expression of the cytoplasmic dynein IC74. Furthermore, given the number of ic74 gene products identified in cortical neurons, neurons must contain populations of cytoplasmic dyneins which have different IC74 polypeptide compositions and differences in polypeptide phosphorylation. However, all the cytoplasmic dynein molecules in glia have the same IC74 polypeptide and differ only in the extent of its posttranslational modification. These results have

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§The abbreviations used are: IC, intermediate chain; IC69, IC74, etc., 69-kDa, 74-kDa intermediate chains, etc.; RT, reverse transcriptase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; 1EF, isoelectric focusing; bp, base pair(s).
important implications for the mechanism of cytoplasmic dynein function.

**MATERIALS AND METHODS**

Immunoprecipitation, Electrophoresis, and in Vitro Phosphatase Treatment of Cytoplasmic Dynaein—Immunoprecipitation and electrophoresis were performed as described previously (16). Western blotting was as described previously, except a poly(vinylidene difluoride) membrane was used instead of nitrocellulose (16). Mouse monoclonal antibody 74.1 (16) and rabbit polyclonal anti-IC74-2 antibody were used to screen the cDNA library. Cytoskeletal changes were performed as described previously (16). A, the IC74 region of the two-dimensional gel, showing the six resolved isoforms. B, diagram identifying the IC74 isoforms. The arrow points to the region known as the B1 spot, the arrowhead points to the A2 spot. The acidic and basic ends of the IEF pH gradient are indicated with a “+” and “–“ on the top of Panel A.

The oligonucleotides were used in the following four combinations: G1/S and G1A/S, G2/S1 and G2A/S1, G2/S1 and G2A/S2, and G2/S2 and G2A/S1. To distinguish the different PCR products generated by these primers, the products of each PCR were separated on 8% acrylamide gels, 1 × TBE (89 mM Tris, 89 mM boric acid, 25 mM sodium EDTA) buffer. A Hadid digest of pBR322 DNA (Marker V, Boehringer Mannheim) was used to determine the sizes of the PCR products.

**RESULTS**

Modification of the Adult Brain IC74 Isoform Pattern by Phosphatase Treatment—At least six isoforms of the adult brain cytoplasmic dynein IC74 subunits can be resolved on two-dimensional gels (Fig. 1, A and B) (16). The isoforms are arranged in two arcs of three spots each. We refer to the acidic arc of spots on the IEF gradient as the A, A1, and A2 spots, and the basic arc of spots as the B, B1, and B2 spots. In order to clearly resolve and visualize all of the spots, the A2 and B2 spots are generally fainter and less well resolved than the other spots, and they tend to appear as "tails" of the A1 and B1 spots. Often the A spot is not well resolved from the A1 spot (Fig. 2A). This pattern of spots was not unique to a specific region of the adult brain, as the same pattern was found in dynein immunoprecipitated from the cerebral cortex, cerebellum, medulla, and pons (not shown).

When brain cytoplasmic dynein, labeled with 32P in vivo, was analyzed on two-dimensional gels, label was found over all the spots, except the B spot (16). However, the extent to which this posttranslational modification contributed to the diversity of the IC74 isoforms seen in adult brain was unknown. To identify and characterize the unphosphorylated IC74 isoforms, we treated immunoprecipitated dynein with λ phosphatase in vitro and analyzed the resulting IC74 isoform pattern on two-dimensional gels (Fig. 2). To demonstrate the effectiveness of the phosphatase treatment, brain cytoplasmic dynein was first labeled in vivo with 32P, then immunoprecipitated and resolved on two-dimensional gels. As previously reported (16), label was observed over all the spots except the B spot (Fig. 2, A and B). λ Phosphatase treatment of the 32P-labeled brain dynein removed all of the detectable 32P (Fig. 2D). Coincident with the removal of the 32P, the A2 and B2 spots were no longer seen (Fig. 2C). Therefore, some of the adult rat brain IC74 isoform diversity is solely the result of posttranslational modification of other isoforms. While four distinct polypeptides corresponding to the A, A1, B, and B1 spots remain after dephosphorylation, the A2 and B2 spots are generated by phosphorylation of the other polypeptides.

Comparison of the IC74 Isoforms of Adult Rat Brain and Primary Cell Cultures—Brain tissue is a mixture of cell types. To identify IC74 isoforms at the cellular level we investigated cytoplasmic dynein from two primary cell cultures, glia and cortical neurons. As shown in Fig. 3A, the IC74 isoform pattern of 3S-labeled dynein isolated from glia and neurons are very distinctive. The similarity of the cortical neuron (Fig. 3A,
Phosphatase treatment of the cytoplasmic dynein eliminates the IC74-A2 and -B2 spots. Adult brain cytoplasmic dynein was labeled in vivo as described under “Materials and Methods.” The radiolabeled dynein was immunoprecipitated and divided into two aliquots. One aliquot was treated with the general protein phosphatase, λ phosphatase, as described under “Materials and Methods,” and the other aliquot was treated in the same manner, except that phosphatase was not added to the reaction mixture. The IC74 isoforms were resolved by two-dimensional gel electrophoresis, visualized by silver staining, and then analyzed by autoradiography. Only the IC74 portion of the gel is shown. A, silver-stained gel of control dynein immunoprecipitated from adult brain; B, autoradiograph of 32P-labeled control dynein; C, silver-stained gel of dynein treated with λ phosphatase; D, autoradiograph of 32P-labeled dynein treated with λ phosphatase. The arrows point to the B1 spot and the arrowheads point to the A1 spot. After phosphatase treatment, the A2 and B2 spots are not found, and no spots are labeled with 32P.

Differential Expression of Dynein IC74 Subunits

Center panel) and brain IC74 isoform patterns suggested that the observed cortical neuron spots corresponded to B and B1, as well as poorly resolved A and A1 spots. Similar results were also obtained with cultured hippocampal neurons (not shown). To more precisely correlate the cultured cell isoforms with those identified in brain, 35S-labeled glial dynein was mixed with an excess of unlabeled dynein from brain and analyzed by two-dimensional gel electrophoresis. When the stained gel and autoradiograph were compared, we found that the major and minor glial IC74 isoforms co-migrate with the brain dynein B and B1 spots, respectively (Fig. 3B).

To directly compare the neuronal and glial isoforms, approximately equal counts/min of metabolically labeled glial and neuronal dynein were mixed, and the combined sample was resolved by two-dimensional gel electrophoresis (Fig. 3A, MIXED). Two major spots of approximately equal intensity, as well as a small amount of a slightly more acidic and faster migrating spot, are identified on the autoradiograph of the combined samples. This demonstrated that the glial and neuronal IC74 isoform patterns overlap. From our analysis of 35S-labeled glial dynein, as well as the general pattern of the spots in the combined sample, we conclude that the majority of the B spot was contributed by the glial dynein and that the faint A spots and majority of the B1 spot were derived from the cortical neuron dynein.

In these initial studies, the glia and neurons were labeled for a relatively short time period. When glia were labeled for longer times (see Fig. 5C), the same IC74 isoform pattern was observed. Furthermore, the relative amounts of the glial IC74 isoforms seen in all the metabolic labeling experiments closely resembled the results observed when the gels were stained for protein (Fig. 4A). However, when cortical neurons were labeled for longer time periods the B2 spot was clearly identified, and the relative amount of the A isoforms appeared to increase (Fig. 5D). Nevertheless, dynein from cultured neurons still had a

FIG. 2. Phosphatase treatment of the cytoplasmic dynein eliminates the IC74-A2 and -B2 spots. Adult brain cytoplasmic dynein was labeled in vivo as described under “Materials and Methods.” The radiolabeled dynein was immunoprecipitated and divided into two aliquots. One aliquot was treated with the general protein phosphatase, λ phosphatase, as described under “Materials and Methods,” and the other aliquot was treated in the same manner, except that phosphatase was not added to the reaction mixture. The IC74 isoforms were resolved by two-dimensional gel electrophoresis, visualized by silver staining, and then analyzed by autoradiography. Only the IC74 portion of the gel is shown. A, silver-stained gel of control dynein immunoprecipitated from adult brain; B, autoradiograph of 32P-labeled control dynein; C, silver-stained gel of dynein treated with λ phosphatase; D, autoradiograph of 32P-labeled dynein treated with λ phosphatase. The arrows point to the B1 spot and the arrowheads point to the A1 spot. After phosphatase treatment, the A2 and B2 spots are not found, and no spots are labeled with 32P.

FIG. 3. Cultured glia and cortical neurons have different IC74 isoforms. Cultured rat glia and cortical neurons were metabolically labeled with 1 mCi of Trans35S-label for 1 h as described under “Materials and Methods.” Cytoplasmic dynein from the radiolabeled cultures was immunoprecipitated, resolved by two-dimensional gel electrophoresis, and analyzed by autoradiography. A, the IC74 region of three autoradiographs showing cytoplasmic dynein from glia (left), neurons (center), and a mixture of equal counts/min of dynein from glia and neurons (right). B, 35S-labeled dynein from glia was mixed with cold carrier dynein from rat brain, and the combined sample was analyzed by two-dimensional gel electrophoresis. The gel was stained with Coomassie Blue and then analyzed by autoradiography. Left, Coomassie Blue-stained gel; right, autoradiograph of the gel showing the position of 35S-labeled glial dynein IC74 isoforms. The arrows point to the B1 spot, and the arrowheads point to the poorly resolved A1 spot. The glial spots co-migrate with the B and B1 spots. Cortical neurons, but not glia, have A spots.

FIG. 4. Analysis of the phosphorylation of cortical neuron and glial cytoplasmic dynein IC74 isoforms. A, cultured rat glia were labeled with 1.0 mCi of [32P]orthophosphate for 12 h, and the dynein was immunoprecipitated and analyzed by two-dimensional gel electrophoresis. The gel was silver-stained and analyzed by autoradiography. Left panel, IC74 region of the stained gel; right panel, autoradiograph of the gel, showing the labeling of the B1 spot. B, cultured cortical neurons were labeled with 2 mCi of [32P]orthophosphate for 12 h, the radiolabeled dynein was immunoprecipitated and mixed with cold carrier dynein from brain, and the combined sample was analyzed by two-dimensional gel electrophoresis. The gel was stained with Coomassie Blue and analyzed by autoradiography. Left panel, IC74 region of the stained gel; right panel, autoradiograph of the gel showing that the neuronal dynein B1 spot is lightly labeled and the B2 spot is heavily labeled. There is faint labeling over the A spots. The arrows point to the position of the B1 spot, the arrowheads to the position of the A1 spot.
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Fig. 5. Phosphatase treatment of glial cytoplasmic dynein eliminates the IC74 B1 spot. Cultured rat brain glia were radiolabeled for 12 h with either 3 mCi of Trans-35S-label or 3.0 mCi of [32P]orthophosphate, then the cytoplasmic dynein was immunoprecipitated. Portions of each sample were treated with λ phosphatase, and all of the samples were analyzed by two-dimensional gel electrophoresis and autoradiography, as above. The relative amount of 32P in the IC74 region of the glia gels was calculated as described under "Materials and Methods." Only the IC74 regions of the gels are shown. A, autoradiograph of cytoplasmic dynein from glia labeled with 32P showing the control 32P-labeling pattern. B, autoradiograph of a portion of the same sample as in Panel A, treated with phosphatase, showing the removal of 32P from the immunoprecipitated dynein. C, autoradiograph showing 35S-labeled glial cytoplasmic dynein. D, autoradiograph of a portion of the same sample as in Panel C, treated with phosphatase, showing the loss of the B1 protein spot upon removal of the phosphate from the dynein polypeptides. The A and B, and C and D pairs of images were exposed to the same storage phosphor screen and were printed with the same exposure settings. Rat cortical neuron cultures were labeled with 4 mCi of Trans-35S-label for 18 h, then the dynein was immunoprecipitated analyzed by two-dimensional gel electrophoresis and autoradiography. E, autoradiograph of the IC74 region of control cytoplasmic dynein from neurons. F, autoradiograph of the IC74 region of phosphatase-treated cytoplasmic dynein from neurons. The arrows point to the position of the B1 spot, the arrowheads to the A1 spot.

lesser amount of the A spots compared to dynein from adult brain. These results demonstrate that different cell types from brain have different cytoplasmic dynein IC74 isoforms.

Phosphorylation of Cortical Neuron and Glial Cytoplasmic Dynein IC74—To further understand the basis for the different IC74 isoforms in neurons and glia, and their relation to the isoforms found in brain, we analyzed the in vivo phosphorylation of the IC74 polypeptides from the cultured cells (Fig. 4). Glia and cortical neurons were metabolically labeled with 32P, and the cytoplasmic dynein was immunoprecipitated and then analyzed by two-dimensional gel electrophoresis and autoradiography. Analysis of the glia protein gel and corresponding autoradiograph demonstrated that the glial B1 spot was labeled with 32P (Fig. 4A). There was no 32P associated with the B spot. Furthermore, no B2 spot or "tail" was seen in cytoplasmic dynein immunoprecipitated from glia. In dynein from cortical neurons, 32P labeling was observed over the B1 spot, and heavy labeling extended into the region corresponding to the B2 spot (Fig. 4B). Faint labeling was also observed over the A spots of dynein from cortical neurons.

To determine if the IC74 isoform resolved at the B1 spot was a phosphoisoform of the polypeptide resolved at the B spot, glial cytoplasmic dynein was treated with phosphatase in vitro. Cytoplasmic dynein was immunoprecipitated from cultures metabolically labeled with either [32P]orthophosphate or 35S-labeled amino acids. One half of each of the labeled dynein samples was treated with λ phosphatase. Then the control and treated samples were analyzed by two-dimensional gel electrophoresis. The results are shown in Fig. 5. When the extent of 35S-labeling of the glial dynein IC74 subunit before and after λ phosphatase treatment was quantified, we found that the phosphatase removed ~95% of the 35S from the immunoprecipitated glial dynein (Fig. 5, A and B). Next, the 35S-labeled IC74 polypeptides were analyzed. Following phosphatase treatment, only the B1 spot was observed. No B1 spot was found (Fig. 5, C and D). This shows that, in glial cells, the cytoplasmic dynein polypeptide migrating with the B1 spot is a phosphorylated form of the polypeptide migrating with the B spot. Therefore, in contrast to brain, glial cells have only one unmodified IC74 subunit. Quantitation of the relative amounts of 35S-label in the B and B1 spots of dynein from cultured glia indicates that only ~20% of the glial IC74 polypeptide is phosphorylated at any one time.

When cytoplasmic dynein from cortical neuron cultures was treated with λ phosphatase the IC74 isoform pattern resembled that of similarly treated dynein from brain (Fig. 5F). Four distinct spots were observed, B, B1, A, A1. Interestingly the spots are more clearly resolved than those observed for the untreated control (Fig. 5E). It is likely that this enhanced resolution results when the protein spread out in the A2 and B2 spots migrates with the other spots after the removal of the phosphate. Similar changes in the resolution of the A spots are observed when embryonic brain dynein, which also has relatively little of the A spots, is treated with λ phosphatase.2 Taken together, the phosphatase treatment of cytoplasmic dynein from brain, glia, and neurons indicates that two polypeptides co-migrate with the B1 spot, a phosphate-modified form of the polypeptide resolved at the B spot and an additional polypeptide.

Identification of the Protein in the B Spots as Products of the ic74-2 Gene—Two genes for the cytoplasmic dynein IC74 subunit, ic74-1 and ic74-2, and five alternative splice variants of those two genes have recently been identified (19). We therefore sought to correlate the observed two-dimensional gel protein isoforms with the IC74 mRNAs. First, we used an antibody specific for the carboxyl terminus of the ic74-2 gene products to screen blots of brain cytoplasmic dynein resolved on two-dimensional gels. As is seen in Fig. 6A, the anti-ic74-2 antibody reacted with only a single arc of spots, which from their shape appeared to be the B spots. The blot was then probed with the 74.1 antibody. With this antibody an additional IC74 polypep-

2 Pfister, K. K., Salata, M. W., Dillman, J. F., III, Torre, E., and Lye, R. J. (1996) Mol. Biol. Cell., in press.
mRNAs which are generated by two alternative splice sites, three combinations of primers were utilized. The first set of primers encompasses both of the alternative splice regions. Therefore when they are used in PCR, a different size product for each IC74-2 mRNA is produced; a product of 348 bp results from the full-length IC74-2A mRNA, a product of 330 bp is produced from IC74-2B, which has region 5 spliced out, and a product of 270 bp results, if there is splicing (mRNAs IC74-2B and IC74-2C), a product of 108 bp is produced. Lane 4, the products of primers S2/AS1 are diagnostic for splicing of region 7. mRNAs IC74-2A and IC74-2B, which contain region 7, will generate products of 245 bp, while IC74-2C will generate a product of 185 bp. From the three IC74-2 reactions analyzed together, mRNA for IC74-2A produces products of 348 bp in lane 2, 126 bp in lane 3, and 245 bp in lane 4; IC74-2B mRNA gives products of 330 bp in lane 2, 108 bp in lane 3, and 245 bp in lane 4; IC74-2C gives products of 270 bp in lane 2, 108 bp in lane 3, and 185 bp in lane 4. Therefore, with this analysis, each IC74 mRNA is uniquely identified. C, ethidium-stained gel of the PCR products produced from adult rat brain mRNA isolated as described under "Materials and Methods," using the primers described in A and B. Lane 1, using the probes for IC74-1, products of 140 and 77 bp are obtained, diagnostic of mRNA IC74-1A and IC74-1B, respectively. The band at >348 bp is unidentified and presumably an artifact. Lane 2, using probes for IC74-2, products of 348, 330, and 270 bp are obtained, diagnostic of the mRNAs, IC74-2A, -2B, and -2C. The products of lanes 3 and 4 confirm the interpretation of the lane 2 results. mRNA from adult rat brain has messages from each of the five known IC74 splice variants.
DISCUSSION

The cytoplasmic dynein protein complex is believed to contain two or three IC74 polypeptides per molecule (4, 7, 14, 15). However, we previously reported that at least six IC74 isoforms are resolved on two-dimensional gels of dynein isolated from adult rat brain (16). Furthermore, five alternatively spliced IC74 mRNAs have been identified (19). We therefore initiated this study to investigate the relationships of the various IC74 isoforms and their association in the dynein complex. Our analysis has identified several factors which account for the two-dimensional gel IC74 spot pattern. First, individual cell types express different IC74 mRNAs and their polypeptide products. One mRNA, IC74-2C, is expressed in glia, while four mRNAs are found in neurons. Second, a fraction of the molecules in each IC74 polypeptide pool is phosphorylated. In brain and neurons, the A2 and B2 spots originate by the phosphorylation of the other isoforms. Finally, in at least one instance, different IC74 isoforms co-migrate at the same spot on two-dimensional gels. This is the first report of differences at the cellular level in the subunits of cytoplasmic dynein from the same species and tissue and demonstrates the existence of different pools of cytoplasmic dynein in neurons.

The cytoplasmic dynein IC74 isoforms from cultured glia and cortical neurons are remarkably different. One IC74 mRNA is transcribed in glia, the IC74-2C message. The polypeptide product of this mRNA migrates at the B spot. We will hereafter refer to this polypeptide as the IC74-2C isoform. Previously we reported that the isoelectric point of the B spot was pH 4.9 (16). This is in reasonable agreement with the value of pH 5.16 calculated by a computer program for the product of the IC74-2C mRNA (Expasy Server, University of Geneva). Approximately 20% of the IC74-2C isoform is phosphorylated in vivo. This produces a phospho-IC74-2C isoform that co-migrates with the B1 spot. Cultured glia show no evidence of the phosphoisoform that migrates with the B2 spot. The two-dimensional gel IC74 spot pattern of dynein from cultured cortical neurons is generally similar to that of dynein from adult brain. Treatment of cytoplasmic dynein from cortical neurons with phosphatase in vitro yielded the same four discrete IC74 spots observed in phosphatase-treated brain dynein. However, a lesser amount of the proteins migrating in the A spots was observed when cytoplasmic dynein is immunoprecipitated from cultured cortical neurons than was found in adult brain. This is not an artifact of labeling cultured cells with 35S-labeled amino acids. Rather, the expression levels of several of the neuronal IC74 polypeptides which resolve at the A, A1, B, and B1 spots change during brain development. The relative amounts of the IC74 isoforms of cytoplasmic dynein isolated from cultured cortical neurons, prepared from cerebral cortices obtained on the 18th day of gestation, are very similar to those of dynein immunoprecipitated from 18th day of gestation brains or cortices. The RT-PCR assay demonstrated that four of the five known brain IC74 mRNAs are expressed in the cultured cortical neurons. The IC74-2A mRNA was not found in mRNA prepared from cultured cortical neurons. Interestingly, it was also not found in mRNA prepared from embryonic or newborn brain through P5, indicating that the expression of this IC74 isoform is also developmentally regulated, although with a different time period than that of the other neuronal IC74 isoforms.

Analysis of these results allowed us to deduce a correlation between the IC74 mRNA products and the two-dimensional gel spot pattern (summarized in Table I). As shown above, the product of IC74-2C migrates at the B spot, and its phosphoisoform, which is eliminated by phosphatase treatment, migrates at the B1 spot. In contrast, the B1 spot of cytoplasmic dynein isolated from neurons and adult brain is still observed after phosphatase treatment. This indicates that some IC74 isoforms co-migrate on two-dimensional gels. The B1 spot observed...
when dynein is isolated from adult rat brain or cultured neurons must contain an unphosphorylated polypeptide as well as the phospho-IC74–2C isoform. Since the antibody specific for ic74-2 gene products recognizes all the B spots, this neuronal IC74 polypeptide co-migrating with the B1 spot is most likely the product of an IC74–2 mRNA. Two IC74 mRNAs are expressed in cortical neurons, IC74–2C and IC74–2B. Therefore, it is likely that the unphosphorylated polypeptide co-migrating with the B1 spot is the product of the IC74–2B mRNA. Evidence supporting this conclusion comes from the demonstration that the IC74–2B message is found in all tissues that have a B1 spot after phosphatase treatment, including those with no IC74–1 messages.2

We were unable to identify the B2 spot in analyses of either 35S-labeled or 32P-labeled cytoplasmic dynein from glia. This suggests that phosphorylation of the IC74–2C isoform does not generate the B2 spot. The B2 spot was observed in cytoplasmic dynein from neurons. It therefore appears likely that the B2 spot is the phospho-IC74–2B isoform. However, the possibility that a cortical neuron kinase, not present in glia, generates the B2 spot by hyperphosphorylating the IC74–2C isoform cannot be ruled out. The observation that polypeptides migrating in the A arc of spots are not detected by the IC74–2-specific antibody suggests that the polypeptides resolved in the A arc of spots are the products of the two IC74–1 mRNAs. Given that cortical neurons have both the IC74–1A and IC74–1B messages, it appears likely that one corresponds to the protein of the A spot and the other to the A1 spot. This was confirmed by further study.2 The polypeptide resolved at the A spot is the IC74–1B isoform and the A1 spot is the IC74–1A isoform.

The best studied dynein ICs are the outer arm dynein ICs of Chlamydomonas flagella, which serve as a model for understanding the role of the cytoplasmic dynein ICs (8, 9, 12, 13, 22). There are two ICs per outer arm dynein, IC68 and IC79 (22, 23). These are the products of distinct, although related genes (9–11). The two ICs associate with one another are located at the cargo binding end of the dynein molecule and contribute to the assembly of the dynein complex (12, 13, 22). The flagellar ic69 and ic78 genes are related to the ic74 genes of cytoplasmic dynein (8, 9). The flagellar IC78 binds the dynein complex to its sole cargo, the A microtubule (12). Interestingly, only one isoform of each IC is observed on two-dimensional gels (24, 25). Since the cargo of cytoplasmic dynein includes a variety of membranous organelles, it seemed possible that the different IC74 isoforms were involved in binding cytoplasmic dynein to specific organelles. However, glia express only the IC74–2C mRNA. Therefore, the presence of only the IC74–2C polypeptide in the dynein complex is sufficient for the constitutive dynein-powered movement of membranous organelles including mitochondria, endosomes, and endoplasmic reticulum–Golgi traffic in cultured cells (26–30). The results presented here further demonstrate that, unlike flagellar dynein, the products of both ic74 genes are not necessary for general cytoplasmic dynein function.

The observation that 20% of the single IC74 polypeptide found in glia, the IC74–2C isoform, is phosphorylated is consistent with our previous identification of differences in the phosphorylation of cytoplasmic dynein associated with anterograde organelles and that of the whole cell pool (16). Since cytoplasmic dynein from glia has a single IC74 polypeptide, the study of glial dynein should simplify further investigations into the role of phosphorylation of the IC74 subunit on the functional properties of dynein. Recent studies suggest that phosphorylation of the kinesin heavy and light chains in vivo correlates with the association of kinesin with membranous organelles (31, 32). Genetic studies on the outer arm flagellar dynein support the hypothesis that the IC subunits regulate dynein function (22). Furthermore, it was recently realized that the conserved portions of the flagellar and cytoplasmic genes are a series of WD repeats in the COOH-terminal portion of all three molecules (9, 10). WD repeats are believed to be important for subunit–subunit interactions in protein complexes and, interestingly, all the other proteins with WD repeats are regulatory proteins (33).

Since dynein-based membrane trafficking in a glial cell functions with only the ic74-2C gene product, the roles of the three additional neuronal IC74 polypeptides and their phosphorysosforms in cytoplasmic dynnein function remain to be determined. Interestingly, multiple isoforms of both the heavy and light chains of bovine brain kinesin have also been identified on one- and two-dimensional gels (34). In an analysis of kinesin from rat brain, Cyr et al. (35) identified a single kinesin light chain gene and three alternative splice variants and suggested that the alternative isoforms may be involved in binding to different organelles. Subsequent work on the light chains from other species has yielded similar results (36–38). Elluru et al. (39) have examined the anterograde axonal transport of the two kinesin heavy chain isoforms. They find that one kinesin heavy chain isoform is predominantly associated with anterogradely moving synaptic vesicles, while the other is associated with mitochondria. While two of the neuronal IC74 polypeptides are found in at least one other tissue, one is specific to neurons.2 Cytoplasmic dynein has two to three IC74 subunits per molecule. Therefore, there must be distinct populations of cytoplasmic dynein; however, the mechanisms underlying this diversity are not yet known.

### Table I

| mRNA | Protein product | Two-dimensional gel spot | Present in |
|------|----------------|--------------------------|------------|
| IC74–1B | IC74–1B | A | Glia  | Neurons  | Adult brain |
|       | Phospho-IC74–1B | A1 | No | Yes | Yes |
| IC74–1A | IC74–1A | A | No | Yes | Yes |
|       | Phospho-IC74–1A | A2 | No | Yes | Yes |
| IC74–2A | IC74–2A | B1 | No | No | Yes |
| IC74–2B | IC74–2B | B1 | No | Yes | Yes |
|       | Phospho-IC74–2B | B2 | No | Yes | Yes |
| IC74–2C | IC74–2C | B | Yes | Yes | Yes |
|       | Phospho-IC74–2C | B1 | Yes | Yes | Yes |

The identification of the other mRNA products with two-dimensional gel spots is from unpublished results,2 except that the position of the IC74–2A mRNA sequence.

The alignment of the IC74–2B and -2C mRNA protein products and their phosphoisoforms with the indicated two-dimensional gel spots is described in the text. The identification of the other mRNA products with two-dimensional gel spots is from unpublished results,2 except that the position of the IC74–2A protein product is derived from a computer calculation (Expasy Server, University of Geneva) of its pI and molecular mass from the IC74–2A mRNA sequence.
mic dynein in neurons. It is tempting to speculate that the additional IC74 isoforms may confer a regulatory or functional specificity on the dynein complex needed in neurons and some other cells, but not glia. Neurons are specialized for long-distance axonal transport, and the presence of additional IC74 isoforms may be related to the specialized cargo moved in retrograde axonal transport, or the regulation of retrograde axonal transport. Interestingly, we find that all the neuronal dynein IC74 isoforms are present in axons (16). However, understanding the role of the various neuronal cytoplasmic dynein IC74 isoforms remains a challenge. The demonstration of basic differences in the IC74 subunits of cytoplasmic dynein from glia and neurons should also raise a cautionary note concerning biochemical studies of dynein from mammalian brain, as dynein from this source is a mixed population of the various neuronal cytoplasmic dynein IC74 isoforms remains a challenge. The demonstration of basic differences in the IC74 subunits of cytoplasmic dynein from glia and neurons should also raise a cautionary note concerning biochemical studies of dynein from mammalian brain, as dynein from this source is a mixed population of the motor protein.

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