

Hexabrachion Proteins in Embryonic Chicken Tissues and Human Tumors

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Abstract. Cell cultures of chicken embryo and human fibroblasts produce a large extracellular matrix molecule with a six-armed structure that we called a hexabrachion (Erickson, H. P., and J. L. Iglesias, 1984, *Nature (Lond.)*, 311:267-269. In the present work we have determined that the myotendinous (M1) antigen described by M. Chiquet and D. M. Fambrough in chicken tissues (1984, *J. Cell Biol.*, 98:1926-1936), and the glioma mesenchymal extracellular matrix protein described by Bourdon et al. in human tumors (Bourdon, M. A., C. J. Wikstrand, H. Furthmayr, T. J. Matthews, and D. D. Bigner, 1983, *Cancer Res.* 43:2796-2805) have the structure of hexabrachions. We also demonstrate that the M1 antigen is present in embryonic brain, where it was previously reported absent, and have purified hexabrachions from brain homogenates. The recently described cytotactin (Gruet, M., S. Hoffman, K. L. Crossin, and G. M.

Edelman, 1985, *Proc. Natl. Acad. Sci. USA*, 82:8075-8079) now appears to be identical to the chicken hexabrachion protein.

In a search for functional roles, we looked for a possible cell attachment activity. A strong, fibronectin-like attachment activity was present in $(\text{NH}_4)_2\text{SO}_4$ precipitates of cell supernatant and sedimented with hexabrachions in glycerol gradients. Hexabrachions purified by antibody adsorption, however, had lost this activity, suggesting that it was due to a separate factor associated with hexabrachions in the gradient fractions. The combined information in the several, previously unrelated studies suggests that hexabrachions may play a role in organizing localized regions of extracellular matrix. The protein is prominently expressed at specific times and locations during embryonic development, is retained in certain adult tissues, and is reexpressed in a variety of tumors.

IN a previous paper (11) we described the structure of an extracellular matrix protein produced by chicken embryo and human fibroblasts. The protein was a disulfide-bonded oligomer of 220 kD (chicken) or 285 kD (human) subunits. Electron microscopy of both the chicken and the human oligomers revealed a distinctive six-armed structure that we called a hexabrachion. These oligomers had previously been considered a form of fibronectin (1). There were several distinctive structural features, however, that suggested this was a different protein.

Two proteins extensively characterized by other laboratories seemed very similar to the hexabrachion proteins. Chiquet and Fambrough (7, 8) described a protein from chicken tissues and cell cultures that they called myotendinous (M1) antigen. This protein was a disulfide-bonded oligomer of 220-kD subunits, with variable components of lower molecular mass. It had the same sedimentation coefficient as our chicken hexabrachions. Bourdon et al. (2-4) described a protein called glioma-mesenchymal extracellular matrix (GMEM)¹ protein that was prominently expressed in gliomas and some other human tumors. The antigen was not detected in normal brain tissue but was present at specific lo-

cations in several other tissues. This protein was a disulfide-bonded oligomer of 250-kD subunits, similar to the hexabrachion protein from human fibroblasts. For the present study we obtained the M1 and 81C6 monoclonal antibodies, which originally identified the myotendinous antigen and GMEM protein, and used them to purify the oligomers from chicken and human cell cultures. Electron microscopy confirmed that both these antigens are hexabrachions, and we will argue that they must be homologous.

More recently a protein called cytotactin has been identified in embryonic chicken brain and several other chicken tissues (10, 15). We noted that the distinctive band pattern of cytotactin on gel electrophoresis was identical to that of the M1 antigen. The tissue distributions were also quite similar, with one important exception: whereas the previous study of M1 antigen reported no detectable protein in brain, embryonic chicken brain was the primary source for cytotactin. Here we address this apparent discrepancy and show that M1 antigen is actually abundant in embryonic chicken brain.

In our previous study we identified a strong hemagglutination activity associated with the hexabrachions (11). Because hemagglutination is frequently associated with cell attachment, we wanted to determine directly whether the hexabrachions could mediate cell attachment. While preliminary results demonstrated a strong cell attachment activity very similar to fibronectin (12), it now appears that this activity

1. *Abbreviations used in this paper:* CAPS, 2-(cyclohexylamino)-1-propanesulfonic acid; GMEM, glioma-mesenchymal extracellular matrix; pFN, plasma fibronectin.

is associated with but not an integral part of the hexabrachions.

Brachionectin has been proposed as a general name for the group of proteins with the hexabrachion structure (12, 19). Tenascin has been proposed as a new name for myotendinous antigen of chicken and immunologically related proteins from other species (9). Here we will avoid controversy over nomenclature and refer to the proteins as hexabrachions or hexabrachion proteins, a usage that will emphasize the remarkable structural homology.

Materials and Methods

Cell Lines and Culture Techniques

Primary cultures of chicken embryo fibroblasts were prepared from skin of 10-d embryos. Cultures were used for protein preparation from the fourth passage until they stopped growing, usually tenth passage. U-251 MG, clone 3, an established line of human glioma (astrocytes), were obtained from Dr. D. D. Bigner, Duke University. Mouse fibroblast 3T3 cells were obtained from the Lineberger Cancer Center, University of North Carolina. NIL.8M hamster fibroblasts were obtained from Dr. Richard Hynes, Massachusetts Institute of Technology.

Cells were grown in DME supplemented with 10% FCS in a 5% CO₂ atmosphere. After the cells reached confluence they were fed with serum that was pretreated with 33% (saturated) (NH₄)₂SO₄ to remove serum proteins that would precipitate.

Purification of Hexabrachions from Cell Supernatant

Cells were grown to confluence in 150-cm² flasks (Corning Glass Works, Corning, NY) with 25 ml of medium per flask. Typically five flasks were used for each preparation. Cell supernatant was removed for processing on the day of confluence and at 2–3 d intervals afterwards. At each of these times fresh medium was added. Protein was harvested from fibroblast cultures until 10–12 d after confluence, and from the U-251 MG glioma cells until 18 d.

Immunoaffinity columns were prepared by coupling 5 mg of M1 or 81C6 antibody to 1 g (3.5 ml) of cyanogen bromide-activated Sepharose. Typically 150 ml of cell supernatant was centrifuged at 20,000 rpm for 20 min to remove cellular debris, and then passed over the column. The column was washed with 50 ml of 0.1 M NaBO₃, 0.5 M NaCl, pH 8.4 and then eluted with 100 mM CAPS (2[cyclohexylamino]-1-propanesulfonic acid), 0.5 M NaCl, pH 11 as described by Chiquet and Fambrough (7). Immunoaffinity chromatography was done at room temperature.

Hexabrachions were alternatively isolated from cell supernatants by precipitation with (NH₄)₂SO₄ and gradient sedimentation. 20 g (NH₄)₂SO₄ was added per 100 ml initial volume, giving a 33% saturated solution. The pH was adjusted to ~7.4 with NH₄OH, the material stirred for 30 min at room temperature and centrifuged 30 min at 30,000 rpm in a Beckman-type 35 rotor (Beckman Instruments, Inc., Palo Alto, CA). The pellets were resuspended in 5 ml of 200 mM CAPS, 0.15 M NaCl, pH 11. The protein was precipitated a second time by adding (NH₄)₂SO₄ (with no pH adjustment) and the pellet resuspended in 0.6–1.5 ml CAPS buffer.

Gradient sedimentation was performed by layering a 0.5-ml sample of protein in CAPS buffer on a 15–40% (vol/vol) glycerol gradient containing 0.2 M NH₄HCO₃, pH 9.5. The gradients were centrifuged at 41,000 rpm in a Beckman SW41Ti rotor, 20°C, for 18 h. 20 fractions were collected from a hole in the bottom of the tube. Sedimentation coefficients were estimated from standards (fibrinogen at 8 S in fraction 12, catalase at 11.3 S in fraction 9, and alpha-2 macroglobulin at 18.5 S in fractions 3 and 4) run in a separate gradient.

Preparation of Hexabrachion Protein from Tissue Extracts

Brain, gizzard, or wings from 11-d chicken embryos were homogenized in 200 mM CAPS, 0.15 M NaCl, pH 11 (5 ml buffer per gram of tissue) for 1 min in a Sorvall Div. Omnimixer (Newton, CT). The homogenate was left on ice for 30–60 min, and then neutralized to pH 7–7.5 by adding 1 M N₂H₂PO₄. Phenylmethylsulfonyl fluoride was added to a concentration of 1 mM. Brain homogenates were processed with no further additions, but giz-

zard and wing homogenates were usually treated with DNAase and hyaluronidase to reduce viscosity. The homogenate was centrifuged at 35,000 rpm for 45 min to remove particulates, and the supernatant was filtered through Whatman No. 1 paper (Whatman Inc., Clifton, NJ) to remove floating debris. The solution was then passed over the M1 antibody column, the column was washed, and the protein eluted as described above.

Protein Assay and Gel Electrophoresis

Protein concentrations were determined by the method of Bradford (5), using reagents from Bio-Rad Laboratories (Richmond, CA). Values were referred to a standard curve of highly purified human plasma fibronectin (pFN). The concentration of this pFN was established by UV spectroscopy, using an extinction coefficient of 1.28 cm²/(mg/ml).

PAGE was performed according to Laemmli (18), using a 5% running gel and a 3% stacking gel. Samples were dialyzed into CAPS buffer and mixed 1:1 with sample buffer containing 1% SDS and 1% beta-mercaptoethanol. Electrophoresis was for ~4 h at 200 v, to bring the dye front to the bottom of the 13.5-cm gel. The gel was washed overnight in 50% methanol, 12% acetic acid and stained with silver by the method of Merrill et al. (20).

Immunoblotting was performed after transfer to nitrocellulose using a semi-dry apparatus as described by Gibson (14). Protease-facilitated transfer (14) gave the most efficient transfer of the ~600-kD band, but it was only useful when blotting with the polyclonal antibody. The epitopes for the monoclonal antibodies were destroyed by the pronase.

Electron Microscopy

10-μl samples of protein in 0.2 M NH₄HCO₃, 30% glycerol, pH 9.5 were sprayed onto mica, dried in vacuum, and rotary shadowed (13). Micrographs were taken at 50,000× and printed at a final magnification of 150,000×.

Immunocytochemistry

An 11-d chicken embryo brain was fixed with 2% paraformaldehyde and glutaraldehyde. 50-μm sections were cut with a vibratome, stained with M1 or control antibody at 10 μg/ml for 18 h at 4°C, and then with peroxidase-coupled anti-mouse IgG (Boehringer Mannheim Diagnostics, Inc., Houston, TX). The peroxidase was developed with diaminobenzidine, the section was postfixated with osmium tetroxide and embedded in Embed-812 (EM Sciences, Cherry Hill, NJ).

Cryostat sections (6 μm) of frozen, unfixed chicken brain were stained with M1 antibody and fluorescein-conjugated second antibody.

Cell Attachment Assay

Cell attachment was assayed by the microtiter well method described by Ruoslahti et al. (22). Serial dilutions of fibronectin or the protein fractions to be tested were incubated in the plastic wells for 2 h at room temperature, the wells were blocked with 1% heat (80°C, 3 min) denatured BSA and washed. Cells were left on the substrate at 37°C for 1 h. After washing off unattached cells and staining, the assay was scored by visual inspection of the wells in a low power microscope, looking for the minimum protein concentration that would give significant attachment.

Results

Purification of Hexabrachions by Immunoabsorption

Fig. 1 shows analysis by PAGE of the proteins purified from cell supernatants and tissue homogenates using the M1 antibody for chicken and the 81C6 antibody for human material. Human and mouse hexabrachions purified by (NH₄)₂SO₄ precipitation and gradient sedimentation, without antibody adsorption, are also shown in Fig. 1 and discussed in a later section.

The M1 antigen purified from chicken fibroblast cell supernatant by antibody adsorption (M1-CEF) comprises a prominent band at 220 kD and a closely spaced doublet near 200 kD. This is the pattern previously described by Chiquet and Fambrough (8) for the M1 (myotendinous) antigen, and

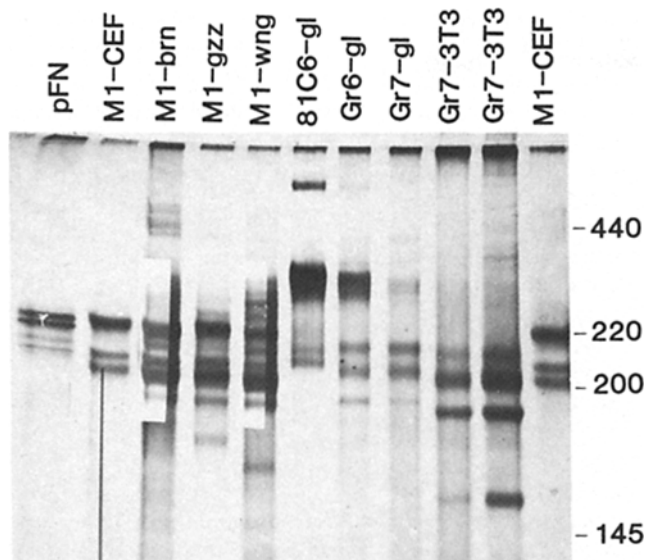


Figure 1. Gel electrophoresis of hexabrachion proteins purified by antibody adsorption (lanes *M1* and *81C6*) or by glycerol gradient sedimentation (lanes *Gr6*, *7*). The lanes are given descriptive labels. *pFN*, human plasma fibronectin (0.5 μ g protein loaded); *M1-CEF*, M1 antigen from cell supernatant of chicken embryo fibroblasts (duplicate samples shown in lane 2 and far right). The next three lanes show M1 antigen purified from tissue homogenates of 11-d chicken embryos. *M1-brn*, brain; *M1-gzz*, gizzard; *M1-wng*, wing (the brain and wing lanes are partly covered to show heavily stained bands). *81C6-gl*, 81C6 antigen purified from cell supernatants of U-251 MG human glioma cultures. *Gr6-gl* and *Gr7-gl*, fractions 6 and 7 from a glycerol gradient of $(\text{NH}_4)_2\text{SO}_4$ precipitate of glioma cell supernatant. *Gr7-3T3*, fraction 7 from $(\text{NH}_4)_2\text{SO}_4$ precipitate of cell supernatant from mouse 3T3 cells. Two different preparations of *Gr7-3T3* are shown. Subunit $M_r \times 10^{-3}$, shown on the right, were estimated from the following standards: 440, laminin, upper band; 220, pFN, upper band of doublet; 200, myosin; 145, muscle C protein.

also the pattern reported by Grumet et al. (15) for cytotactin. Both of these reports assigned a value of 190–200 kD to this doublet so we will continue to use these numbers, although on our gels the doublet actually ran slightly above myosin (usually assigned a value of 200 kD as an M_r standard). The intensity of the doublet relative to the 220-kD band was variable, as previously noted by Chiquet and Fambrough (8).

The M1 antigen was also isolated from homogenates of several chicken tissues, all from 11-d embryos. Brain homogenates gave the same three bands as the fibroblast cell supernatant, although with different relative intensities, plus new bands. The 190-kD band, the lower band of the doublet, was strong in the brain protein. A cluster of bands of higher mass, \sim 500 kD, was apparent in some preparations. M1 antigen was also obtained from gizzard and wing homogenates. The 190-kD band was prominent, the other bands of brain extracts were present, and there were additional bands at higher and lower mass. The heterogeneity of subunit sizes is particularly evident in the antigen from wing homogenates (M1-Wng).

The 81C6 antigen from U-251 MG glioma cells was previously identified only in the extracellular matrix, and was thought to be absent from the cell supernatant (4). In our present study we found it to be abundant in the cell superna-

tant. Protein isolated from cell supernatant by adsorption to the 81C6 antibody column showed a prominent and somewhat diffuse band (Fig. 1, 81C6-gl) significantly higher than the 220-kD band of the chicken antigen. We previously estimated a mass of \sim 285 kD for this band from human fibroblasts (11), while Bourdon et al. (4) and Carter and Hakomori (6) both designated it 250 kD. The band ran about half-way between reduced and nonreduced plasma fibronectin (220 and 440 kD), so the higher value seems more appropriate. The 81C6 antigen also showed a closely spaced doublet that ran slightly above the chicken doublet. This doublet was always very weak in the human protein. Finally, a sharp band of much higher mass, running above nonreduced pFN and the upper band of laminin, was apparent in most preparations. We estimate a value of \sim 600 kD for this band, but there are no standards in this region. Neither this high molecular mass band nor the doublet was observed in the previous study of GMEM protein (4). The sensitive silver stain and the larger quantities of protein obtained by our methods were important to demonstrate these weaker bands.

Purification of Hexabrachions from Cell Supernatants without Antibody Adsorption

We had previously purified hexabrachions from cell supernatants using $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by gradient sedimentation (11). This procedure was especially useful with human hexabrachions, which sedimented faster than those from chicken and were well separated from fibronectin. Hexabrachions from human U-251 MG glioma cells sedimented at 14–17 S. A 15-S fraction (lane *Gr6-gl* in Fig. 1) showed a prominent band at 285 kD, a weak band at \sim 600 kD, and two bands near 200 kD. In the next fraction (13.5 S, lane *Gr7-gl*) only this doublet is prominent, Fraction 6 showed a high concentration of hexabrachions by electron microscopy; fraction 7 had far fewer hexabrachions. Thus the strong band at 285 kD corresponds to the presence of hexabrachions, while the doublet at 200 kD (which is much stronger and also more widely spaced than the doublet in antibody-adsorbed protein) may be contaminating proteins.

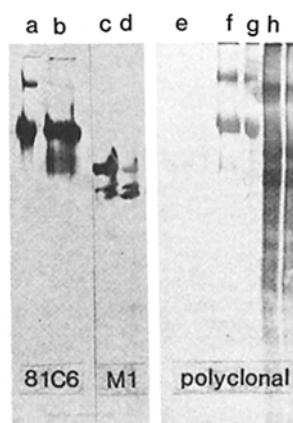


Figure 2. Immunoblots stained with the 81C6 monoclonal against GMEM (*a* and *b*); M1 monoclonal against chicken myotendinous antigen (*c* and *d*); and Chiquet-Fambrough polyclonal against M1 antigen (*e*–*i*). The proteins loaded are: (*a*) gradient purified human hexabrachions, a fraction similar to *Gr6-gl* (Fig. 1) but with a heavier \sim 600-kD band; (*b*) human hexabrachions purified by 81C6 antibody adsorption, similar to 81C6-gl (Fig. 1) but with a lighter \sim 600-kD band; (*c*) chicken hexabrachions

purified from fibroblast cultures by M1 antibody adsorption; (*d*) chicken hexabrachions purified from brain homogenates by M1 antibody adsorption; (*e*) human fibronectin, 2 \times more protein than lanes *f*–*i*; (*f*) gradient purified human hexabrachions; (*g*) antibody purified human hexabrachions; (*h* and *i*) two concentrations of chicken embryo fibroblast M1 antigen.

Cell supernatant from mouse 3T3 cells was processed by $(\text{NH}_4)_2\text{SO}_4$ precipitation and gradient sedimentation. The 13.5-S fractions showed a set of bands near 200 kD (Fig. 1, lanes *G7-3T3*) and electron microscopy showed hexabrachions (Fig. 4 *E*). This purification scheme, although not as rigorous as antibody adsorption, is quite useful for screening cell cultures for production of hexabrachions.

Another advantage of the gradient purification is the high yield of protein. The peak hexabrachion fractions from glioma cultures had a protein concentration up to 0.5 mg/ml (from 200 ml U-251 MG cell supernatant), in which the 285 kD appeared to account for >80% of the band intensity on silver-stained gels. In contrast the 5-ml antibody columns appeared to be saturated at ~ 200 μg of protein, and the maximum concentration of eluted protein was ~ 50 $\mu\text{g}/\text{ml}$.

Immunological Cross-Reactivity and Immunocytochemistry

Immunoblots of human and chicken hexabrachion proteins are shown in Fig. 2. The 81C6 antibody stained both the main 285-kD band and the ~ 600 kD of human hexabrachion protein (Fig. 2, *a* and *b*). A diffuse patch from 260–220 kD also

stained, but the pronounced doublet in gradient fraction Gr6-gl did not stain. The M1 antibody stained the main 220-kD band and the 190–200-kD doublet of M1 antigen from fibroblast cell cultures (*c*), as previously demonstrated by Chiquet and Fambrough (8). These same bands, as well as a weak 210-kD band, were stained in M1 antigen prepared from brain homogenate (*d*). The ~ 500 -kD bands seen in Fig. 1, M1-brn, were missing from this preparation, but when present they also stained with the M1 antibody. Neither the 81C6 nor the M1 monoclonal antibodies cross-reacted with other species.

Chiquet and Fambrough prepared a polyclonal antibody to the chicken M1 antigen and showed that it did not cross-react with fibronectin. In Fig. 2, *h* and *i* we show that this polyclonal antibody stains the main band and the doublet of M1 antigen from chicken cultures, as well as a ~ 500 -kD band and several lower M_r bands, which were scarcely visible on the silver-stained gel of this preparation. Most important, this polyclonal antibody cross-reacts with the human hexabrachion protein, staining both the 285- and the ~ 600 -kD bands.

The presence of M1 antigen in embryonic chicken brain was confirmed by immunocytochemistry. Fig. 3 *A* shows a

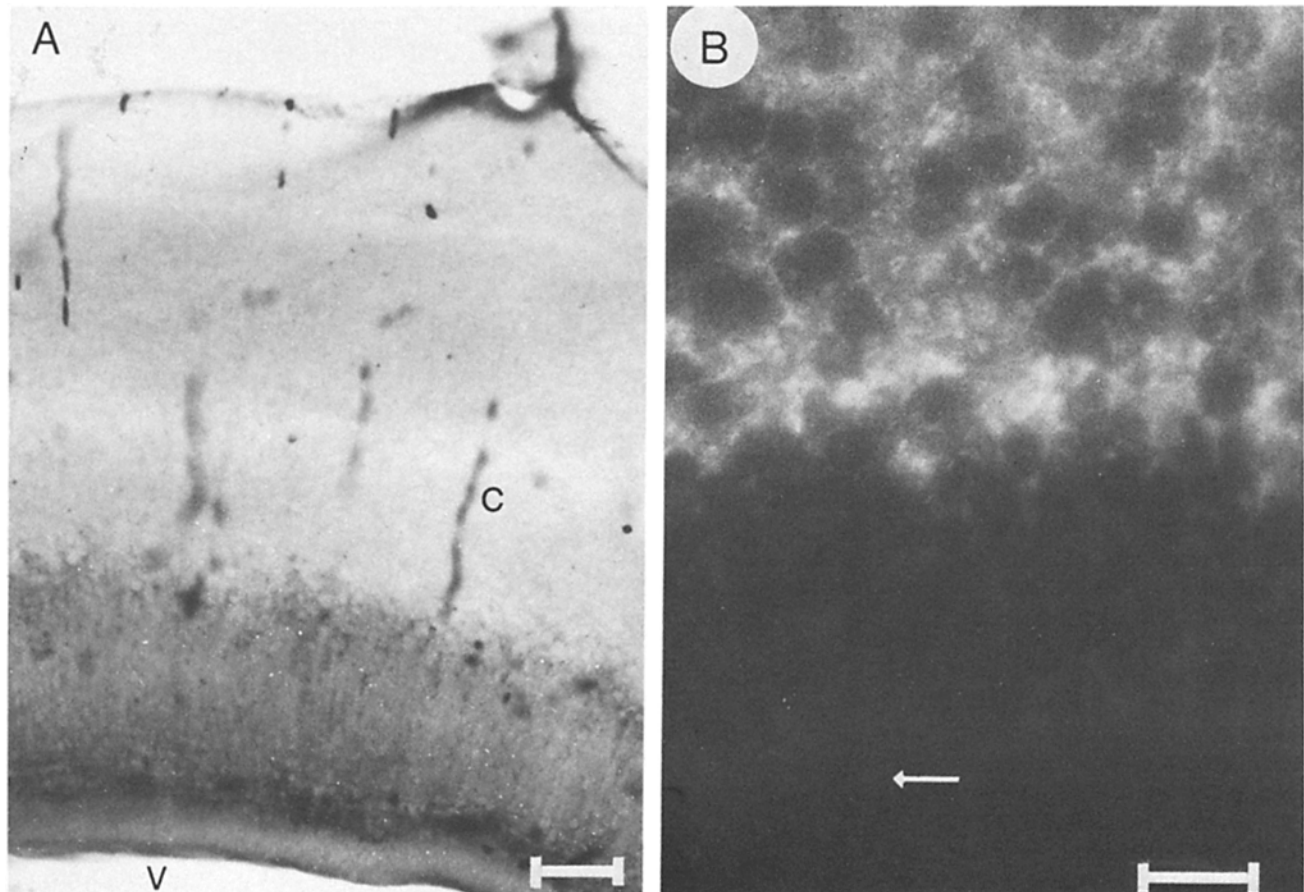


Figure 3. (A) A horizontal section through the optic tectum of an 11-d chicken embryo stained with the M1 monoclonal antibody and visualized with peroxidase-conjugated second antibody. The antibody stain is dark. Staining is pronounced in two layers adjacent to the neuroepithelium (the letter *v* indicates the ventricle). Staining of the external layers is much lighter, but darker than the control. The dark lines are capillaries (*c*) which stain because of endogenous peroxidase activity of blood cells. (B) A frozen section of brain tissue stained with M1 antibody and a fluorescein-conjugated second antibody. The (white) fluorescence is intense in punctate patches between cell bodies on the right, but is virtually absent from the ~ 20 - μm surface layer at the bottom. The arrow indicates the ventral edge of the brain tissue. Bar in *A*, 100 μm ; in *B*, 10 μm .

horizontal section through the optic tectum of an 11-d chicken embryo. This thick section, stained with the M1 antibody and a peroxidase-conjugated second antibody, shows a concentration of M1 antigen in certain layers of the tectum, probably layers II and III as defined by LaVail and Cowan (17), with much lighter staining of other layers. Fig. 3 B shows a thin section of brain tissue stained with a fluorescent second antibody. The M1 antibody did not stain the outermost (neuroepithelial) layer but was localized in patches around the cell bodies in the deeper layers.

Electron Microscopy of Purified Hexabrachions

Proteins purified from chicken embryo fibroblasts or human glioma cells by antibody adsorption and/or by gradient sedimentation were examined by rotary shadowing electron microscopy (Fig. 4). Protein eluted from the antibody columns always showed a high concentration of hexabrachions, but these preparations were frequently contaminated with irregular debris, perhaps shed from the column. Fractions from a subsequent gradient sedimentation showed clean hexabrachions, their concentration corresponding to the intensity of the bands on gels. Thus the M1 and the 81C6 antigens are identified as chicken and human hexabrachion proteins, respectively.

In addition to typical hexabrachions, the chicken protein showed a significant number of three-armed oligomers. These trimers were separated from hexabrachions by gradient sedimentation. Hexamers were obtained in a 13-S peak, and trimers and some single strands were found in a 9-S peak. The trimers (Fig. 4 C) are clearly half-hexabrachions: the arms are arranged in the "T" configuration, usually with a central nodule (or part of the central nodule) attached above the cross of the "T." Each arm has the knob on the end, and has the characteristic thickening of the distal segment. We can't yet say whether these half-hexabrachions are products of proteolysis or of incomplete assembly.

The binding of the M1 antibody to chicken hexabrachions was visualized directly in electron microscope specimens. In this experiment hexabrachions were prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation and gradient sedimentation, without exposure to the antibody column. The peak fraction of hexabrachions as assayed by electron microscopy was incubated with M1 antibody in the gradient buffer. An antibody concentration of 10–30 $\mu\text{g/ml}$ gave the cleanest specimens (the concentration of hexabrachions was estimated to be $\sim 10 \mu\text{g/ml}$). After overnight incubation at 4°C and 1 h at room temperature, samples were sprayed for rotary shadowing. Antibody molecules could be seen clustered near the center of the hexabrachion, apparently binding to the arms about one-third of the distance from the center (Fig. 4 D). Similar attempts to localize the 81C6 antibody on human hexabrachions have not been successful, perhaps because of low affinity.

A mouse hexabrachion from 3T3 cell supernatant, isolated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and gradient sedimentation (Fig. 1, lane *Gr7-3T3*), is shown in Fig. 4 E.

Two points of structure should be noted in the examples shown in Fig. 4. First, the arms are usually curved or sharply kinked, and in almost all cases this bend is in the clockwise direction. This means that the hexabrachions have a preferred orientation when they flatten on the mica, probably because they are cup shaped rather than flat. Second, pairs

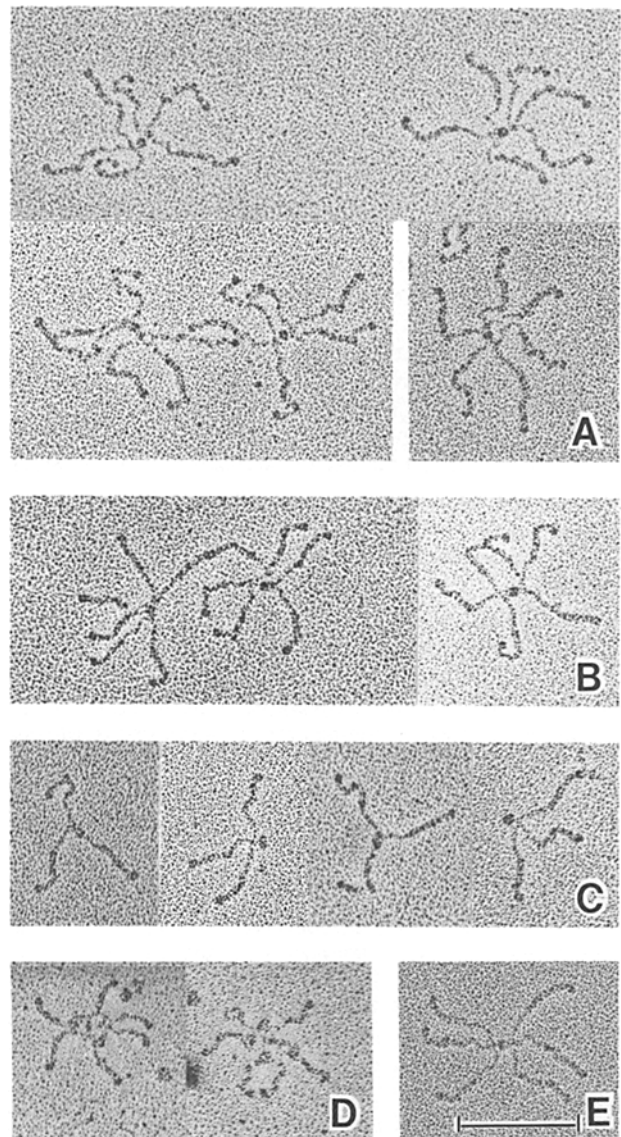


Figure 4. Electron micrographs of hexabrachions from human, chicken and mouse. Bar, 100 μm . 10- μl samples of glycerol gradient fractions were sprayed onto mica and rotary shadowed (11). (A) Hexabrachions from human glioma cells, purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and gradient sedimentation (Fig. 1, lane *Gr6-gl*). Human hexabrachions purified by antibody adsorption were identical in structure. (B) Hexabrachions from chicken embryo fibroblasts, purified by immunoaffinity followed by gradient centrifugation (13-S peak). (C) Trimers (half-hexabrachions) from chicken embryo fibroblasts (9 S peak). (D) M1 antibody (10 $\mu\text{g/ml}$) reacted with chicken embryo fibroblast hexabrachions (concentration not determined, $\sim 10 \mu\text{g/ml}$) in gradient buffer, overnight at 4°C. Antibody molecules are bound to the arms about one-third of the distance from the center. (E) A hexabrachion from 3T3 mouse fibroblasts (Fig. 1, lane *Gr7-3T3*).

of hexabrachions are sometimes found to be attached to each other, as in the left hand panels in Fig. 4, A and B. These complexes are not frequent, but the morphology is very reproducible: a reciprocal contact of a distal knob of one hexabrachion at a midpoint of an arm on the other. These images suggest the possibility that hexabrachions can self-associate to form pairs and eventually more extended networks.

Cell Attachment Activity

Using the microtiter cell attachment assay of Ruoslahti and Pierschbacher (23) we obtained a reproducible titration of cell attachment activity with pFN. Wells coated at 5–10 $\mu\text{g/ml}$ pFN were mostly covered with flattened cells, and substantial cell attachment was obtained down to 2.5 $\mu\text{g/ml}$ pFN. NIL.8M hamster fibroblasts and U-251 MG glioma cells gave virtually identical results in attaching to pFN.

Human hexabrachions prepared from cell supernatants by $(\text{NH}_4)_2\text{SO}_4$ precipitation and gradient sedimentation reproducibly showed a high level of cell attachment activity (12). The hexabrachion fractions promoted cell attachment down to a protein concentration of 3.5 $\mu\text{g/ml}$, and the attachment was inhibited by the peptide GRGDSP, which is known to inhibit cell attachment to fibronectin and other proteins (23). The 285-kD hexabrachion band accounted for $\sim 80\%$ of the intensity seen on silver-stained gels (Fig. 1, lane GR6-g1). Bands ~ 200 kD, not seen on antibody purified hexabrachions, are the most obvious contamination.

Hexabrachion protein purified by immunoaffinity chromatography, however, gave no cell attachment up to the highest concentrations tested, 20–50 $\mu\text{g/ml}$. Both NIL.8M and U-251 MG cells, and hexabrachions from chicken and human, were tested. To address the possibility that the cell attachment activity was being destroyed by the pH 11 elution buffer, separate experiments were performed in which hexabrachions were eluted from the antibody column with 2 M urea or with 2 M potassium thiocyanate. These preparations also had no cell attachment activity. We conclude that purified hexabrachions have no cell attachment activity, but an active factor is associated with them in the gradient fractions.

Hexabrachion Synthesis by Different Cell Lines

The U-251 MG human glioma cultures were the richest cellular source of hexabrachions we have found, 200 ml of cell supernatant yielding up to 1 mg of protein in the hexabrachion gradient fractions. Several other cell types were assayed for hexabrachion production by preparing gradient fractions from cell supernatant and looking for hexabrachions by electron microscopy. Primary cultures of chicken embryo and human fibroblasts produced much less hexabrachion protein and relatively more fibronectin than the U-251 MG. Established fibroblast lines, 3T3 from mouse and normal rat kidney from rat, also produced hexabrachions. Cultures of virally transformed chicken embryo fibroblasts, 3T3 and normal rat kidney cells all produced hexabrachions, essentially the same as the parent cultures. This establishes an important point, that hexabrachion production does not seem to be linked to viral transformation.

Discussion

The myotendinous (M1) antigen from chicken (7, 8), and the GMEM protein from human tumors (2–4) have each been well characterized biochemically and histologically, but not previously associated with each other. The evidence we present here shows that these two proteins are structurally and immunologically homologous. Correlating these previously unrelated studies, and the more recent studies of cytactin, suggests that hexabrachion protein is prominently expressed in specific locations during embryonic development, includ-

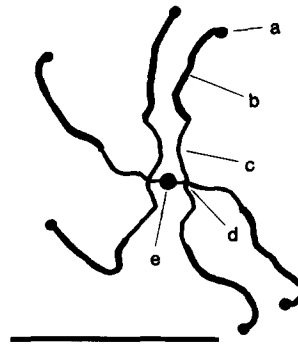


Figure 5. A diagram of a human hexabrachion (reprinted from reference 11 with permission) designating five characteristic features. Each of the arms has (a) a terminal knob, (b) a thicker distal segment, and (c) a thinner inner segment. Three arms come together to form a "T" junction (d) at which two arms extend colinearly in opposite directions and the third is perpendicular. Each of these trimers

is attached by a short link to a central globular particle (e) As shown in Fig. 4 C the trimers can be isolated as separate entities. Bar, 100 nm.

ing development of the brain, and is reexpressed by several types of tumors.

The strongest argument for homology of the chicken embryo and human tumor proteins is their distinctive and elaborate hexabrachion structure. With most protein molecules a low resolution structure would not be sufficient basis for suggesting homology. The hexabrachion, however, has at least five distinctive features, illustrated in Fig. 5. It is difficult to imagine that all the features of this complex structure could arise in unrelated proteins.

The homology is confirmed by immunological cross-reactivity. The monoclonal antibodies stain all the major bands of their own antigen (8; and Fig. 2), but are not cross-reactive with other species. A polyclonal antibody against the chicken M1 protein stained the main 285-kD and the ~ 600 -kD bands of human hexabrachions.

A curious feature of the human protein is that its major subunit is much larger than that of chicken, 285 kD vs. 220 kD. In our previous study (11) we showed that this size difference corresponded to the length of the arms: the arms of chicken hexabrachions were 68-nm long and those of human, 87-nm long. The smaller subunits of the chicken doublet might correspond to still shorter arms but we have not yet been able to demonstrate this small difference.

The multiple bands seen especially with the chicken protein may be the result of alternative RNA splicing. The bands differ in M_r by 5–20 kD, and the larger ones all stain with the monoclonal antibodies. Data of Chiquet and Fambrough (8) suggest that the heterogeneity is not due to proteolysis. Fibronectin, which has a strand-like structure similar to the hexabrachion arm, is a string of small (5–10 kD) globular domains, some of which are omitted by alternative RNA splicing (21). An analogous mechanism seems an attractive hypothesis to explain the heterogeneity of hexabrachion bands.

We can now make a strong argument that the recently named cytactin is the same as the M1 antigen, the chicken hexabrachion protein. The band pattern on gel electrophoresis reported for cytactin (15), both reduced and nonreduced, is identical to that reported here and previously (8) for the M1 antigen. Our present finding that M1 antigen is prominent in embryonic chicken brain resolves the only apparent discrepancy between the two proteins. The identification of hexabrachions in embryonic brain adds a new dimension of interest to the protein—developmental neurobiology. The detailed studies of cytactin have already demonstrated

a changing pattern of expression at specific times and locations during neural development, possibly related to cell migration (10, 15).

Bourdon et al. (2) reported that the human GMEM protein was prominent in most gliomas but absent from normal brain tissue. We have not yet reinvestigated the presence of hexabrachion protein in human brain, but we would expect it to be present, at least in fetal tissue.

The J1 antigen from mouse brain (16, 24) has been less extensively characterized than the other antigens, but it may be the mouse hexabrachion protein. Some similarities to cytactin have been noted (15). The GP-250 protein, previously characterized as a component of the extracellular matrix of human fibroblasts (6), is almost certainly the same protein as the human hexabrachion. An important finding from this study, also supported by experiments of Bourdon et al. (4), is that hexabrachions can be extracted from the matrix by detergent in the absence of reducing agent. This is in contrast to fibronectin, most of which is covalently bound into the matrix by disulfide bonds.

Our present finding that hexabrachions can be extracted intact from homogenates of chicken tissue makes two important points. First, it confirms that the association of hexabrachions with cells or other matrix components in tissues is noncovalent, as it appears to be in cell culture. Second, it shows that at least a significant fraction of the protein exists in tissue with the intact hexabrachion structure, i.e., it is not degraded or processed to other forms.

Hexabrachion protein purified by antibody adsorption had no cell attachment activity comparable to fibronectin. There is, however, a strong, fibronectin-like cell attachment activity associated with hexabrachions in glycerol gradient fractions of cell supernatants (12). The simplest explanation is that this strong attachment activity is due to a separate factor, associated with hexabrachions in the gradient fractions but washed off during antibody adsorption. We should note that the assay used in these studies (22) measures cells that are strongly attached by a combination of initial attachment and spreading over the 1-h incubation. We are currently investigating the possibility that hexabrachions have a weak or restricted cell attachment activity not demonstrated by this particular assay.

Grumet et al. (15) reported that the cytactin antibody inhibited neuron-glial attachment, but in the experiment shown the fraction of cells affected was very small, ~1% of the total probe cells. Kruse et al. reported a much larger effect with their J1 antibody (16) but details of their assay have not yet been published. Both these studies report a limited number of assays (two or three) and are using a complex mixed cell assay. We are hesitant to ascribe a cell attachment activity until it can be demonstrated with purified protein using a solid phase assay.

In a recent paper Chiquet-Ehrismann et al. (9) found no detectable cell attachment activity for the M1 antigen, which they renamed tenascin, using an assay involving 2 d of growth on a protein-coated substrate. They did, however, observe that the protein stimulated growth and cell division in the absence of added serum, a finding of potential significance to the role of the protein in tumors. They identified an immunologically cross-reactive protein in rat and found that this protein was present in embryonic breast tissue, disappeared in adult, and was prominently reexpressed in experimentally

induced breast tumors. Their study thus demonstrated the same association with embryonic and cancerous tissue that we have argued here.

A central question is the function of this extracellular matrix protein. The developmental regulation of its appearance in embryos (7, 10), and its apparent reexpression in a variety of tumors (2, 9) are especially intriguing. One would hope that a comprehensive catalogue of its highly restricted tissue distribution (2, 7, 9, 10, 19) would correlate with an obvious function, but this is not the case. As an extracellular matrix molecule it is reasonable to think that it must be binding to cell surface receptors and/or other matrix molecules. The highly specific tissue localization, e.g., in tendons, perichondrium, and perineureum of chicken embryos (7, 10), in a very narrow sheath around bird muscle spindle (19), and around capillaries and extracellular matrix fibers in human tumors (2) suggest that hexabrachions may be binding to other matrix molecules and possibly self-associating to establish the restricted distribution.

The structure of the hexabrachion suggests three points that may be important in relation to function. First, it is big. Each arm is 87-nm long (for the human; 68 nm for the chicken, reference 11), so the arms can easily span a distance of 150 nm. Second, the arms have a thin elongated structure. The similar structure in fibronectin is thought to be based on a linear arrangement of multiple small domains (21). If the hexabrachion arms are also a linear array of small domains, each arm could contain multiple independent binding functions. Third, the molecule is apparently multivalent. Assuming each arm is identical, the hexabrachion should be able to bind six ligands into a single complex, or self-associate with up to six other hexabrachions. This might easily lead to formation of an extended network. Whatever functions are eventually determined, it would be surprising if they did not use some or all of the possibilities provided by the elaborate hexabrachion structure.

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