Characterization of Chartins, Cytoplasmic Microtubule Associated Proteins

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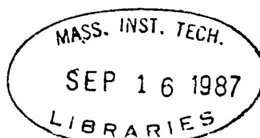

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ABSTRACT

Microtubule associated proteins are minor components of the microtubule organelles of eukaryotic cells. As such, these proteins may be involved in the regulation of microtubule structure, function and assembly. We have studied the structures and functions of several microtubule associated proteins. First, some physical properties of four approximately 200kd associated proteins were compared by antibody crossreactivity, thermostability and two-dimensional tryptic peptide mapping. The peptide maps showed that two of the microtubule associated proteins isolated from different species were not related. Our analysis of microtubule associated proteins then shifted to one complex family of proteins, the chartins. This study of chartins proceeded first at the protein and then the nucleic acid level. Chartins are highly modified proteins and work done by Pallas and Solomon (1982) suggested that the phosphorylated chartins may function in microtubule assembly. These workers found that the differential phosphorylation of chartins determined their biochemical fractionation with respect to the assembled microtubules of cells. We have extended these results with an analysis of chartins in systems where microtubule assembly can be varied. We found that the relationship between phosphorylated chartins and assembled microtubules was not simple. In particular, the mechanism by which microtubules were assembled seemed to affect the phosphorylation pattern of the chartins. We decided that some aspects of chartin structure and function would be more effectively studied at the nucleic acid level. We therefore began such studies. We first isolated chartin specific cDNA from a λgt11 mouse brain expression library. A structural analysis of the fusion proteins derived from this isolated cDNA showed that it was in fact chartin-specific. This cDNA was then used to identify both chartin genomic sequences and messenger RNA transcripts in mouse brain tissue and cultured cell lines.

Thesis Supervisor: Dr. Frank Solomon

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I grew up in Weston, Massachusetts, where I graduated from high school in 1976. I then attended Swarthmore College and received a Bachelor of Arts in Chemistry in 1980. During the summers of 1978 and 1979, I worked in the laboratory of Dr. Jon Beckwith, at the Harvard Medical School. While in the Beckwith laboratory, I attempted to generate spontaneous mutations in the signal sequence of the maltose binding protein of E.coli as part of a genetic analysis of the function of this sequence. During the summer of 1980, I worked in the laboratory of Dr. Nigel Fraser at the Wistar Institute. There, in collaboration with Dr. Margaret Miovic, I searched for Herpes Simplex Virus type II sequences in the DNA of human brain tissue. In the fall of 1980, I began graduate studies in the Biology department at M.I.T. In the summer of 1981, I joined the laboratory of Dr. Frank Solomon and have spent the past six years investigating various aspects of microtubule chemistry and biology.

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Chapter one

Identification and Function of Microtubule Associated Proteins

Minor components of microtubules *in vitro*

Microtubules are one of the major fiber systems of the eukaryotic cytoskeleton. These fibers are hollow tubules of approximately 250 angstroms in diameter whose major component is the dimeric protein tubulin. The α and β subunits of this dimer are structurally related proteins of 50-55kd molecular weight. Not only are α and β closely related, but both subunits have highly conserved primary structures across the spectrum of eukaryotic species. Microtubules are the major components of several cell organelles, such as the mitotic spindle, the marginal band of many vertebrate erythrocytes, cilia, flagella, centrioles and the fibrous microtubular network of interphase cells.

The existence of multiple microtubule organelles, each having different functions, and the highly conserved nature of their basic structural component, the microtubule itself, raises the question of how the cell specifies the formation and function of these organelles. One potential source of such information could be the minor structural components of microtubules; i.e. proteins noncovalently bound to microtubules. Perhaps the first protein which exhibited such an association was dynein (Gibbons, 1965). Dynein could be selectively released and rebound to flagellar axonemes isolated from Tetrahymena. Efforts to identify other minor components of microtubules (microtubule associated proteins) rapidly expanded when assembly of

tubulin into polymers was achieved in vitro (Weisenberg, 1972). Microtubules formed in vitro look (as determined by electron microscopy) like microtubules in vivo (Weisenberg, 1972). On the basis of this result, in vitro polymerization was assumed to proceed somewhat like in vivo polymerization and therefore to involve similar components. Any component of in vitro assembled microtubules was therefore considered a candidate component of microtubules in vivo.

Initial analysis of in vitro assembled microtubules showed the presence of many non-tubulin proteins. Since some of the proteins detected may have adhered nonspecifically to the microtubules, another "purification" step was added to the in vitro assembly of microtubules (Weisenberg, 1972). Cell extracts were cycled through several rounds of temperature dependent microtubule assembly and disassembly. The non-tubulin proteins which persisted in constant stoichiometry to tubulin through the several cycles of assembly and disassembly were defined as microtubule associated proteins. This in vitro purification of microtubules as well as a taxol-dependent (see below) variation of this procedure (Vallee, 1982) have been extensively used to identify associated proteins.

Hereafter, the term microtubule associated protein will be abbreviated as m.a.p. and used to describe minor microtubule components isolated by a variety of methods (see below). Several m.a.p.s have been given the specific name MAP followed by an identifying number (i.e. MAP1). The abbreviation m.a.p. does not refer only to this subset of associated proteins. Rather, m.a.p. is meant to be a generic term.

Although these proteins are reasonable candidates for in vivo m.a.p.s, the in vitro isolation procedures have some limitations. First, a true m.a.p. may either not associate at all with microtubules, or at least not with constant stoichiometry to tubulin under the nonphysiological conditions used in vitro. Also, proteins which do assemble to a constant stoichiometry may not associate with microtubules in vivo; these proteins may fill some nonphysiological requirement for tubulin in vitro. Two experiments support this latter possibility. First, purified tubulin, largely devoid of m.a.p.s assembles in vitro under certain buffer conditions. For example, Mg^{2+} levels of 10-16mM induce microtubule assembly. Microtubules formed under these conditions look normal by electronmicroscopy and depolymerize when exposed to cold, Ca^{2+} or colchicine (Lee and Timasheff, 1975; Herzog and Weber, 1977). Although 10-16mM Mg^{2+} seems to be nonphysiological, the constitution of microenvironments in vivo is difficult to determine. Also, Mg^{2+} could substitute in vitro for several different ions in vivo. Second, purified tubulin can be induced to assemble in buffers which are close to physiological. In these experiments, the concentration of tubulin is high, about 1.0-1.5mg/ml (Lee et al, 1978). This is nearly 5 times the concentration of tubulin required for assembly in the presence of m.a.p.s (Lee et al, 1978). These conditions may or may not mimic the tubulin concentration in an in vivo microenvironment.

The m.a.p.s identified in vitro could interact with microtubules as nonspecific polycations. Some polycations (for example RNase, lysozyme, polylysine, histones and DEAE-dextran) have been able to induce the assembly of purified tubulin in vitro (Erickson, 1976;

Erickson and Voter, 1976; Murphy *et al*, 1977; Lee *et al*, 1978). Although the microtubules formed under these conditions were often aberrant, some studies report the formation of large numbers of normal microtubules (Murphy *et al*, 1977; Erickson, 1976). All of the above experiments raise the possibility that under physiological conditions, microtubules may not require associated proteins to assemble.

The above limitations of *in vitro* analyses can be simply summarized. *In vitro* conditions need not mimic *in vivo* conditions. In fact, one experiment to be presented demonstrates that the *in vivo* behavior of one set of associated proteins is not reproduced *in vitro*. Therefore, the *in vivo* relevence of all m.a.p.s identified by *in vitro* analyses of microtubules remains to be demonstrated. With these qualifications in mind, the following is a summary of some of the m.a.p.s identified to date by *in vitro* methods. I have not attempted a comprehensive review of all reported m.a.p.s since their numbers have grown rapidly in the past few years. Rather, I primarily discuss m.a.p.s identified in vertebrate species and review some experiments which characterize these proteins.

Microtubule associated proteins identified *in vitro*

The first proteins identified as microtubule associated proteins coassembled with calf or porcine brain tubulin *in vitro*. These proteins are the HMW (high molecular weight) (Borisy *et al*, 1975) and tau (Weingarten *et al*, 1975) proteins. The HMWs have been separated into two proteins called MAP1 and MAP2, and more recently into more subgroups (see below). Both the HMWs and tau, when purified and added

to tubulin preparations which are incompetent to assemble, stimulate microtubule assembly (Murphy et al, 1977; Sloboda et al, 1975; Weingarten et al, 1975; Cleveland et al, 1977b). While these experiments suggest properties theoretically expected of microtubule associated proteins, the results must be qualified since the protein preparations that contain them are not pure. The possibility exists that a minor contaminant of the HMW or tau preparations is responsible for the observed stimulation of assembly.

All of these proteins have been partially purified and characterized. MAP1 is a protein of about 350kd molecular weight (Sloboda et al, 1975). Recently, three closely migrating polypeptides have been distinguished and are presently designated MAP1A, 1B, and 1C (Bloom et al, 1984; Hermann et al, 1985). However, MAP1 has been described as proteolysis sensitive (Sloboda et al, 1975; Bloom et al, 1984) and two of the three polypeptides may be specific degradation products. MAP1 has also been purified away from MAP2 and this preparation can stimulate microtubule assembly in vitro (Vallee and Davis, 1983). MAP2 is a heat stable protein of about 270kd molecular weight (Sloboda et al, 1975). This protein has also been resolved into more than one polypeptide called MAP2A and 2B. By peptide mapping, these proteins are identical (Hermann et al, 1985). MAP2 is phosphorylated in vivo (Sloboda et al, 1975); this phosphorlyation has been studied both in vitro and in vivo. Some of this work will be discussed below. Tau is actually a series of proteins. These proteins mostly range in size from 47-80kd, although at least one protein of 125kd crossreacts with an anti-tau serum (Weingarten et al, 1975; Cleveland et al, 1977a; Drubin et al, 1984 and 1986). The

number of tau proteins varies among species. All of the tau proteins are heat stable and, of those analyzed by partial cleavage peptide maps, all are structurally related (Cleveland et al, 1977b and 1979). The number and broad size range of the tau proteins raise the possibility that at least some of these proteins are degradation products.

Other associated proteins initially defined by their ability to co-purify with tubulin through in vitro assembly include 125k and 210kd HeLa proteins (Bulinski and Borisy, 1979) and a protein derived from neuroblastoma, later detected as a triplet of polypeptides of molecular weights 220-240kd (Olmsted and Lyon, 1981; Parysek et al, 1984). These three proteins have been named MAP4 (Parysek et al, 1984).

One microtubule associated protein from rat brain was actually identified by its reactivity with a monoclonal antibody (Huber et al, 1985). A series of such antibodies were raised to a fraction of microtubule proteins assembled and disassembled through three temperature dependent cycles. One of these antibodies stained a protein of 180kd molecular weight. This protein was only found in fractions containing in vitro assembled microtubules and has been designated MAP3.

One final set of microtubule associated proteins has been identified through analysis of in vitro polymerized brain microtubules. These are the STOPs (stable tubule only proteins) (Margolis and Rauch, 1981); they associate specifically with the cold stable fraction of microtubules which are pelleted after a low temperature depolymerization cycle in vitro. Tubulin as well as the

STOPs are released when these microtubules are exposed to Ca^{2+} and calmodulin. The STOPs have molecular weights of 56, 70-82 and 135kd.

The discovery that the drug taxol can stabilize or promote microtubule assembly both in vitro and in vivo (reviewed in Horwitz et al, 1982) led to another in vitro procedure for the isolation of m.a.p.s (Vallee, 1982). This procedure has been used to define associated protein fractions from cells whose tubulin had previously been incompetent to assemble in vitro. Briefly, whole cell homogenates are first centrifuged, then taxol is added to the supernatant and the solution is incubated at 37°C. The microtubules assembled during this incubation are then pelleted. These microtubules are not easily disassembled, but proteins noncovalently bound to the microtubules can be washed off with high salt buffers (Vallee, 1982). The protein fraction eluted in this way is called the microtubule associated protein fraction.

Several proteins previously identified by in vitro coassembly experiments assemble with taxol induced tubulin. These proteins include MAP1, MAP2, tau, the HeLa 210kd protein and MAP4. The HeLa 125kd protein was found in salt eluted fractions only after the original purification scheme was modified (Vallee, 1982). Taxol-induced microtubule assembly therefore provides a method for the rapid partial purification of some previously identified m.a.p.s. However, as a means to identify new candidate in vivo microtubule components, the procedure involves all the assumptions of the previously described in vitro purification and at least one more. The mechanism of action of taxol is not known. Although the drug seems not to interfere with the binding of MAP1, MAP2, tau and others, this

may not be the case for all true microtubule associated proteins. New proteins identified by this method are proteins associated with taxol induced microtubules and not necessarily in vivo components of microtubules.

Actually, association of proteins with taxol stabilized microtubules has not been the sole criteria used to define new microtubule associated proteins. Goldstein and workers (1986) used taxol to polymerize microtubules from Drosophila melanogaster and found a 205kd protein in the salt eluate from these polymers. Both rabbit polyclonal and mouse monoclonal antibodies were made to this protein; these two antibodies were specific for different epitopes on the protein. Both sera also stained microtubules in vivo (by immunofluorescence). This result reduced the possibility that the 205kd protein was not associated with microtubules in vivo but that the antibodies fortuitously recognized epitopes common between the 205kd protein and microtubules. The use of antibodies to determine the in vivo association of m.a.p.s with microtubules is discussed in greater detail below.

A set of proteins from sea urchin eggs was also initially identified by the taxol method of microtubule purification (Vallee and Bloom, 1983). The major proteins identified in the salt eluate from stabilized microtubules had molecular weights of 77, 100, 120 and 205-300kd. Subsequently, a series of monoclonal antibodies raised against the proteins of this salt wash were shown to stain sea urchin mitotic spindles. When some of these sera were used to probe the proteins of the salt wash, four proteins of molecular weights 37,150, 200 and 235kd were identified. These proteins were defined as

m.a.p.s. The fact that the four antisera specific for the four salt eluted proteins also stain mitotic spindles is consistent with the notion that these proteins are m.a.p.s. However, some or all of these antisera may simply recognize common epitopes on the proteins and spindles; the proteins themselves may not be associated with microtubules in vivo.

In vivo identification of m.a.p.s isolated in vitro

The identification of proteins associated with microtubules in vitro was followed by experiments designed first to identify these proteins in vivo and also to determine their distribution among microtubule organelles as well as different cell types. The localization of some m.a.p.s to particular organelles or cell types would suggest possible roles for these proteins in microtubule function. These studies have relied heavily on antibodies. Proteins in both fixed whole cells and tissues as well as whole cell extracts and tissue homogenates have been stained by antisera raised to various m.a.p.s. This work shows that antisera to in vitro isolated m.a.p.s recognize microtubule fibers in vivo. In addition, some antisera show specialized distributions of their associated antigens among organelles or cell types. A brief summary of results from some of these studies is presented below.

The in vivo distributions of MAP1, MAP2, MAP4, tau and the two HeLa proteins have been examined across several cell types and species. Various antisera, both monoclonal and polyclonal in origin have been used in these studies. All of these m.a.p.s seem to be

present in a variety of cell types, both of neural and noneural origin. In nervous tissue, MAP1 may be more prevalent in white matter (Vallee, 1982). MAP2 seems to localize predominantly in cell bodies and dendrites (Matus et al, 1983), but its presence in axons is disputed (Matus et al, 1983; Papasozomenes et al, 1985). Tau protein epitopes localize only to axons, not to cell bodies or dendrites (Binder et al, 1985). The MAP4 antiserum identifies proteins across a spectrum of tissues, but appears to be specific to mouse (Parysek et al, 1984b). Similarly, the 125kd and 210kd proteins may be speicific to primates and marsupials (Bulinski and Borisy, 1980; DeBrabander et al, 1981).

The above experiments are subject to the limitations of all antibody-based studies. Most of these limitations are due to the nature of antibodies themselves. The binding sites are highly specific; that is, each antibody binds to one epitope. However, this epitope may be present in the structure of unrelated proteins. Thus, an antiserum can recognize a protein which is not the original immunogen. The specificity of antibodies is particularly problematic when the antiserum used as a probe is monoclonal. These sera contain antibodies to only one epitope. In contrast, polyclonal antisera may contain antibodies specific to more than one epitope on the protein of interest. This increases the possibility that the serum will recognize only the antigen to which it was raised. With respect to the studies on identification and distribution of m.a.p.s _in vivo_, this specificity of antibodies means that a m.a.p. is not necessarily present in a cell structure simply because this structure is labeled by an antiserum raised to that m.a.p.

The process of antibody induction also limits their usefulness. The production of antibodies can be induced by any immunogenic material, even minor components of the injected antigen. Since protein preparations are rarely pure, the exact identity of the immunogen may be difficult to determine. Furthermore, antisera from one species (i.e. a rabbit) may not contain antibodies to epitopes common to the rabbit and injected antigen. Antibodies to epitopes specific to the source of the antigen (e.g. human) are more likely to be induced. Thus, reported species specific distributions may simply be due to use of a species-specific antiserum.

The roles of microtubule associated proteins in microtubule function

M.a.p.s may not only help determine microtubule formation into particular organelles, but also help regulate their assembly into these structures. Results from some early in vitro experiments are consistent with such a role for m.a.p.s. Many associated proteins identified in vitro stimulate microtubule assembly in vitro. In fact, this property is sometimes used as one criterion to define m.a.p.s (Weingarten et al, 1975; Cleveland et al, 1977b). More recently, the role of m.a.p.s during microtubule assembly in vivo has been investigated. Some of these in vivo experiments are discussed below. In general, all yield results consistent with the notion that m.a.p.s function in microtubule assembly. However, few of these experiments provide strong evidence to support this hypothesis.

To investigate whether or not m.a.p.s function in the assembly of microtubules in vivo, cultured cell lines which undergo morphological

differentiation have been examined. Specifically, rat pheochromocytoma (PC12) cells have been extensively studied. These cells will flatten and eventually extend neurites, a process known to require microtubule assembly (Seeds _et al_, 1970), when incubated in the presence of nerve growth factor (NGF). Microtubule associated proteins have been identified and monitored during this morphological differentiation. Several of these proteins are altered during the course of neurite extension.

One of the first reports of an altered m.a.p. in differentiating PC12 cells was an observed increase in the level of MAP1 of cells exposed to NGF for two days (Greene _et al_, 1983). The time course of this increase in MAP1 correlated with the time course of neurite extension. However, PC12 cells grown in suspension and thus inhibited in neurite extension also had greater levels of MAP1 over those of control cells not treated with NGF. Drubin and coworkers (1986) confirmed and extended this observation. Both tau and MAP1 protein levels increased in NGF-treated PC12 cells in concert with both neurite outgrowth and increasing levels of assembled microtubules; all of these changes were detected after 2-3 days in NGF. These workers also observed an increase in the total tubulin content of NGF-treated cells, but these levels increased with a different time course; i.e. tubulin levels increased steadily from day 0 to day 7 in NGF.

The observed correlation between synthesis of m.a.p.s and microtubule assembly suggests that these proteins function in assembly. However, this conclusion assumes that the action of NGF on these cells is limited to induction of neurite extension. This is not the case; PC12 cells incubated in NGF also cease to divide. A

separation of the biochemical correlates of neurite outgrowth and cessation of cell division is virtually impossible. Cell division can be inhibited by various other drugs (Greene et al, 1982), but other potential effects of these drugs limit the value of cells exposed to them as controls.

Another microtubule associated protein, MAP2, has also been reported in PC12 cells exposed to NGF. This m.a.p. is not present in untreated cells, but MAP2 specific antibodies have detected the protein in cells exposed to NGF for 21 days (Black et al, 1986). The protein was further characterized as MAP2-like by virtue of its thermostability. The cause for MAP2 induction in these cells is unclear. Not only have these cells ceased to divide, but greater than 50% of PC12 cell populations incubated in NGF for 21 days are dead (D. Pallas, unpublished observations). Too many changes have occurred in these cells to justify the claim that the appearance of MAP2 is linked to the cells' high levels of assembled microtubules.

The role of MAP2 in microtubule assembly has been more extensively studied in vitro than in vivo. In particular, MAP2 is phosphorylated in vivo (Sloboda et al, 1975), and investigations have focused on the role of this modification in MAP2 function. To summarize, cAMP-dependent protein kinase type II has been reported to co-purify with MAP2 through both cycles of microtubule assembly as well as phosphocellulose purification of MAP2 (Vallee, 1980). It is thought that this protein kinase associates exclusively with the projection domain of MAP2 (a 200kd chymotryptic fragment of MAP2) (Vallee et al, 1981). In addition, MAP2 can be phosphorylated in vitro by both cAMP-dependent and independent mechanisms. In both

cases, *in vitro* phosphorylated MAP2 decreases the rate of microtubule assembly and the affinity of MAP2 for assembled microtubules (Burns *et al*, 1984; Murthy *et al*, 1983).

The significance of these studies to the *in vivo* role of MAP2 phosphorylation is unclear. In addition to the question of how the nonphysiological *in vitro* conditions alter assembly, the phosphorylation of MAP2 *in vitro* may not reflect its *in vivo* state of modification. In fact, Murthy and workers (1983 and 1985) have shown that the sites of endogenous phosphorylation on MAP2 do not turn over *in vitro*. In contrast, sites on MAP2 phosphorylated *in vitro* by a cAMP-dependent mechanism do turn over and are subject to phosphatase removal (Murthy *et al*, 1983 and 1985). These results suggest that the *in vitro* phosphorylation of MAP2 may not mimic its *in vivo* phosphorylation and such nonphysiological modification of MAP2 could affect its functional properties.

Several experiments (some discussed above) provide data which correlate the presence of or change in a m.a.p. with microtubule function or assembly, but these correlations are often complicated by other cell events. These experiments have not demonstrated a direct effect of a m.a.p. on microtubule function or assembly *in vivo*. Recently, Drubin and Kirschner (1987) demonstrated such an effect. These workers microinjected RAT1 fibroblast cells with a purified bovine brain tau protein fraction. These cells do not normally contain detectable levels of tau (Drubin and Kirschner, 1987). The microtubule mass of both uninjected and injected cells was quantitated. Cells microinjected with tau contain a greater microtubule mass than the control cells. In addition, both uninjected

and injected cells were treated the microtubule depolymerizing drug nocodazole. Microtubule mass in cells injected with tau protein declined much more slowly than this mass in control cells. These experiments strongly suggest that at least some of the proteins in the purified tau fraction promote microtubule assembly and stabilize microtubules *in vivo*.

Isolation of microtubule associated protein-specific nucleic acid sequences

A new approach to the study of m.a.p.s and their function has begun. This approach is the isolation of nucleic acid sequences specific for these protein. cDNA sequences corresponding to tau proteins may have been isolated (Drubin *et al*, 1984). In addition, sequences for MAP1, MAP2 (Lewis *et al*, 1986a and b) and a 205kd m.a.p. in Drosophila melanogaster (Goldstein *et al*, 1986) have been isolated. Candidate tau cDNA was isolated from a cDNA library constructed with tau specific mRNA. This mRNA was isolated from polysomes which had been procipitated using a polyclonal anti-tau serum. The isolated cDNA was judged to be tau-specific by the following criterion. Mouse brain mRNA which selectively hybridized to the cDNA was translated into proteins. These proteins were recognized by the anti-tau serum. This criterion is not sufficient to show the tau specificity of the cDNA because the experiment is circular. The antiserum should recognize epitopes on proteins made from this selected mRNA; the cDNA used to selct the mRNA is complementary to mRNA originally isolated by precipitation with this antiserum. The *in vitro* translated proteins

carry the epitopes of interest, but may not be tau. Thus, these clones may not be tau-specific.

The MAP1 and MAP2 clones were isolated from a λgt11 mouse brain library using polyclonal anti-MAP1 and anti-MAP2 sera. They were shown to be MAP1 and MAP2 specific in the following way. Two nonoverlapping sister clones for both MAP1 and MAP2 were isolated and inserted into plasmids so as to create bacterial-mouse fusion proteins. Both fusion proteins were stained by the relevent anti-MAP serum. Therefore, two MAP-specific epitopes were present in protein fragments translated from one mRNA. The results indicate that the clones are MAP1 and MAP2 specific.

The MAP1 and MAP2 clones have been used in some initial Northern and Southern blot experiments. Analysis of mouse genomic DNA shows that there is one gene for each of the two proteins. Furthermore, these genes are expressed predominantly in brain tissue, although low levels of MAP1 transcripts were detected in several cell types. MAP2 mRNA seems entirely brain specific (Lewis et al, 1986a and b).

Finally, cDNAs specific for the 205kd D. melanogaster m.a.p. have been cloned (Goldstein et al, 1986). cDNA inserts were isolated by screening a λgt11 Drosophila expression library with a polyclonal anti-205kd serum. One of three isolated fusion proteins was also stained by a monoclonal anti-205kd serum, suggesting that the two sera are specific for different epitopes. The DNA corresponding to the above fusion protein is likely to be 205kd m.a.p.-specific.

The isolation of genes encoding m.a.p.s will enhance the characterization of these proteins along several fronts. Knowledge of gene transcription across cell types as well as through development

may suggest specific functions for these proteins. In addition, structural information at the level of both nucleic and amino acids may identify regions of homology (e.g. potential microtubule binding sites) and divergence (e.g. sites important for specialized function) across the range of associated proteins.

All the microtubule associated proteins thus far discussed were originally identified by the analysis of microtubules *in vitro*. The major limitation to these techniques is that the relationship of these proteins to microtubules *in vivo* is not known and must be subsequently proved. Such proof has relied heavily on specific antibody decoration of microtubules *in vivo*. Interpretation of these experiments is limited by the specificity of the antiserum for the protein of interest.

Direct Identification of microtubule associated proteins *in situ*

In order to avoid the limitations of the *in vitro* identification techniques, a method for the analysis of microtubules formed *in vivo* was developed in our laboratory (Solomon *et al*, 1979). This analysis involves the detergent extraction of cells to yield intact cytoskeletons. Cells are lysed with a nonionic detergent in a buffer containing glycerol (Solomon *et al*, 1979) or polyethylene glycol (Osborn and Weber, 1977) and a calcium chelator such as EGTA. The original cellular microtubules are stabilized under these conditions and have been visualized by immunofluorescence (Osborn and Weber, 1977, Solomon *et al*, 1979; Spiegelman *et al*, 1979a and b), electron microscopy (Solomon *et al*, 1979) and immunoelectronmicroscopy (Webster

et al, 1978). By these techniques, extracted cell microtubules are comparable in number and distribution to those of unextracted cells.

The extraction conditions used to isolate cellular microtubules are similar to those used for *in vitro* microtubule polymerization. The visualized microtubules may have therefore polymerized *in vitro*. This possibility is ruled out by results from several experiments. First, GTP is necessary for microtubule assembly *in vitro*. However, cytoskeletal microtubules can be isolated in the absence of GTP (Solomon *et al*, 1979). Second, the presence of microtubules in extracted cytoskeletons depends on the integrity of microtubules in cells prior to lysis. Cells pretreated with colcemid (Osborn and Weber, 1977), colchicine (Solomon *et al*, 1979) or nocodazole (Duerr *et al*, 1981) yields cytoskeletons devoid of microtubules upon extraction. Finally, when unlabeled cells are extracted with an extract from radiolabeled cells, the labeled tubulin is not incorporated detectably into the unlabeled cytoskeletons (Solomon *et al*, 1979).

This isolation of intact cellular microtubules has been used to identify several microtubule associated proteins in cultured cell lines. The strategy used in our laboratory to define such proteins is as follows. Populations of cells, one pretreated with a microtubule depolymerizing drug, are first lysed into a microtubule stabilizing buffer. Seventy-five per cent of all cell proteins are released in this step, including all the unassembled components of microtubules (such as tubulin). Proteins released from the drug-treated cells include all microtubule components since these cells are devoid of assembled microtubules. The remaining cytoskeletons are then extracted with a buffer containing calcium to depolymerize all

remaining microtubules. Proteins released by this second extraction include the assembled microtubule components of intact cells as well as many background polypeptides. This protein fraction from drug-treated cells contains only the background polypeptides. Microtubule components are defined as proteins present in the cytoskeletal extract of untreated cells but absent from the same extract of drug-treated cells. Microtubule associated proteins, nontubulin microtubule components, have been identified by comparison of the proteins from these two extracts.

Initially, the large number of background polypeptides obscured differences other than tubulin between the cytoskeletal extracts in analysis by SDS-PAGE. Therefore, a second purification step was necessary (Solomon *et al*, 1979). Proteins from these extracts which will assemble with heterologous tubulin (unlabeled calf brain tubulin) were selected by repeated cycles of temperature dependent assembly and disassembly. This step significantly reduced the number of background proteins and the minor microtubule components were then detected. Analysis of the cytoskeletal extracts by two-dimensional separation techniques allowed the detection of associated proteins without this further purification of the cytoskeletal extracts (Duerr *et al*, 1981; Pallas and Solomon, 1982).

Our laboratory has identified several microtubule associated proteins from a number of cultured cell lines using this two-step detergent extraction (Solomon *et al*, 1979; Duerr *et al*, 1981; Pallas and Solomon, 1982; Zieve and Solomon, 1982). All rodent cell lines examined as well as chick embryo fibroblast cells contain a microtubule associated protein of 69kd molecular weight. The NIL8

69kd protein is related to a brain protein of similar molecular weight as determined by one-dimensional partial peptide maps (Duerr et al., 1981). All the rodent cells also contain a 220kd associated protein. This protein, by virtue of its mass and rodent origin may be related to MAP4. This possibility is discussed further in chapter two. Two more microtubule associated proteins were found in rodent cells, but only in those of neural origin. These proteins have molecular weights of 55 and 80kd. The 69 and 220kd proteins are not present in the extracts from primate cells, but two associated proteins of 125 and 210kd were detected. Two proteins of analogous sizes were also identified by in vitro analysis of HeLa microtubule protein (Bulinski and Borisy, 1979; Weatherbee et al, 1980). Finally, 150kd and 235kd proteins were found in hamster cells. These proteins associate specifically with microtubules of the mitotic spindle (Zieve and Solomon, 1982).

The 69 and 80kd proteins of rodent cells have been extensively characterized (Pallas and Solomon, 1982). A modification in the two-dimensional gel analysis of the proteins in cytoskeletal extracts showed that several microtubule associated proteins having molecular weights of 69 and 80kd were present in these cells. Another set of 72kd associated proteins was also detected (These results are shown schematically in Figure 2). Analogous extraction and analysis of rat sympathetic ganglia showed that these multiple proteins are also microtubule components of primary neurons (Black and Kurdyla, 1983). Two-dimensional tryptic peptide map analysis of many of these associated proteins showed that they are structurally related. All the proteins of one molecular weight are identical. In addition, all

three molecular weight classes of proteins are related. The maps of the 80kd proteins contain all the peptides of the 69kd protein along with three additional spots. The 72kd proteins share about half their peptides with the 69 and 80kd proteins. All of these proteins, then, make up a family of related microtubule associated proteins. This family has been named chartins (Magendantz and Solomon, 1985). Recently, two antisera specific to chartins have been raised (Magendantz and Solomon, 1985). One polyclonal antiserum, 4-81, stains all chartins by immunoblot as well as microtubule networks in cells by immunofluorescence. A polyclonal antiserum, 3417, specifically stains only the 80kd chartins.

Further analysis of the chartins has shown that the isoelectric variation among the proteins is at least in part due to phosphorylation. Measurement of the ^{32}P-phosphate to ^{35}S-methionine ratios incorporated into several chartin variants demonstrated that the more acidic proteins are also the more highly phosphorylated. At least one other modification of the chartins must occur, because there is at least one isoelectric variant in the 69 and 72kd classes that is not phosphorylated. Such extensive post-translational modification of the chartins raises the question of function and the possibility that the variants may have separate or only partially overlapping functions (Pallas and Solomon, 1982).

Differences in function among the chartins are more strongly suggested by differences in the variants' fractionation properties (Pallas and Solomon, 1982). The two or three most highly phosphorylated chartin variants preferentially associate with the assembled microtubules of a cell. That is, these variants are only in

cell fractions which include assembled microtubule components. The less phosphorylated variants distribute between the unassembled and asembled pools of microtubule components in the same ratio as tubulin, approximately 4:1.

Other laboratories have started to investigate the role of chartins in microtubule function. In particular, modifications of chartins have been observed during NGF-induced extension of rat pheochromocytoma (PC12) cells. Experiments have been done which demonstrate a direct correlation between the assembly of microtubules and increased chartin phosphorylation. PC12 cells grown in the presence of NGF for 21 days have increased levels of both assembled tubulin and the highly phosphorylated chartins (Black et al, 1986). This effect of long-term NGF treatment on chartins may depend on the presence of assembled microtubules. Aletta and Greene (1987) report a loss of acidic chartins and an increase in the levels of the more basic variants when NGF-treated cells are exposed to nocodazole. Paradoxically, the same effects on chartin modification occur when NGF-treated cells are exposed to taxol for 2.5-24 hours. Chartins of cultured rat sympathetic neurons also respond to taxol treatment in the same way (Black and Peng, 1986). The loss of acidic variants may be less dramatic in taxol treated cells than in nocodazole treated cells, although this observation was not confirmed by a quantitative analysis (Aletta and Greene, 1987).

Interpretation of the above experimental results is problematic for two reasons. First, as discussed earlier, NGF-treatment results in the cessation of cell division as well as neurite extension, and the biochemical correlates of these two cellular responses cannot be

distinguished after long term NGF exposure. Second (also previously discussed), the mechanism of action of taxol on microtubules is unclear. In particular, its binding site on microtubules is unknown and may interfere with chartin binding.

Attempts have been made to extend the correlation between NGF-induced neurite extension and chartin phosphorylation. Neurite extension in NGF-treated PC12 cells can be blocked by a variety of agents such as Li^+ ion, forskolin, and cholera toxin (Burstein et al, 1985; Greene et al, 1986). PC12 cells pretreated with any one of these agents do not extend neurites when exposed to NGF. Phosphorylation of the cells' chartins is also blocked. Results from these experiments are difficult to interpret because the mechanism of action of these agents is unclear. One important effect of these agents on cells is to increase their intracellular levels of cyclic AMP. However, dibutyryl cAMP, which also increases intracellular levels of cyclic AMP in cells, is reported to enhance the rate of NGF-induced neurite extension (Gunning et al, 1981; Heidemann et al, 1985). Therefore, the observed prevention of neurite extension and chartin phosphorylation may be due to some other effects(s) of these agents on the cells.

This thesis describes experiments designed to further characterize several microtubule associated protein, although the majority of the work primarily concerns analysis of the chartins. Chapter two first describes a structural comparison of two approximately 200kd m.a.p.s, one from human cells and one from rodent cells. The second section of this chapter discusses experiments designed to yield more information about chartin phosphorylation and

its role in microtubule assembly. These experiments follow directly from the observation of Pallas and Solomon (1982) that highly phosphorylated chartins associate exclusively with assembled microtubules. Finally, chapter three describes further characterization of the chartins by the isolation of chartin-specific cDNA.

Chapter two

Biochemistry of microtubule associated proteins

Introduction

Several microtubule associate proteins have been identified from a large variety of tissues and cultured cells. These proteins have been defined as associated proteins according to different criteria, but the property they have in common is that they all do associate physically with assembled microtubules under some experimental conditions. This property among the proteins suggests that they are related to one another in some way. Experiments presented in this chapter investigate the structural and functional relationships among several microtubule associated proteins.

This work is divided into two sections.

First, four associated proteins identified by three different laboratories are further characterized. All of the proteins have similar molecular weights and therefore may be homologous. The proteins are a HeLa 210kd associated protein identified by Bulinski and Borisy (1979), a thermostable mouse 220kd protein presently named MAP4 (Parysek _et al_, 1984; Olmsted and Lyon, 1981), and two analogous proteins identified from HeLa and rodent cell lines by Duerr _et al_, (1981). These proteins were not assumed to be related for two reasons. First, the 210kd and 220kd proteins not only had slightly different molecular weights, but also were distributed differently among different species. Second, the proteins were defined by

different experimental criteria. The 210kd and 220kd proteins identified by Duerr *et al* fractionated in the same way as tubulin when cultured cells were detergent extracted (see chapter 1). The 210kd HeLa protein found by Bulinski and Borisy and MAP4 copurified with tubulin through temperature dependent cycles of *in vitro* microtubule assembly and disassembly (Bulinski and Borisy, 1979; Olmsted and Lyon, 1981). For these reasons, further characterization of these proteins was necessary to determine the relationship(s) among them.

The experiments presented below investigated which, if any, of these four proteins were homologous. The crossreactivities between the 210kd proteins and between the 210kd and the rodent proteins were tested by immunoprecipitation using a rabbit polyclonal antibody raised in the Borisy laboratory to the 210kd HeLa protein. Also, the heat stability of the detergent extracted 220kd protein was determined. Finally, the two associated proteins isolated by detergent extraction of cells were analyzed by two-dimensional limit tryptic digest peptide maps.

In the second section of this chapter, some possible functions of chartins are studied. In particular, the significance of the chartins' phosphorylation to their role in microtubule assembly is further explored. Such a role was originally suggested by Pallas and Solomon (1982), who showed that differentially phosphorylated chartins fractionate differently with respect to the assembled microtubules of cells. That is, the highly phosphorylated chartins fractionate exclusively with the assembled microtubule components.

To understand better the relationship between phosphorylation and chartin function, this family of proteins was analyzed both *in vitro*

and *in vivo*, in systems where the levels of microtubule assembly could be varied. First, the chartins from calf brain tissue were examined. Proteins from this tissue were studied because the level of assembled tubulin in the tissue is high, about 70% of the total tubulin pool. In contrast, only 20-25% of the tubulin of cultured cells is assembled into microtubules. Second, chartin variants were monitored throughout the induction of microtubule assembly, both *in vitro* and *in vivo*. *In vitro*, microtubule assembly and disassembly was induced by changes in temperature. *In vivo*, microtubule assembly occurred when cultured neural cells were induced to extend neurites. Analysis of the chartins in each case involved, where possible, determination of the array of chartin variants present (i.e. their number and degree of modification), as well as each variant's biochemical fractionation with respect to the unassembled and assembled microtubule pools.

Materials and Methods

Cell culture, metabolic labeling and induction of neuroblastoma cells

Mouse neuroblastoma N115 cells (Seeds et al, 1970) were obtained from M. Nirenberg. Mouse neuroblastoma NB2A and N115 cells were both maintained in Dulbecco's modified Eagles' medium (DME) supplemented with 10% fetal calf serum. Rat pheochromocytoma (PC12) cells were obtained from L. Greene and grown in DME with 10% fetal calf serum on collagen-coated dishes (Greene and Finkler, 1976). HeLa cells, obtained from P. Sharp, were grown in alpha medium with 5% fetal calf serum. To incorporate label, cells were incubated either with ^{32}P-phosphate at 1mCi/ml (New England Nuclear) for 2-4 hours in phosphate free DME and 10% dialyzed fetal calf serum, or with ^{35}S-methionine (50μCi/ml, NEN) in DME medium containing 1/10 the normal methionine concentration and 10% fetal calf serum for 18-24 hours. Cells to be labeled in suspension were first incubated overnight at a density of $2\text{-}4 \times 10^6$ cells/ml and then labeled as above at a density of $1\text{-}3 \times 10^6$ cells/ml. Serum starved cells were labeled in media containing 0.1% dialyzed fetal calf serum (NB2A cells) or 0% serum (N115 cells).

Cells were induced to extend neurites by one of three changes in culture conditions. Either 1mM $N^6,O^{2'}$-dibutyryladenosine 3':5'-cyclic monophosphate (dibutyryl cAMP) or 10μM isoproterenol plus 5mM 3-isobutyl-1-methyl-xanthine (all from Sigma) was added to the culture or labeling medium. Incubations of cells with these inducing agents ranged from 30 minutes to 24 hours. Radioisotope-labeling and

induction of cells was simultaneous in all experiments. Cells also extended neurites when shifted into medium containing 0.1% fetal calf serum (NB2A cells) or 0% fetal calf serum (N115 cells). Incubation of NB2A cells in low serum ranged from 30 minutes to 72 hours. N115 cells were maintained without serum for up to 7 days. Cells were radiolabeled at various times during maintenance in low serum media.

The time course for neurite extension after induction was examined in parallel populations of cells plated from an overnight suspension culture into tissue culture dishes. At 30 minute time points after plating, random fields of the cultures were examined; each cell which had extended a process equal to the diameter of its soma was scored as positive.

Preparation of detergent-extracted cytoskeletons

Extraction of cells was performed at room temperature. Cells maintained and labeled while attached to tissue culture dishes were extracted from 75-100% confluent dishes. Prior to extraction, populations of cells were preincubated with either 1µg/ml of Nocodazole diluted from a 100X stock solution in dimethylsulfoxide (DMSO) or with an equivalent concentration of DMSO alone for 60-120 minutes. Cells were first washed twice with PBS_A (phosphate buffered saline, pH 7.2), and once with PM2G (0.1M piperazine-N,N'-bis(ethane-sulfonic acid)(PIPES), 1mM $MgSO_4$, 2mM EGTA and 2M glycerol, pH 6.9), containing 1mM phenylmethylsulfonyl fluoride and 0.20 TIU/ml aprotinin (both from Sigma). Several culture dishes (usually 5-10) of cells were then serially extracted. PM2G plus 0.1%

NP-40 (British Drug House) was added to lyse the cells. After 5 minutes, the buffer was removed and held on ice. This supernatant contains 90% of the cell lipid and 75% of cell proteins (Solomon _et al_, 1979), including those from the unassembled pool of microtubule components. The remaining cellular material was washed once with PM2G plus 0.1% NP-40 and then incubated in a small volume (see below) of 0.1M PIPES, 1mM $MgSO_4$ and 1mM $CaCl_2$ (PM plus Ca^{2+}) plus protease inhibitors. The buffer volume was just sufficient to cover the surface area of the culture dish (e.g. about 1ml per 10cm dish). After 10 minutes, this buffer was removed and added to a second dish of cells already lysed with PM2G plus 0.1% NP-40. This supernatant was finally removed from the last dish and held on ice. Cell proteins extracted by this buffer include cytoskeletal proteins solubilized in the absence of glycerol and presence of Ca^{2+}; i.e. the fraction contains the assembled microtubule components. This protein fraction extracted from control cell populations (those not treated with drug) will referred to as the mt+ fraction, while the same fraction from drug-treated cells will be the mt- fraction. SDS to 1.5% and β-mercaptoethanol to 5% were added to all fractions, the samples were boiled for 5 minutes and then analyzed on gels as described below.

The detergent extraction procedure was modified for cells grown in suspension. Populations of cells, preincubated with Nocodazole or DMSO alone, were collected by centrifugation for 3 minutes at 2000 rpm, washed once with PBS_A (containing Nocodazole or DMSO alone as appropriate), and suspended in PM2G at a concentration of 10^6 cells/ml. NP-40 was added to a final concentration of 0.1% and the mixture was gently agitated. After extraction at 25°C for 10 minutes,

the cytoskeletons were pelleted and the supernatant removed carefully and held on ice. The cytoskeletons were resuspended in three or four times the volume of PM2G and allowed to extract for 5 minutes. Again, the cytoskeletons were pelleted and the supernatants removed. Pellets were then suspended in PM plus Ca^{2+} at a concentration of 10^7 cell equivalents/ml, and incubated at 25°C for 10 minutes. Insoluble material was pelleted in an Eppendorf centrifuge for 5 minutes. Supernatants were removed and held on ice. Again, SDS and β-mercaptoethanol were added to all fractions, the samples were boiled for 5 minutes and then analyzed on gels.

Immunoprecipitation

Samples for immunoprecipations were originally in a buffer of 0.1M Pipes, pH 6.9, 1mM $MgSO_4$, 1mM $CaCl_2$. This buffer was first adjusted to 70mM NaCl, 2mM EDTA, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1mM PMSF and 0.2% aprotinin, then normal rabbit serum was added to a final concentration of 10% and the solution incubated overnight at 4°C. Before use, the normal rabbit serum (provided by P. Sharp) was centrifuged at 10k rpm for 15 minutes at 4°C and filtered through a 0.2μ Nalgene filter. To precipitate antibody-antigen complexes, a suspension of Staphylococcus Aureus bacteria (Staph. A.) were added to the solution in a ratio of 10 volumes Staph. A. suspension to 1 volume rabbit serum, and the solution rocked at 4°C for 30 minutes. The Staph. A. cells were then pelleted by centrifugation in an Eppendorf centrifuge. The resulting supernatant was the "precleared" sample. The anti-210kd serum was added to this

sample at a dilution of 1 to 22 and the sample incubated at 4°C. After 2 hours, a Staph. A. cell suspension was added as described and the mixture rocked at 4°C for 30 minutes. The Staph. A. cells were pelleted and the supernatant removed. The pellet was kept at 0-4°C through five washes, the first two in 10mM Tris-HCl pH 7.2, 500mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 2mM EDTA, 0.2% aprotinin, 1mM PMSF (high salt RIPA), and the last three washes in RIPA buffer (the same as high salt RIPA, except that the NaCl concentration was 15mM). The pellet was then suspended in PBS_A, 10% v/v glycerol, 0.1M dithiothreitol, 0.5% SDS, boiled for 5 minutes and the Staph. A. cells were pelleted. The supernatant was removed and the Staph. A. cells were again suspended in the same buffer, boiled and pelleted. The two supernatants were combined; this sample was then analyzed by one- or two-dimensional SDS-PAGE to identify the immunoprecipitated proteins.

The bacteria, Staphylococcus Aureus, were prepared for use by suspension in 0.15M NaCl, 1mM Tris-HCl, pH 7.2, 2mM EDTA, 0.5% NP-40 containing protease inhibitors as described (STE with 0.5% NP-40). The cells were then incubated at 25°C for 15 minutes, pelleted by centrifugation and washed twice in STE with 0.05% NP-40. Cells were finally suspended for use in a solution of STE, 0.05% NP-40 and 1mg/ml BSA (fraction V, Sigma).

Preparation of whole cell extracts at -30°C

Cells were pelleted and washed twice in DME and 0.1% fetal calf serum. Pellets were then suspended in 100µl of lysis buffer (1%

deoxycholate, 1% NP-40, 0.1% SDS, 45% v/v ethylene glycol, 5mM EDTA, 50mM PPi, 50mM NaF, 50mM Tris-HCl pH 8.9 at -30°C (Foulkes and Rosner, 1985), and transferred to a Dounce homogenizer containing 1ml lysis buffer cooled to -30°C in an ethanol/dry ice bath. The cell suspension was then subjected to seven strokes in the Dounce homogenizer and the lysed cell solution put on ice to warm for 15 minutes. The solution was then centrifuged in an Eppendorf centrifuge to pellet the nuclei, the supernatant removed, SDS added to 1.5%, the sample boiled for 5 minutes and analyzed by two-dimensional gel electrophoresis (as described below).

Calf brain microtubule protein

Calf brain microtubule protein was prepared by two cycles of temperature dependent assembly and disassembly in reassembly buffer without glycerol (Borisy _et al_, 1975) for use _in vitro_. Fractions from each cycle were analyzed by two-dimensional gel electrophoresis (as described below).

Quantitation of assembled tubulin

Neuroblastoma cells were extracted with detergent in the presence of 5μg/ml of the microtubule stabilizing drug taxol, as described (Zieve and Solomon, 1982). Taxol was added to the extraction buffer in order to minimize any loss of tubulin from the assembled microtubules which might occur _in vitro_. Cells were extracted at 25°C for 10 minutes in PM2G containing 5μg/ml taxol and 0.1% NP-40. Cells

were collected off the dishes, spun at 2000 rpm for 3 minutes, resuspended in PM2G containing 100µg/ml DNase, incubated at 25°C for 5 minutes and finally centrifuged. Pellets were then suspended in 0.1M PIPES, 1mM $MgSO_4$ and 2mM EGTA containing protease inhibitors. SDS to 1.5% and β-mercaptoethanol to 5% were then added, the samples were boiled, analyzed by one dimensional SDS-PAGE and the gels were silver-stained by the method of Morrissey (1981). The tubulin in each extract was quantitated by densitometry and normalized for cell number using vimentin as a standard, since none of that protein is extracted from cells under the conditions used.

Quantitation of labeled microtubule associated proteins

NB2A cells were incubated in suspension as described above with ^{32}P-phosphate at 1mCi/10^6 cells (NEN). Half of the cells were simultaneously treated with 1mM dibutyryl cAMP. After 4 hours, cells were extracted by the detergent extraction procedure described and 50µl of the soluble and cytoskeletal protein fractions from each cell population were analyzed on two-dimensional gels. Using the corresponding autoradiographs as guides, each 69 and 80kd variant from all fractions were cut from the gels. Regions from the gels near each variant and having similar size and shape but not containing protein were also cut out to measure background radioactivity. All gel pieces were immersed in scintillation fluid, and the ^{32}P-phosphate signal in each variant was corrected for background.

Two-dimensional gel electrophoresis

Cell extracts were analyzed on two-dimensional gels, first by isoelectric focusing and then by apparent molecular weight on SDS-PAGE (7.5% acrylamide) (O'Farrell, 1975). Separation by isoelectric point over a gradient range of pH 4.8 to 7.4 was obtained by using 400µl of pH 3.5-10.0 ampholines (LKB) with 150µl of pH 6-8 ampholines (LKB) per 10ml of gel solution. Gels of ^{35}S-methionine-labeled proteins were pretreated with EN^3HANCE (NEN) before exposure. Gels of ^{32}P-phosphate-labeled proteins were exposed using Dupont Lightning-Plus Cronex screens. All exposures were on XAR-5 film (Kodak) at -70°C. Gels of unlabeled proteins were silver-stained (Morrissey, 1981).

Peptide mapping

Proteins to be peptide mapped in two dimensions were excised from dried polyacrylamide gels and labeled with ^{125}I. Gel pieces containing the proteins were rehydrated in 0.5M Na_2HPO_4/NaH_2PO_4, pH 7.5. These pieces were then placed in Eppendorf tubes and 0.5mCi $Na^{125}I$ (Amersham) was added directly onto the gel piece. 5µg chloramine T in enough 0.5M Na phosphate buffer to cover each gel piece was then added and the reaction mixtures were incubated at 25°C. After one hour, the reactions were quenched by the addition of 1ml of 1mg/ml $NaHSO_3$ and incubation for 15 minutes. The labeled proteins, still in the gel pieces were washed extensively with 0.17M NaI, 0.003M KI, 0.01M Na_2HPO_4, 0.0011M KH_2PO_4 (phosphate buffered iodine). The

labeled proteins were further purified away from the unincorporated label by electrophoresis through another acrylamide gel. Proteins were excised from these dried gels with the aid of autoradiograms and peptide mapped.

The labeled proteins were then mapped by modifications of published procedures (Gibson, 1974; Elder et al, 1977). Proteins in gel slices were digested with 0.5ml of fresh 50µg/ml TPCK-trypsin (250 TIU/µg, Worthington) in 50mM ammonium bicarbonate (MCB reagent grade) for 18-24 hours at 37°C. The supernatants were removed and the ^{125}I incorporated into the peptides measured. Peptide maps of $1X10^4$ cpm of sample were visualized by autoradiographs after one week of exposure. The samples were lyophilized, suspended in distilled deionized water and lyophilized again. This step was repeated four times. The final pellet was suspended in distilled deionized water at a concentration of $1X10^4$ cpm/5µl. 5µl samples were then spotted onto 10cmX10cm cellulose-coated TLC plates (EM Laboratories). To keep the sample spot small, application of the sample was performed by repeated application of about 0.3µl of sample. Electrophoresis of samples occurred for 20 minutes across a drop of 1kV in a thin-layer electrophoresis double chamber Thin-layer Isoelectric Focusing Apparatus (DESAGA; West Germany). The electrophoresis buffer was formic acid: acetic acid: water, 1:3:16, pH 1.9. The TLC plates were air dried and the second dimension chromatography performed at 25°C. The chromatography buffer was n-butanol: pyridine: acetic acid: water in a ratio of 6.5:5:1:4. Migration of the dye front to the top of the plate required about 2 hours. The plates were air dried and exposed to X-ray film at -70°C, with an intensifying screen.

For partial cleavage peptide mapping, proteins were prepared as follows. For ^{125}I protein labeling, calf brain microtubule protein was first carried through two cycles of temperature dependent assembly and disassembly. Then the brain proteins of the final warm pellet as well as the mouse neuroblastoma proteins of the assembled microtubule pool were precipitated by the addition of four volumes of ice cold acetone. Proteins were precipitated overnight at -20°C, pelleted and resuspended at a concentration of 5-10mg/ml in 0.1M Na borate buffer, pH 8.5. 0.25mCi of mono-iodinated Bolton-Hunter reagent (NEN) was dried down in the vial and 10μl of protein solution added to the dried label. After two hours reaction on ice, the unreacted ester was quenched by the addition of 0.4M glycine in 0.1M sodium borate for 20 minutes. Each sample was then run over a G-25 Sephadex column previously blocked with 2% hemoglobin (Bolton and Hunter, 1973). The approximate yield of TCA precipitable counts was 20%. ^{32}P-phosphate lebeled proteins were prepared by extraction from ^{32}P-phosphate labeled NB2A cells. For peptide mapping, both ^{32}P-phosphate and ^{125}I-iodine labeled proteins were cut from two-dimensional gels, subjected to partial digestion with chymotrypsin (1.5μg per sample) and resolved on 15% SDS acrylamide gels (Cleveland et al, 1977b).

Results

A. Structural comparison of four microtubule associated proteins

The human 210kd associated proteins are antigenically related

Two 210kd proteins from HeLa cells have been identified as microtubule components by different experimental criteria. One 210kd protein was shown by Bulinski and Borisy (1979) to co-purify with tubulin through temperature dependent cycles of assembly *in vitro*. The second protein fractionated in the same way as assembled tubulin when HeLa cells were detergent extracted (Duerr *et al*, 1981). To determine the antigenic relationship between the two m.a.p.s, immunoprecipitation was attempted using an anti-210kd serum raised to the coassembling associated protein (antiserum provided by J.C. Bulinski). Parallel populations of HeLa cells were radiolabeled with ^{35}S-methionine and detergent extracted (see Materials and Methods). Two hours prior to extraction, one cell population was treated with a microtubule depolymerizing drug. As described in Materials and Methods, extraction of the untreated cell population yielded a protein fraction containing assembled microtubule components, hereafter abbreviated as the mt+ fraction. A similar fraction extracted from the drug-treated cells contained no microtubule proteins. This fraction will be designated the mt- fraction. These two protein fractions were then incubated with the anti-210kd serum. The antigen-antibody complexes formed were precipitated with Staphylococcus A. bacteria. The analysis of these protein complexes

is shown in Figure 1, lanes A and B. One protein of approximately 210kd is precipitated by the antiserum from the mt+ fraction but not the mt- fraction. Normal rabbit serum does not specifically precipitate proteins from either fraction (Figure 1, lanes C and D). Therefore, the two 210kd proteins are antigenically related. This result, along with the fact that the two proteins were isolated from the same cell line and that both assemble with tubulin to constant specific activity in vitro (Bulinski and Borisy, 1979; Duerr et al, 1981) suggests that the two laboratories identified the same microtubule component by different techniques.

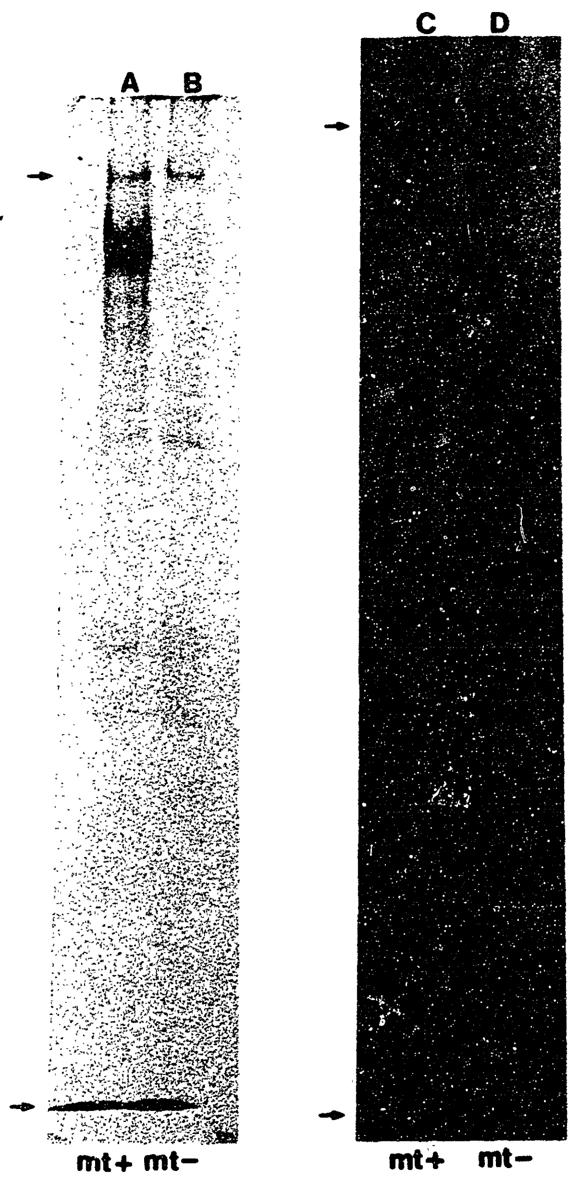
The two 220kd rodent-specific associated proteins may be distinct

220kd m.a.p.s have been identified in mouse neuroblastoma cells in two laboratories by two experimental techniques. One of these proteins is MAP4, a protein identified as a component of mouse neuroblastoma microtubules assembled in vitro (Olmsted and Lyon, 1981; Parysek et al, 1984a). The other 220kd protein fractionates with tubulin when rodent cells are detergent extracted (Duerr et al, 1981). As discussed, these proteins share some biochemical properties. Both proteins have similar molecular weights; they also have similar isoelectric points (about pI 5.4) as determined by two-dimensional gel electrophoresis (Olmsted and Lyon, 1981; D. Pallas, unpublished results). Furthermore, the 220kd protein found in detergent extracts has been shown to coassemble with tubulin to constant specific activity, as does Map 4 (Duerr et al, 1981; Olmsted and Lyon, 1981).

Figure 1.

Identification by immunoprecipitation of the 210kd HeLa protein in the assembled microtubule cell fraction.

Proteins of the assembled microtubule pool from untreated (mt+) and nocodazole-treated (mt-) HeLa cells were immunoprecipitated using either an anti-210kd serum (A and B) or normal rabbit serum (C and D). The autoradiograms show the ^{35}S-methionine labeled proteins precipitated from each extract. One protein of high molecular weight is specifically precipitated from the mt+ but not the mt- cell extract (compare A and B). In order to compare the results from the normal rabbit serum precipitation and the anti-210kd serum precipitation (run on different gels), arrows indicate a common high molecular weight protein as well as the bottom of the gels.


A
B
C
D
mt+ mt−
mt+ mt−

One additional reported property of MAP4 is its heat stability; when NB2A microtubule protein isolated from four cycles of temperature dependent assembly and disassembly *in vitro* is boiled for 5 mintues, MAP4 remains soluble (Olmsted and Lyon, 1981). The stability of the detergent extracted 220kd protein when boiled was unknown. To characterize further the rodent 220kd protein, its heat stability was tested. Mt+ and mt- fractions from NB2A cells were boiled for 5 minutes, the aggregated protein removed by centrifugation and the supernatants analyzed by one- and two-dimensional gel electrophoresis. No 220kd protein specific to the untreated cell extract was found (data not shown). Thus, the 220kd rodent m.a.p. identified by the detergent extraction of cells precipitates upon boiling.

The difference in heat stability between MAP4 and the detergent extracted 220kd m.a.p. can be explained in one of two possible ways. First, the proteins are, in fact, not identical. Second, MAP4 and the detergent extracted protein are one and the same, but the differences in the experimental conditions of the heat stability tests are sufficient to change the stability properties of the protein. This stability of the proteins when boiled can be affected by a number of factors such as protein concentration or association with other proteins and small molecules in the solution. If the experimental conditions are made more similar, that is, if the detergent extracted protein is cycled with tublin *in vitro*, and then this protein fraction is boiled, the 220kd protein might remain soluble. Due to these differences in the conditions of the experiments, no conclusions can be drawn as to the identity or nonidentity of MAP4 and the detergent extracted 220kd protein. A polyclonal antibody to MAP4 has been

raised (Parysek et al, 1984a), but at the time the above experiments were done, the antibody was not available.

The human and rodent associated proteins are not homologous

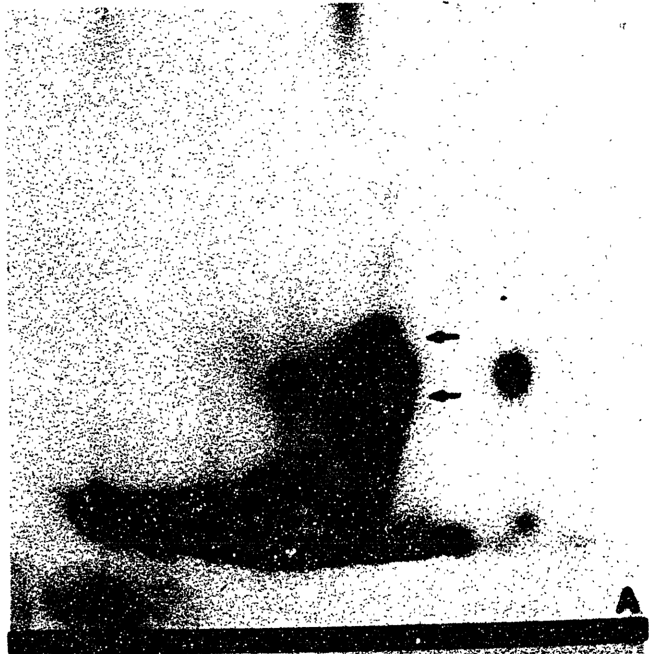
Previous immunofluorescence results have shown that the anti-210kd serum does not recognize proteins in rodent protein extracts (Bulinski and Borisy, 1980). This result was corroborated in our laboratory; all attempts to precipitate an approximately 210kd protein from the assembled microtubule fraction of NB2A cells with the anti-210kd serum failed; the human protein must carry at least one epitope not found on any NB2A cytoskeletal protein.

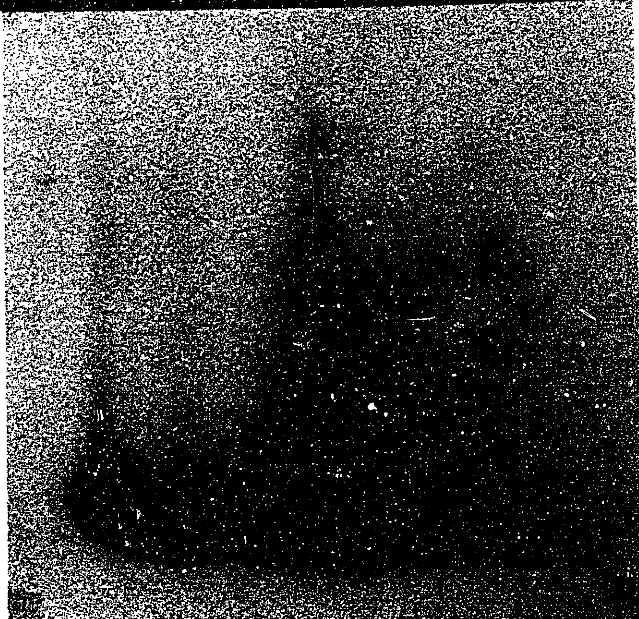
However, the 220kd protein could still be related to the 210kd HeLa protein. To determine the structural relationship between these two proteins, both were analyzed by complete two-dimensional tryptic peptide maps. The 210kd protein was immunoprecipitated from assembled microtubule fractions, the fractions were separated by SDS-PAGE and the protein excised from the gel. The 220kd protein was excised from two-dimensional gels of proteins from assembled microtubule fractions. Both were then labeled with ^{125}I, digested to completion with trypsin and the resulting peptides separated in two-dimensions (see Materials and Methods). The maps are shown in Figure 2. The reproduction of the autoradiograms results in some loss of the detail seen in the original maps. There are at least three peptides present in the 210kd HeLa protein map which are barely visible in Figure 2A. However, both the original autoradiograms and the reproductions show two peptide maps which are markedly different. The arrows in Figure 2 identify

Figure 2.

Two-dimensional tryptic peptide maps of the 210 and 220kd associated proteins from mt+ cell extracts.

The autoradiograms show the two-dimensional separation of ^{125}I-labeled peptides from limit tryptic digests of the HeLa 210kd (A) and NB2A 220kd (B) proteins. The directions of the electrophoretic (E) and chromatographic (C) migration of the peptides are indicated. The small arrows mark peptides from each protein which may co-migrate.


HeLa
210kd
A


NB2A
220kd
C
E

the only two spots in the 220kd protein's peptide map which may co-migrate with spots in the 210kd protein's map. However, the intensity of the signals from the peptides in the identified region of the 210kd protein's map prevents detection of separate spots. Thus, no conclusions about the co-migration of spots in this region of the two maps can be drawn. The two peptide maps indicate that the proteins from which they are derived are not structurally related.

B. Phosphorlyation and assembly of chartins

Chartin variants are phosphorylated *in vivo*

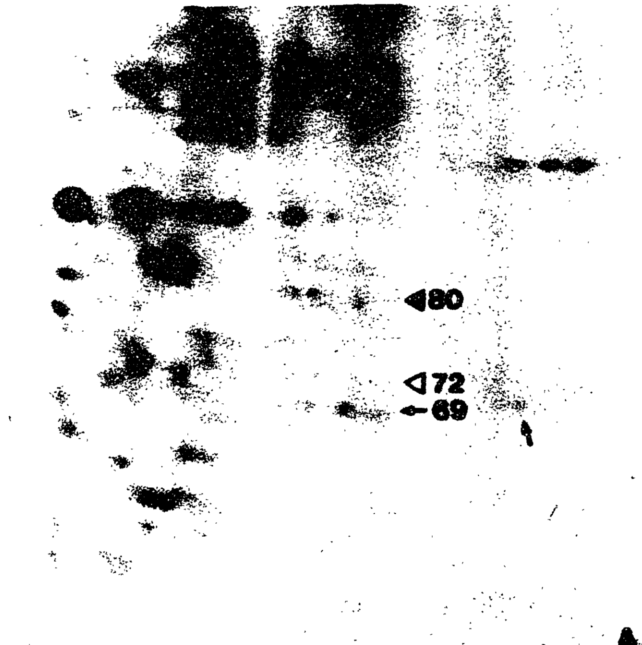
The first step in the study of the chartin variants was to determine that such modifications occur *in vivo*. Protein phosphatases and kinases may be released or activated during the detergent extraction of cells and alter the phosphorylation of the chartins. To identify any potential *in vitro* activity, parallel populations of ^{32}P-phosphate labeled mouse neuroblastoma NB2A cells were either detergent extracted or homogenized at -30°C in a buffer containing kinase and phosphatase inhibitors (see Materials and Methods; Foulkes and Rosner, 1985). The proteins extracted by each method were analyzed by two-dimensional gel electrophoresis. Autoradiograms of the chartin containing portions of these gels are shown in Figure 3. The characterisic two-dimensional pattern of chartins analyzed in this way is shown in Figures 4B and 4C of this chapter (see also Pallas and Solomon, 1982). Although the 72kd chartins, marked by open deltas, are not easily detected in the figure, the original autoradiograms

Figure 3.

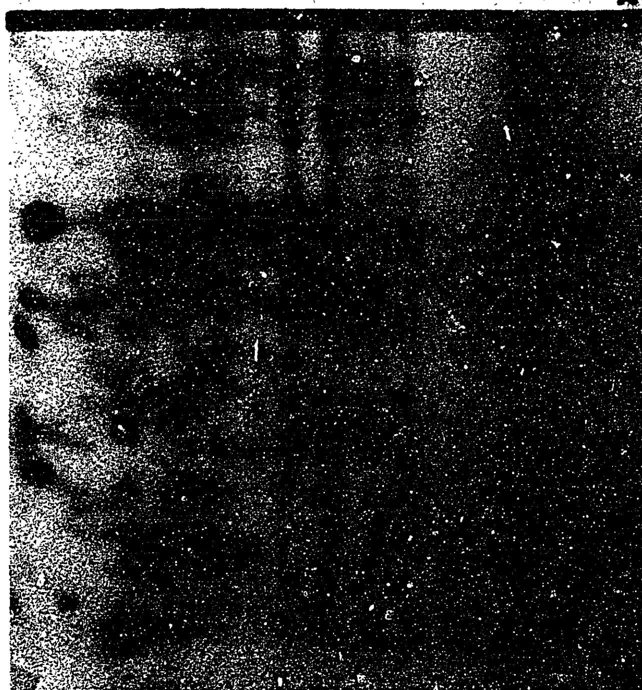
Chartins in $-30^{o}C$ whole cell lysate.

^{32}P-phosphate labeled mouse neuroblastoma NB2A cells were either lysed with nonionic detergent at $-30^{o}C$ into a buffer containing phosphatase inhibitors or detergent extracted in two steps to separate the assembled and unassembled microtubule pools. The autoradiograms show the chartin-containing regions from two-dimensional gels of the whole cell lysate (A) and the unassembled microtubule pool (B). This pool contains 80% of the total cell microtubule protein. Several variants of the 69kd (arrows), 72kd (open deltas) and 80kd (closed deltas) proteins are present in both cell extracts.

-30°C whole cell lysate



unassembled mt pool



show that all of the variants present in the detergent extracts were also found in the -30°C homogenate. Thus, the multiple phosphorylation of chartins is not an in vitro artifact, but occurs in vivo.

Chartin variants in calf brain cells

Work done previously has shown by two different criteria that proteins related to chartins are present in calf brain. First, calf brain microtubule proteins (MTP) contain a polypeptide of about 69kd that is homologous to the 69kd chartin from NIL8 hamster fibroblasts; this result is based on partial cleavage peptide mapping (Duerr et al, 1981). Second, an antibody raised against mouse neuroblastoma chartins binds to two bands on a one-dimensional Western blot of calf brain MTP (Magendantz and Solomon, 1985). The apparent sizes of these bands are the same as chartins isolated from other neural cells, 69 and 80kd.

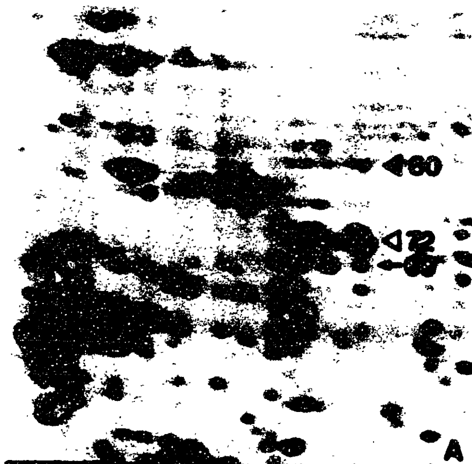
To characterize further the chartins of calf brain, protein extracts from the tissue were analyzed by two-dimensional gel electrophoresis. This analysis of calf brain protein extracts demonstrates the presence of presumptive chartin isoelectric variants. The silver-stained gel in Figure 4A shows calf brain proteins which have two properties similar to those of the chartins from neuroblastoma cells (see Figures 4B and 4C). First, the brain proteins have gel mobilities that correspond to molecular weights of approximately 69, 72 and 80kd (shown by the arrow, open delta and filled delta). Second, these proteins form a distinctive

Figure 4.

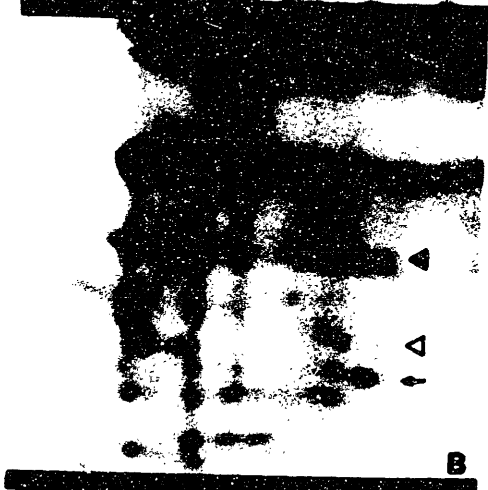
The two-dimensional pattern of calf brain and NB2A cell chartins.

The chartin-containing regions from two-dimensional gels of proteins from calf brain homogenate (A), NB2A unassembled microtubule pool (B) and NB2A assembled microtubule pool (C) are shown. The calf brain proteins are visualized by silver stain (A) while the neuroblastoma proteins are detected by autoradiography of ^{32}P-phosphate label (B and C). Proteins of all three molecular weight classes are detected in both the calf brain and neuroblastoma cells, and they closely co-migrate. The relative proportion of the chartin classes as well as the ratio of the acidic to basic variants within each class differ between the tissue and cell type.

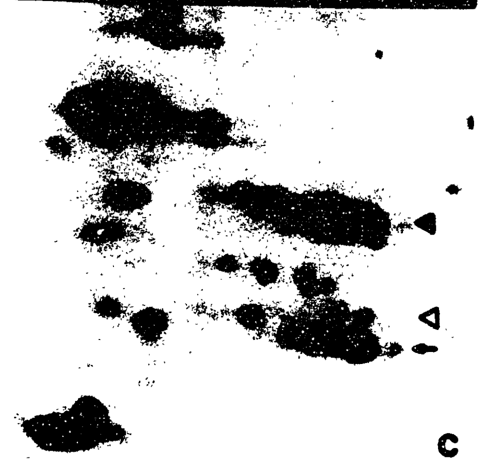
calf
brain
homogenate



NB2A
unassembled
mt pool



NB2A
assembled
mt pool



two-dimensional pattern, essentially the same pattern formed by neuroblastoma chartin variants.

The calf brain proteins were more rigorously compared to neuroblastoma chartins by peptide mapping. Brain and neuroblastoma variants of 69kd were excised from two-dimensional gels, iodinated, partially cleaved with chymotrypsin and analyzed by one-dimensional SDS-PAGE. The peptide maps of the two calf brain proteins shown in Figure 5, lanes B and C, are virtually identical. These maps are also very similar to the map of the 69kd neuroblastoma chartin (Figure 5, lane A). These results suggest that the chartins in calf brain exist as a family of structurally related isoelectric variants.

The calf brain chartins differ from the neuroblastoma chartins in the abundances of two subsets of the protein family. First, the 72kd protein is the least abundant molecular weight class of chartin in neuroblastoma cells (see Figure 4B and 4C). In calf brain, however, the pattern of proteins in Figure 4A suggests that the 72kd chartin is more abundant than either the 69 or the 80kd proteins. Second, the proportion of the more acidic variants relative to the other chartins differs between the cell and tissue type. All the variants within each molecular weight class of the chartins in calf brain are present in nearly equal amounts. In contrast, no acidic variants are detected in the NB2A protein fraction containing the unassembled microtubule components (Figure 4B). This unassembled microtubule pool contains 80% of the total microtubule components of the cell. This lack of acidic variants is not due to the use of ^{32}P-phosphate as radioactive label. These same variants are not detected in the unassembled pool when amino acids are used as the source of radioactive label (Pallas

Figure 5.

One-dimensional peptide maps of 69kd chartins.

Partial cleavage peptide maps of the major 69kd chartin from NB2A cells (A) and two of the 69kd chartins from calf brain (B and C) are shown. The proteins were cut from two-dimensional gels, labeled with ^{125}I, partially digested with chymotrypsin and the peptides separated by SDS-PAGE. The maps are visualized by autoradiography. The peptide maps of all three proteins are nearly identical.

A
B
C
NB
calf brain

and Solomon, 1982). Thus, the relative proportion of NB2A chartins that are acidic is far less than the proportion of calf brain chartins that are acidic. The possible significance of this difference between the cell and tissue type chartins is discussed at the end of this chapter.

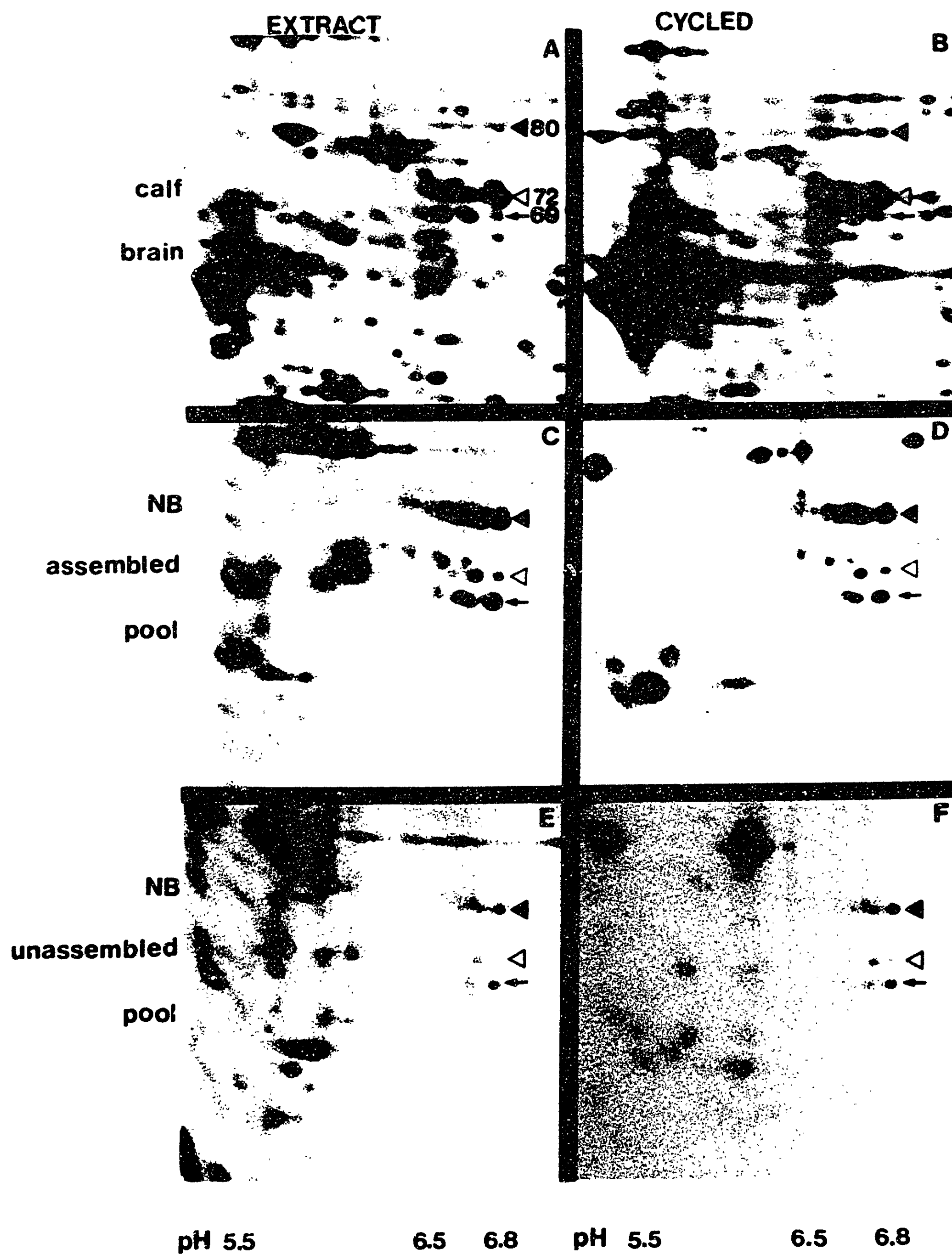
Affinity of chartins for microtubules *in vitro*

We wanted to assay the behavior of chartin variants *in vitro*. In particular, we wanted to ask whether the preferential association of highly phosphorylated chartins with assembled microtubules seen *in vivo* also occurred *in vitro*. Two experiments were done to address this question. First, calf brain homogenate was carried through two cycles of temperature dependent assembly and disassembly in a medium similar to that used in the *in vivo* extraction analysis (see Materials and Methods). As shown by comparison of the two-dimensional gels in Figures 6A and 6B, the ratio of acidic to more basic chartins did not change. In a similar experiment, ^{32}P-phosphate labeled chartins from either the unassembled or assembled microtubule pools of neuroblastoma cells were co-assembled with carrier calf brain tubulin. Again, no enrichment of any chartin species occurred after two full cycles of assembly and disassembly (compare Figures 6C with 6D and 6E with 6F); the relative proportion of the variants in the extracts is the same after cycling. Thus, the acidic chartin variants which preferentially assembled *in vivo* are not preferentially bound to microtubules *in vitro* by this affinity purification procedure. In the above experiments, the ratio of radioactive proteins from cells to the

Figure 6.

In vitro assembly and coassembly of chartins

The six panels show the chartin-containing regions from two-dimensional gels of proteins from calf brain homogenate (A and B), the NB2A assembled microtubule pool (C and D) and the NB2A unassembled microtubule pool (E and F). The protein fractions were either analyzed directly ("EXTRACT"; A,C and E) or after two cycles of temperature dependent assembly ("CYCLED"; B,D,and F). The calf brain protein is visualized by silver stain (A and B) while the protein from the cultured cells is ^{32}P-phosphate labeled, and visualized by autoradiography (C-F). The NB2A cell protein was cycled in the presence of unlabeled carrier microtubule protein. Although the two or three more acidic chartins of each class (69, 72 and 80kd) are highly enriched in both the assembled microtubule fraction of NB2A cells and the brain homogenate, there is no detectable preferential assembly of those variants during *in vitro* assembly.


EXTRACT
CYCLED
A
B
80
72
68
calf
brain
C
D
NB
assembled
pool
E
F
NB
unassembled
pool
pH 5.5
6.5
6.8
pH 5.5
6.5
6.8

unlabeled carrier proteins drops after one cycle of assembly, but remains constant thereafter. The efficiency of assembly in each cycle is 50-60% under the experimental conditions.

The in vitro assembly reaction described failed to show preferential association of some acidic chartin variants with assembled microtubules. One of several explanations could account for this result. First, the number of binding sites for chartins along the microtubules assembled in vitro may not be limiting. That is, any chartin present could bind to the microtubules. Second, the conditions of the assay may not be sufficiently stringent to detect differences in the affinity of the chartin variants in vitro. Finally, chartins may play a role and therefore associate with microtubules during the assembly process in vivo, but in vitro these proteins may only associate with microtubules once they are assembled. In this case, the observed in vivo preferential association of highly phosphorylated chartins with assembled microtubules may not be detected in vitro.

Modifications of chartins during dibutyryl cAMP induction of neurite extension in cultured cells.

The behavior of chartins during in vitro microtubule assembly raised the question of how chartin variants associate with microtubules during assembly in vivo. To address this question, we analyzed the microtubule components of several established neuronal cell lines. These cell lines will flatten and extend neurites, a process known to be microtubule dependent, when exposed to media

containing one of several possible inducers. Some of these inducers, are agents that increase intracellular levels of cAMP (Stull and Mayer, 1972). One drawback to the use of these inducers of neurite extension is that they also result in the cessation of cell division. This fact complicates identification of the biochemical events relevent to neurite extension. However, if the study of differentiated cells is limited to early times after induction of neurite extension, then the biochemical differences between cell populations can be more readily correlated with both each other and induction. The experiments of this section analyze the microtubule components of mouse neuroblastoma NB2A cells induced to extend neurites by the addition of dibutyryl cAMP to the growth medium.

After three hours in media containing dibutyryl cAMP, differentiation of the NB2A cells is sufficiently marked that a comparative study is possible. For example, when the number of cells with neurites are determined in several (3-5) fields of about 100 cells each, less than 10% of an untreated population have neurites. In contrast, three hours after the addition of $5X10^{-3}M$ dibutyryl cAMP, 70% (±15%) of cells had neurites at least one cell body diameter in length. These neurites were microtubule dependent; they retracted rapidly upon exposure to microtubule depolymerizing drugs.

Exposure of cells to dibutyryl cAMP changes the state of the tubulin pools in these cells differentiating cells. Over the same time course of induction, the proportion of tubulin which assembles into microtubules increases. The assembled tubulin pools are assayed by a modification of the detergent extraction procedure described (see Materials and Methods; Zieve and Solomon, 1982). Three hours after

induction of neurite extension, the percentage of tubulin in the assembled form increases 1.6 fold from 20% to 33%. Figure 7 shows densitometric scans of the tubulin regions from one-dimensional SDS-PAGE of the assembled tubulin fractions from control and dibutyryl cAMP treated NB2A cells. The total amount of tubulin in these cells does not increase detectably. This result differs from that obtained when cells are held in inducing media for long periods of time. After 7-21 days in media containing nerve growth factor, both the amount of tubulin assembled and the total tubulin pool increase (Drubin _et al_, 1985; Black _et al_, 1986).

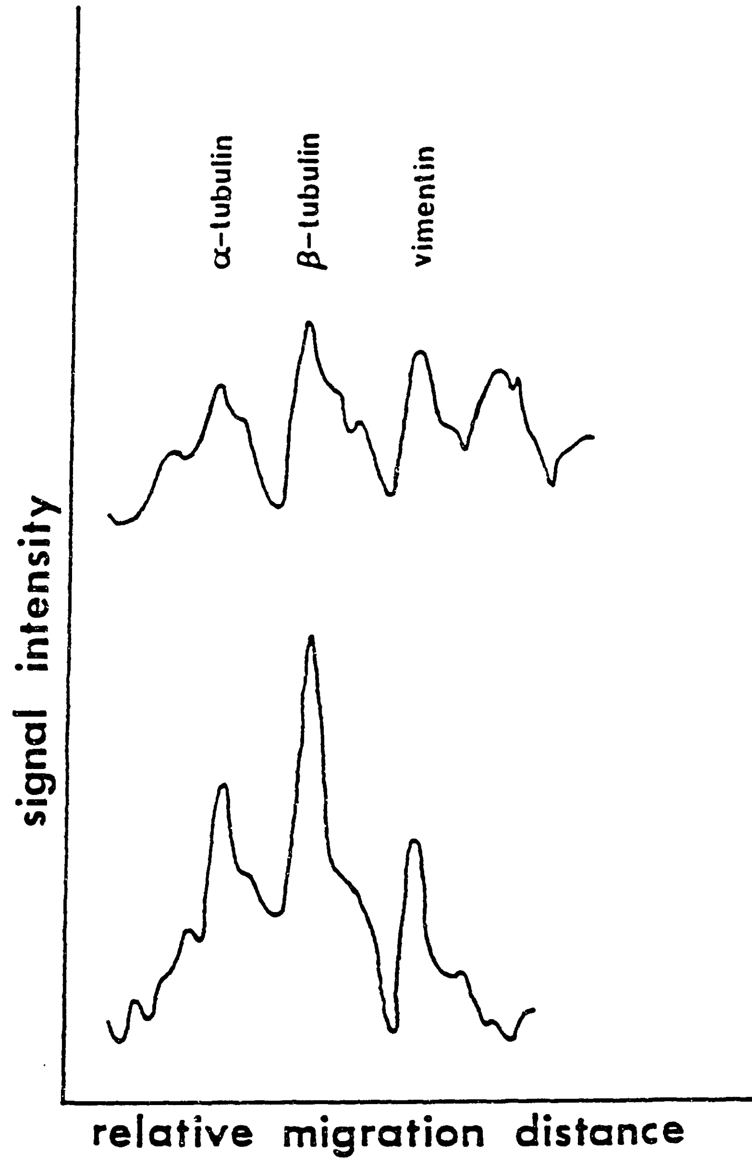
Another microtubule component, the chartins, is also affected when cells are treated with dibutyryl cAMP. Figure 8 shows two-dimensional gel analysis of chartins from ^{32}P-phosphate labeled control and dibutyryl cAMP-treated cells. The pattern of chartins from dibutyryl cAMP treated cells is markedly different from that of untreated cells. That is, the chartins' are altered when cells are exposed to dibutyryl cAMP. First, the proportion of phosphorylated chartins of each molecular weight class is diminished in both the assembled and unassembled microtubule pools. The highly phosphorylated variants are in fact not detected in extracts from dibutyryl cAMP-treated cells. In addition, a new prominent set of phosphorylated proteins appears; these proteins have an apparent molecular weight of 80kd and occur as a series of phosphorylated variants having several isoelectric points. These proteins are chartins as data presented below indicates, and will be called cAMP-dependent chartins.

The panels of Figure 8 also show that the cAMP-dependent chartins

Figure 7.

Densitometric scans of assembled tubulin from neuroblastoma cells.

The densitometric scans are of the assembled tubulin fractions from NB2A cells analyzed by one-dimensional SDS-PAGE. The fractions were prepared by a modification of the detergent extraction procedure (Zieve and Solomon, 1982). Scans are over only the tubulin and vimentin regions of the gels. Dibutyryl cAMP-treated cells contain higher levels of assembled tubulin than untreated cells.

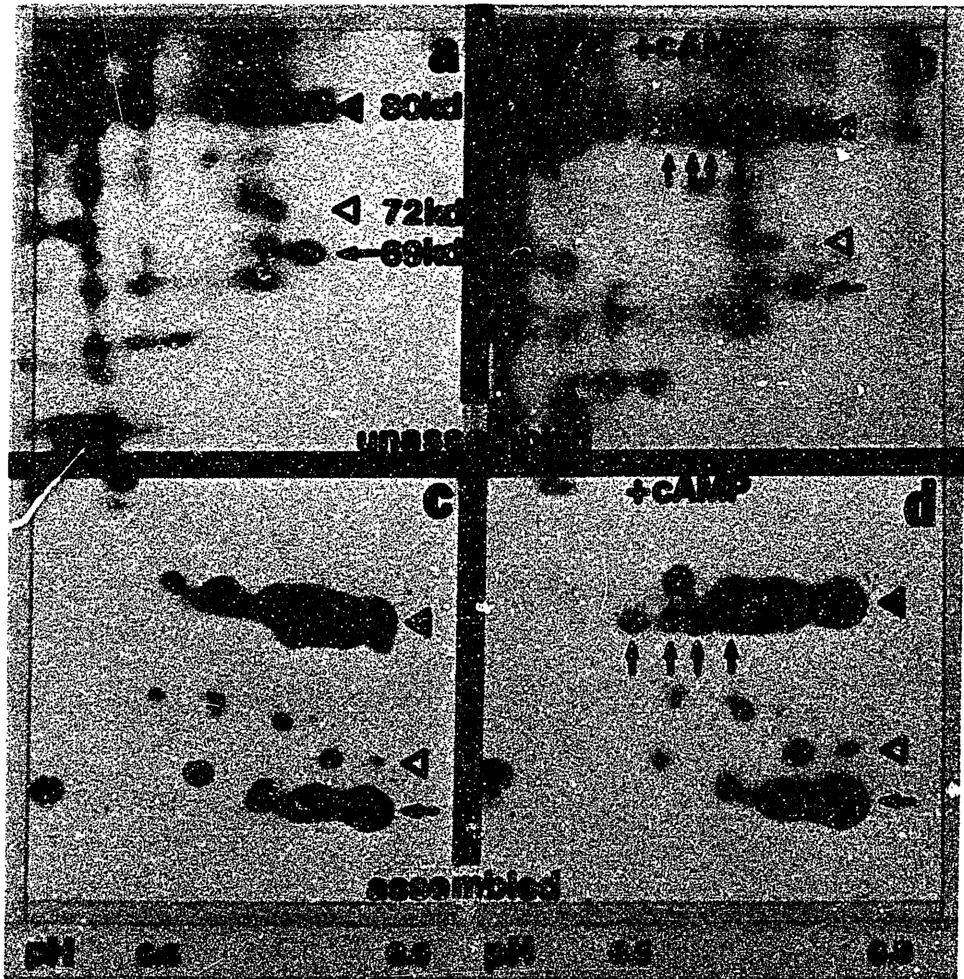


α-tubulin
β-tubulin
vimentin
signal intensity
relative migration distance

Figure 8.

Chartin phophorylation patterns in dibutyryl cAMP-treated neuroblastoma cells.

Proteins from both NB2A mouse neuroblastoma cells exposed to $5X10^{-3}M$ dibutyryl cAMP (b and d) and control cells (a and c) were analyzed on two-dimensional gels. The autoradiograms shown are of the ^{32}P-phosphate label in the assembled (a and b) and the unassembled (c and d) microtubule pools. All three molecular weight classes of chartins are detected in all fractions. The 69kd proteins are marked by arrows, the 72kd proteins by open deltas and the 80kd proteins by filled deltas. After cells are incubated in dibutyryl cAMP, the more acidic variants are diminished and a new set of phosphorylated proteins appears in the 80kd size range (marked by vertical arrows in panels b and d). The same results are obtained when cells are incubated with isoproterenol and 3-isobutyl-1-methyl-xanthine (data not shown).



a
80kd
72kd
69kd
+cAMP
b
unassembled
c
+cAMP
d
assembled
pH
pH

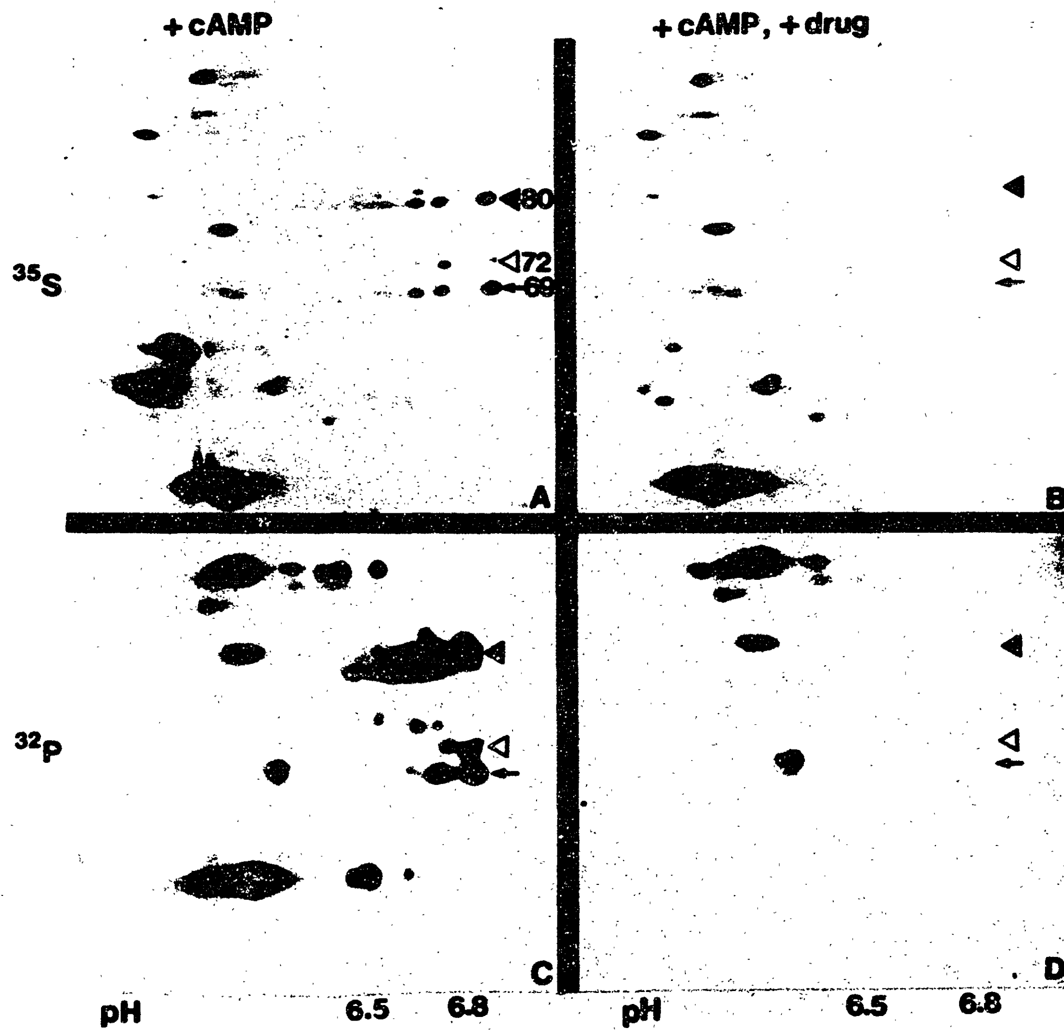
localize in cells differently from the highly acidic chartins. None of the cAMP-dependent variants preferentially associate with assembled microtubules. All the cAMP-dependent chartins appear in both the unassembled and assembled microtubule pools. Data will be presented which substantiate this result by quantitation of the ^{32}P-phosphate incorporated into the variants of both microtubule protein pools. The cAMP-dependent chartins co-extract with the assembled tubulin when NB2A cells are fractionated by detergent extraction. As shown in Figure 9A and 9C, these chartins are present in the mt+ fraction from dibutyryl cAMP-treated cells. The analogous extract from cells preincubated with both dibutyryl cAMP and a microtubule depolymerizing drug contains no tubulin, chartins, or the cAMP-dependent chartins (see Figures 9B and 9D). Therefore, these proteins fit the criteria for microtubule components (Solomon et al, 1979).

The cAMP-dependent proteins were compared to some of the previously identified 80kd chartins by partial cleavage peptide mapping (Cleveland et al, 1977). Three prominent cAMP-dependent species as well as the two most basic 80kd chartins ("80-1" and "80-2"; these variants are identified in Figure 10) were cut from two-dimensional gels of ^{32}P-phosphate labeled extracts. The proteins were briefly exposed to chymotrypsin and the cleavage products analyzed by one-dimensional SDS-PAGE. Figure 11 demonstrates that all five of the peptide maps have at least six cleavage products in common (marked by arrowheads). However, there are two significant differences among these peptide maps. First, although the maps of the three cAMP-dependent chartins are essentially identical, at least three of the phosphorylated peptides differ in intensity of label

Figure 9.

Identification of the cAMP-dependent phosphorylated proteins as microtubule associated proteins.

The autoradiograms show the two-dimensional pattern of chartins in the cytoplasmic fraction from control (A and C) and drug-treated (B and D) neuroblastoma cells. Panels A and B show ^{35}S-methionine labeled proteins while panels C and D show ^{32}P-phosphate labeled proteins. The phosphoprotein present in the mt+ but not in the mt- extract and which approximately co-migrates with ^{35}S-methionine labeled tubulin is probably β-tubulin (Gard and Kirschner, 1985).



+ cAMP
+ cAMP, + drug
80
72
69
35S
32P
A
B
C
D
pH
6.5
6.8
pH
6.5
6.8

Figure 10.

Schematic diagram of the phosphorylated chartin variants

The diagram shows the phosphorylated chartins present in the mt+ fraction of NB2A cells. The phosphate incorporated into each labeled variant in the diagram is quantitated by scintillation counting (see Table 1). The peptide map of the 69-1 variant is shown in Figure 5. The peptide maps of 80-1 and 80-2 are shown in Figure 11.

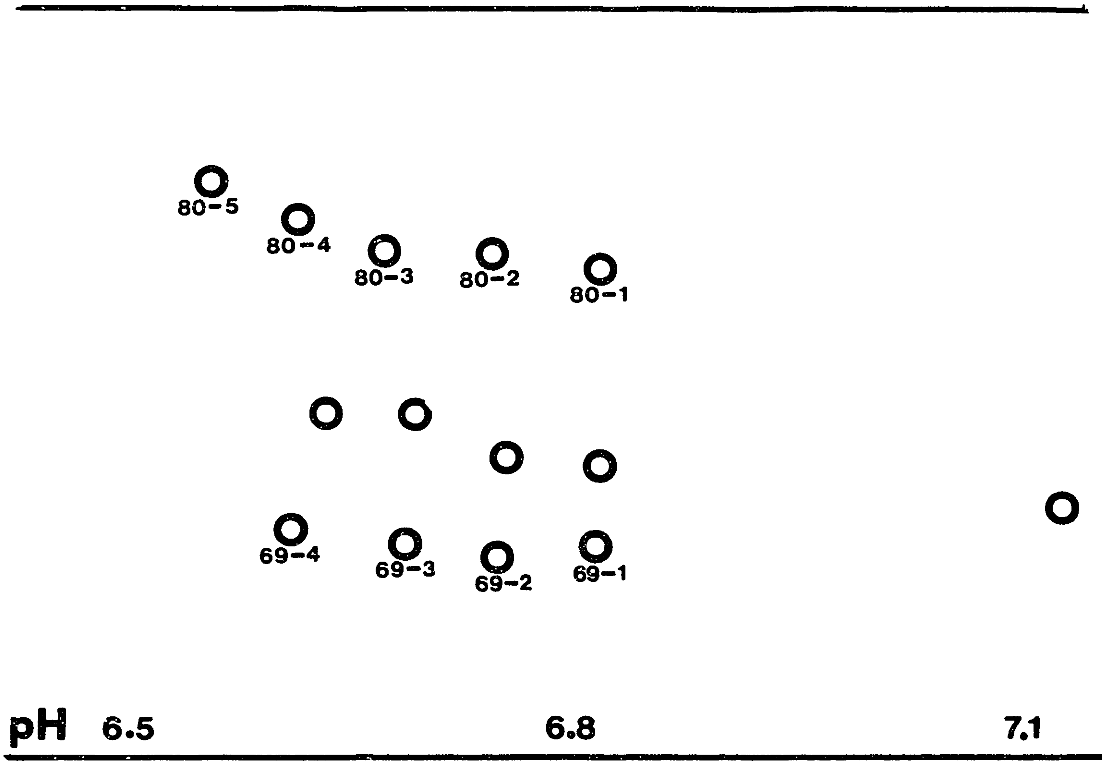

80-5
80-4
80-3
80-2
80-1
69-4
69-3
69-2
69-1
pH
6.5
6.8
7.1

Figure 11.

Peptide maps of five 80kd phosphorylated chartins.

The autoradiogram shows the partial cleavage peptide maps of two of the phosphorylated chartins from untreated cells (lanes a and b) and three of the new 80kd variants from cAMP-treated cells (lanes c,d and e). Proteins were excised from two-dimensional gels, cleaved and analyzed as described in the text. The arrowheads mark common bands in the maps of all species, while the small arrows mark bands that are of different intensityamong the three cAMP-dependent variants.

cAMP
induced

a b c d e

(marked by arrows). These differences in intensity of label may reflect differences in the degrees of phosphorylation among the variants; such differences could explain the existence of isoelectric variants. Second, there are phosphorylated peptides in the cAMP-dependent variants that are not seen in the maps of "80-1" and "80-2". The cAMP-dependent chartins also do not show an apparent increase in molecular weight with decreasing isoelectric point, as do chartins in untreated cells. This observation, as well as the results from the peptide mapping suggest that at least some of the sites of phosphorylation of the chartins differ in untreated and dibutyryl cAMP-treated cells.

The changes in phosphorylation of the chartins seen in Figure 8 can be described quantitatively. All of the chartins in the 69kd and 80kd regions were excised from two-dimensional gels and the ^{32}P-phosphate incorporated into each variant was determined by scintillation counting. The chartins in the 72kd region are insufficiently labeled and could not be quantitated. The results of this analysis are shown in Table 1. These data provide direct quantitation of the cAMP-induced changes in chartin phosphorylation first detected by two-dimensional gel analysis.

After a three hour treatment with dibutyryl cAMP, several quantitative changes occur in the phosphorylation of the chartins. The most acidic of the 69kd ("69-4"; see Figure 10) and the two most acidic of the 80kd chartins ("80-4" and "80-5"; see Figure 10) are no longer detected in cell extracts. The ^{32}P-phosphate incorporated into the other phosphorylated variants is also diminished. Finally, cAMP-dependent chartins are detected and the label incorporated into

Table I. Quantitation of ^{32}P-phosphate incorporated into microtubule associated proteins.

Protein	Unassembled Pool		Assembled Pool		Percent of Label in Assembled Chartins	
	-cAMP	+cAMP	-cAMP	+cAMP	-cAMP	+cAMP
69-1	4,410	3,120	880	1,010	17	24
69-2	3,920	2,200	800	390	17	15
69-3	*	*	260	140	(100)	(100)
69-4	*	*	90	*	(100)	-
80-1	8,700	6,360	880	1,020	9	14
80-2	7,440	1,760	1,830	860	20	33
80-3	*	*	500	220	100	100
80-4	*	*	210	*	100	-
80-5	*	*	50	*	100	-
cAMP-induced Proteins	*	8,900	*	1,510	-	13
Total cpm	24,470	23,420	5,500	5,150	18%	18%

* Not Detected

Legend: The proteins were cut from gels and ^{32}P-phosphate incorporation was determined by scintillation counting and corrected for background as described in Materials and Methods. Proteins are identified first by their molecular weight and then in order of increasing acidity (see Figure 10). Thus, protein 80-1 is the least phosphorylated 80kd cyclic AMP-independent protein and 80-5 is the most acidic 80kd variant. The data in the table are from a single representative experiment. Similar results were obtained in four independent experiments.

these variants compensates for the label lost from the others. Thus, the total label incorporated into chartins does not change significantly during the three hour exposure of cells to dibutyryl cAMP.

The numerical data in Table 1 confirm the observation that the cAMP-dependent proteins are not preferentially compartmentalized as are the heavily phosphorylated chartins found in untreated cells. About 13% of the label in the cAMP-dependent chartins fractionates with the assembled microtubule components; that is, about 13% of these chartins assemble with tubulin. This fraction is equal to or less than either the fraction of cell tubulin or the fraction of the major chartin species ("69-1", "69-2", "80-1" and "80-2"; see Figure 10 and Table 1) that assembles.

Exposure of cells to dibutryrl cAMP may result in increased levels of intracellular cAMP. This, in turn, could be one triggerwhich induces neurite extension. Results from the following two experiments suggest that dibutyryl cAMP is acting on neuroblastoma cells by raising their intracellular levels of cAMP. First, both the morphological and biochemical effects induced by dibutyryl cAMP can be reproduced by treatment of NB2A cells with isoproterenol and 3-isobutyl-1-methyl-xanthine (data not shown); these reagents increase intracellular cAMP by stimulating adenylate cyclase and inhibiting phospho-diesterase, respectively. Second, incubation of NB2A cells in comparable concentrations of sodium butyrate, a breakdown product of dibutyryl cAMP, neither induces neurite extension nor affects the chartins during a three hour incubation (data not shown). This result means that one potential complication of dibutyryl cAMP treatment,

i.e. effects due to sodium butyrate, is not a factor in the experiments presented here.

The appearance of cAMP-dependent chartins can be separated from neurite outgrowth

The results described above indicate that dramatic alterations in the covalent modifications of chartins occur with the same time course as neurite extension in cells induced with dibutyryl cAMP. However, the induction of neurite extension in NB2A cells does not require modification of the chartins. When these cells are incubated for three hours in medium containing 0.1% fetal calf serum, they extend neurites morphologically identical to neurites produced in dibutyryl cAMP containing media. These cells also show a significant increase in microtubule assembly (data not shown). In contrast, the chartins remain unchanged from those of untreated cells. There is no decrease in the acidic phosphorylated variants and no appearance of new 80kd phosphorylated variants. Changes in the covalent modifications of chartins do not occur even in cells held in low serum for up to 72 hours.

Conversely, the appearance of the cAMP-dependent chartins does not require neurite extension. These proteins appear when cells grown in suspension are incubated with dibutyryl cAMP. The chartins of rat pheochromocytoma PC12 cells show a similar response to dibutyryl cAMP. That is, PC12 cells, whether grown attached or in suspension, have both diminished levels of the phosphorylated chartins normally present

in untreated cells, and the cAMP-dependent 80kd chartin variants (data not shown).

These results are summarized in Table 2.

Table II. Neurite extension, microtubule assembly, and alterations of chartin patterns in cultured neuronal cells.

Cell Line	Growth Conditions	Neurite Extension	Increase in Per Cent Assembled Tubulin	Alteration the Pattern of Chartin Species
NB2A	dBcAMP	Yes	Yes	Yes
	dBcAMP, grown in suspension	No	No	Yes
	isoproterenol+ 3-isobutyl-1-methyl-xanthine	Yes	Yes	Yes
	0.1% Serum	Yes	Yes	No
PC12	dBcAMP	Yes	N.D.	Yes
	0.1% Serum	Yes	N.D.	No

Legend: "Neurite extension" is scored as described, three hours after change to inducing medium. "Assembled tubulin" was measured using the extraction procedure of Zieve and Solomon (1982); the resulting cytoskeletons were analyzed on one-dimensional gels, and the tubulin peak measured by scanning densitometer. In induced NB2A cells, the proportion of assembled tubulin increased from 20% to 33%. The changes in the two-dimensional pattern of chartin species are depicted in Figure 8. The decrease in all the normal acidic variants and appearance of new 80kd variants always occurred together.

Discussion

The number of microtubule organelles combined with the regulation of their formation and dissolution suggest a need for great diversity among the proteins involved in these functions. This diversity exists in microtubule associated proteins at two or more levels. First, the number of different associated proteins may be high. Second, the proteins may be multifunctional. Experiments presented in this chapter have provided more evidence that both levels exist among associated proteins.

In the first section of this chapter, four microtubule associated proteins of similar molecular weight are compared. The results show that while some associated proteins of similar size are related, homology cannot be assumed. Two 210kd HeLa proteins identified by different criteria (Bulinski and Borisy, 1979; Duerr et al, 1981) are probably related, if not identical. Both proteins assemble with tubulin into microtubules in vitro. Furthermore, an antiserum raised against one recognizes the other by immunoprecipitation. In contrast, the two 220kd NB2A proteins may be different proteins. Although MAP4 and the 220kd protein identified by the selective extraction of cells share several properties, only MAP4 is thermostable. This difference in heat stability may be explained by discrepancies in experimental conditions, but it is possible that the proteins are not identical.

In one final set of experiments, the 210kd HeLa and thermolabile 220kd NB2A protein were compared. Results from our laboratory as well as others (Bulinski and Borisy, 1980) show that the anti-210kd serum does not crossreact with the mouse 220kd protein. Structural

analysis of these two proteins by two-dimensional tryptic peptide maps suggests that they are not related. No in vivo function for either protein has yet been demonstrated, but their structural differences are likely to impart some functional specificity to each protein.

The chartins, a set of related associated proteins, are discussed in the second half of this chapter. In particular, the role of chartins in the regulation of microtubule assembly was investigated. Evidence consistent with chartin involvement in assembly originally resulted from the analysis of unassembled and assembled microtubule components of neuroblastoma cells. The highly phosphorylated chartins associate only with assembled microtubules (Pallas and Solomon, 1982). Experiments presented above further characterize the relationship between chartin modification and microtubule assembly; chartins were analyzed under conditions in vivo and in vitro where microtubule assembly varied. The data from these experiments show that no simple relationship exists between modified chartins and the assembled microtubules of a cell.

In brain tissue, where the level of microtubule assembly is much higher than in cultured cells, the relative proportion of acidic chartins is also much higher. The differences between the chartin variants of brain and cultured cell lines may reflect functional differences necessary for the creation and/or maintenance of the microtubule organelles of each cell type. Such functions could involve not only roles in assembly, but also in shape and location of the cells' microtubules.

The effect on the chartins in cells induced to extend neurites is complex. The most highly phosphorylated chartins are no longer

detected, and the levels of all chartins normally found in untreated cells is diminished. In addition, the 80kd chartin seems to be a substrate for a cAMP-dependent protein kinase; a new set of modified chartins appears after cAMP treatment. Peptide maps show that these proteins are 80kd chartins. Some differences in the maps, however, suggest that the cAMP-dependent proteins are phosphorylated at new sites. Finally, the cAMP-dependent chartins are not compartmentalized; they distribute between the unassembled and assembled microtubule pools stoichiometrically with tubulin. All the described changes in the chartins of cAMP-induced cells occur with the same time course as neurite extension and microtubule assembly.

The above results show that phosphorylation of chartins at some sites correlates with their cytoplasmic localization. That is, the correlation suggests that the extent of phosphorylation alone may not determine which chartin variants associate with assembled microtubules.

The close correlation between the cAMP-induced initiation of neurite extension and changes in chartin phosphorylation suggests that chartins play a role in this process of neurite extension. If so, such a role is not likely to be quantitatively related to the assembly of tubulin into microtubules. Both the loss of phosphorylated variants previously limited to the assembled pool and the presence of cAMP-dependent chartins in both microtubule pools argue against such a model. These two modifications of the variants leave no subset of the chartin family specifically associated with microtubules.

One requirement of proteins which help regulate microtubule structure, function and assembly is diversity. The experiments

presented have shown such diversity among microtubule associated proteins at two levels. First, two proteins previously thought to be related were shown to be structurally distinct; this increases the long list of different associated proteins by one. Second, cAMP-induced changes in phosphorylation of some closely related proteins, the chartins, alter some of the proteins' biochemical fractionation properties. This change in the fractionation of some variants could reflect changes in the functions of these variants. If so, changes in post-translational modification are another method by which diversity among microtubule associated proteins can be achieved.

Chapter three

Isolation of chartin-specific DNA

Introduction

Results from many experiments have characterized chartins biochemically and suggested function(s). However, new experimental directions are needed if structural and functional studies are to progress. Fine structure study of the chartin proteins themselves is nearly impossible. The proteins represent only 0.01% of total cell protein and variants can only be purified by two-dimensional gel electrophoresis. Experiments which examine chartin modification and distribution have also yielded all the useful information possible at the protein level.

One first step toward new directions of investigation is the isolation of chartin specific nucleic acid. Chartin specific cDNA, for example, can be used to identify the proteins' genes and study their expression. Also, the determination of the sequence(s) which code for chartins may both decipher the structural differences among the family members and lead to the isolation of variant specific nucleic acid and immunologic probes.

This chapter describes the isolation and some characterization of probable chartin cDNA sequences. A chartin-specific antibody, 4-81 (Magendantz and Solomon, 1985), was used to identify potential chartin sequences in a mouse brain λgt11 expression library. These cDNA inserts were mapped, sequenced and expressed <u>in vivo</u> as part of

bacterial fusion proteins. Antibodies were raised to one fusion protein and tested for reactivity with the native chartins. An attempt was made to compare two native chartins to a fusion protein by analysis of their partial cleavage peptide maps. Finally, the fusion protein was tested for crossreactivity with two anti-chartin antisera.

In the last section of the chapter, the cDNA insert corresponding to the mouse brain portion of one fusion protein was used as a probe in initial studies of chartin genes and transcription.

Materials and Methods

Antibody screening of recombinant λgt11 phage libraries

250,000 recombinant phage from the mouse brain library (Citri _et al_, 1987; provided by Y. Citri) were screened with the anti-chartin serum 4-81 (Magendantz and Solomon, 1985) by a modification of the method of Young and Davis (1983). Phage were adsorbed to the E.coli strain Y1090 in 10mM $MgSO_4$ at a multiplicity of infection of $1x10^{-4}$ plaque forming units (pfu)/cell and plated at a density of 25,000 pfu per 15cm Luria broth (LB) plate containing 40μg/ml ampicillin (Sigma). The phage were grown for five hours at 42°C. 137mm nitrocellulose filters (Schleicher and Schuell, 0.45μ) were soaked in 10mM isopropyl-β-D-thiogalactoside (IPTG) for one hour and air dried. The filters were overlaid on the plaques and the plates incubated at 37°C for two hours to induce synthesis of the β-galactosidase fusion proteins. The nitrocellulose filters were then keyed to the plates with a 20 gauge needle containing India ink and screened with serum 4-81 by one of two methods. In most experiments, the filters were first washed for one hour in 2% hemoglobin (Sigma), 0.05M Tris-HCl pH 7.5, 0.15M NaCl (Hb/TBS). Serum 4-81 diluted 1 to 300-500 in Hb/TBS was then added to the filters and they were gently agitated at 25°C for four hours. Unbound antibody was removed by three 10 minute washes in Hb/TBS and ^{125}I-protein A (Dupont-NEN) diluted to $1x10^5$ cpm/ml in Hb/TBS was added. The filters were agitated at 25°C for two hours. Finally, the filters were washed three times for 10 minutes with Hb/TBS, air dried and exposed to X-ray film at -70°C using a

Dupont Lightning Plus-Cronex screen.

A less frequently followed screening procedure used a horseradish peroxidase-conjugated second antibody. In this procedure, the keyed filters were washed in a 50mM Tris-HCl pH 8.0, 0.15M NaCl, 0.05% Tween-20 (Sigma) buffer (TBST), then gently agitated in TBST containing 1% BSA for 10 minutes at 25°C. The filters were agitated in serum 4-81 diluted as above in TBST/1%BSA for four hours. Unbound antibody was removed by three 10 minute washes with TBST. The remaining steps in this procedure use reagents from the Vectastain ABC kit (Vector Laboratories). Solution A containing avidin and solution B containing biotinylated horseradish peroxidase were diluted into TBST as directed and added to the filters. After agitation for 30 minutes, the filters were washed 3 times for 10 minutes in TBST. Finally, the filters were placed in a solution of TBS (no Tween-20) containing 4-chloro-napthol (6 mg per filter) and 0.015% hydrogen peroxide. The filters were monitored and when the colorimetric reaction had developed satisfactorily, they were washed rapidly several times in distilled H_2O to halt the reaction. Filters were stored wet at 4°C and protected from the light.

Purified phage were screened once with a partially purified fraction of antibodies from serum 4-81. This antibody fraction was purified by elution off nitrocellulose bound chartins. Details of this procedure are presented under "Protein-antibody blots". The screening procedure was as described except that the antibody fraction was diluted only 1 to 10 into the appropriate buffer.

Strain construction

Lysogens of the isolated phage were prepared by infection of the E.coli strain Y1089. About 10^4 phage were spotted onto LB top agarose (0.7%) containing 40µg/ml ampicillin and 200µl of stationary phase Y1089 cells; the plates were incubated overnight at 30°C. Cells from the infected areas were streaked onto LB-amp plates containing 80µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and previously spread with lambda cI phage; the plates were incubated overnight at 30°C. White colonies from these plates were tested for lysogeny by growth at 30°C and 42°C on LB-amp-X-gal plates. Bacteria which grew only at 30°C carry lysogenized phage.

The mouse brain cDNAs of interest were subcloned into the plasmid pGEM-2 (Promega). The cDNA was purified by elution off glass beads (Vogelstein and Gillespie, 1979) and ligated overnight at a molar ratio of 1 to 1 with the EcoR1 digested plasmid backbone. E.coli strain HB101 LM1035 was transformed with the constructed plasmids.

For DNA sequencing, the 2.3kb 15B1 cDNA was cloned into phage M13. Procedures and materials for the phage construction were from the New England Biolabs M13 Cloning and Sequencing System and corresponding manual.

The 2.3kb 15B1 cDNA was also subcloned into the path N plasmids (obtained from T. Koerner; Spindler et al, 1984) in order to construct a trpE-15B1 fusion protein. The purified insert was ligated at a molar ratio of 1 to 1 into the EcoR1 sites of path 1,10 and 11. These plasmids carry EcoR1 sites in the three different reading frames with respect to the trpE coding region. Six 15B1 containing plasmids were

constructed and transformed into the E.coli strain RR1. One such transformed strain produced a trpE-15B1 protein.

Fusion protein purification

The β-galactosidase-mouse brain fusion proteins 11A4,12A2 and 15B1 were all purified as follows. 500ml of LB media containing 40μg/ml ampicillin was inoculated with 15ml of an overnight culture and grown aerobically at 30°C until the culture reached mid-log phase. The cells were then induced at 43°C for 15 minutes, isopropylthiogalactoside (IPTG) was added to a final concentration of 2mM and the culture grown aerobically for 3 hours at 37°C. Cells were then harvested, pelleted and suspended in 30ml 0.2M Tris-HCl pH 7.5, 0.2M NaCl, 1mM DTT, 1mM EDTA and 5% glycerol (breaking buffer). This cell suspension was sonicated at 4°C to rupture the cell walls and cell debris was pelleted by centrifugation at 10k rpm, 4°C, for 15 minutes. SDS to 1.5% and β-mercaptoethanol to 5% were added to the resulting supernatant and the proteins were separated by SDS-PAGE (7.5%). The gels were briefly stained in a 50% methanol, 7% acetic acid solution containing 0.0125% Coomassie brillant blue. Gel slices containing the fusion proteins were excised and the proteins eluted and concentrated at 4°C overnight across a drop of 100 volts in an ISCO electrophoretic sample concentrator. The inner chambers of the apparatus contained 0.025M Tris, 0.192M glycine and 0.1% SDS (running buffer) and the outer chambers 10x running buffer. The concentrated proteins were used immediately or stored frozen at -30°C.

Preparation of the trpE-15B1 protein was according to the method of Koerner (T.J. Koerner, personal communication and Spindler et al, 1984). 10ml of M9 media containing 0.5% casamino acids (Difco), 20μg/ml tryptophan (Sigma) and 40μg/ml ampicillin was inoculated with cells grown on LB/tryptophan plates and aerated at 37°C until the culture reached the mid-log phase of growth. These cells were then added to 100ml of M9 media as described, but without tryptophan. Cells were aerated at 37°C for one hour, indoleacrylic acid (Sigma) was then added to 20μg/ml and the cells aerated again for 4 hours. This culture was left overnight at 4°C. The cells were then harvested, pelleted, washed in 10mM Tris-HCl pH 7.5, suspended in 20ml of 50mM Tris-HCl pH 7.5, 5mM EDTA, 3mg/ml lysozyme and left on ice for 2 hours. 1.4ml of 5M NaCl was then added, followed by 1.5ml 10% NP-40. This mix was left on ice for 30 minutes, after which the solution was sonicated to shear the DNA and cell debris was pelleted by centrifugation at 10K rpm for 10 minutes at 4°C. Although both the trpE and trpE fusion proteins are expected to fractionate with the insoluble material, most of the trpE-15B1 protein remained soluble. The protein was further purified from the supernatant by separation on SDS-PAGE followed by elution and concentration as described above.

Immunizations

The antigen preparations were mixed with Freund's complete adjuvant and injected subcutaneously into New Zealand White rabbits at multiple sites (three to five sites). Antigen for secondary injections was mixed with incomplete adjuvant, injections started

three weeks after the primary injection and they occurred every 2-3 weeks thereafter.

Preparation of microtubule associated proteins from calf brain

Calf brain microtubule protein (MTP) was first prepared by two cycles of temperature dependent microtubule assembly and disassembly (Borisy et al, 1975). This MTP fraction was then diluted to a concentration of 5 mg/ml in 0.025M MES (2[N-Morpholino]ethanesulfonic Acid) (Sigma), 1mM EDTA, 5mM $MgCl_2$, 1mM β-mercaptoethanol (PC buffer) and passed over a phosphocellulose column (Millipore). This column was pre-equilibrated with PC buffer. The m.a.p.s were eluted off the column in 0.35M NaCl PC buffer. In addition to chartins, this fraction contains MAP1, MAP2 and tau proteins. The fraction is called the PC MAPs fraction.

Protein-antibody blots

Protein preparations were separated by SDS-PAGE and transferred to nitrocellulose paper (Schleicher and Schuell, 0.2μ pore size, BA85). After electrophoresis, the gels were washed three times for 10 minutes each in 0.05M Na_2HPO_4/NaH_2PO_4 pH 6.8 (Na phosphate buffer) and the proteins transferred to nitrocellose paper in a Hoeffer Transfer apparatus (TE42) at 0.4 volts overnight (12-16 hours) in 0.05M Na phosphate buffer. After transfer, the nitrocellulose sheets were washed and probed with antibody by one of the three following procedures.

Some nitrocellulose bound proteins were probed with antibody and washed by the method of Towbin et al (1979), except that all the blocking and washing solutions contained 2% Hemoglobin (Sigma). Bound antibody was detected by incubating nitrocellulose in a solution containing 10^6 cpm/ml of ^{125}I-protein A (Dupont-NEN) and exposing the air dried nitrocellulose to X-ray film with an intensifying screen at -70°C. The second procedure for antibody incubation and washes was as described in Paul et al (1986), except that the incubation of the nitrocellulose with the antibody solution was overnight (10-14 hours). The third method for immunoblotting made use of the Vectastain kit reagents and the procedure was as described above.

The whole antisera 4-81 and 6-99 were diluted for immunoblotting into buffers at a ratio of 1:50. Partially purified antibody fractions from these sera were prepared by the following method and were diluted at ratios of 1:10-20.

The method of Olmsted (1981) was used to affinity elute antibodies from serum 4-81 and 6-99. Either PC MAPs or bacterial lysate containing the 15B1 fusion protein were separated by SDS-PAGE and transferred to nitrocellulose as described above. The nitrocellulose sheets were blocked and probed with antibody overnight by one of the methods described above. Regions of the nitrocellulose which contained bound chartins (of the PC MAPs) or fusion protein were then cut out using autoradiographs as guides. Antibodies were eluted from these nitrocellulose pieces with a 0.23M glycine buffer, pH 2.6. 1M Tris-HCl pH 8.1 was added to the eluate to neutralize the solution. Eluted antibodies were active and could be used after storage at 4°C for up to one week.

DNA sequencing

DNA sequence was determined by dideoxynucleotide chain termination sequencing as described in the New England Biolabs manual (Sanger et al, 1977). All materials for the dideoxynucleotide chain termination sequencing were obtained from New England Biolabs. The DNA sequence of both strands was determined by sequencing overlapping fragments of the 2.3kb 15B1 cDNA.

Overlapping fragments of the insert were isolated by two methods and cloned into the M13 phage as described in the above manual. Overlapping 15B1 DNA fragments of the complementary DNA strand (the strand not transcribed) were obtained by the method of Dale et al (1985). Procedures and materials were from the IBI Cyclone System. This method did not yield enough overlapping fragments to sequence through all 2.3kb. Furthermore, the exonuclease digestion of the transcribed DNA strand by this method was completely unsuccessful. The sequence of the transcribed strand as well as the remaining sequence of the complementary strand were determined by analysis of fragments generated by restriction endonuclease digestions.

Preparation of DNA and DNA fragments

Three confluent 15cm tissue culture dishes of mouse NB2A or 3T3 cells were washed with PBS_A and then the cells collected in a total of 3ml 10mM Tris-HCl pH 8.0, 0.15M NaCl, 10mM EDTA, 0.2% SDS and 200μg/ml Proteinase K (Sigma). The cells were incubated in this buffer at 37°C overnight (10-14 hours). An equal volume of phenol was added to the

cells and the mixture gently rocked for at least one hour at 25°C. The phenol used in all nucleic acid extraction was saturated with 100mM Tris-HCl pH 7.6, 10mM EDTA and contained 0.2% w/v 8-hydroxyquinoline. The organic and aqueous layers were separated by centrifugation for 10 minutes at 2k rpm. An equal volume of chloroform was added to the aqueous layer and the mixture again rocked at 25°C for at least one hour. Chloroform for nucleic acid extraction was a mixture of chloroform and isoamyl alcohol at a ratio of 24:1. The phases were separated and one tenth volume 3M Na acetate and 2.5 volumes 100% ethanol were added to the aqueous phase. The solution was kept at -70°C overnight to precipitate the DNA. DNA was removed from solution with a sealed pasteur pipette, dried and suspended in approximately 0.2ml of 10mM Tris-HCl pH 7.5, 1mM EDTA (TE).

Phage DNA was prepared from induced lysogen cultures by the method of Pirrota *et al* (1971).

Plasmid DNA was isolated by two methods, one yielding small analytical quantities of plasmids and the other preparative amounts. In the small scale prep (mini-prep), plasmid DNA was extracted from a 2ml overnight culture of transformed bacteria (2ml LB with 40μg/ml ampicillin). The cells were pelleted, suspended in 100μl of 50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA. 200μl of 0.2M NaOH, 1% SDS in TE was added, the suspension mixed manually and left on ice. After 5 minutes, 150μl of 5M K acetate, 5M acetic acid pH 4.8 was added, mixed in manually and the suspension again left on ice for 5 minutes. Cell debris was pelleted and the nucleic acid in the supernatant precipitated with 0.6 volume of isopropanol. This precipitate was pelleted, air dried,and the pellet suspended in 40μg/ml RNase in TE.

The RNA was digested for 15 minutes at 25°C. The plasmid DNA was finally extracted twice with equal volumes of phenol and chloroform, once with chloroform and precipitated as described. The pelleted, dried DNA was suspended in TE. This procedure yields enough plasmid to analyze the banding patterns of DNA fragments from two or three restriction enzyme digests.

Larger quantities of plasmid DNA (approximately 1-3mg) were prepared by a scaled up version of the above procedure. A 100ml overnight culture of bacteria was harvested and nucleic acid extracted precisely as described except that solution volumes were twenty-fold greater. After precipitation of the nucleic acids with isopropanol, the dried pellets were suspended in 4.5ml TE. CsCl to a concentration of 1.1g/ml and 100µl of 10mg/ml ethidium bromide in distilled H_2O were added, the solution loaded into VTi 65 Quick Seal tubes (Beckman), the tubes sealed and centrifuged at 45k rpm, 20°C for a minimum of 9 hours. Bands of plasmid DNA were collected with a 20 gauge needle. The ethidium bromide was removed by extraction and phase separation with H_2O- and NaCl-saturated isopropanol. The aqueous phase was then diluted with three volumes of TE and the DNA precipitated as described. The final pellet was suspended in TE and stored at 4°C.

Fragments of DNA created by restriction enzyme digestions were isolated by agarose gel electrophoresis followed by glass bead elution (Vogelstein and Gillespie, 1979).

Preparation of RNA

Total cellular RNA was extracted from mouse tissue by a procedure adapted from Chirgwin et al (1979). Tissues were dissected out of adult Balb/C mice, rinsed with cold PBS_A and frozen in a dry ice/ethanol bath until use. The frozen tissues were minced and thawed in 4M guanidinium thiocyanate, 25mM Na citrate pH 7.0, 0.5% sarkosyl and 0.1M β-mercaptoethanol. This tissue suspension was passed several times through a 16 1/2 gauge needle and then through a 23 gauge needle. The tissue homogenate was loaded onto a 2.5ml 5.7M CsCl, 0.1M EDTA pH 7.0 cushion and centrifuged at 35k rpm, 15°C for 15-18 hours. The CsCl supernatant was aspirated off and the pellets air dried. Pellets were suspended in sterile 1% SDS/TE and precipitated on dry ice as described. The precipitated RNA pellets were washed with 70% ethanol, air dried, suspended in RNase free H_2O and stored at -80°C.

Cytoplasmic RNA was isolated from tissue culture cells by a phenol-chloroform extraction procedure (Bond and Farmer, 1983). Twenty 10cm confluent tissue culture dishes of cells were washed once with ice cold PBS_A and then scraped into a total of 40ml ice cold PBS_A. Cells were pelleted, resuspended in 1.8ml cold PBS_A, pelleted again, the supernatant asperated and the pelleted was dried. The pellet was then suspended in 1.8ml of cold 10mM NaCl, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.3, 10mM vanadyladenosine, vortexed and held on ice. NP-40 was added to a concentration of 1%, the suspension was vortexed for 15 seconds and the nuclei pelleted in an Eppendorf centrifuge for 30 seconds. The supernatant was transferred to eppendorf tubes containing 10% SDS for a final concentration of 1% SDS. An equal

volume of phenol and chloroform was added, the mixture vortexed and centrifuged for five minutes. The aqueous phase was extracted twice more as above, then once with chloroform and precipitated as described. The precipitated RNA was finally suspended in RNase-free H_2O and stored at -80°C.

Isolation of polyadenylic acid-containing RNA (polyA$^+$ RNA) was accomplished by passage over an oligodeoxythymidylic acid (oligo dT) cellulose (Pharmacia) column. Cytoplasmic or total cellular RNA was precipitated, suspended in 0.5M NaCl, 10mM Tris-HCl pH 7.5, 0.5% SDS, 1mM EDTA (binding buffer) and heated to 55^oC. The RNA was then passed over the oligo dT column 3-5 times and the column washed with 10 volumes of binding buffer. PolyA$^+$ RNA was eluted from the column with six 250µl volumes of 10mM Tris-HCl pH 7.5, 0.05% SDS, 1mM EDTA. RNA was precipitated as described from each 250µl eluate as soon as it came off the column. The precipitated RNA was suspended in RNase-free H_2O and stored at -80^oC.

Nucleic acid filter hybridization

The procedure for Northern blot analysis was exactly as described in Bond and Farmer (1983). Southern blot analysis was by the method of Maniatis et al, (1982). ^{32}P-α-ATP-labeled cDNA was prepared by nick translation as described in Spiegelman and Farmer (1982). Although both a pGEM-2 plasmid containing the fragment of interest as well as the isolated fragment were used as probes, the labeled fragment alone resulted in lower background signal on the filters.

^{32}P-α-ATP labeled DNA was not used more than one week after radiolabeling.

Peptide mapping

Proteins were cut from dried one-dimensional polyacrylamide gels, rehydrated in 0.2M Tris-HCl pH 6.8, 0.1% SDS, 10% glycerol, and 1mM EDTA, partially digested with either alpha chymotrypsin, trypsin-TPCK (both from Millipore Corp.) or SV8 protease (Boehringer Mannheim Biochemicals) and analyzed on 12% polyacrylamide gels (Cleveland _et al_, 1977). Partial digestion was achieved by incubation of the proteins for 30 minutes with 10,30 or 100μg of chymotrypsin or trypsin-TPCK and 0.01, 0.03, or 0.1μg of SV8 protease.

Results

Isolation and characterization of cDNA from a mouse brain λgt11 expression library

The rabbit polyclonal anti-chartin serum 4-81 (Magendantz and Solomon, 1985) was used to screen a mouse brain λgt11 library (provided by Y. Citri; Citri _et al_, 1987) by the method of Young and Davis (1983). Phage plaques stained by serum 4-81 were purified and screened again. All of the stained phage were screened through three rounds of purification. The pure phage were finally screened with an affinity eluted fraction of serum 4-81. The antibody fraction was eluted off of the 69kd and 80kd regions of phosphocellulose purified calf brain microtubule protein (PC MAPs) immunoblots (Olmsted, 1981). Such purification of the anti-chartin antibodies minimized the possibility that an irrelevent antibody in the 4-81 serum reacted with a phage protein, resulting in a falsely positive signal. This final screen eliminated all but three phages. Each phage was isolated from different, separately amplified size fractions of the mouse brain library and are therefore independent isolates. Data to be presented show that two of these phage probably contain identical cDNA inserts.

All three phages were lysogenized into the E.coli strain Y1089 and protein extracts of the respective induced and uninduced cell lysates were analyzed by immunoblots. Figure 12 shows that serum 4-81 stains the β-galactosidase-mouse brain fusion protein from all three induced lysogens. All other stained bands are also present in the uninduced cell extracts (lanes b,d,f,h,j,l and n). Serum 4-81

Figure 12.

Recognition of β-galactosidase-mouse brain fusion proteins by an anti-chartin serum.

Proteins from whole cell lysates of induced bacterial lysogens (lanes a,c,e,g,i,k and m) and uninduced bacterial lysogens (lanes b,d,f,h,j,and n) were separated by one-dimensional SDS-PAGE and analyzed by immunoblot. Serum 4-81 specifically stains the induced fusion proteins from lysogens 15B1, 12A2 and 11A4. Neither the fusion protein of a fourth lysogen, 11B7 (lane e), nor β-galactosidase itself (lane m) are stained by the antibody. The 10-1B phage (and lysogen) was isolated from a λgt11 rat liver library and shown to be unrelated to chartins.

serum 4-81

undoubtedly contains may anti-bacterial protein antibodies as well as the anti-chartin antibodies of interest; multiple stained bands were anticipated.

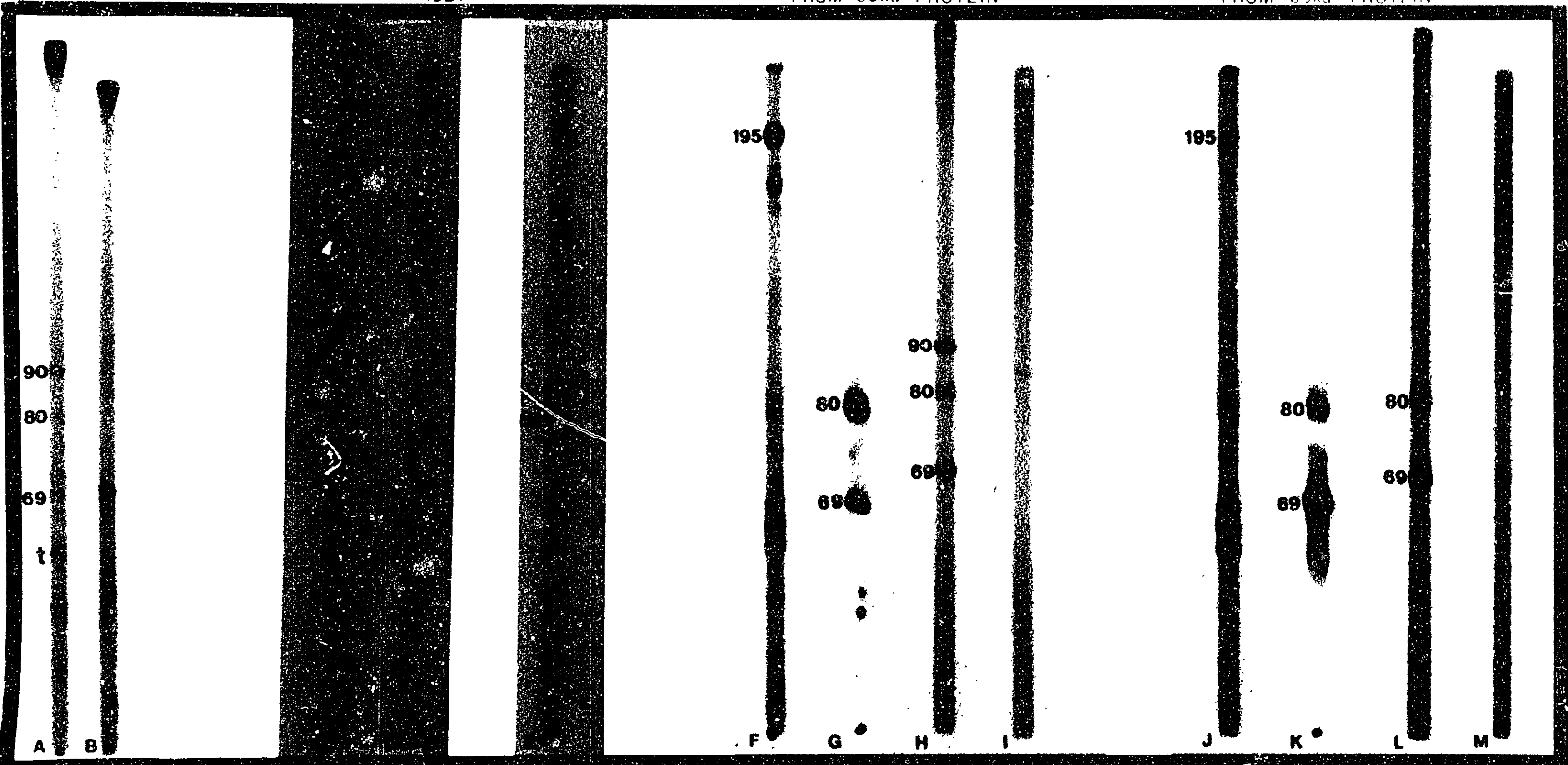
These three fusion proteins, called 11A4, 12A2, and 15B1 were all purified out of induced cell lysates for use as antigen. All three proteins are fairly large; 11A4 and 15B1 each have molecular weights of about 200kd and 12A2 one of 160kd. The proteins were purified by excision from Coomassie blue-stained gels followed by electro-elution and concentration from the gel slices (see Materials and Methods).

The protein eluates were mixed one to one with Complete Freund's adjuvant and injected into rabbits. After a total of three injections with antigen, antisera from the rabbits were screened for reactivity to both the fusion protein antigen and chartins. Only one rabbit and thus one fusion protein, 15B1, yielded an anti-fusion protein/anti-chartin serum. This serum, named 6-99, stained both the β-galactosidase-mouse brain fusion protein originally used as antigen and several bands in neuroblastoma microtubule protein extracts. Figure 13, panel 1 shows mt+ and mt- protein extracts from NB2A cells; there are at least four bands stained only in the mt+ extract. These protein bands have approximate molecular weights of 55, 69, 80 and 90kd. The lowest band is probably tubulin. Tubulin is one of the most abundant proteins in the mt+ extracts and frequently binds IgG molecules in such preparations. Further dilution of the serum eliminates staining of only the 55kd band. The bands of 69 and 80kd could be chartins. The identity of the 90kd protein is unclear, but the protein fractionates as a microtubule associated protein. Serum 6-99 apparently recognizes several microtubule proteins.

Figure 13.

Identification of several microtubule associated proteins by and anti-15B1 fusion protein serum.

The autoradiograms show strips of nitrocellulose-bound proteins analyzed by immunoblot. Panel 1 shows NB2A proteins from the mt+ (A) and mt- (B) fractions of cells. Antibodies from the whole anti-fusion protein serum 6-99 stain four microtubule proteins: tubulin, the 69 and 80kd chartins and a 90kd protein band. Tubulin is nonspecifically stained (see text). Panel 2 shows proteins stained by a purified fraction of antibodies from serum 6-99. The antibodies were purified by elution off the 15B1-fusion protein. These antibodies stain both a 90kd protein in a NB2A mt+ fraction (C) and an 80kd band in calf brain PC Maps (E). Panels 3 and 4 show strips of proteins from 15B1 IPTG-induced bacterial lysate (F and J), calf brain PC Maps (G and K), mt+ NB2A cell fractions (H and L) and mt- NB2A cell fractions (I and M). The strips of panel 3 are probed with serum 6-99 antibodies eluted off the 80kd region of PC Maps proteins strips. These antibodies stain the 195kd β-galactosidase-mouse brain fusion protein (15B1) (F), the 69 and 80kd bands of PC Maps (G) and the 69, 80 and 90kd bands of the NB2A mt+ fraction (H). The strips of panel 4 are probed with serum 6-99 antibodies eluted off the 69kd region of PC Maps protein strips. This antibody fraction stains the same proteins stained in panel 3, with the exception of the 90kd protein of neuroblastoma cells (L).


ANTISERUM 6-99
ANTI- 15B1 FUSION PROTEIN ANTISERUM
WHOLE SERUM
AFFINITY ELUTED FROM FUSION PROTEIN 15B1
AFFINITY ELUTED FROM CALF BRAIN MAPs
FROM 80kd PROTEIN
FROM 69kd PROTEIN
90
80
69
t
195
A
B
F
G
H
I
J
K
L
M
MT+ MT-
NBA1
MT+ MT-
NBA1
CALF BRAIN
PC MAPs
BACTERIAL LYSATE
PC MAPs
MT+ MT-
NBA1
BACTERIAL LYSATE
PC MAPs
MT+ MT-
NBA1
1
2
3
4

In order to confirm that the antibodies in serum 6-99 which stain the fusion protein also stain the microtubule proteins of mouse, serum 6-99 antibodies were fractionated by affinity elution. Antibodies were eluted off both nitrocellulose bound 15B1 protein and the chartins of PC MAPs. These antibody preparations were used to probe protein blots of induced lysogen extract, PC MAPs and the assembled microtubule protein fraction from NB2A cells. Figure 13 shows the results of this experiment.

Panel 2, lanes C, D and E show that 6-99 antibodies eluted off of the fusion protein recognize primarily the highest molecular weight band in both NB2A assembled microtubule fractions (90kd) as well as PC MAPs (80kd). Faint bands corresponding to NB2A proteins of 69 and 80kd do appear when the immunoblot is exposed for long times. The high background may obscure the appearance of a 69kd band in longer exposures of the PC MAPs strips. No band corresponding to tubulin is stained. In fact, virtually no bands at 55kd were seen when any cell extract was probed with a purified antibody fraction.

Lanes G, H and I show nitrocellulose strips analogous to those in lanes C,D and E. Lanes F and J contain extract from induced λgt11-15B1 lysogen. These nitrocellulose strips have been probed with 15B1 antibody eluted from the chartins in PC Maps. The strips of panel 3 were probed with antibody eluted from the 80kd region while those of panel 4 were probed with antibody eluted from the 69kd region. The fusion protein as well as the 69 and 80kd chartins in PC Maps are stained by both eluted antibody preparations. Tubulin is not stained. The 90kd protein of NB2A cells is only identified by the antibody eluted off the 80kd region of PC Maps. Careful examination

of PC Maps probed with either the whole 15B1 antiserum or 4-81 shows that two closely migrating bands are stained in the 80kd region. It is possible that the upper band of the doublet is a protein related to the 90kd protein of NB2A cells.

The staining of chartins and the 15B1 fusion protein by affinity eluted antibodies from serum 6-99 demonstrates that the fusion protein carries at least one epitope in common with the chartins. These same antibodies have also identified a new microtubule associated protein of about 90kd in NB2A cells. Because this protein is stained by affinity eluted antibodies, it shares at least one epitope with the 15B1 and chartin proteins; the 90kd protein may be related to the chartins.

Structural comparison of chartin and mouse brain fusion proteins

The 69 and 80kd chartins from PC MAPs and a 15B1 fusion protein were analyzed by one-dimensional partial cleavage peptide mapping. Such maps, if similar, would suggest that 15B1 cDNA is chartin specific. However, results from this experiment could be misleading for several reasons. First, analysis of the fusion protein map will be complicated by bacterial peptides and bacterial-mouse peptides not present in the chartin map. Second, the folding of the mouse brain portion may differ from that of the native protein. Finally, the low abundance of the chartins in mouse culture cells and the small size of mouse brains prevented analysis of mouse chartins. Calf brain chartins were mapped; these chartins may differ from mouse chartins enough to complicate analysis of the data.

These potential complications of the experiment were addressed in two ways. First, problems which could be caused by the bacterial portion of the fusion protein were minimized by the construction of a trpE-15B1 protein. The trpE bacterial protein has a molecular weight of 37kd, about 78kd less than β-galactosidase. Therefore, the trpE-15B1 protein contains approximately twice as much mouse protein as bacterial protein. This protein may be more likely than the β-galactosidase fusion protein to allow native protein-like folding of the mouse derived amino acid sequence. The constructed trpE-15B1 fusion protein, like the β-galactosidase-15B1 protein, is stained by serum 4-81. Figure 14 shows whole cell lysates of E.coli strain RR1 transformed with the plasmids path 11, 11-1 and 11-6. The plasmid path 11 carries no 15B1 insert, while plasmids 11-1 and 11-6 carry the insert in opposite orientations. One band of approximately 120kd is stained in only the path 11-1 transformed cell lysate. This band is therefore the plasmid-specific trpE-15B1 fusion protein. Second, comparisons of the chartin and trpE-15B1 peptide maps were limited to peptides having some chartin native structure; peptides which carried the reactive NB2A chartin epitope were specifically stained by serum 4-81 in immunoblot analysis of the peptide maps. This immunoblot analysis stained several peptides (up to five) in maps of the trpE-15B1 protein as well as those of the 69 and 80kd chartins. Some of the peptides among the maps had similar molecular weights, some did not. Thus, the results of this experiment were uninterpretable.

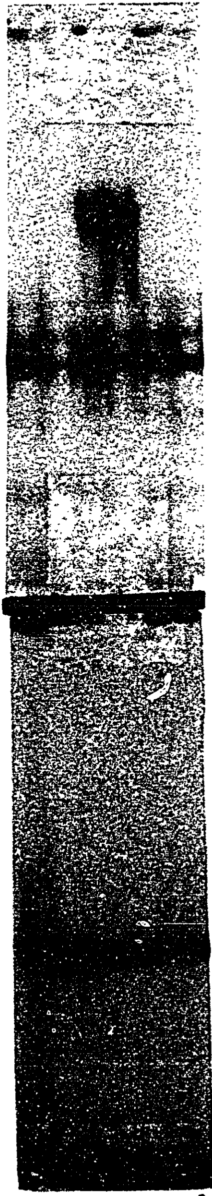
Figure 14.

Identification of the trpE-15B1 fusion protein, 11-1, by serum 4-81.

The autoradiograms show blots of proteins from uninduced whole bacterial lysates (lanes a,c and e) and induced whole bacterial lysates (lanes b,d and f). These blots are probed with either serum 4-81 or pre-immune rabbit serum. The lysates are from the E.coli strain RR1 transformed with the empty Path 11 plasmid (a and b), the 11-1 plasmid carrying the trpE-15B1 gene (c and d) and the 11-6 plasmid carrying 15B1 DNA inserted in the opposite orientation to that in 11-1. Serum 4-81 specifically stains a band in the 11-1 lysates which is enhanced in the induced cell lysate. This 120kd protein is the trpE-15B1 fusion protein.

PATH 11
PATH 11 INDUCED
11-1
11-1 INDUCED
11-6
11-6 INDUCED

4-81



PRE-IMMUNE

a b c d e f

Two chartin epitopes are present on the trpE-15B1 fusion protein

One recent experiment done in collaboration with M. Magendantz strongly suggests that 15B1 is chartin-specific. The trpE-15B1 protein was identified as such because it carries one chartin-like epitope; immunoblot analysis of induced bacterial lysates show that serum 4-81 specifically stains the trpE-15B1 protein (Figure 14, lanes C and D). This same fusion protein is also stained by another anti-chartin serum in immunoblot analysis. This serum, called 3417 (Magendantz and Solomon, 1985), was raised against proteins cut from the 69kd region of calf brain PC MAPs separated on gels. This serum stains only the 80kd chartins of both NB2A cells and PC MAPs (Magendantz and Solomon, 1985; Figure 15, lanes A and B). Figure 15, lanes C and D show that this serum also stains the trpE-15B1 protein. Therefore, two antisera raised against chartins and specific for different epitopes recognize a 15B1 fusion protein.

Structural analysis of the isolated cDNA inserts

In parallel with the characterization of the mouse brain fusion proteins by antigen/antibody studies, their corresponding cDNAs were also analyzed. The cDNA inserts of all three isolated phage were excised by EcoR1 digestion and subcloned into the high copy number plasmid pGEM-2. This subcloning allowed the insert DNA to be isolated in large quantity for restriction map and sequence analysis. The three inserts are named in the same way as their corresponding fusion proteins: 11A4, 12A2 and 15B1. 15B1 and 11A4 are approximately 2.3kb

Figure 15.

Identification of the 11-1 protein by a second anti-chartin serum, 3417.

The autoradiograms show immunoblots of the mt+ fraction (A) and mt- fraction (B) from NB2A cells, the bacterial cell lysate of strain 11-1 (C and E) and the purified protein 11-1 (D). The nitrocellulose strips are probed with either serum 3417 (A,B,C,D) or pre-immune serum (E). Serum 3417 stains only the 80kd chartin (A) and the 11-1 fusion protein (C,D). There is probably some degradation of the fusion protein in the whole cell lysate (C).

A
B
C
D
E
mt+
mt−
whole cell
11−1
whole cell

while 12A2 is about 1.2kb in length. Their restriction maps are shown in Figure 16 (work done in collaboration with M. Magendantz).

The maps of the subcloned EcoR1 fragments of 11A4 and 15B1 are identical, while that of 12A2 is entirely different; the two maps shown have no pattern of restriction sites in common. Consistent with this result, the 15B1 insert does not hybridize to the 12A2 insert. The restriction map of 11A4 and 15B1 strongly suggests that these EcoR1 fragments of the two independently isolated phage inserts are identical.

Both the antigen/antibody studies and the restriction map analyses suggest that further characterization of the mouse brain DNA be confined to the 15B1 protein and cDNA. As mentioned above, the restriction maps of 11A4 and 15B1 show that the phage isolates carry identical inserts; further work on the 11A4 clone would therefore be redundant. Study of the 12A2 protein and insert was discontinued because rabbits injected with the 12A2 protein did not yield antisera of interest; thus, a common epitope with chartins could not be confirmed.

The direction of translation of 15B1 was determined by restriction map analysis of the λgt11-15B1 phage. λgt11 DNA with no insert is restricted by the endonuclease Kpn1 into 1.5 and 41.5kb fragments. The 15B1 insert is restricted by Kpn1 into 400, 700 and 1200bp fragments (see Figure 17). When the λgt11-15B1 phage is restricted by Kpn1, the 1200bp fragment disappears; this result oriented the 15B1 DNA in the phage and determined its direction of translation (shown in figure 17).

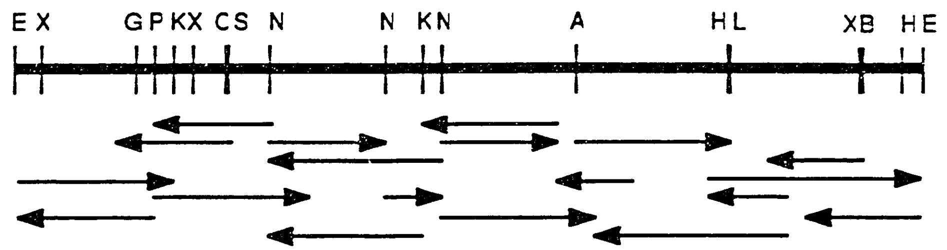
The next step in the study of the 15B1 clone was to determine its

Figure 16.

Restriction maps of cDNA inserts 15B1,11A4 and 12A2.

The restriction maps of 15B1 and 11A4 (A) and 12A2 (B) are shown. The sites of restriction enzyme cleavage are designated by the following one letter code: A-AhaII, B-BamHI, C-ClaI, E-EcoRI, G-BglII, H-HincII, K-KpnI, L-SalI, N-NsiI, P-PstI, S-SacI and X-XhoII. 12A2 was mapped using enzymes B,C,E,G,H,L and P. 15B1 was more extensively mapped. The two maps demonstrate that the cDNA inserts are not closely related. The arrows below the map of 15B1 and 11A4 (A) show the strategy used for sequencing 15B1 from its various restriction fragments.

15B1 & 11A4



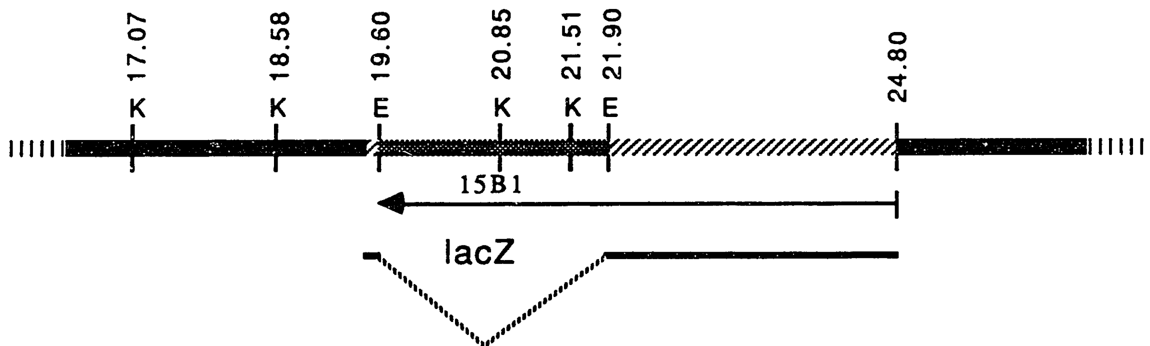
12A2

Figure 17.

Orientation of the 15B1 cDNA insert in the λgt11 phage.

The restriction map of the λgt11 15B1 phage shows the orientation of the cDNA insert in the phage. This orientation was determined by cleavage of the phage with the endonucleases EcoRI (E) and KpnI (K). Cleavage of the phage with both endonucleases releases fragments of approximately 400, 700 and 1200bp specific to the 15B1 insert, as well as fragments of about 1.5 and 1.1kb specific to the phage. Cleavage with KpnI alone releases only the 700bp 15B1 fragment and one new fragment of about 2.3kb containing λgt11 and 15B1 sequence.

LAMBDA GT11-15B1



DNA sequence. These data are important for at least three reasons. First, a comparison of the 15B1 sequence with the GenBank databank can determine whether or not 15B1 is specific for a protein other than chartin. Second, this sequence will help to more rapidly identify sister clones presently being isolated from the mouse brain library (K. Bupp, work in progress). Finally, as previously discussed, one future goal of this work is to better understand the structural differences among members of the chartin family. The sequence of 15B1 is probably at least a partial sequence of one chartin.

To obtain sequence, the 15B1 insert was excised and cloned into phage M13. DNA sequence was obtained by the dideoxynucleotide chain termination procedure from N.E. Biolabs. The sequence was determined off both DNA strands and is shown in Figure 18 (work done by M. Magendantz). No stop codons are present in the sequence. A search of the GenBank databank showed that neither the 15B1 DNA sequence nor its predicted amino acid sequence is homologous to any of the published sequences in the databank.

Identification of genomic DNA and mRNA homologous to 15B1 cDNA

Genomic DNA and mRNA sequences from mouse cultured cells and brain tissue homologous to 15B1 cDNA were identified by Southern (Maniatis _et al_, 1982) and Northern (Bond and Farmer, 1983) blot hybridization. For Southern blot analysis, DNA was extracted from both mouse 3T3 and NB2A cells, aliquots were digested separately with three restriction endonucleases and processed for analysis. Figure 19 shows such a blot probed with nick-translated ^{32}P-α-ATP-labeled 15B1

Figure 18.

DNA and predicted amino acid sequence of the 15B1 mouse brain cDNA.

The 15B1 cDNA was subcloned into the phage M13 in both orientations in order to sequence both DNA strands. The strategy for sequencing the approximately 2.3kb insert is shown in figure 16. The sequence may consist entirely of open reading frame. The predicted amino acid sequence is shown directly below the DNA sequence.

```
    GAATTCCGCCCCGGCACCCCCGGACCGACCATGGGCAGATCCCAGGGCAGCCCGATGGAC
1   ---------+---------+---------+---------+---------+---------+ 60
    CTTAAGGCGGGGCCGTGGGGGCCTGGCTGGTACCCGTCTAGGGTCCCGTCGGGCTACCTG

    E  F  R  P  G  T  P  G  P  T  M  G  R  S  Q  G  S  P  M  D  -

    CCAATGGTGATGAAGAGACCTCAGTTGTATGGGATGGGTACTCACCCCCACTCCCAGCCA
61  ---------+---------+---------+---------+---------+---------+ 120
    GGTTACCACTACTTCTCTGGAGTCAACATACCCTACCCATGAGTGGGGGTGAGGGTCGGT

    P  M  V  M  K  R  P  Q  L  Y  G  M  G  T  H  P  H  S  Q  P  -

    CAGCAGAGCAGCCCATACCCAGGAGGCTCCTACGGTCCCCCAGGTGCACAGCGGTATCCC
121 ---------+---------+---------+---------+---------+---------+ 180
    GTCGTCTCGTCGGGTATGGGTCCTCCGAGGATGCCAGGGGGTCCACGTGTCGCCATAGGG

    Q  Q  S  S  P  Y  P  G  G  S  Y  G  P  P  G  A  Q  R  Y  P  -

    CTTGGCATGCAGGGCCGGGCTCCAGGGGCCCTGGGAGGCTTGCAGTACCCGCAGCAGCAG
181 ---------+---------+---------+---------+---------+---------+ 240
    GAACCGTACGTCCCGGCCCGAGGTCCCCGGGACCCTCCGAACGTCATGGGCGTCGTCGTC

    L  G  M  Q  G  R  A  P  G  A  L  G  G  L  Q  Y  P  Q  Q  Q  -

    ATGCCACCGCAGTACGGACAGCAAGCTGTGAGTGGCTACTGCCAGCAAGGCCAGCAGCCA
241 ---------+---------+---------+---------+---------+---------+ 300
    TACGGTGGCGTCATGCCTGTCGTTCGACACTCACCGATGACGGTCGTTCCGGTCGTCGGT

    M  P  P  Q  Y  G  Q  Q  A  V  S  G  Y  C  Q  Q  G  Q  Q  P  -

    TACTACAACCAGCAGCCGCAGCCCTCGCACCTCCCGCCCCAGGCACAGTACCTGCAGCCG
301 ---------+---------+---------+---------+---------+---------+ 360
    ATGATGTTGGTCGTCGGCGTCGGGAGCGTGGAGGGCGGGGTCCGTGTCATGGACGTCGGC

    Y  Y  N  Q  Q  P  Q  P  S  H  L  P  P  Q  A  Q  Y  L  Q  P  -

    GCGGCGGCGCAGTCCCAGCAGAGGTACCAGCCACAGCAGGACATGTCTCAAGAAGGCTAT
361 ---------+---------+---------+---------+---------+---------+ 420
    CGCCGCCGCGTCAGGGTCGTCTCCATGGTCGGTGTCGTCCTGTACAGAGTTCTTCCGATA

    A  A  A  Q  S  Q  Q  R  Y  Q  P  Q  Q  D  M  S  Q  E  G  Y  -

    GGAACTAGATCTCAGCCTCCTCTGGCCCCTGGAAAATCCAACCATGAAGACTTGAATTTA
421 ---------+---------+---------+---------+---------+---------+ 480
    CCTTGATCTAGAGTCGGAGGAGACCGGGGACCTTTTAGGTTGGTACTTCTGAACTTAAAT

    G  T  R  S  Q  P  P  L  A  P  G  K  S  N  H  E  D  L  N  L  -
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481 ---------+---------+---------+---------+---------+---------+ 540
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    I  Q  Q  E  R  P  S  S  L  P  D  L  S  G  S  I  D  D  L  P  -

    ACGGGAACAGAAGCAACTCTGAGCTCAGCAGTCAGTGCATCCGGGTCTACAAGCAGCCAG
541 ---------+---------+---------+---------+---------+---------+ 600
    TGCCCTTGTCTTCGTTGAGACTCGAGTCGTCAGTCACGTAGGCCCAGATGTTCGTCGGTC

    T  G  T  E  A  T  L  S  S  A  V  S  A  S  G  S  T  S  S  Q  -

    GGAGATCAGAGCAACCCAGCGCAGTCTCCTTTCTCCCCACATGCATCACCTCACCTCTCC
601 ---------+---------+---------+---------+---------+---------+ 660
    CCTCTAGTCTCGTTGGGTCGCGTCAGAGGAAAGAGGGGTGTACGTAGTGGAGTGGAGAGG

    G  D  Q  S  N  P  A  Q  S  P  F  S  P  H  A  S  P  H  L  S  -
```

```
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661  ---------+---------+---------+---------+---------+---------+ 720
     TCGTAGGGACCTCCCGGCAGTGGAAGAGGACAACCGAGAGGACACCCTTCGTTGGTTAGC

     S  I  P  G  G  P  S  P  S  P  V  G  S  P  V  G  S  N  Q  S  -

     AGGTCTGGTCCGATCTCCCCTGCGAGTATTCCAGGTAGCCAGATGCCTCCGCAACCACCT
721  ---------+---------+---------+---------+---------+---------+ 780
     TCCAGACCAGGCTAGAGGGGACGCTCATAAGGTCCATCGGTCTACGGAGGCGTTGGTGGA

     R  S  G  P  I  S  P  A  S  I  P  G  S  Q  M  P  P  Q  P  P  -

     GGAAGCCAGTCAGAATCCAGTTCCCATCCTGCCTTGAGCCAGTCACCAATGCCACAGGAA
781  ---------+---------+---------+---------+---------+---------+ 840
     CCTTCGGTCAGTCTTAGGTCAAGGGTAGGACGGAACTCGGTCAGTGGTTACGGTGTCCTT

     G  S  Q  S  E  S  S  S  H  P  A  L  S  Q  S  P  M  P  Q  E  -

     AGAGGTTTTATGACAGGCACTCAGAGAAACCCTCAGATGTCTCAGTACGGACCTCAGCAG
841  ---------+---------+---------+---------+---------+---------+ 900
     TCTCCAAAATACTGTCCGTGAGTCTCTTTGGGAGTCTACAGAGTCATGCCTGGAGTCGTC

     R  G  F  M  T  G  T  Q  R  N  P  Q  M  S  Q  Y  G  P  Q  Q  -

     ACAGGACCATCCATGTCGCCTCACCCATCTCCTGGGGGCCAGATGCATCCTGGGATCAGT
901  ---------+---------+---------+---------+---------+---------+ 960
     TGTCCTGGTAGGTACAGCGGAGTGGGTAGAGGACCCCCGGTCTACGTAGGACCCTAGTCA

     T  G  P  S  M  S  P  H  P  S  P  G  G  Q  M  H  P  G  I  S  -

     AACTTTCAGCAGAGTAACTCAAGTGGCACGTACGGCCCACAGATGAGCCAGTATGGACCC
961  ---------+---------+---------+---------+---------+---------+ 1020
     TTGAAAGTCGTCTCATTGAGTTCACCGTGCATGCCGGGTGTCTACTCGGTCATACCTGGG

     N  F  Q  Q  S  N  S  S  G  T  Y  G  P  Q  M  S  Q  Y  G  P  -

     CAAGGCAACTACTCCAGAACCCCAACATATAGCGGGGTACCCAGTGCAAGCTACAGCGGC
1021 ---------+---------+---------+---------+---------+---------+ 1080
     GTTCCGTTGATGAGGTCTTGGGGTTGTATATCGCCCCATGGGTCACGTTCGATGTCGCCG

     Q  G  N  Y  S  R  T  P  T  Y  S  G  V  P  S  A  S  Y  S  G  -
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1081 ---------+---------+---------+---------+---------+---------+ 1140
     GGTCCCGGGCCATACCCGTAGTTACGGTTGTTGGTCTACGTACCTGTTCCCGGTCGGGTC

     P  G  P  G  M  G  I  N  A  N  N  Q  M  H  G  Q  G  P  A  Q  -

     CCATGTGGTGCTATGCCCCTGGGACGAATGCCTTCAGCTGGGATGCAGAACAGACCATTT
1141 ---------+---------+---------+---------+---------+---------+ 1200
     GGTACACCACGATACGGGGACCCTGCTTACGGAAGTCGACCCTACGTCTTGTCTGGTAAA

     P  C  G  A  M  P  L  G  R  M  P  S  A  G  M  Q  N  R  P  F  -

     CCTGGAACCATGAGCAGCGTCACCCCCAGTTCTCCTGGCATGTCTCAACAGGGAGGGCCA
1201 ---------+---------+---------+---------+---------+---------+ 1260
     GGACCTTGGTACTCGTCGCAGTGGGGGTCAAGAGGACCGTACAGAGTTGTCCCTCCCGGT

     P  G  T  M  S  S  V  T  P  S  S  P  G  M  S  Q  Q  G  G  P  -

     GGAATGGGCCCACCAATGCCCACTGTGAACCGGAAGGCCCAGGAAGCTGCCGCAGCTGTG
1261 ---------+---------+---------+---------+---------+---------+ 1320
     CCTTACCCGGGTGGTTACGGGTGACACTTGGCCTTCCGGGTCCTTCGACGGCGTCGACAC

     G  M  G  P  P  M  P  T  V  N  R  K  A  Q  E  A  A  A  A  V  -
```

```
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1321 ---------+---------+---------+---------+---------+---------+ 1380
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     M  Q  A  A  A  N  S  A  Q  S  R  Q  G  S  F  P  G  M  N  Q   -

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1381 ---------+---------+---------+---------+---------+---------+ 1440
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     S  G  L  V  A  S  S  S  P  Y  S  Q  S  M  N  N  N  S  S  L   -

     ATGAGCACCCAGGCCCAGCCCTACAGCATGACGCCCACAATGGTGAACAGCTCCACAGCA
1441 ---------+---------+---------+---------+---------+---------+ 1500
     TACTCGTGGGTCCGGGTCGGGATGTCGTACTGCGGGTGTTACCACTTGTCGAGGTGTCGT

     M  S  T  Q  A  Q  P  Y  S  M  T  P  T  M  V  N  S  S  T  A   -

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1501 ---------+---------+---------+---------+---------+---------+ 1560
     AGATACCCAGAACGTCTATACTACAGAGGGTCACTCAGGTTTAACAGACACGGAGAATTT

     S  M  G  L  A  D  M  M  S  P  S  E  S  K  L  S  V  P  L  K   -

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1561 ---------+---------+---------+---------+---------+---------+ 1620
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     A  D  G  K  E  E  G  V  S  Q  P  E  S  K  S  K  D  S  Y  G   -

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1621 ---------+---------+---------+---------+---------+---------+ 1680
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     S  Q  G  I  S  Q  P  P  T  P  G  N  L  P  V  P  S  P  M  S   -
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1681 ---------+---------+---------+---------+---------+---------+ 1740
     GGGTCGAGACGGTCGTAGAGGAGGAAAGTACCTCTACTCTCACTGTCGTAATCGTCGGGT

     P  S  S  A  S  I  S  S  F  H  G  D  E  S  D  S  I  S  S  P   -

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1741 ---------+---------+---------+---------+---------+---------+ 1800
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     G  W  P  K  T  P  S  S  P  K  S  S  S  S  S  T  T  G  E  K   -

     ATCACGAAGGTCTATGAGCTGGGGAATGAGCCGGAGAGGAAGCTGTGGGTCGACCGTTAC
1801 ---------+---------+---------+---------+---------+---------+ 1860
     TAGTGCTTCCAGATACTCGACCCCTTACTCGGCCTCTCCTTCGACACCCAGCTGGCAATG

     I  T  K  V  Y  E  L  G  N  E  P  E  R  K  L  W  V  D  R  Y   -

     CTAACGTTCATGGAAGAGAGGGGCTCCCCGGTGTCCAGTCTGCCAGCAGTGGGCAAGAAG
1861 ---------+---------+---------+---------+---------+---------+ 1920
     GATTGCAAGTACCTTCTCTCCCCGAGGGGCCACAGGTCAGACGGTCGTCACCCGTTCTTC

     L  T  F  M  E  E  R  G  S  P  V  S  S  L  P  A  V  G  K  K   -

     CCCCTGGACCTGTTCCGACTGTATGTCTGCGTCAAGGAGATTGGAGGTTTGGCGCAGGTT
1921 ---------+---------+---------+---------+---------+---------+ 1980
     GGGGACCTGGACAAGGCTGACATACAGACGCAGTTCCTCTAACCTCCAAACCGCGTCCAA

     P  L  D  L  F  R  L  Y  V  C  V  K  E  I  G  G  L  A  Q  V   -
```

```
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1921 ---------+---------+---------+---------+---------+---------+ 1980
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     P  L  D  L  F  R  L  Y  V  C  V  K  E  I  G  G  L  A  Q  V   -

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1981 ---------+---------+---------+---------+---------+---------+ 2040
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     N  K  N  K  K  W  R  E  L  A  T  N  L  N  V  G  T  S  S  S   -

     GCAGCCAGCTCTCTGAAAAAGCAGTATATTCAGTACCTGTTCGCCTTTGAGTGCAAAACT
2041 ---------+---------+---------+---------+---------+---------+ 2100
     CGTCGGTCGAGAGACTTTTTCGTCATATAAGTCATGGACAAGCGGAAACTCACGTTTTGA

     A  A  S  S  L  K  K  Q  Y  I  Q  Y  L  F  A  F  E  C  K  T   -

     GAGCGCGGGGAGGAGCCCCCACCTGAAGTCTTCAGCACCGGGGATTCGAAGAAGCAGCCA
2101 ---------+---------+---------+---------+---------+---------+ 2160
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     E  R  G  E  E  P  P  P  E  V  F  S  T  G  D  S  K  K  Q  P   -

     AAGCTCCAGCCGCCATCTCCTGCTAACTCAGGATCCTTACAAGGCCCACAGACTCCACAG
2161 ---------+---------+---------+---------+---------+---------+ 2220
     TTCGAGGTCGGCGGTAGAGGACGATTGAGTCCTAGGAATGTTCCGGGTGTCTGAGGTGTC

     K  L  Q  P  P  S  P  A  N  S  G  S  L  Q  G  P  Q  T  P  Q   -

     TCAACTGGGAGCAATTCGATGGCAGAGGTTCCAGGTGACCTGAAGCCACCAACCCCAGCC
2221 ---------+---------+---------+---------+---------+---------+ 2280
     AGTTGACCCTCGTTAAGCTACCGTCTCCAAGGTCCACTGGACTTCGGTGGTTGGGGTCGG

     S  T  G  S  N  S  M  A  E  V  P  G  D  L  K  P  P  T  P  A   -
     TCGAATTC
2281 -------- 2288
     AGCTTAAG

     S  N  ?  -
```
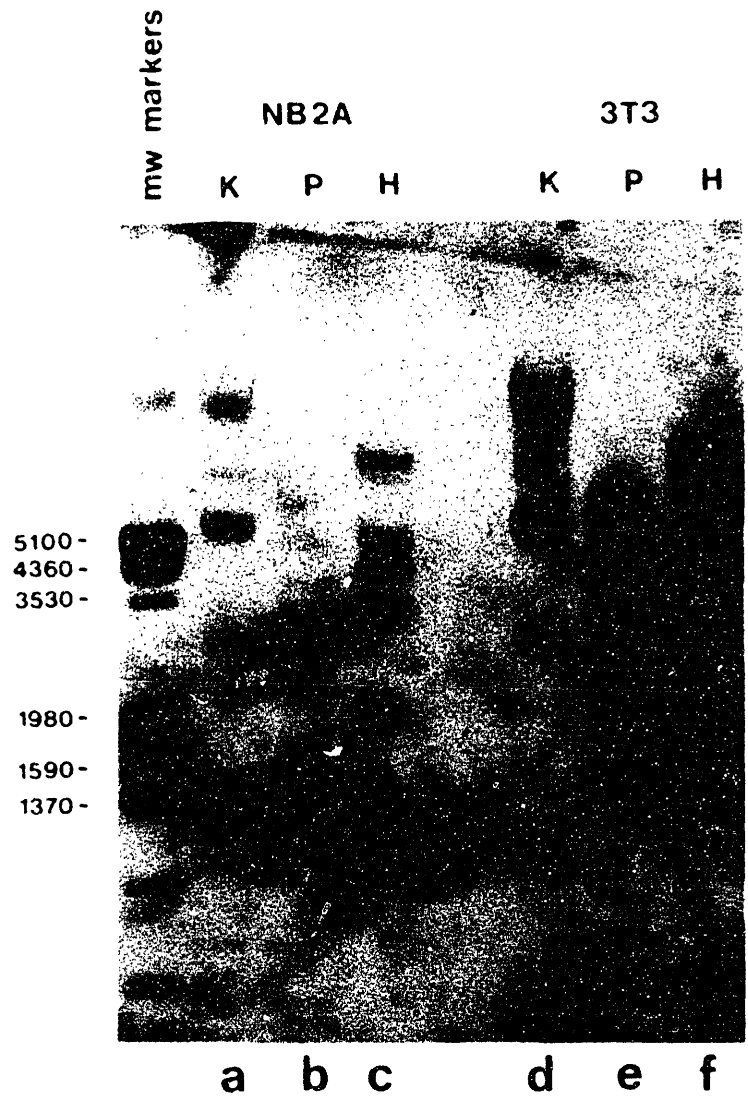

Figure 19.

Chartin-specific sequences in the mouse genome.

DNA from two mouse cultured cells lines, NB2A and 3T3, was digested with KpnI (K), PstI (P) or HindIII (H), separated by gel electrophoresis and transferred to nitrocellulose. The autoradiogram shows a Southern blot of these DNA fragments probed with nick-translated 15B1 insert.


mw markers
NB2A
3T3
K
P
H
K
P
H
5100 -
4360 -
3530 -
1980 -
1590 -
1370 -
a
b
c
d
e
f

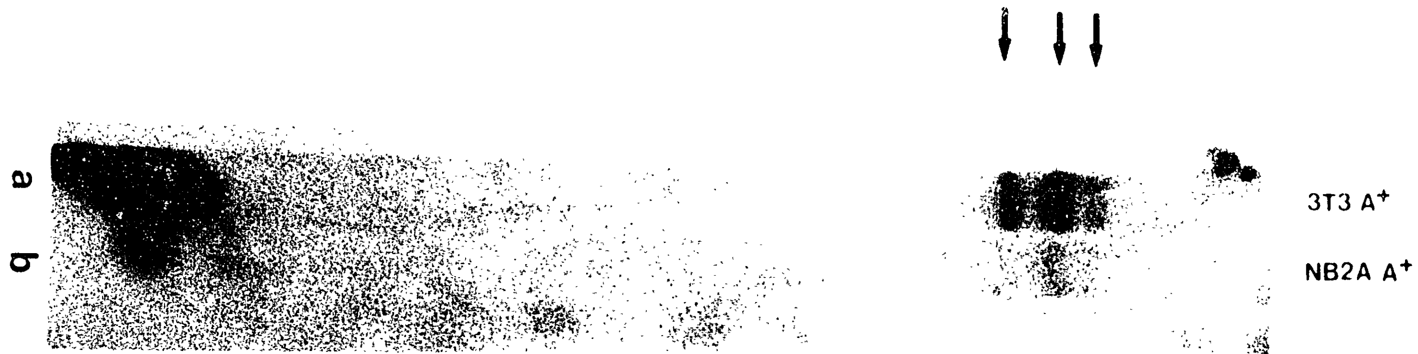
insert. The autoradiogram shows that sequences homologous to 15B1 do exist in the mouse genome. There are several bands recognized by the probe in each enzymatic diggestion of the mouse DNAs. At least two possible arrangements in the chartin genomic DNA can explain these numerous bands. First, the mouse genome may contain more than one chartin gene. Also, the cDNA sequence used as probe may be interrupted by introns in teh genomic DNA. If these introns contain relevent restriction enzyme sites, then the DNA sequence homologous to the probe may be found in more than one genomic DNA fragment. The structure of the chartin genomic DNA is unknown; multiple genes and introns may both exist.

Northern blots of polyA$^+$ mRNA from 3T3 and NB2A cultured cells as well as mouse brain tissue were also probed with ^{32}P-α-ATP-labeled 15B1 probe. The results from one such hybridization experiment are shown in Figure 20. The probe hybridized to three bands in both of the cultured cell lines' mRNA (lanes A, B, C and D). Two messages corresponding to two of the three bands in the cultured cells are present in mouse brain total RNA (lane E). The sizes of the three messages recognized by the probe are all well over 5kb; this length mRNA could easily code for a protein of 69 to 80kd. Thus, the Northern blot analysis shows that at least two or three messages homologous to 15B1 and of sufficient length exist in mouse cells and brain tissue.

Figure 20.

Chartin-specific messenger RNA transcripts in mouse cells and brain tissue.

Poly A+ mRNA from mouse NB2A and 3T3 cells and total cell mRNA from mouse brain was hybridized to nick-translated 15B1 cDNA by Northern blot analysis. The autoradiogram of the blot shows that three large messages from both 3T3 and NB2A cells hybridize to the 15B1 CDNA (a,b,c and d). Two large mRNA messages are identified from mouse brain (e). These two messages are the same size as the largest messages found in the cultured cells.


3T3 A+
NB2A A+
a
b

3T3 A+
NB2A A+
m. brain
c
d
e

Discussion

In order to isolate chartin-specific cDNA, phage carrying mouse brain cDNA were isolated from expression libraries by means of the anti-chartin serum 4-81. These phage produced β-galactosidase-fusion proteins which were recognized by serum 4-81. When these same proteins were injected into rabbits, one caused the production of anti-chartin antibodies. This immunogenic fusion protein as well as its corresponding cDNA were considered potentially chartin specific and were further characterized.

Antibodies to the mouse brain fusion protein 15B1 stained chartins by immunoblot analysis. DNA sequence analysis of the 15B1 insert showed that the 2.3kb fragment is not homologous to published sequences and may consist entirely of open reading frame sequence. This final result was anticipated due to the size of both the β-galactosidase- and trpE-15B1 proteins.

Although the 15B1 insert was not identified as an already published sequence, it need not be chartin specific. Sufficient proof that the 15B1 clone is chartin DNA requires identification of some chartin-specific property in the nucleic acid or its corresponding protein fragment. Since chartin nucleic acid has not been previously identified and no definitive function for chartins is known, this property has to be the primary structure of the chartin proteins.

Chartin structure is characterized by two types of experiments. First, structure of the proteins has been analyzed by peptide mapping. Also, two chartin specific regions of three dimensional structure have been identified by two different anti-chartin sera, 4-81 and 3417.

The structure of the trpE-15B1 protein was compared to that of the chartins by both methods of analysis. First, the trpE-15B1 protein was compared to that of the 69 and 80kd chartins by immunoblot analysis of their respective peptide maps. This experiment was uninformative. Also, the trpE-15B1 protein was shown have two epitopes in common with native chartins. Two anti-chartin sera, specific for different epitopes both stain the trpE-15B1 protein by immunoblot analysis. This result constitutes strong evidence that the mouse brain portion of the fusion protein is chartin-specific.

Although no one experiment proves that 15B1 cDNA is chartin specific, the combined data from all experiments strongly suggest that this is the case. For this reason, studies were initiated in which 15B1 cDNA was used as a probe to identify genomic DNA and mRNA. The filter hybridization studies presented show that 15B1 homologous genomic DNA as well as three large mRNA species exist in mouse cultured cells. In addition, at least two mRNA species are recognized in mouse brain; these mRNAs correspond by size to two of the three RNA species of cultured cells. Future hybridization experiments are planned which will determine gene number, as well as the expression and number of messages present across several animal species and tissue types.

Other experiments have also been initiated which involve the use of 15B1 cDNA or fusion proteins as tools to create more useful chartin probes. The mouse brain library is being screened in order to isolate sister clones (K. Bupp, work in progress). The objective of these experiments is to obtain a full length chartin cDNA. Also, the trpE-15B1 protein will be used as antigen for the production of

monoclonal antibodies. Some of these antibodies may be specific for epitopes found only in a subset of proteins in the chartin family. Both a full length cDNA and chartin subset-specific antibodies will be useful probes to study the structure and expression, and therefore function of chartins.

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