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Expression and localization of α-adaptin isoforms

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SUMMARY

There are two α-adaptin genes, α_A and α_C, which in brain encode proteins of of M_r 108×10^3 and 104×10^3, respectively. Although both mRNAs can be detected on northern blots of brain and liver, the higher molecular mass polypeptide can only be detected on western blots of brain. Here we explain these observations by showing that α_A is alternatively spliced and that the protein product in most tissues is different from the one expressed in brain in that it is missing 21 amino acids within the hinge region, giving it a similar mobility to that of α_C. Monospecific antibodies were raised against the various α-adaptin isoforms and used to compare their distribution in cells and tissues. Both α_A and α_C are co-assembled into the same coated pits, and the larger isoform of α_A is co-assembled with the smaller isoforms of α-adaptin, both in cells that naturally express it and in transfected cells. Examination of brain and spinal cord sections, labelled either for the larger isoform of α_A or for α_C, reveals that that the two are to some extent differentially distributed, consistent with previous in situ hybridisation studies. This finding, combined with the observation that there is considerable variability in the relative expression of the two isoforms in different tissues, indicates that the two genes are switched on in response to different stimuli. Moreover, the larger isoform of α_A appears to be more efficiently concentrated in the nerve terminals than α_C, which is found not only at the terminals but also diffusely distributed in the cell bodies and dendrites. This suggests that α_C may play more of a role in the recycling of membrane components throughout the cell.

Key words: α-adaptin, brain, endocytosis

INTRODUCTION

Clathrin-coated vesicles are responsible for the selective endocytosis of a number of different membrane proteins, including receptors for various extracellular ligands, and proteins that need to cycle between the plasma membrane and an intracellular compartment. This cycling pathway is particularly important in neuronal cells, which must retrieve synaptic vesicle membrane components within seconds after exocytosis (Heuser and Reese, 1973). Coated vesicles are also thought to play a major role in the removal of cell adhesion molecules from the plasma membrane, which in neurons would allow new cell contacts to be made and new synapses formed (Hu et al., 1993). In addition to endocytic coated vesicles associated with the plasma membrane, the cell also contains clathrin-coated vesicles associated with the *trans*-Golgi network (TGN), which facilitate the delivery of newly synthesised lysosomal enzymes to a pre-lysosomal compartment.

The major component of the coat is clathrin, which in its native form consists of a triskelion containing three copies of clathrin heavy chain and three copies of clathrin light chain (Kirchhausen and Harrison, 1981). In mammalian cells there is only one type of clathrin heavy chain so far identified, but there are two types of clathrin light chains in most cells, called LC_a and LC_b, which are encoded by separate genes. In brain there are two additional isoforms of LC_a and one additional isoform of LC_b, all of which are somewhat larger than those expressed in other tissues. The complete conservation of the flanking sequences between the brain and non-brain forms of the light chains indicates that the different isoforms are produced by alternative splicing of the mRNAs (Jackson et al., 1987; Kirchhausen et al., 1987). The brain-specific sequences are exposed and are thought possibly to interact with a specific factor in brain cytoplasm (Jackson et al., 1987).

The inner layer of the coat is made up of adaptors, protein complexes that are in close proximity to the membrane. The adaptors are believed to interact with the cytoplasmic domains of selected membrane proteins, causing these proteins to become concentrated in coated vesicles (Pearse, 1988; Glickman et al., 1989). Different adaptors are associated with the plasma membrane and the TGN (Robinson, 1987; Ahle et al., 1988). Both types of adaptors are composed of four distinct subunits: two of M_r ~100×10^3, called adaptins (α- and β-adaptin at the plasma membrane, γ- and β′-adaptin at the TGN), one of M_r ~50×10^3, and one of M_r ~20×10^3 (Ahle et al., 1988). Structural studies show that the adaptors consist of a central brick-like mass (the 'head') flanked by two symmetrically placed appendages (the 'ears'), which are connected to the head by protease-sensitive hinges (Heuser and Keen, 1988). The ears have been found to correspond to the C-terminal domains of the adaptins, while the hinges are proline- and glycine-rich stretches of sequence found in all three classes of

adaptins between amino acids ~600 and ~700-750 (Robinson, 1989, 1990; Kirchhausen et al., 1989).

Cloning and sequencing of the adaptins has revealed the existence of different isoforms which could potentially have different functions. Although so far only a single γ-adaptin gene has been cloned, Southern blotting suggests that there may be at least one other gene (Robinson, 1990; Ball and Robinson, unpublished data). Three β-type adaptins have been identified: two from rat brain (Kirchhausen et al., 1989), which are encoded by separate genes and have been shown to correspond to β- and β′-adaptins (Galluser and Kirchhausen, 1993), and the third from rat lymphocytes (Ponnambalam et al., 1990). This is identical to rat brain β-adaptin except that it lacks a 42 bp insert in the hinge, indicating that β-adaptin, like the clathrin light chains, is alternatively spliced, giving rise to both brain and non-brain isoforms. Two α-adaptins have been cloned from brain, and are called α_A and α_C. They are encoded by different genes and show 84% identity at the amino acid level (Robinson, 1989). From their sequences, α_A-adaptin has a deduced size of 108×10^3 while α_C-adaptin has a deduced size of 104×10^3. Western blots of various tissues probed with a monoclonal antibody that reacts with both isoforms of α-adaptin show two bands in brain and spinal cord with apparent molecular masses of ~112×10^3 and ~105×10^3, presumably α_A- and α_C-adaptins, but only a single band of ~105×10^3 in other tissues (Robinson, 1987). These observations suggest that α_C is universally expressed while α_A is only expressed in neuronal tissues. However, northern blots of RNA from brain and liver probed for the two α-adaptin messages show that a small but significant amount of α_A-adaptin mRNA is present in liver as well as in brain, even though the protein cannot be detected on western blots (Robinson, 1989).

The experiments described in this paper were designed to find out why α_A-adaptin can be detected in liver on northern blots but not western blots; to investigate the patterns of expression of both α-adaptin genes in different cells and tissues; and to localise the protein products of the two genes to find out whether the distribution of the different α-adaptin isoforms might be correlated with possible differences in function.

MATERIALS AND METHODS

DNA manipulations

Most DNA manipulations were carried out using techniques described by Sambrook et al. (1989). α_A-Adaptin sequences were amplified by PCR from rat liver cDNA (generously provided by Paul Guest) and from mouse genomic DNA prepared from liver. The forward primer corresponded to bases 1895 to 1920, while the reverse primer corresponded to bases 2374 to 2399. The amplified α_A-adaptin DNA was reamplified using similar primers but with *Bam*HI and *Eco*RI sites added to the 5′ ends of the forward and reverse primers, respectively. These sites were then used to ligate the reamplified DNA into pBluescript, and the DNA was sequenced using the Sequenase Version 2.0 kit (United States Biochemicals).

For northern blotting, poly(A)-containing RNA was purified from 3T3 tissue culture cells by the lithium/urea method (Auffray and Rougeon, 1980), subjected to electrophoresis (9 μg/lane), and blotted onto Hybond paper. An additional northern blot containing mRNA from mouse tissues was bought from Clontech. Three antisense oligonucleotides were used to probe the blots. Probe 1 was taken from nucleotides 2115 to 2180 of α_A-adaptin, probe 2 was taken from nucleotides 2175 to 2240 of α_A-adaptin, and probe 3 was taken from nucleotides 2818 to 2883 of α_C-adaptin (part of the 3′ untranslated region). The three oligonucleotides were end-labelled and the blots were probed following the instructions supplied by Clontech, then washed at room temperature in four changes of 2× SSC, 0.1% SDS, 1 mM EDTA, followed by a wash at 65°C for 30 minutes in the same solution.

Production of antibodies

Fusion proteins were made in pGEX-3X and expressed in MC1061 cells. The proteins were purified as soluble fusion proteins as described by Smith and Johnson (1988). Regions to be expressed were amplified from plasmid DNA, using primers that would allow them to be inserted in frame into the *Bam*HI and *Eco*RI sites of the vector. Fusion protein A706-727 contains the sequence of α_A-adaptin that is removed in the splicing event; fusion protein A620-655 contains a sequence from α_A-adaptin that is not removed in the splicing event; and fusion protein C619-656 contains a sequence from α_C-adaptin corresponding to the sequence of α_A-adaptin used for fusion protein A620-655 (see Figs 1 and 3).

Rabbits were injected subcutaneously along the flank with 0.5 mg fusion protein made up in complete Freund's adjuvant, and boosted with the same amount of protein, in incomplete Freund's adjuvant, after 2 weeks and then after a further 6 weeks. One week after the final boost, a test bleed was performed, and the rabbits were bled out after an additional week.

The antibodies were absorbed with glutathione S-transferase (GST) fusion proteins to ensure that they would be specific for the protein for which they had been designed. Antiserum C619-656 was absorbed with fusion protein A620-655, antiserum A620-655 with fusion protein C619-656, and antiserum A706-727 with GST alone. The antisera were then affinity purified (Robinson and Pearse, 1986) using the fusion proteins against which they had been made. In every case 1 mg of protein was used per ml of serum. (In a previous study, antiserum A706-727 was used as a probe for brain α-adaptin and was called C4; Seaman et al., 1993.)

Immunoprecipitation and western blotting

A 20 μl sample of pig brain plasma membrane adaptors at a concentration of 1 mg/ml (prepared as described by Pearse and Robinson, 1984) was boiled in 1% SDS for five minutes, then diluted into 180 μl RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP40) made without the SDS to give a final concentration of 0.1% SDS. The solution was pre-cleared by rotating with 50 μl of a 50% slurry of Protein A-Sepharose (Pharmacia) in PBS for one hour, after which the Sepharose was removed by centrifugation. The antibodies were added to the supernatants and the tubes rotated for a further hour. A 50 μl sample of Protein A Sepharose slurry was added and the tubes rotated again for one hour, then the Sepharose was collected by centrifugation, washed six times with 500 μl RIPA buffer, resuspended in 40 μl sample buffer (0.2 M CHES, pH 9.5, 40% glycerol, 8% SDS, 5% mercaptoethanol, 0.25% Bromophenol Blue), and boiled for two minutes. SDS-PAGE, western blotting, and antibody labelling were carried out as described (Robinson and Pearse, 1986). Antibodies included the three antisera against fusion proteins described above, and the mouse monoclonal anti-α-adaptin antibodies AC1-M11 and AC2-M15. ^{125}I-Protein A was used to visualise all the antibodies directly except AC2-M15, which needed to be incubated with rabbit anti-mouse Ig (Sigma) before the ^{125}I-Protein A incubation step. Western blotting of total plasma membrane adaptors was performed in the same manner.

Cell culture

Cos cells were maintained in DMEM supplemented with 10% foetal calf serum, 4 mM L-glutamine, 50 i.u./ml penicillin, 50 μg/ml streptomycin. Transfection and immunofluorescence were carried out as

described previously for Rat1 cells (Robinson, 1990). The plasmid used for transfection contained cDNA encoding the longer form of α_A-adaptin (Robinson, 1989), ligated into the *Eco*RI site of the vector pHYKS3 (Robinson, 1990), which contains the SV40 early promoter. The cells were examined two days after transfection.

Primary neuronal cells were cultured on sterile coated coverslips, prepared by incubating the coverslips in 100 μg/ml polyornithine in 0.15 M sodium borate buffer, pH 8.4, overnight, washing in PBS, incubating in 5 μg/ml laminin in PBS for four hours, and washing again in PBS. The cells were prepared from the brains of 17-day rat embryos. The brains were washed in EBSS (Gibco), the two lobes separated, and the meninges peeled away. The lobes were gently pushed through a 100 μm nylon mesh into EBSS to separate the cells, and the cells were centrifuged at 1000 rpm for five minutes and resuspended in 2 ml medium (DMEM supplemented with 10% foetal calf serum, 10% horse serum, 20 i.u./ml penicillin, 20 μg/ml streptomycin, and 4 mM glutamine). The cells were then passed through a 20 μm nylon mesh and plated at 0.6×10^6 cells/ml onto the coated coverslips. After 24 hours, the medium was changed to defined medium (DMEM supplemented with, for each litre, 5 mg insulin, 100 mg transferrin, 16.1 mg putrescine, 6.3 μg progesterone, 5.36 μg selenium, 18 mM HEPES, pH 7.4) and the cells were left to grow for a further 5-8 days before the coverslips were fixed and labelled.

For some experiments, the cells were double labelled with mouse monoclonal antibodies including the α-adaptin antibodies AC2-M15 (Robinson, 1987) and AP6 (Chin et al., 1989) (generously provided by Frances Brodsky), and commercially available antibodies against MAP5 and GFAP (Sigma).

Labelling of rat brain sections

Rats were terminally anaesthetized with saturated aqueous chloral hydrate (1.5 ml i.p.) and perfused intracardially with 200 ml of normal saline followed by 400 ml of freshly prepared paraformaldehyde, 4% in 0.05 M sodium phosphate buffer (PB), pH 7.4. Brains were removed, post-fixed in the latter solution for 2 hours, and washed overnight in 0.1 M PB containing 30% sucrose and 0.01% sodium azide. Sections 40 μm thick were cut and incubated free-floating in antibody A706-727 or C619-656 diluted in TTBS (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl, 0.3% Triton) at 4°C for 48 hours, then washed in four changes of 0.1 M PB. The sections were then incubated in biotinylated anti-rabbit IgG (Vector Laboratories) diluted into TTBS for two hours at room temperature, washed in PB, and then incubated in fluorescein/avidin D (Vector Laboratories), diluted into TTBS for two hours at room temperature and washed again. The sections were mounted onto microscope slides and viewed with an MRC 600 confocal microscope.

RESULTS

A novel isoform of α_A-adaptin

Although α_A-adaptin mRNA can be detected in liver by northern blotting, an immunoreactive protein of the expected size is only found in brain and spinal cord. This suggests either that α_A-adaptin is not translated in liver in sufficiently high amounts to be detected, or that it is made as a smaller isoform which co-migrates with α_C-adaptin and is therefore effectively masked. Fig. 1 shows partial amino acid sequences of α_A (upper sequence) and α_C (lower sequence), including the hinge regions, where the two proteins are most divergent (the hinge is defined as amino acids 621-746 in α_A and 620-707 in α_C; see Robinson, 1993). In particular, there is an extra stretch of 41 amino acids in α_A. To investigate whether all or part of this region might be missing in liver, PCR primers corresponding to amino acids 632-640 and 792-800 of α_A (see italics in Fig. 1) were used to amplify cDNA from rat liver. The major PCR product migrated with an apparent size of ~440 bp, smaller than the expected size for α_A-adaptin of 504 bp. Sequencing revealed that 66 bp of the DNA was missing from the extra stretch, encoding the boxed-in region in Fig. 1. This indicates that α_A-adaptin, like clathrin light chains and β-adaptin, is alternatively spliced, with a smaller isoform expressed in liver than in brain.

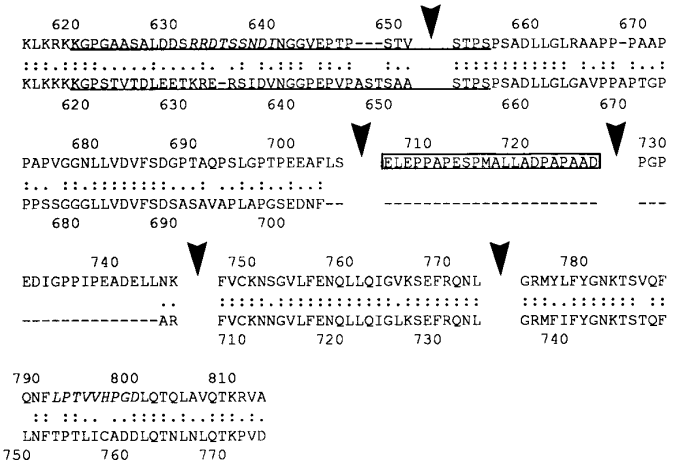

Fig. 1. Comparison of part of the sequences of α_A-adaptin (above) and α_C-adaptin (below). The amino acids in italics are those used to design PCR primers to amplify α_A-adaptin from rat liver cDNA and from mouse genomic DNA. The boxed-in sequence is the region that was found to be present in brain but absent in liver and other tissues because of alternative splicing. This sequence was expressed as a fusion protein and used to raise antibody A706-A727 (the numbers refer to the amino acids). The underlined sequences further upstream were also expressed as fusion proteins and used to raise antibodies A620-655 and C619-656. The arrowheads indicate the positions of introns.

To confirm that the difference between liver and brain α_A-adaptin is due to alternative splicing, the genomic structure of the region around the putative splice site was investigated, using the same PCR primers to amplify α_A-adaptin from mouse genomic DNA. Sequencing the PCR product showed that the extra 66 bp in brain is a single exon with introns on either side of it. Three additional introns were also found in the amplified DNA (indicated with arrowheads in Fig. 1), with all intron/exon boundaries conforming to the consensus sequences for splice sites (Padgett et al., 1986).

Tissue-specific expression of the different α-adaptins

Is the smaller isoform of α_A-adaptin expressed in other tissues as well as liver? To investigate the patterns of expression of the different α-adaptin isoforms, three oligonucleotide probes were used to label northern blots of different tissues and also of 3T3 cells (Fig. 2). Probe 1 was designed to hybridize to the DNA removed in the splicing event, probe 2 to the DNA just downstream from the spliced sequence, and probe 3 to the 3′ untranslated region of α_C-adaptin. Probe 1 (Fig. 2a) labelled only brain (lane 2) and skeletal muscle (lane 6). In contrast, probe 2 (Fig. 2b), like probe 3 (Fig. 2d), labelled all the tissues on the blot as well as the 3T3 cells (Fig. 2c). These results show that, far from

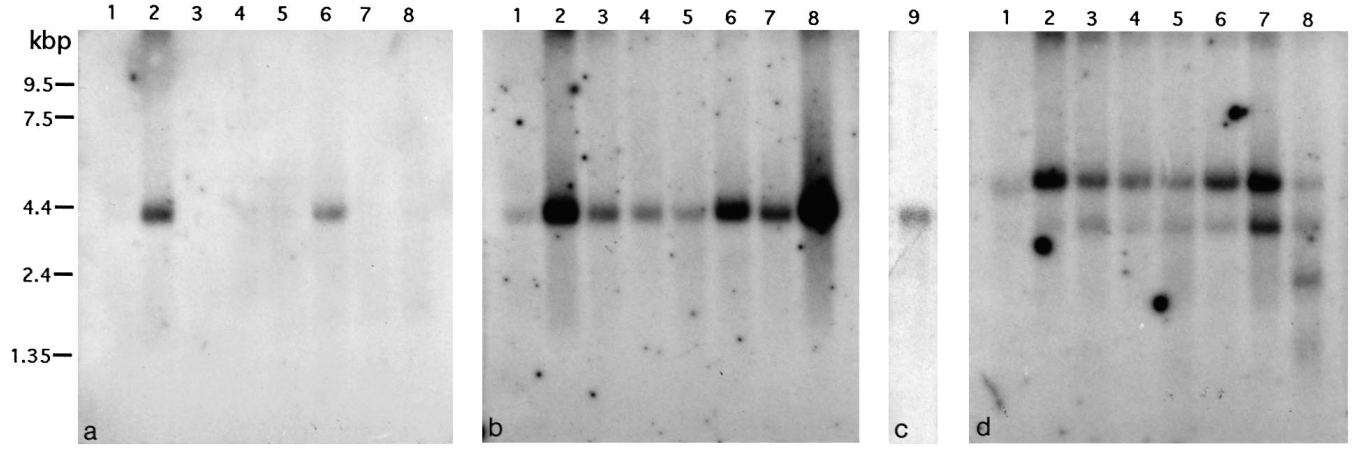

Fig. 2. Northern blots of mRNA from a variety of tissues, labelled with three different probes: (a) probe 1, which recognises the larger isoform of α_A-adaptin; (b and c) probe 2, which recognises both isoforms of α_A-adaptin; (d) probe 3, which recognises α_C-adaptin. Lane 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis; 9, 3T3 cells. The two bands in the blot probed for α_C-adaptin (d) appear to be the result of differences in the 3′ untranslated region (Robinson, 1989).

being brain-specific, α_A-adaptin is ubiquitously expressed along with α_C-adaptin, although the relative amounts of the two vary (e.g. compare kidney (lane 7) and testis (lane 8)). However, the larger isoform of α_A-adaptin only appears to be expressed in neuronal tissue and, unexpectedly, in skeletal muscle.

Production of monospecific antibodies

To study the subcellular distribution of the different α-adaptins, it is necessary to have antibody probes that can distinguish between the various isoforms. A number of monoclonal anti-α-adaptin antibodies have been raised, including two that appear to be specific for α_C-adaptin (Robinson, 1987). However, these are of limited use, since they only recognise the SDS-denatured protein. All other α-adaptin antibodies that have so far been raised appear by western blotting to cross-react with all isoforms of the protein. Thus, we set out to raise monospecific antibodies against fragments of the different α-adaptins expressed as fusion proteins which could be used for immunolocalisation and immunoprecipitation experiments. The hinge region was chosen for these studies for two reasons: first, because it is here that there is least homology between α_A and α_C; and second, because the hinge is known to be exposed in the native adaptor complex. Three fusion proteins were constructed, using the expression vector pGEX-3X: A706-727, A620-655, and C619-656 (see Figs 1 and 3). A706-727 was designed to elicit antibodies that would be specific for the larger isoform of α_A-adaptin; A620-655 was designed to elicit antibodies that would be specific for both α_A-adaptins but not α_C-adaptin; and C619-656 was designed to elicit antibodies that would be specific for α_C-adaptin.

Affinity-purified and cross-absorbed antibodies against all three fusion proteins were tested by labelling western blots of purified pig brain plasma membrane adaptors (Fig. 4). Antibody A706-727 binds only to the upper α-adaptin band (lane 1), while C619-656 binds only to the lower α-adaptin band (lane 3). However, A620-655, which should recognise both forms of α_A-adaptin, binds to the upper band and a lower band, although the upper band is much more heavily labelled than the lower band (lane 2). In addition, the mobility of the lower band is not identical to that of α_C-adaptin, although it is very close to it. This indicates that in brain, a small amount of the smaller, 'non-brain' form of α_A-adaptin is expressed as well as the larger isoform, and it confirms that the smaller isoform of α_A-adaptin runs on SDS-polyacrylamide gels with a very similar mobility to that of α_C-adaptin.

Two mouse monoclonal anti-α-adaptin antibodies were also included on the blot as positive controls: AC1-M11 and AC2-M15. AC1-M11 is known to recognise both α_A-adaptin and α_C-adaptin, since it reacts with both recombinant proteins (Robinson, 1989, and unpublished results). On the blot, it labels both bands with approximately equal intensity (lane 4). However, AC2-M15 (lane 5), which was also thought to bind both α_A-adaptin and α_C-adaptin (Robinson, 1987), produces a pattern more similar to that of antibody A620-655, suggesting that AC2-M15 may in fact be specific for α_A-adaptin.

To test whether AC2-M15 is an α_A-specific antibody, purified pig brain adaptors were immunoprecipitated and western blotted. The four lanes in Fig. 5 contain immunoprecipitates carried out with no first antibody (lane 1), A706-727 (lane 2), A620-655 (lane 3), and C619-656 (lane 4) (A620-655 precipitated less efficiently than the other antibodies). When the blot was probed with AC2-M15 (a), the α_A-adaptin bands in lanes 2 and 3 were labelled but not the α_C-adaptin band in lane 4. A similar pattern was seen when the blot was probed with A620-655 (c) or with A706-727 (not shown). In contrast, the universal anti-α-adaptin antibody AC1-M11 was found to label bands in all three lanes (b), while C619-656 labelled the α_C-adaptin band in lane 4 only (d). These results confirm that AC2-M15 is specific for α_A-adaptin and does not cross-react with α_C-adaptin.

Colocalization of the different α-adaptins

Are the different α-adaptin isoforms incorporated into the same coated pits? To compare the distribution of α_C-adaptin and α_A-adaptin, Cos cells were double labelled with the rabbit α_C-specific antibody C619-656 (Fig. 6a) and the mouse α_A-specific antibody AC2-M15 (Fig. 6b). The staining patterns show almost complete coincidence, indicating that, at least in these cells, all of the plasma membrane coated pits appear to contain both α-adaptins.

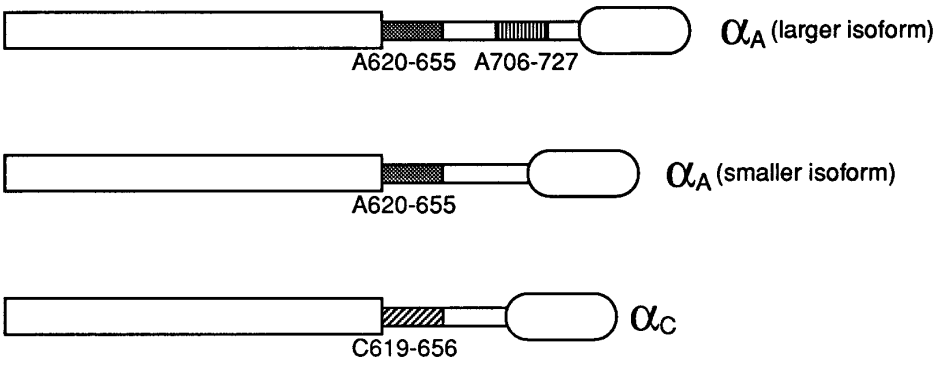

Fig. 3. Diagrams of the three α-adaptin isoforms showing the positions of the sequences used to raise antibodies (see also Fig. 1). Epitope A620-655 is located in the proximal part of the α_A-adaptin hinge and thus is expressed in both the larger and the smaller isoforms of α_A. Epitope A706-727 corresponds to the α_A-adaptin exon that is spliced in in brain but spliced out in most other tissues. Epitope C619-656 is located in the proximal half of the α_C-adaptin hinge; thus, antibodies against this sequence react with α_C but not with α_A.

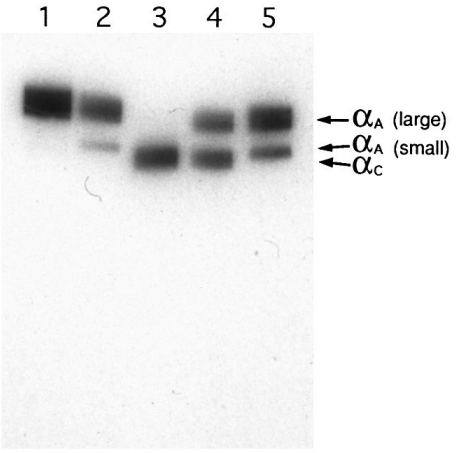

Fig. 4. Western blot of pig brain plasma membrane adaptors probed with three affinity-purified antibodies raised against fusion proteins: A706-727 (lane 1), A620-655 (lane 2), and C619-656, (lane 3); and with mouse monoclonal antibodies AC1-M11 (lane 4) and AC2-M15 (lane 5). A706-727 binds only the larger isoform of α_A-adaptin; A620-655 binds both isoforms of α_A-adaptin, C619-656 binds only α_C-adaptin, and AC1-M11 binds all isoforms of α-adaptin. AC2-M15, which originally was also thought to bind all isoforms of α-adaptin, appears from the blot to be α_A-specific.

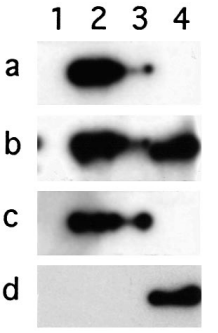

Fig. 5. Western blots of immunoprecipitates of purified pig brain plasma membrane adaptors. Lane 1, control immunoprecipitate (no first antibody); lane 2, A706-727 immunoprecipitate; lane 3, A620-655 immunoprecipitate; lane 4, C619-656 immunoprecipitate. The immunoprecipitates were then probed with antibodies AC2-M15 (a), AC1-M11 (b), A620-655 (c), and C619-656 (d). These results confirm that AC2-M15 is α_A-specific, since it binds only to the proteins precipitated by antibodies A706-727 and A620-655.

Is the larger isoform of α_A-adaptin able to be incorporated into the same coated pits and vesicles as the smaller isoforms? This question was addressed by transfecting Cos cells with a plasmid containing the original α_A-adaptin cDNA cloned from brain. The cells were then double labelled with A706-727 and AP6, a mouse monoclonal antibody believed to recognise both α_A-adaptin and α_C-adaptin (Chin et al., 1989). Fig. 7 shows that the larger isoform of α_A-adaptin (a), which is not normally expressed in these cells, is incorporated into the same structures as the endogenous α_A- and α_C-adaptins (b). However, to look at the natural distribution of the protein it is necessary to use cells in which it is normally expressed.

Distribution of α-adaptins in cultured neuronal cells

To localise the larger isoform of α_A-adaptin under more physiological conditions, primary cultures were prepared of rat foetal brain cells. Double labelling with A706-727 (Fig. 8a) and AP6 (Fig. 8b) reveals that only a subpopulation of the cells express the larger isoform of α_A-adaptin. All of the positive cells have the typical appearance of neuronal cells, consisting of small cell bodies and long neurites. The protein appears to be uniformly distributed in the endocytic coated pits and vesicles in these cells, since the two antibodies show very similar labelling patterns in the positive cells.

To identify the positive cells definitively, double labelling was carried out using antibodies against proteins with restricted distributions. Double labelling with A706-727 (Fig. 8c) and an antibody against microtubule-associated protein 5 (MAP5), a neuronal-specific protein (Fig. 8d), shows that the two proteins are expressed in the same cells. In contrast, double labelling with A706-727 (Fig. 8e) and an antibody against glial fibrillar associated protein (GFAP), a protein found in glial cells (Fig. 8f), shows that different cells express the two antigens. Thus, the larger isoform of α_A-adaptin, like the larger isoforms of the clathrin light chains (Wong et al., 1990), is specifically expressed in neurons.

Distribution of α-adaptins in tissue sections

Do all neurons express the larger isoform of α_A-adaptin, and do they all express similar amounts of α_A and α_C? Previous studies making use of in situ hybridization to localise α_A- and α_C-adaptin mRNAs suggest that the relative amounts of expression of the two messages may vary in different parts of the brain (Robinson, 1989). To study the localization of the α-adaptin isoforms at the protein level, and to identify the regions where the larger isoform of α_A is concentrated, sections of spinal cord and brain were labelled with either A706-727 or

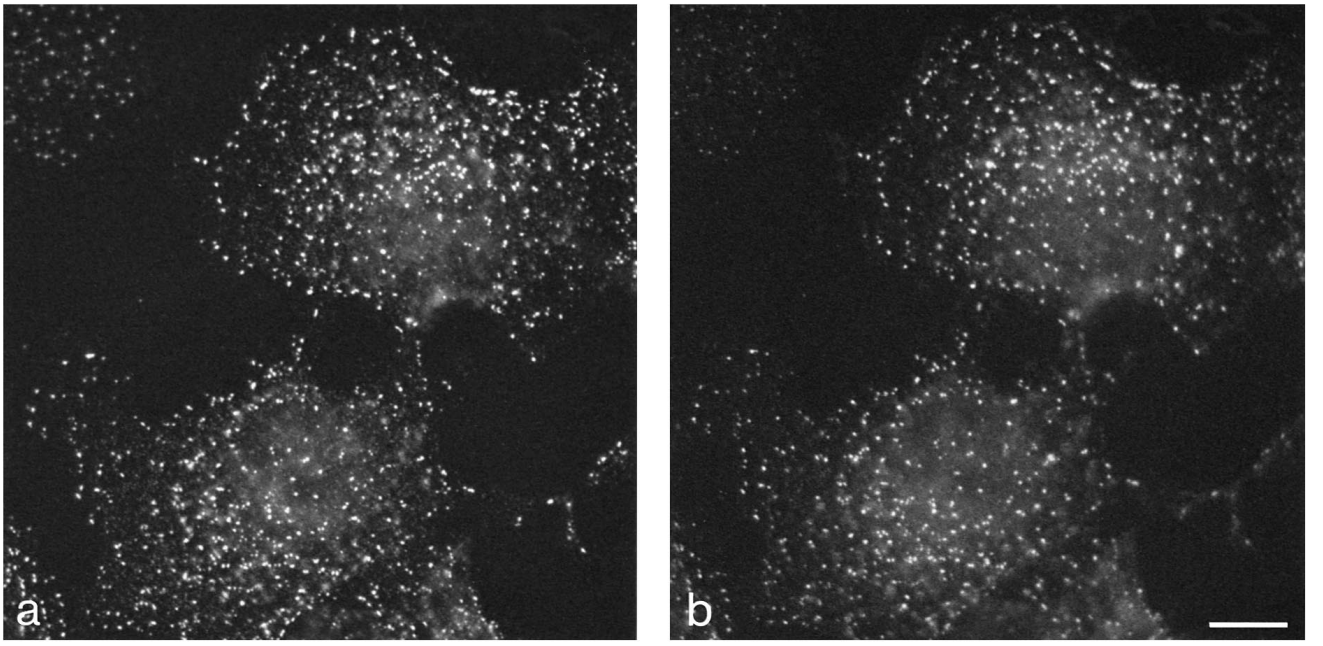

Fig. 6. Immunofluorescence micrographs of Cos cells double labelled with (a) antibody C619-656, which recognises α_C-adaptin; and (b) AC2-M15, which recognises α_A-adaptin. The complete coincidence of the two labelling patterns shows that α_A- and α_C-adaptins are present within the same coated pits. Bar, 10 μm.

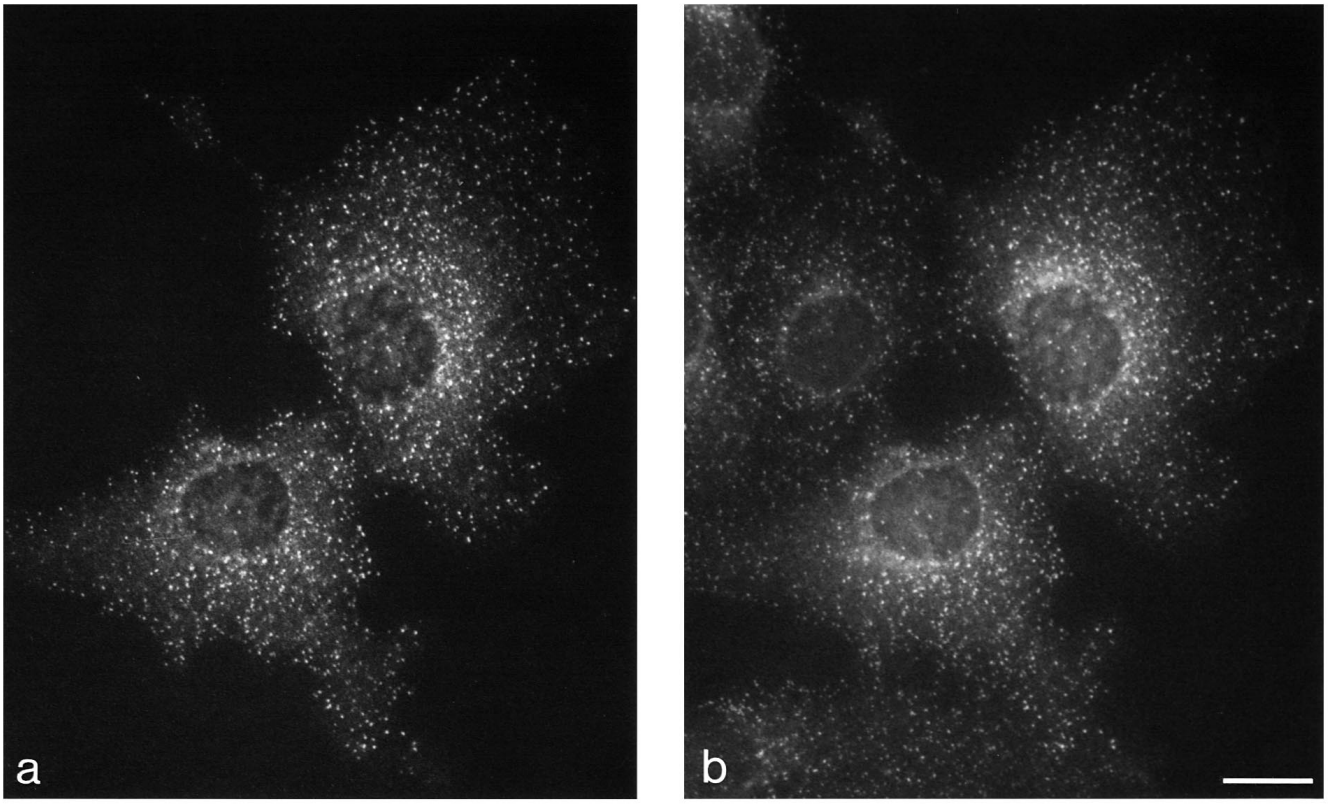

Fig. 7. Immunofluorescence micrographs of Cos cells transfected with the larger isoform of α_A-adaptin double labelled with (a) A706-727, which is specific for that isoform; and (b) AP6, an antibody that recognises all α-adaptin isoforms. The coincidence in staining patterns in the transfected cells shows that the larger isoform of α_A-adaptin can be incorporated into the same coated pits and vesicles as the endogenous α_A- and α_C-adaptins. Bar, 15 μm.

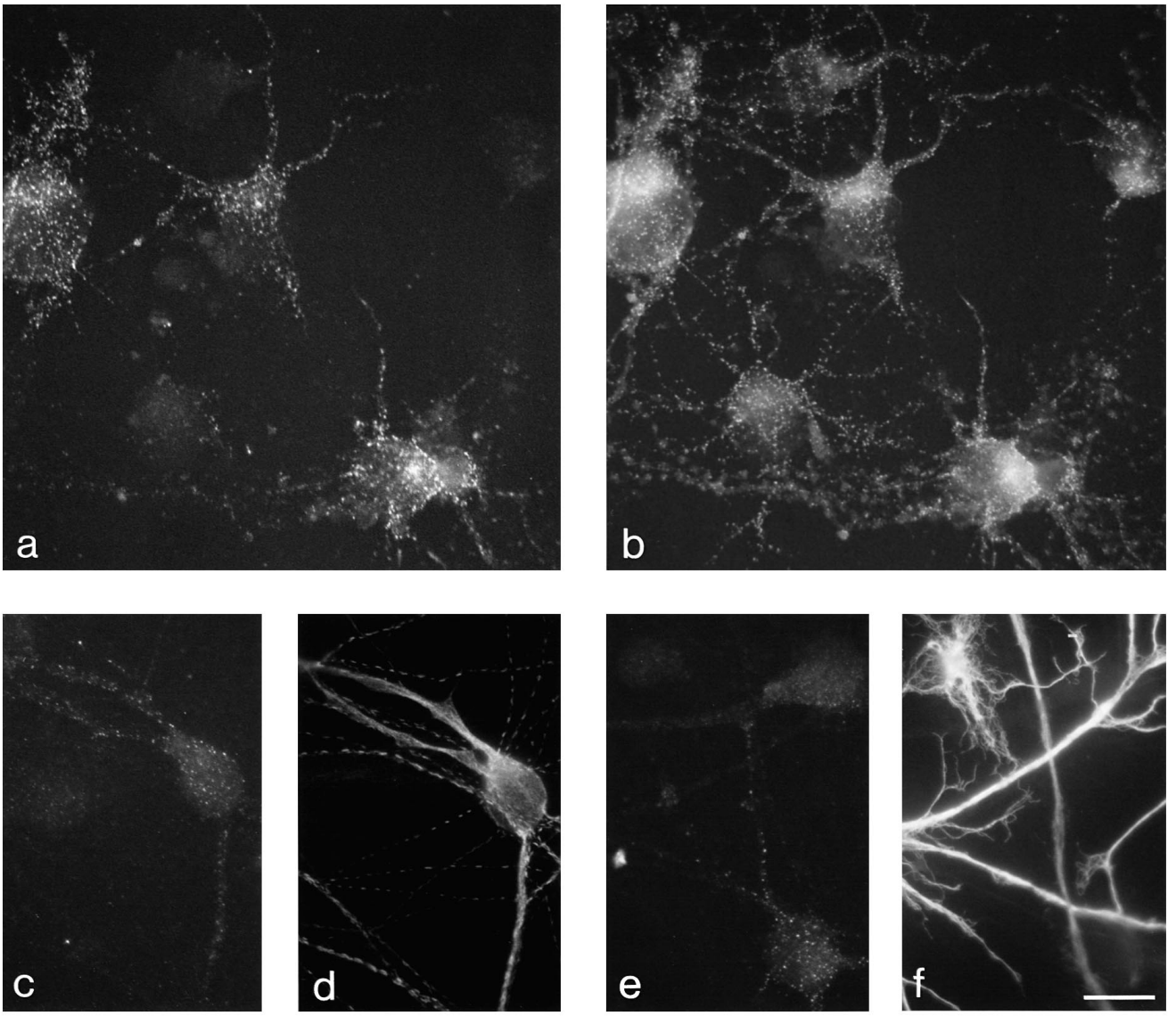

Fig. 8. Immunofluorescence micrographs of cells cultured from foetal rat brains. (a and b) Cells were double labelled with antibodies A706-727 (a) and AP6 (b). Only a subset of the cells express the larger isoform of α_A-adaptin. (c and d) Cells were double labelled with antibody A706-727 (c) and an antibody against MAP5 (d), a protein found only in neurons. The same cells express the two antigens. (e and f) Cells were double labelled with antibody A706-727 (e) and an antibody against GFAP (f), a protein found in glial cells. Different cells express the two antigens. Thus, the larger isoform of α_A-adaptin is specifically expressed in neurons. Bar, 15 μm.

C619-656. Immunoreactivity was seen with both antibodies throughout both tissues, and was particularly concentrated within the grey matter of the spinal cord, the hippocampus, the cerebellum, and the ventral forebrain.

Fig. 9 shows sections of spinal cord labelled with either A706-727 (a and c) or C619-656 (b and d). At low magnification (a and b), the two antibodies produce fairly similar patterns, both primarily labelling the grey matter. However, in the region of the dorsal horn (substantia gelatinosa) where the small diameter sensory neurons of the peripheral nervous system terminate (arrowheads), strong labelling can be seen with antibody C619-654 (b) but not with antibody A706-727 (a). Since clathrin-coated vesicles are known to be highly concentrated in nerve terminals, where they are involved in membrane recycling, the lack of labelling with A706-727 suggests that the larger isoform of α_A-adaptin is not expressed in peripheral neurons. In contrast, the rest of the dorsal horn and the ventral horn, which receive input from other neurons of the central nervous system, are strongly labelled with both antibodies.

Close examination of the immunoreactive pattern at higher magnification suggests that the axon terminals are labelled. This is particularly obvious around motor neurons using A706-727 (Fig. 9c), where multiple discrete patches can be seen associated with the cell surface. C619-654 also labels surface patches on the motor neurons (Fig. 9d), but in addition produces diffuse cytoplasmic labelling.

Fig. 10 shows sections of brain labelled with either A706-727 (a and c) or C619-654 (b and d). In situ hybridization studies have shown that the α_C-adaptin message is particularly strongly expressed in the pyramidal cells (CA3) of the hippocampus. Similarly, labelling with C619-654 (b) reveals that

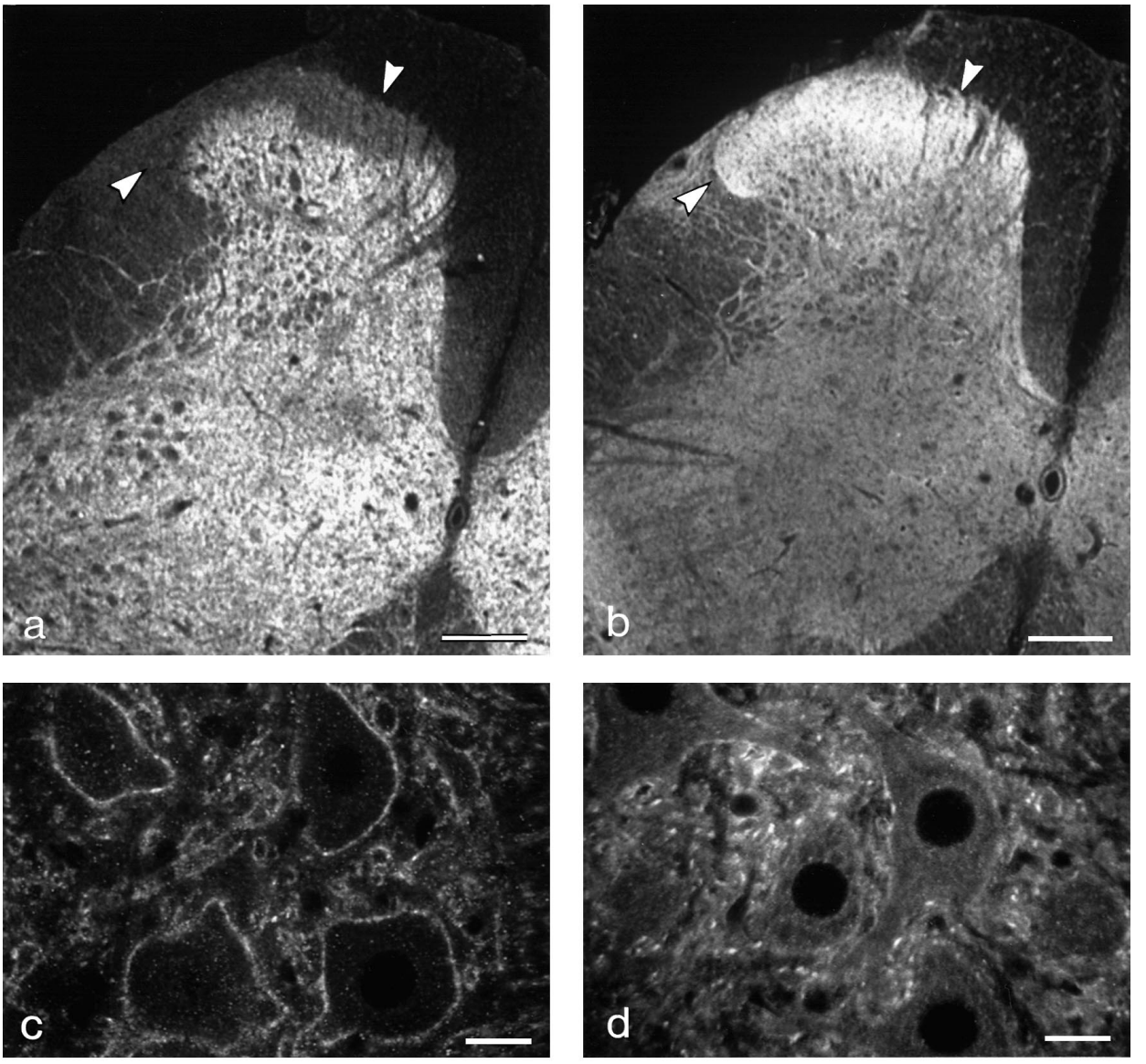

Fig. 9. Confocal micrographs of sections of spinal cord. (a and b) Low magnification views of spinal cord labelled with either A706-727 (a) or C619-656 (b). Both antigens are concentrated in the grey matter, but the highest concentration of labelling with C619-656 is in the superficial dorsal horn (laminae I and II, the substantia gelatinosa), a region that is poorly stained with A706-727 (arrowheads). This indicates that the larger isoform of α_A-adaptin may not be expressed in the small diameter sensory neurons that terminate in this region. Bars, 200 μm. (c and d) Higher magnification views of the large motor neurons in the ventral horn labelled with either A706-727 (c) or C619-656 (d). Both antibodies label surface patches, presumably corresponding to the terminals of other neurons; in addition, the cytoplasm of these cells is relatively heavily labelled with C619-656 but not with A706-727. Bars, 20 μm.

the cell bodies and the dendrites (indicated with arrowheads) of these cells contain high levels of the protein. In contrast, A706-727 (a) labels patches on the pyramidal cell surface but relatively little cytoplasmic staining is observed. Throughout the brain, both antigens are concentrated in regions where nerve terminals are abundant. Fig. 10c and d show comparable regions of the cerebellum. In both cases, the strongest labelling is of the granular (upper) layer, specifically staining the mossy fibre terminals (some indicated with asterisks). In addition, however, C619-656, but not A706-727, labels the cell bodies of the Purkinje cells that border the granular layer (arrowheads), particularly those in the ventral folia.

Thus, taken together these observations indicate that the larger isoform of α_A-adaptin is only expressed in the CNS neurons, and is mainly confined to the nerve terminals, so that cells tend to be outlined by bright patches where other neurons form synapses. In contrast, some cytoplasmic staining can be seen with the α_C-specific antibody C619-656, particularly in the motor neurons, hippocampal pyramidal cells, and Purkinje cells.

DISCUSSION

Previous studies have suggested that α_A-adaptin is expressed only in neural tissues while α_C-adaptin is expressed univer-

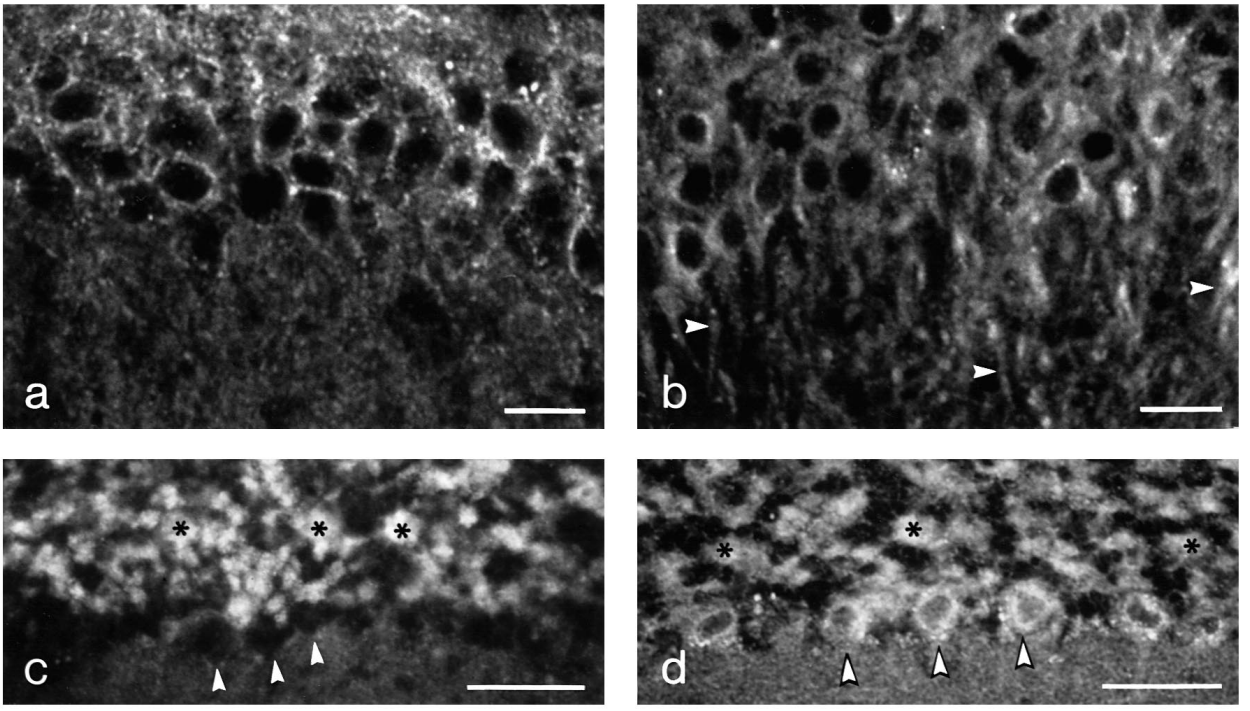

Fig. 10. Confocal micrographs of sections of brain. (a and b) CA3 pyramidal cells of the hippocampus labelled with either A706-727 (a) or C619-656 (b). A706-727 primarily labels surface-associated patches, while C619-656 labelling fills the cytoplasm of the cell bodies and of the dendrites that project downwards (indicated with arrowheads). Bars, 25 μm. (c and d) Cerebellum (folium XIII) labelled with either A706-727 (c) or C619-656 (d). The granular layer is labelled with both antibodies in a patchy pattern corresponding to the terminals of the mossy fibres (asterisks). Both antibodies also give diffuse labelling of the molecular layer. The cell bodies of some of the Purkinje cells between these two layers, indicated with arrowheads, are labelled with C619-656 but not with A706-727. Bars, 50 μm.

sally. Results described in this paper show that this is not the case as α_A-adaptin was successfully amplified from rat liver cDNA. However, the amplified α_A-adaptin differs from the cDNA cloned from mouse brain in that it has 66 bp missing from the coding sequence. The smaller protein to which this spliced message gives rise has escaped prior detection probably because it runs with a similar mobility to that of α_C-adaptin on SDS-polyacrylamide gels and any antibody seen to be labelling this band on western blots was assumed to be recognising α_C. The 22 amino acid sequence removed by splicing is found within the hinge region, at the proximal end of a stretch of 41 amino acids which has no homologue in α_C-adaptin.

Sequencing of genomic DNA from this region revealed that the spliced piece is a separate exon. There is a second exon corresponding to the distal part of the α_A-specific 41 amino acid stretch, allowing for the possibility of other alternatively spliced isoforms of α_A-adaptin. The other introns in the region appear to some extent to divide the gene into blocks with greater or lesser homology with α_C-adaptin. It will be interesting to analyse the genomic structure of the two α-adaptins further and to compare them with the more distantly related γ-adaptin.

Northern blots were used to investigate the expression of the different isoforms of α-adaptin in different tissues. α_A-Adaptin was found to be expressed in all the tissues that were examined, as well as in 3T3 cells. The larger isoform of α_A-adaptin was only detected in two of the tissues tested, brain and skeletal muscle. α_A-Adaptin is not the only coat protein to be alternatively spliced in neural tissue. β-Adaptin cDNA cloned from brain contains an extra 42 bp of sequence, also in the hinge region, which is not present in lymphocytes (Kirchhausen et al., 1989; Ponnambalam et al., 1990), and clathrin light chains are also alternatively spliced in brain (Jackson et al., 1987; Kirchhausen et al., 1987). However, there has been no published report of any of the other coat proteins existing in the larger isoform in skeletal muscle. Although it is possible that the signal in skeletal muscle could come from the motor neurons that innervate the tissue, this seems unlikely, since the signal is relatively strong and the mRNA would be expected to be concentrated in the neuronal cell bodies, which are found in spinal cord, rather than in the nerve terminals.

α_A-Adaptin and α_C-adaptin have proved in the past to be very difficult proteins against which to make antibodies as they do not elicit a strong immune response and most of the antibodies that have been made against the α-adaptins have been unable to distinguish between the different isoforms. Moreover, most of these antibodies only recognise the denatured proteins and are not very useful for immunolocalisation studies. The design of the fusion proteins A706-727, A620-655, and C619-656 has allowed these difficulties to be overcome. The antibodies raised against these fusion proteins can be seen to be entirely specific for the proteins they were designed to recognise (see Fig. 4), and two of them, A706-727 and C619-656, work well for immunofluorescence and even for immunoelectron microscopy (Seaman et al., 1993, and unpublished observations). The use of all three antibodies for immunoprecipitation has shown that the monoclonal antibody AC2-M15, previously thought to bind to both α_A- and α_C-

adaptin, in fact binds only to α_A-adaptin, the shorter form of α_A having previously been mistaken for α_C. This finding helps to explain the species specificity of AC2-M15 (Robinson, 1987), since it seemed unlikely a monoclonal antibody against the products of two distinct genes would recognise both of them in some animals and neither of them in others. Moreover, because AC2-M15 is a mouse antibody specific for α_A-adaptin, it can be used in conjunction with the rabbit antiserum C619-656 to compare the distribution of α_A-adaptin and α_C-adaptin in the same cells by double labelling.

Cos cells double labelled with these two antibodies show virtually identical staining patterns, indicating that, at least within these cells, α_A-adaptin and α_C-adaptin are being used within the same coated pits as each other. Similarly, Cos cells transfected with the larger isoform of α_A incorporate it into the same coated pits as their endogenous, shorter α-adaptins; and in cultured neuronal cells, which naturally express the larger isoform, the protein appears to be uniformly equilibrated amongst all the plasma membrane coated pits and not concentrated in any particular part of the cell.

So do these observations indicate that the three α-adaptin isoforms are functionally equivalent? It is possible that there are more subtle differences between the different isoforms which are not reflected in their subcellular localisation. For instance, α_A and α_C could be preferentially binding to different subsets of membrane proteins in coated pits. However, all of the studies to date indicate that plasma membrane adaptors containing α_A and those containing α_C bind to the same membrane proteins equally well. Thus, when purified adaptors are passed over affinity columns bearing the cytoplasmic domains of the LDL receptor (Pearse, 1988) or the mannose 6-phosphate receptor (Glickman et al., 1989), the adaptors that bind and the adaptors in the starting material look similar when compared by SDS-PAGE. Similarly, studies on interactions between adaptors and the EGF receptor, which involve treating cells with EGF, immunoprecipitating receptors plus associated proteins, and probing western blots with the anti-α-adaptin antibodies AC1-M11 and AC2-M15, indicate that the relative amounts of α_A and α_C that co-immunoprecipitate with the receptor are directly in proportion to the relative amounts of α_A and α_C in the cell as a whole (Sorkin and Carpenter, 1993).

Is the larger isoform of α_A-adaptin performing a specialised role? The finding that α_A-adaptin, β-adaptin, and the clathrin light chains all have brain-specific inserts suggests that the coat proteins in neurons might need to be different from the coat proteins in other cells: for instance, to allow them to be transported along the axon or to facilitate the very rapid endocytosis that must occur in the nerve terminal. It may be significant in this regard that the brain-specific isoform of α_A-adaptin is very highly concentrated in nerve terminals, while in at least some neurons much of the α_C-adaptin has a cytoplasmic distribution in the cell body and dendrites, suggesting that it is less efficiently transported. It is also worth noting that the extra sequences in both α_A-adaptin and β-adaptin are found in the hinge region, where they might help to increase the flexibility of the brain adaptor complexes, and where they would be exposed and accessible to cytoplasmic factors.

One observation that tends to argue against a specialised role for alternatively spliced coat in the axon or nerve terminal is our finding that β′-adaptin is also alternatively spliced: β′ cDNA amplified by PCR from rat liver was found to have 21 bp of the brain sequence missing, again within the hinge region (Ball and Robinson, unpublished observations). As β′-adaptin is a component of the TGN adaptor complex, it would be expected to reside in the cell body. Thus, it is possible that the presence of brain-specific coat proteins may simply be a reflection of differences in splice site selection between brain and other tissues. On the other hand, β-adaptin and β′-adaptin appear to be somewhat promiscuous, with a fraction of the plasma membrane adaptor complexes containing β′ instead of β (Page and Robinson, unpublished data); thus, it might be important for those complexes also to have a brain-specific insert in their β subunits.

The finding that different tissues express different amounts of α_A-adaptin and α_C-adaptin relative to each other might be seen as evidence that the two proteins fulfil different roles. However, there are precedents for tissue-specific isoforms of proteins being functionally equivalent. In particular, there are numerous isoforms of both α- and β-tubulin, encoded by different genes, with much more restricted distributions than the α-adaptins, yet in most cases no functional differences have been detected between the proteins. Instead, it has been proposed that differences in the upstream elements of the various tubulin genes allow them to be switched on in response to different stimuli (Sullivan, 1988). The same may be true for the α-adaptins. For instance, the strikingly strong expression of α_A-adaptin in testis relative to α_C-adaptin might be due to a particular transcription factor in testis which activates the expression of α_A for a testis-specific role. It is not clear what that role might be, but a clathrin heavy chain mutation has been described in *Drosophila* which specifically causes sterility in males (Bazinet et al., 1993). Similarly, in neurons α_A and α_C may be switched on in response to different factors; e.g. although both might be switched on in response to increased synaptic activity, α_C might be preferentially switched on in response to a need to break pre-existing cell-cell interactions. Hu et al. (1993) have shown that clathrin light chain is one of the main proteins to be expressed at increased levels in sensory neurons during long-term facilitation in *Aplysia*. They propose that clathrin light chain (and presumably coat proteins in general) are switched on to enable the cells to internalise cell adhesion molecules, a first step in the process of new synapse formation. It is interesting to note that in mammalian brain, α_C-adaptin is concentrated in the cell bodies and dendrites of many of the neurons, and is particularly strongly expressed in the pyramidal cells of the hippocampus (Robinson, 1989), where it may play a similar role in the generation of long-term memory.

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