An Epithelium-type Cytoskeleton in a Glial Cell: Astrocytes of Amphibian Optic Nerves Contain Cytokeratin Filaments and Are Connected by Desmosomes

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Abstract. In higher vertebrates the cytoskeleton of glial cells, notably astrocytes, is characterized (a) by masses of intermediate filaments (IFs) that contain the hallmark protein of glial differentiation, the glial filament protein (GFP); and (b) by the absence of cytokeratin IFs and IF-anchoring membrane domains of the desmosome type. Here we report that in certain amphibian species (Xenopus laevis, Rana ridibunda, and Pleurodeles waltlii) the astrocytes of the optic nerve contain a completely different type of cytoskeleton. In immunofluorescence microscopy using antibodies specific for different IF and desmosomal proteins, the astrocytes of this nerve are positive for cytokeratins and desmoplakins; by electron microscopy these reactions could be correlated to IF bundles and desmosomes. By gel electrophoresis of cytoskeletal proteins, combined with immunoblotting, we demonstrate the cytokeratinous nature of the major IF proteins of these astroglial cells, comprising at least three major cytokeratins. In this tissue we have not detected a major IF protein that could correspond to GFP. In contrast, cytokeratin IFs and desmosomes have not been detected in the glial cells of brain and spinal cord or in certain peripheral nerves, such as the sciatic nerve. These results provide an example of the formation of a cytokeratin cytoskeleton in the context of a nonepithelial differentiation program. They further show that glial differentiation and functions, commonly correlated with the formation of GFP filaments, are not necessarily dependent on GFP but can also be achieved with structures typical of epithelial differentiation; i.e., cytokeratin IFs and desmosomes. We discuss the cytoskeletal differences of glial cells in different kinds of nerves in the same animal, with special emphasis on the optic nerve of lower vertebrates as a widely studied model system of glial development and nerve regeneration.

Cell architecture is largely based on cytoplasmic structures, collectively referred to as “the cytoskeleton,” which include the microfilaments, the microtubules, the intermediate-sized filaments (IFs), as well as localized densities such as the membrane-associated plaques of desmosomes, hemidesmosomes, adherent junctions, and focal adhesions. Biochemical and immunological analyses have shown that many cytoskeletal elements are formed by proteins of multigene families whose members are differentially expressed, a certain pattern being characteristic of a given differentiation pathway. These different expression patterns of cell type-specific cytoskeletal proteins have led to the idea that the organization of a given type of cell and tissue is architecturally and functionally correlated with, and perhaps dependent on, a specific ensemble of cytoskeletal components. Among the different kinds of filament proteins, the IF protein family is particularly well known for its complexity and for the differential synthesis of IF proteins in cell type-specific patterns (for reviews see 32, 55, 62, 74, 78). Epithelial organization is typically associated with the expression of cytokeratin IF proteins and the formation of desmosomes; mesenchymally derived cells usually express vimentin IFs and lack desmosomes; muscle cells contain desmin IFs with—as in myocardium—or without—as in other muscles—desmosomal junctions (for exceptions see 10, 29, 45, 81). The two major cell differentiation pathways in the nervous system of avian and mammalian species have been shown to be characterized by the synthesis of two special kinds of IFs: neurofilaments are the hallmark of neuronal differentiation; whereas glial differentiation is typically associated in astrocytes but also in certain ependymal and Schwann cells by the synthesis of large amounts of a specific IF protein, the glial fibrillary protein (GFP; 5, 8, 17–20, 24, 25, 42, 48, 69, 75, 76, 83).

Numerous studies of embryogenesis of the avian and mammalian nervous system and of in vitro differentiation of neural cells have shown that differentiating glial and neuronal cells are characterized by the absence of cytokeratin IFs and...
the appearance of neurofilaments (in neurons) and of GFP
(in certain glial elements, often in combination with vimen-
tin) (7, 17-20, 23, 49, 64, 68, 69, 75, 79). A portion of the
nervous system particularly intensely studied with respect to
glial development is the optic nerve where the same princi-
ple approach has been found (for reviews see 40, 58). These observations of a glia-
specific expression of GFP have led to the view that glial fila-
ments are important for the development and cell type-specific functions of astrocytes. Here we show that the astroglial cytoskeleton of the optic nerve of various amphi-
bian species consists primarily of cytokeratin IFs and that the cells of this tissue are interconnected by true desmo-
somes.

Material and Methods

Animals and Tissue Preparations

African clawed toads (Xenopus laevis) were obtained from the South African Snake Farm (Fish Hoek, South Africa); frogs (Rana ridibunda), captured in Turkey and on the Balkan Peninsula, were from Fivaz SA (Vallorbe, Switzerland); and newts (Pleurodeles waltlii) were from the Station d'accli-
matation et d'élevage (Bouilh-St. Paul, France). After decapitation, halves of enucleated posterior eye cups containing the optic nerve were frozen by immersion in melting isopentane (−130°C) or were fixed for electron mi-
croscopy. For comparison, whole tadpoles of X. laevis (stage 42) were fro-
zen for immunocytochemistry. For both immunocytochemistry and bio-
chemical analyses of cytoskeletal proteins, various tissues (brain, spinal cord, and sciatic and optic nerves) were snap-frozen and stored at −70°C. For comparison, X. laevis kidney epithelial cells of line A6 were grown as described (XLKE cells; cf. 31, 39) often after labeling overnight with [35S]-methionine (150 μCi/6-ml culture dish; added in minimal essential medium containing one fifth of the normal methionine concentration).

Electron Microscopy

Pieces of eye tissue, including samples containing the optic nerve, were
fixed as described (cf. 63) or in the presence of 0.2% tannic acid as de-
scribed previously for retinaiæ (71). Samples were washed, postfixed, de-
hydrated, embedded, and processed for ultrathin section electron micro-
scopy as described (63, 71).

Antibodies

The following primary antibodies were used: (a) murine monoclonal anti-
body lu-5 which reacts with a broad range of cytokeratins (29, 41; Boehringer Mannheim GmbH, Mannheim, FRG). (b) Murine monoclonal antibody Kp.an 1-8.136 (from Progen Biotechnics, Heidelberg, FRG) which reacts with most basic type II cytokeratins of mammals as well as of amphibia (for Xenopus cytokeratins see specifically Figs. 10 and 6 of references 28 and 41, respectively). This antibody reacts with all Xenopus epithelia examined and with certain other tissues known to contain cyto-
keratin 1/8 (41). (c) Guinea pig antibodies raised against purified bovine cytokeratins 8 and 18 (cf. 30, 74; Progen Biotechnics); these antibodies re-
act with the same tissues as the murine antibody Kp.an 1-8.136. (d) Murine monoclonal antibody VIM 3B4 specifically reacting with vimentin from amphibia, birds and mammals (39; Progen Biotechnics). (e) Guinea pig an-
tisera against bovine vimentin (cf. 30, 31). (f) Murine monoclonal antibod-
ies against desmoplakin I and II (DP la2-2.19) alone or in mixture with DP la2-2.15 (14). (g) Guinea pig antibodies against purified bovine neurofilament polypeptides (cf. 2, 33, 74). (h) Murine monoclonal antibod-
ies against GFP were antibodies GFP2.24 (C; Progen Biotechnics) and anti-
body GA-A-5 (21; Boehringer Mannheim GmbH). (i) A rabbit antiserum against GFP (from Dakopatts, Hamburg, FRG). Secondary antibodies were goat immunoglobulins against mouse and guinea pig immunoglobulins and were coupled to Texas red or FITC (Dianova, Hamburg, FRG).

Immunofluorescence and Immunoelectron Microscopy

Cryostat sections of nerve-containing tissues and eye cups were prepared and reacted with antibodies as described (63). For immunoelectron micro-
scopy, the preembedding procedure for cryostat sections (cf. 15, 44) was ap-
plicated. Secondary antibodies were coupled to colloidal gold particles (Jans-
sen Pharmaceutica, Beerse, Belgium).

Gel Electrophoresis of Cytoskeletal Proteins and Immunoblotting

Defined tissue regions were dissected using a binocular microscope, snap-
frozen, and then extracted with detergent and high-salt buffer (“cytoskeletal
proteins.”); cf. 1). The cytoskeletal proteins of cultured Xenopus A6 cells have been described (28, 36, 39). SDS-PAGE and two-dimensional gel elec-
trophoresis, transfer to nitrocellulose paper, staining with Poncet-S, and
immunoblotting were as described, using for the latter either 125I-labeled secondary antibodies or protein A (1, 28, 63). Alternatively, cytokeratins were identified by their specific binding of 125I-labeled polypeptides of the complementary cytokeratin subfamily after blotting (for example see 28).

Results

Immunofluorescence Localization of Desmoplakin and Cytokeratin

As in higher vertebrates the nervous tissue of amphibian brain and spinal cord contain neurofilaments in neurons and GFP in glial elements, and the same holds for the diverse types of peripheral nerves (e.g., 3, 20, 38, 66, 67). In addi-
tion, we have recently shown (3) that in some amphibia the perineural cells of the sheaths of peripheral nerves, as well as the arachnoidal cells of the meninges, are interconnected by desmosomes (Fig. 1 a) and contain cytokeratin IFs (Fig. 1 b), an occurrence that has also been reported for some mammals but not for others (3, 61). Moreover, in the numerous amphibian peripheral nerves examined as well as in the brain and the spinal cord, the astrocytes were negative for both desmosomal proteins and cytokeratins (Fig. 1, a and b), with the surprising example of the optic nerve.

In the optic nerves of all amphibian species examined >80% of glial cells were astrocytes (77). Immunofluores-
cence microscopy on cryostat sections through these optic nerves revealed, in all three species examined, the presence of both desmosplakins and cytokeratins (examples of X. laevis

Figure 1. Immunofluorescence microscopy of frozen sections through sciatic (a and b) and optic (c-e) nerves of X. laevis (a-c) and R. ridibunda (d and e). (a, c, and d) Stained for desmosomes with monoclonal antibody to desmosplakin: DP la2-2.19 alone (a and c) or in a mixture with DP la2-2.15 (d). (b and e) Stained for cytokeratins with monoclonal antibody Kp.an 1-8.136 (b) or guinea pig antibod-
ies (e). In the sciatic nerve (a and b), the reactions of both desmosplakins (a) and cytokeratins (b) are restricted to the perineurial cell layers (brackets) and are absent from the nerve interior (asterisks). In addition, the endothelial layer of a nerve-associated blood vessel is positive for cytokeratins (b). In the optic nerve (c and d), desmosomes are abundant in the perineural cell layers of the arachnoid (brackets) but also occur throughout the entire interior of the nerve (central portion in c and below the arachnoidal cell layers in d). Note the higher frequency of desmosomes in the perineural meninges and the subjacent region corresponding to the glia limitans. Reaction for cytokeratins in the optic nerve head (e) is often particularly strong in the arachnoidal cells (upper bracket) and in the glial elements of the nerve interior. Here a relatively weak reaction is seen in the adjacent retinal pigment epithelium (lower right bracket). Bars: (a-c and e) 50 μm; (d) 25 μm. The Journal of Cell Biology, Volume 109, 1989 706
and *R. ridibunda* are shown in Fig. 1, c–e). With antibodies to desmoplakins a punctate pattern reflecting the distribution of desmosomes was found throughout the interior of the optic nerve (Figs. 1c and 2a) and was conspicuously concentrated in the epithelioid cell layers of the arachnoid and in the underlying glia limitans layer of astrocyte endfeet (Fig. 1, c and d). Similarly, intense staining for cytokeratins was observed not only in the meningeal cells but also in the glial elements of the nerve interior (Figs. 1e, 2c, and 3a and d). Similar immunostaining patterns were obtained with various antibodies known to cross react with amphibian cytokeratins: i.e., monoclonal antibodies K,pan 1-8.136 (Fig. 2c) and lu-5 (Fig. 3d) and the guinea pig antibodies against mammalian cytokeratins 8 and 18 (Figs. 1e and 3a). Frequently, the cytokeratin reaction in the optic nerve was even stronger than that in the adjacent retinal pigment epithelium (e.g., Fig. 1e; for a detailed study of retinal pigment epithelium in various species see 63).

When the distribution of cytokeratin-containing cells in the optic nerve was examined by double-label immunofluorescence microscopy using antibodies against various IF proteins, some of the glial elements within the optic nerve stained with both cytokeratin and vimentin antibodies, whereas other regions were positive only for cytokeratins (Fig. 3, a and b). The specificity of the vimentin immunostaining could be seen from the reaction with other vimentin-
Figure 3. Immunofluorescence microscopy of frozen sections through the optic nerve of *X. laevis* after reaction with antibodies to cytokeratins (a and d), vimentin (b and c), and neurofilament protein NF-L (e). (a and b) Double-label staining of the optic nerve head comparing the distribution of cytokeratins (a, guinea pig antibodies) with vimentin (b; murine antibody VIM 3B4). Note that the perineural meninges and certain cell tracts of the nerve interior are stained with both antibodies. (c) Specificity of vimentin staining (VIM 3B4) within the optic nerve (NO) is shown by comparison with the staining of the surrounding interstitial tissue (IT) as well as the erythrocytes, the endothelium, and the smooth muscle wall (bracket) of a blood vessel (BV). (d and e) Double-label staining of an oblique section through the optic nerve for cytokeratins (d; antibody lu-5) and neurofilament protein NF-L (e; guinea pig antibodies). Note that the two patterns are not superimposable. The perineural meningeal cell layer (brackets) is not stained by neurofilament antibodies (e). Arrows point to some cells with prominent neurofilament contents. Bars, 50 µm.
Figure 4. Electron micrographs of sections through the interior of the optic nerve of the frog, R. ridibunda, showing glial filaments and desmosomes. (a) Survey picture showing the abundance of IFs in axonal processes of neurons (N), including myelinated ones, and of glial elements (G), which are exclusively astrocytes in this region. The arrow denotes a desmosome to which IF bundles attach. (b) Higher magnification picture showing an extended junction with typical desmosomal organization; i.e., two membranes and the central midline structure (parallel bars), the two cytoplasmic plaques (brackets), and attached IF bundles (i.e., tonofibrils; T). (c) Cross section of a small desmosome showing the two plaques (brackets) and the numerous IFs that are associated with the plaques, mostly abutting at a low angle. Bars: (a) 0.5 \mu m; (b and c) 0.25 \mu m.

taining cells, such as the fibroblasts of the perineural connective tissue, the endothelium, the smooth muscle cells of the vascular walls, and even the erythrocytes (Fig. 3 c; see also 39). Double-label comparison of the cytokeratin antibody reaction with that of neurofilament protein antibodies (Fig. 3, d and e) shows that the cells containing cytokeratins were different from those positive for neurofilaments; i.e., the optic nerve axons.

Also unexpectedly, monoclonal GFP antibodies, which strongly reacted with some ependymal structures and certain other glial cells of amphibian brain, spinal cord, and various peripheral nerves (not shown; cf. 20, 38), were practically negative on the optic nerve interior. The unspecified commercial rabbit GFP antiserum showed some diffuse staining of the optic nerve; however, this was difficult to assess because the antiserum also reacted with proteins other than GFP (2).

Electron Microscopy

In ultrathin sections through Rana and Xenopus optic nerves, we noted abundant IFs in the glial cell processes surrounding both myelinated and nonmyelinated axons (Fig. 4 a). These IFs were often parallel, forming loosely woven fleeces (Fig. 4, a–c) rather than the tightly packed bundles that are typical of cytokeratin filaments in diverse epithelial cells. In some astrocytic processes, these IFs were the only major cytoplasmic structures seen (Fig. 4, a and c). Adjacent glial cells were connected by numerous desmosomes of various sizes, which revealed characteristic structural elements such as the midline, the pair of plaques, and the laterally attached bundles of IFs (Fig. 4, b and c). Occasionally, we noticed intracytoplasmic vesicles that were associated with plaque structures and IFs (Fig. 5 b) and probably represented endocytosed desmosomal elements, as they have been described in other tissues (44).
Figure 5. Immunoelectron microscopy of sections showing the astrocyte processes in the interior of the optic nerve of *X. laevis* after reaction with cytokeratin antibodies and secondary antibodies coupled to colloidal gold particles. (a) Survey picture showing a desmosome between two astrocytes (bracket) and the myelin sheath–covered axon of a neuronal element (N). Note that the gold particles are exclusively associated with the IF bundles of the astrocytes. (b) Higher magnification of peripheral regions of two adjacent astrocytes connected by a desmosome (bracket) showing immunogold decoration of IF bundles. V, intracytoplasmic vesicle with desmosomal plaques, probably originated by endocytosis. Bars: (a) 0.5 μm; (b) 0.2 μm.

Electron microscopic immunolocalization of cytokeratins showed a specific reaction of immunogold particles with IFs of astroglial cells only, whereas the neuronal cells, including the neurofilaments, were devoid of any label (Fig. 5 a). Immunogold decoration was associated with most of the glial cell IFs (Fig. 5 b), although we could not exclude the possibility that a minor subfraction of the IFs was negative and perhaps not of the cytokeratin type. Remarkably, the immunogold particles were excluded from the desmosomal plaques proper (Fig. 5 b), perhaps reflecting the limited accessibility of the antigenic structures within these dense webs.

**Identification of Cytokeratins by Gel Electrophoresis and Immunoblotting**

To identify the proteins reactive with cytokeratin antibodies, we applied gel electrophoresis with immunoblotting. Monoclonal antibody K.pan 1-8.136, which recognizes a number of basic (type II) cytokeratins in various species, reacted in immunoblots of SDS-PAGE–separated cytoskeletal proteins from optic and sciatic nerves of *R. ridibunda* with a component of ~56,000 *M*₂, (Fig. 6 b), probably the amphibian equivalent to human cytokeratin 8 (35). However, this kind of analysis did not allow definitive identification of the individual cytokeratin polypeptides. Therefore, to further resolve the cytokeratins present in the optic nerve, we separated the cytoskeletal proteins from microdissected optic nerve tissue of *Rana* (Fig. 7, a–d) and *Xenopus* (Fig. 7, e and f) by two-dimensional gel electrophoresis and examined them by immunoblot reactions with different cytokeratin antibodies. The predominant component of the basic (type II) cytokeratin subfamily was the largest immunoreactive polypeptide with an ~56,000 *M*₂, and appeared as a series of isoelectric variants, probably differently phosphorylated forms (Fig. 7, b–d and f). As judged from its electrophoretic mobility and immunoreactivity, this polypeptide corresponded to the "56k/5.8–6.0 protein" described by Quitschke et al. (67) in cytoskeletons of the optic nerve of *Rana catesbeiana* and seemed identical, or closely related, to the well-characterized cytokeratin 8/1 of *X. laevis* (35, 36). Three other major polypeptides reactive with cytokeratin antibodies were noted in the optic nerve cytoskeletons of both species (one with an ~55,000 *M*₂, and two with ~50,000 *M*₂, in *Xenopus* [Fig. 7, e and f]; and 52,000, 49,500, and 49,000
Identification of amphibian basic (type II) cytokeratin(s) related to human cytokeratin 8 among the cytoskeletal proteins of microdissected amphibian nerves by SDS-PAGE and immunoblotting. (a) Coomassie blue staining of SDS-PAGE-separated cytoskeletal polypeptides from cultured kidney epithelial cells of X. laevis of line A6 (lane 2) in comparison with those of microdissected optic (lane 3) and sciatic (lane 4) nerves of R. ridibunda and reference proteins (lane 1 shows from top to bottom: myosin heavy chain, β-galactosidase, phosphorylase A, BSA, ovalbumin, carbonic anhydrase). (b) Autoradiography of an immunoblot of a parallel SDS-PAGE (with a much lower protein loading in lane 2 compared with a, lane 2) obtained after reaction with monoclonal cytokeratin antibody K-pan 1-8.136. The reactive bands in b correspond to a component of ~56,000 Mr (indicated by dots in a).

M., in Rana [Fig. 7, a and c]). Similar proteins, although not identified as IF components, can also be seen in optic nerve tissue preparations of R. catesbeiana (67).

When the cytoskeletal proteins of sciatic nerves (Fig. 7 g) and spinal cord interior (not shown) were examined in the same way, none of the major cytoskeletal polypeptides identified by Coomassie blue or Ponceau S staining was reactive with the cytokeratin antibodies (data not shown). Conversely, none of the major cytokeratins identified in the optic nerve was identical with one of the three very minor cytokeratin-reactive polypeptide spots of the sciatic nerve cytoskeleton (Fig. 7 g, arrowheads). From the known distribution of immunocytochemical reactivity of the sciatic nerve (cf. Fig. 1 b) and the immunoblot-reactive components detected in the optic nerve cytoskeletons after prolonged exposure (not shown), we suggest that these minor cytokeratins are located in the perineurium of this nerve (3) and that the optic nerve astroglial and the perineurial cytokeratin polypeptide complements may be different. Use of the various GFP antibodies (see Materials and Methods) has not identified any of the stainable cytoskeletal polypeptides of the optic nerve as being GFP (not shown).

The cytoskeletal proteins of the optic nerve of Xenopus were also directly compared with those of a well known cultured epithelial cell line from the same species. When [35S]-methionine-labeled cytoskeletal proteins of kidney epithelial A6 cells (for identifications of cytokeratins and vimentin in this line see 28, 36, 39) were coelectrophoresed with optic nerve cytoskeletal proteins, two of the cytokeratins in either sample (Fig. 7, h and i, open circles), in addition to the minor component vimentin, comigrated, whereas two polypeptides, probably cytokeratins, were specific either for the optic nerve or the A6 cells.

Discussion

Astrocyte differentiation is commonly believed to be associated with (a) the formation of abundant IFs containing GFP as the predominant protein, sometimes together with some vimentin; and (b) the absence of epithelium-type cytoskeletal elements (i.e., cytokeratin IFs or desmosomal junctions). In view of the concept that GFP is a typical and major component of the glial differentiation program (8, 20, 23, 25, 37, 42, 69, 75, 83), the results of the present study are unexpected since they show that the IF cytoskeleton and the intercellular connections of a certain type of glial cells—i.e., the astrocytes of the optic nerve in amphibia—are different from the cytoskeletal elements of many other nerves in the same species as well as from those of the astrocytes of the optic nerves of higher vertebrates.

True desmosomes have not been identified in glial and neuronal cells of higher vertebrates, notably mammals (e.g., 65; structures interpreted as desmosomes in reference 46 are probably erroneously classified), nor do these cells react with antibodies to desmosome-specific marker proteins. The situation is obviously different in lower vertebrates. Our immunolocalization of desmosomal marker proteins supports and biochemically substantiates earlier suggestions, based on electron microscopy, that certain plaque-bearing junctions between astrocytes of amphibian optic nerves represent true desmosomes (e.g., 9, 53, 77), although here, as in most tissues, desmosomes cannot be unequivocally distinguished from other kinds of plaque-bearing adherent junctions by morphological criteria alone (cf. 32). In addition, immunostaining with antibodies against plakoglobin (cf. 15) has shown an even more extensive distribution of reactive spots (data not shown), indicating that other—i.e., nondesmosomal plaque-bearing junctions—are also involved in connections of astroglial cells.

The identification of the nature of the connections between astrocytes is important in relation to the demonstrated mechanical and electrical coupling of these cells, their special conductance properties, and the positive effects that the astrocyte-surface system exerts on growth and differentiation of certain subtypes of glial and neuronal cells. It is particularly relevant for our understanding of how neurite-astroglial adhesion and the supracellular architectural order characteristic of the optic nerve are established and maintained (9, 16, 23, 27, 46, 49, 58, 70). Moreover, interactions between a special subtype of astrocytes and the surfaces of certain Schwann cells appear to help form the nodes of Ranvier (58, 82). With this in mind, however, our finding that in these amphibian desmosomes occur only in the optic nerve astroglia, and not in glial elements of other neurological structures, is as puzzling as the apparent absence of desmosomes from all mammalian glial and neuronal elements, including optic nerves. Moreover, the first identification of desmosomes in the glial system of some lower vertebrates should remind one that junctions with "desmosome-like" appearances have often been described in a wide range of invertebrate glial tissues (e.g., 11, 12, 26, 47, 59, 72).

Our observation that most, if not all, astrocytes of a nerve
contain abundant cytokeratin IFs is remarkable. While one of these cytokeratins (~56,000 M_0) comigrates with, and is probably identical to, the major type II cytokeratin found in single-layered ("simple") epithelia, oocytes, early embryos, and certain smooth muscles of X. laevis and appears to be the amphibian homologue to human cytokeratin 8 (28, 35, 36, 41), the other polypeptides are not yet directly comparable with any of the characterized Xenopus cytokeratins (cf. 28, 36, 54).

Some of the cytokeratin-containing astroglial cells appear also to contain vimentin, though only in small amounts, since our gel electrophoretic analyses did not detect vimentin (cf. 39) among the major cytoskeletal proteins from optic nerve tissue. Unexpectedly, we did not detect significant GFP in the amphibian optic nerve despite the use of antibodies that reacted with GFP in other nervous tissues of the same animals; nor was a GFP candidate among the major cytoskeletal proteins identified in our gel electrophoretic analyses of optic nerve proteins. Remarkably, the literature on the occurrence in fish and amphibian tissues of a protein closely related to mammalian GFP is rather controversial: immunoblot results with antibodies against mammalian GFP have been "very weak" or negative (20, 50, 67) or have shown reactions with an unusually large polypeptide (~66,000 M_0) of uncertain nature (38). In addition, the published gel electrophoretic results of optic nerve proteins from various other amphibians also do not show a GFP candidate (e.g., 20, 67).

Correspondingly, immunocytochemical studies have also shown no (60) or only "comparatively few" (20) structures.

II cytokeratin. In addition, the guinea pig antibodies (c) react with two of the other major cytoskeletal polypeptides (denoted by the right arrow and the fork), which are probably acidic (type I) cytokeratins. (e and f) Identification of cytokeratins in optic nerve tissue of X. laevis by immunoblotting. (e) Ponceau S staining of proteins blotted on nitrocellulose filter. Major cytokeratins are denoted by brackets. The uppermost component of ~56,000 M_0 was a type II cytokeratin as shown by its reaction with antibody K, pan 1-8.136 (not shown). (g) Ponceau S staining of cytokeratin polypeptides present in microdissected sciatic nerve tissue of X. laevis. Note the high complexity of polypeptide composition. None of the components stained by the dye reacted with any of the antibodies to cytokeratins used. The positions of the three minor components that did react with cytokeratin antibodies are denoted by the arrowheads and are not visible by protein staining with Coomassie blue or Ponceau S. These are probably components of the perineurial epithelium (for details see Fig. 3). Note the numerous Ponceau S-stained proteins, most of which are probably noncytokeratinous IF proteins, which in this species have not yet been positively identified by two-dimensional gel electrophoresis. (h) Identification of cytokeratins among the cytoskeletal proteins of the optic nerve of X. laevis (h) by coelectrophoresis with [35S]methionine-labeled cytoskeletal proteins from cultured kidney epithelial cells (line A6) of the same species (i); same gel electrophoretic system as in a and f). Brackets in h denote the same components as in e and f; those in i denote the four major cytokeratins of A6 cells. Cytokeratin polypeptides denoted by an open circle comigrate; those denoted by filled circles are exclusive to the specific cytokeratin. V, vimentin (minor component in the nerve tissue shown in h); a, endogenous nonmuscle actin.

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that cross react with antibodies to authentic mammalian GFP (see, however, 38, 57). While our results do not exclude the presence of minor amounts of GFP in optic nerve astrocytes, they obviously allow the conclusion that the large amounts of cytokeratins existing in these cells exceed the cytoskeletal contributions of all other kinds of IF. So GFP could only be a minor component. Interestingly, coexistence of cytokeratin and GFP IFs, has been demonstrated in some human myoepithelial cells of salivary glands and in pleomorphic adenomas (2, 56) as well as in certain cells of ependymomas (51) and astrocytomas (13).

Cytokeratins, which have also been reported immunocytochemically in astroglia of the optic nerve of a fish (52), have not yet been identified in astrocytes of higher vertebrates with the exception of a subpopulation of astrocytes in the rat optic nerve which contain cytokeratins 8 and/or 18, some of them apparently together with GFP IFs (Stumpp, S., and W. W. Franke, unpublished data). In the case of a relatively large polypeptide (~65,000 M\(_{r}\)) that cross reacts with epidermal cytokeratins which has been reported to occur in ependymal cells and in a subclass of brain astrocytes of mouse and hamster (34), the cytokeratinous nature is unclear. In this context, it is also worth mentioning a report (80) that astroglial IFs of degenerating optic nerves of mice contain two polypeptides of 45,000 and 55,000 M\(_{r}\), which so far have not been related to one of the known IF proteins.

Our observations allow several important conclusions. First, they show that the synthesis of cytokeratins and the assembly of cytokeratin IFs, although characteristicly found in lining epithelia, is not restricted to epithelial cells. In this respect, the situation found in the three-dimensional astroglial system of the amphibian optic nerve is similar to findings of cytokeratin IFs in other cells systems that do not border on a basal lamina and/or a luminal space, such as the three-dimensional mesh works of reticulum cells in the thymus and extrafollicular zones of lymph nodes (29, 32, 55, 74). Moreover, small amounts of cytokeratins 8, 18, and 19 (or their homologues) have been recently identified in certain amphibian and human smooth muscle tissues as well as in fetal myocardium of human and chicken (e.g., 10, 41, 45, 81). This shows that the expression of genes encoding certain cytokeratins, which in most cells are not coexpressed with muscle-, neuron-, or glial-type IF proteins, is not always regulated in a way mutually exclusive with the synthesis of these other kinds of IF proteins. The same arguments hold for the cell type-specific expression of desmosomal constituents.

Second, the differentiation of astrocytes and the establishment of a functional astroglia does not depend on the formation of IFs containing GFP, but might as well be effected with cytokeratin IFs. Hence, the formation of glial filaments may not be an indispensable feature of astrocyte differentiation and functions, at least in the optic nerve.

Third, desmosomes can be formed in true astrocytes, at least in the optic nerve of lower vertebrates, where they are abundant and obviously contribute to the astroglial tissue framework, which is known for its high reorganization potential and effective neurite "guidance," particularly after damage to the nerve (58, 70).

Fourth, the astrocytes of different nerves of the same animal differ drastically in their cytoskeletal and junctional complements. This conclusion is also supported by experimental results (43, 66, 67) showing that in fish, newts, and frogs the cytoskeletal protein composition of the optic nerve is profoundly different from that of the spinal cord, whereas only minor polypeptide differences between these two kinds of nervous tissues were noted in mammals. These authors (43, 66, 67) as well as Maggs and Scholes (50), who also described an optic nerve-"specific" IF protein of 56,000 M\(_{r}\), have related these proteins to vimentin and not to cytokeratins. The reason for these differences in the same cell type (i.e., the astrocyte) in different kinds of nerves is not clear. It is possible that the dominance of cytokeratin IFs and desmosomes is a special feature of the optic nerve, which in amphibians and fishes is known for its remarkable potential for remodeling (16) and regeneration, involving astrocyte hypertrophy and neurite outgrowth (9, 67, 70; for regeneration of spinal cord elements in certain fish species see 4).

During embryogenesis the formation of GFP filaments is a rather late addition to astrocyte differentiation, at least in birds (79) and mammals, in which this protein appears in precursor cells containing vimentin IFs (e.g., in several rodent species it is a strictly postnatal event in progenitor cells that have already become committed to glial differentiation in embryonal stages; cf. 6, 7, 17, 19, 23, 75). In contrast, the continual synthesis of desmosomal components and cytokeratins as the predominant IF protein in astrocytes of a special portion of the nervous system, the amphibian and fish optic nerve, may represent a glial cell type that is "archaic" in evolution and "embryonic" in ontogeny (66), reflecting its neuroepithelial origin. Remarkably, in Xenopus embryogenesis, certain simple epithelium-type cytokeratins are continually expressed, together with other cell type-specific IF proteins, in most tissues of the early embryo, the nervous system included (28, 38), whereas synthesis of cytokeratins in immature astrocytes or astrocytic precursors has so far not been noted during development of birds and mammals (cf. 7, 17-20, 69, 75, 79). Recently, however, Bartlett et al. (6) have described the basic fibroblast growth factor-induced synthesis of marker proteins for neuronal and astrocytic differentiation, including GFP, in certain clones of murine neuroepithelial cells taken at embryonic day 10 and immortalized by transfection with the c-myc oncogene, although their report did not specifically examine the coexistence of GFP and cytokeratins in the same cells. Cytokeratins have also been reported to occur, occasionally and focally, in certain human ependymomas, astrocytomas, and "primitive neuroectodermal tumors" (22, 51, 73). Thus, expression of cytokeratins may be characteristic of a certain type of astrocytes that remains in an embryonal proliferative state.

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