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Oxysterol 7α-Hydroxylase (CYP39A1) in the Ciliary Nonpigmented Epithelium of Bovine Eye

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SUMMARY: The CYP39A1 oxysterol 7α-hydroxylase preferentially catalyzes the 7α-hydroxylation of 24-hydroxycholesterol and has been suggested to play a role in the alternative bile acid synthesis pathway in the liver. The presence of CYP39A1 oxysterol 7α-hydroxylase has been reported only in the liver. To investigate the physiologic characteristics of the ciliary processes in bovine ocular tissues, we raised a mAb, 42C, against nonpigmented epithelial (NPE) cells, which have tight junctions that act as a blood-aqueous barrier and are involved in producing aqueous humor and maintaining ocular homeostasis. Immunohistochemical analysis showed that 42C antibody reacted intensely with an antigen in the NPE cells of the ciliary processes but not with other ocular tissues. The SDS-PAGE profile of immunoaffinity-purified antigens from bovine ciliary processes showed a predominant protein of molecular mass of 44.0 kDa. The amino acid sequence of this antigenic protein was identical to human CYP39A1 oxysterol 7α-hydroxylase. Immunoreactivity with 42C antibody was found only in hepatocytes and ocular tissues. These data suggest a new physiologic function for the CYP39A1 oxysterol 7α-hydroxylase in addition to the production of bile acids and provide new insight into the physiologic role of the ciliary NPE cells concerning the metabolism of sterols in the eye. (*Lab Invest 2003, 83:349–355*).

Three different sterol 7α-hydroxylases participate in cholesterol catabolism in the liver and are involved in the conversion of cholesterol into bile acids. The classic pathway of the bile acid synthesis is initiated with the conversion of cholesterol to 7α-hydroxycholesterol by cholesterol 7α-hydroxylase (CYP7A1) (Jelinek and Russell, 1990; Schwarz et al, 1996). The alternate pathway is initiated with hydroxylation of the side chain of cholesterol to form an oxysterol, which is then hydroxylated at the 7α-position by an oxysterol 7α-hydroxylase (Schwarz et al, 1997; Toll et al, 1994). Recent studies show that two kinds of oxysterol 7α-hydroxylases are involved in the alternative pathway: the CYP39A1 oxysterol 7α-hydroxylase, which prefers 24-hydroxycholesterol (24-OH-Chol) (Li et al, 2000a), and the CYP7B1 oxysterol 7α-hydroxylase, which acts preferentially on 25- and 27-hydroxycholesterol (Li et al, 2000a, 2000b; Schwarz et al, 1997).

Cholesterol 24-hydroxylase, which converts cholesterol to 24-OH-Chol, is expressed predominantly in the brain as judged by RNA and protein blotting (Lund et al, 1999). Approximately 80% of the total 24-OH-Chol is reported to be localized in the brain (Lutjohann et al, 1996), and a net flux of 24-OH-Chol from the brain into the circulation is also reported (Kudo et al, 1989; Lutjohann et al, 1996; Pedersen et al, 1989). As the distribution of CYP39A1 oxysterol 7α-hydroxylase seems to be limited to the liver (Li et al, 2000a), 24-OH-Chol synthesized in the brain and excreted into the circulation is suggested to be metabolized by the CYP39A1 oxysterol 7α-hydroxylase in the liver (Bjorkhem et al, 1998).

The nonpigmented epithelial (NPE) cells and the pigmented epithelial cells make up the ciliary epithelium in the ciliary processes and secrete aqueous humor from the circulation into the eye. Aqueous humor supplies nutrients to nonvascularized tissues, such as the lens and the cornea in the eye (Reddy, 2000; Schutte, 1980), and maintains intraocular pressure. The NPE cells that face the posterior chamber are involved in the active secretion of aqueous humor (Usukura et al, 1988) and have tight junctions that act as a blood-aqueous barrier (Hirsch et al, 1977; Raviola, 1974, 1977). In addition, the NPE cells have various steroid hormone receptors (Mirshahi et al, 1996, 1997; Stokes et al, 2000; Suzuki et al, 2001) and are considered to modulate the aqueous humor in the maintenance of ocular homeostasis.

To investigate the function of NPE in the eye, we raised a mAb, 42C, that specifically reacted with bovine NPE cells. Partial amino acid sequence of the antigenic protein purified from bovine ciliary pro-

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cesses were identical to human CYP39A1 oxysterol 7α-hydroxylase (Li et al, 2000a). Immunohistochemical studies confirm the presence of CYP39A1 in NPE cells of the eye and in hepatocytes of the liver.

Results

Selection of 42C mAb-Producing Hybridoma

By indirect immunofluorescence staining, one mAb from clone 42C was found to be highly reactive to the NPE cells of bovine eye. No other ocular tissues demonstrated expression of the antigen. The isotype of 42C mAb was IgG 2a.

Immunohistochemical Analysis of 42C Antigen Expression in Bovine Eye and Other Organs

The 42C antigen was detected in the ciliary NPE cells of the anterior pars plicata (Fig. 1b) and the posterior pars plicata (Fig. 1e) but not in the pigmented epithelial cells. At the ora serrata, the expression of antigen in the NPE cells became weaker and was undetectable in the retina (Fig. 1h). The negative controls stained by antitrinitrophenyl (anti-TNP) mAb showed no expression (Fig. 1, c, f, and i). The weak expression of 42C antigen in the NPE cells of the iris extended for $\sim$1 mm from the root of the iris, and beyond this point iridial cells showed no expression. Immunofluorescence was detectable uniformly in the cell body but not in the nuclei of ciliary NPE cells by confocal laser microscopy (Fig. 2). No other intraocular structures demonstrated the expression of 42C antigen.

42C antigen was moderately expressed in the hepatocytes (Fig. 3). Specific staining with 42C mAb was not detected in bovine adrenal, kidney, duodenum, small intestine, brain (cerebral cortex and medulla), ovary (follicular phase through luteal phase), or spleen (data not shown).

42C Antigen Purified from Bovine Ciliary Processes

42C antigen was purified from bovine ciliary processes by immunoaffinity chromatography with 42C mAb-conjugated gel. The SDS-PAGE profile of the affinity-purified antigen showed a major specific protein at molecular weight 44,000 under reducing conditions (Fig. 4). This purification was performed independently four times with high reproducibility.

Partial Amino Acid Sequencing of the Protein Purified from Bovine Ciliary Processes

The 44.0-kDa protein was affinity-purified from bovine ciliary processes and transblotted onto a polyvinyli-

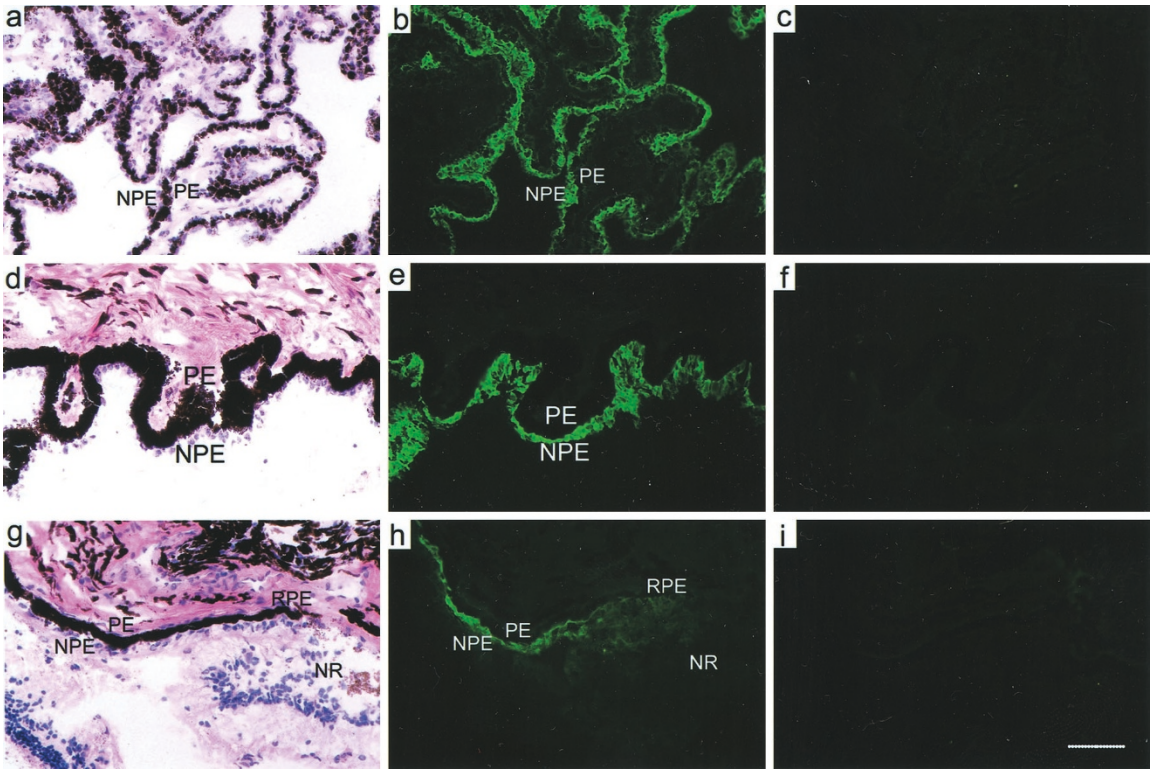

Figure 1.

The expression of 42C antigen in bovine ciliary cells detected by indirect immunofluorescence method. The immunohistochemistry was performed as described in "Materials and Methods." a to c, The anterior pars plicata (the anteriormost portion of the ciliary body including major ciliary processes). d to f, The posterior pars plicata (the anterior portion of the ciliary body including minor ciliary processes). g to i, The ora serrata (the area between the cilia and the retina). a, d, and g, Hematoxylin and eosin staining. b, e, and h, Staining with 42C mAb. c, f, and i, Negative controls staining with antitrinitrophenyl (anti-TNP) mAb. 42C antigen was expressed intensely in the nonpigmented epithelial (NPE) cells but not in the pigmented epithelial cells. Fluorescence intensity was negative in the retina. Separated neural retina from the posterior part of the retina is seen in the left bottom corner of g. PE, pigmented epithelium; NR, neural retina; RPE, retinal pigment epithelium. Scale bar = 100 μm.

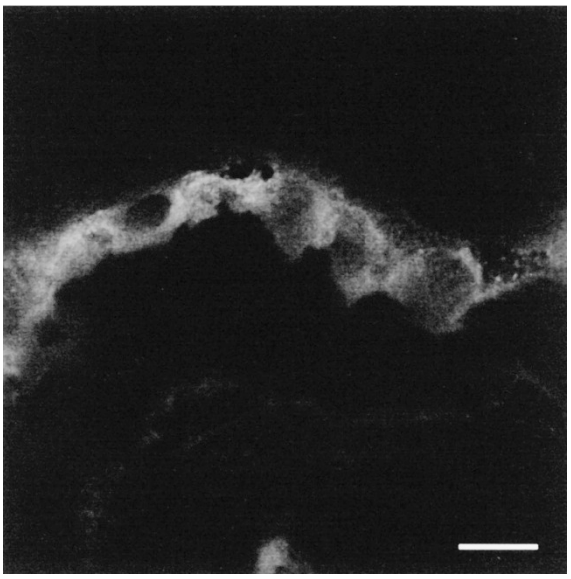

Figure 2.

Fluorescence observation by confocal laser microscopy. The immunohistochemistry was performed as described in "Materials and Methods." Fluorescence was detected uniformly in the ciliary NPE cells of bovine eye by confocal laser scanning microscopy with an argon-ion laser. No fluorescence was detectable in the nucleus of the NPE cells. Scale bar = 10 μm.

dene difluoride (PVDF) membrane. The N-terminal amino acid sequence of 13 amino acids was analyzed (Fig. 5, 42C-1). Next, the 44.0-kDa protein in the gel was treated with lysylendopeptidase, and three polypeptides were obtained. We analyzed the amino acid sequences of each polypeptide (Figs. 5, 42C-2, -3, and -4). A homology search showed that four peptide sequences were nearly identical in sequence to the human CYP39A1 oxysterol 7 α-hydroxylase.

Discussion

We generated a mAb, named 42C, which reacts specifically with antigens in the cytoplasm of bovine ciliary NPE cells. Immunoreactivity was not found in the other ocular tissues. The main protein purified from bovine ciliary processes by immunoaffinity chromatography using 42C mAb showed a molecular mass of 44.0 kDa. The amino acid homology search showed that four peptide sequences from 44.0-kDa protein were nearly identical (75 to 100% identity) to the cDNA-deduced protein sequence of human oxysterol 7α-hydroxylase encoded by Cyp39A1 locus (Li et al, 2000a) (Fig. 5). Any other possible cholesterol or oxysterol hydroxylase was not detected in search for homologous protein (data not shown). According to Li et al (2000a), cholesterol 7α-hydroxylase and CYP7B1 oxysterol 7α-hydroxylase share $< 30\%$ identity with CYP39A1 oxysterol 7α-hydroxylase. These results enable us to discriminate this antigen from any other possible cholesterol or oxysterol hydroxylase including cholesterol 7α-hydroxylase and CYP7B1 oxysterol 7α-hydroxylase. Thus, we conclude that the 42C antigen specific to the ciliary NPE cells is the CYP39A1 oxysterol 7α-hydroxylase.

Li et al (Li et al, 2000a, 2000b) demonstrated that CYP39A1 oxysterol 7α-hydroxylase was a microsomal cytochrome P450 enzyme that acts preferentially on 24-OH-Chol and that it was expressed in the liver. They did not find expression of CYP39A1 oxysterol 7α-hydroxylase in the heart, brain, spleen, lung, muscle, kidney, or testis by RNA blotting (Li et al, 2000a). In the present study, 42C mAb reacted intensely to the NPE cells and moderately with hepatocytes, but specific staining of 42C mAb was not observed in the adrenal, kidney, duodenum, small intestine, brain (cerebral cortex and medulla), ovary (follicular phase through luteal phase), or spleen. Our results suggest that the expression of CYP39A1 oxysterol 7α-hydroxylase is limited to hepatocytes in the liver and NPE cells in the eye.

Cholesterol 24 hydroxylase, which is expressed predominantly in the brain, converts cholesterol to 24-OH-Chol (Lund et al, 1999), and most of the 24-OH-Chol content in the body is contained in the brain (Lutjohann et al, 1996). In addition, 24-OH-Chol is the only oxysterol that has a net flux from the human brain into the circulation (Lutjohann et al, 1996). Excess cholesterol in the brain is converted into 24-OH-Chol, which is secreted across the blood-brain barrier into the circulation, and then 24-OH-Chol has been believed to be metabolized to bile acids in the liver by CYP39A1 oxysterol 7α-hydroxylase (Bjorkhem et al, 1998; Li et al, 2000a). It is interesting that CYP39A1 oxysterol 7α-hydroxylase exists in the NPE cells because ocular tissues are not involved in bile acid synthesis. The present results indicating that CYP39A1 oxysterol 7α-hydroxylase is expressed in the NPE cells of the ocular tissues suggest a new physiologic function for this enzyme in addition to the production of bile acids.

The ciliary epithelial cells of the ciliary processes are well known to secrete aqueous humor to maintain the intraocular pressure. Aqueous humor derives primarily from blood circulating in the capillaries of the ciliary processes, and it passes through the two-layered ciliary epithelial cells, the pigmented epithelial cells, and the NPE cells into the posterior chamber of the eye. The capillaries of the ciliary processes are highly permeable to protein, and they have fenestrations (Hara et al, 1977; Ober and Rohen, 1979) facing the ciliary epithelium (Lutjen et al, 1983). To prevent the passage of blood-borne macromolecules into the posterior chamber, tight junctions of the NPE cells are presumed to act as a blood-aqueous barrier (Hirsch et al, 1977; Raviola, 1974, 1977). The findings of the present study suggest that 24-OH-Chol, transported to the NPE cells via the circulation, would be metabolized into 7α-hydroxylated 24-hydroxycholesterol by CYP39A1 oxysterol 7α-hydroxylase in the NPE cells.

To date, the distribution and activity of few steroid-metabolizing enzymes have been studied in ocular tissues. Immunohistochemical localization of the mineralocorticoid receptor (Mirshahi et al, 1996, 1997; Stokes et al, 2000; Suzuki et al, 2001) and the glucocorticoid receptor (Stokes et al, 2000; Suzuki et al, 2001) was demonstrated in NPE cells. Corticosteroid

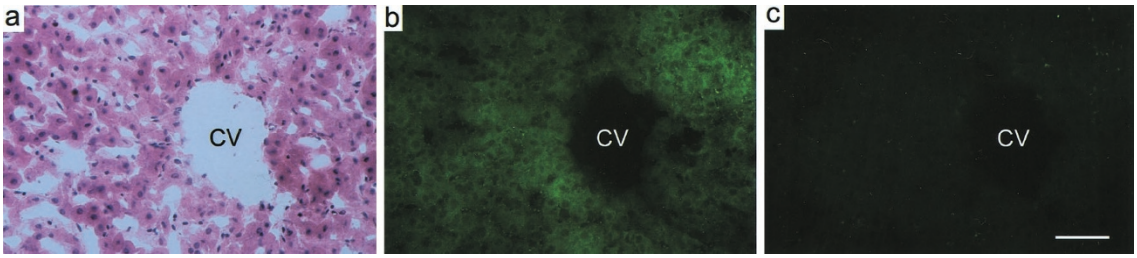

Figure 3.
The expression of 42C antigen in the liver detected by indirect immunofluorescence method. The immunohistochemistry was performed as described in "Materials and Methods." a, Hematoxylin and eosin staining. b, Staining with 42C mAb. c, Negative control staining with anti-TNP mAb. Immunofluorescence for 42C mAb was moderately expressed in the hepatocytes. CV, central vein. Scale bar = 100 μm.

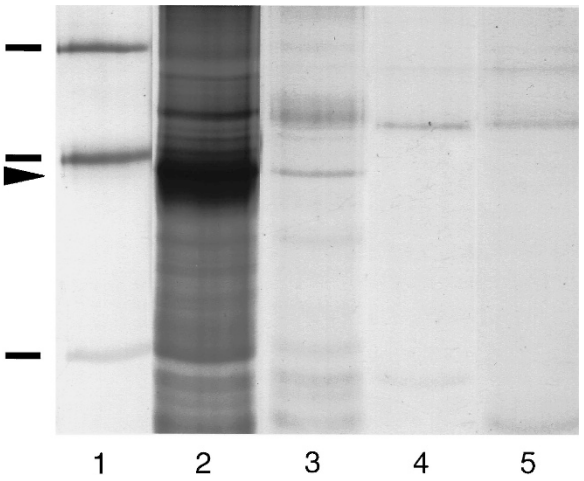

Figure 4.
SDS-PAGE analysis of the antigenic molecules purified with 42C mAb-conjugated Affi-gel 10. Purification of 42C antigen was performed by immunoaffinity chromatography. The purified 42C antigen was visualized by silver staining after 12% SDS-PAGE in reducing conditions. Lane 1, the molecular size markers. Lane 2, the proteins purified with 42C mAb from bovine ciliary processes. Lane 3, the proteins purified with anti-TNP mAb from bovine ciliary processes. Lane 4, the eluate from the 49C-conjugated Affi-gel 10 without incubation in the tissue lysate. Lane 5, the eluate from the anti-TNP–conjugated Affi-gel 10 without incubation in the tissue lysate. In Lane 2, a specific protein band was observed at the molecular mass of 44.0 kDa (*arrowhead*). Bars show gel molecular size markers of 66.2, 45, and 31 kDa from top to bottom.

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Homo sapiens     1  MELISPTVIIILG  13   (92.3%)
                    **.**********
42C-1               MEFISPTVIIILG

Homo sapiens    49  PLEFIEK  55        (100%)
                    *******
42C-2               PLEFIEK

Homo sapiens   135  FTGQLTEELHEQ  146  (92%)
                    *********.**
42C-3               FTGQLTEELQEQ

Homo sapiens   354  ILNYIIPSGDLL  365  (75%)
                    **.* .******
42C-4               ILDYTUPSGDLL
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Figure 5.
Partial amino acid sequencing analysis of the protein purified from bovine ciliary processes. The N-terminal amino acid sequences (42C-1) and internal amino acid sequences of three polypeptides (42C-2, -3, and -4) from the 44.0-kDa protein were compared with the homologous sequences deduced from the cDNA of human oxysterol 7α-hydroxylase (CYP39A1) (Li et al, 2000a). Four amino acid sequences determined from the 44.0-kDa proteins were highly homologous to human oxysterol 7α-hydroxylase (CYP39A1). Identical amino acids are indicated by asterisks, similarities are indicated by dots. The numbers beside the amino acid sequences of human CYP39A1 oxysterol 7α-hydroxylase represent the sequence position of the amino acids from the N-terminus. The percentage of identical amino acid residues is indicated.

hormone action has been examined through the activity of 11β-hydroxysteroid dehydrogenase (Rauz et al, 2001; Stokes et al, 2000; Suzuki et al, 2001). These studies showed that the NPE cells play a pivotal role in hormonal action modulating the aqueous humor or maintaining ocular homeostasis. The presence of CYP39A1 oxysterol 7α-hydroxylase in the NPE cells suggests that oxysterol, 24 OH-Chol, may be metabolized and its product may play important roles in the NPE cells. The NPE cells might be involved in the utilization of 24 OH-Chol for producing various steroid hormones or the prevention of the passage of 24 OH-Chol in the circulation into the posterior chamber.

The retina is also a target tissue of steroid hormones, where the glucocorticoid receptor (Suzuki et al, 2001), mineralocorticoid receptor (Golestaneh et al, 2001), and estrogen receptor (Kobayashi et al, 1998) have been found. Recently, the expression of oxysterol-binding proteins 1 and 2 was demonstrated in monkey retina (Moreira et al, 2001). The presence of oxysterol-binding proteins, which are capable of binding a variety of oxidized forms of cholesterol (Taylor and Kandutsch, 1985), suggests that oxysterols metabolized from 24-OH-Chol in the NPE cells may be used for steroidogenesis in the retina. In the ciliary epithelium, the metabolic pathways and functions of oxysterols are little known. We believe that our study is able to provide a clue to investigate the metabolic pathway of sterol in the eye. Additional investigation will assess the presence of CYP39A1 oxysterol 7α-hydroxylase enzyme activity and the functional significance of this enzyme in NPE cells.

Materials and Methods

Tissues

Bovine eyes were obtained from a local slaughterhouse. A razor blade cut was made around the entire

globe just posterior to the ora serrata. The lens, lens capsule, and zonulae were removed, exposing the ciliary process. Bovine adrenal, kidney, duodenum, small intestine, brain, ovary, liver, and pancreas were obtained from the same slaughterhouse.

Production of mAb, 42C

The ciliary processes were dissected from the excised eyeball and minced with scissors. They were incubated in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 0.2% collagenase (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 0.01% deoxyribonuclease I (Sigma, St. Louis, Missouri), at 37° C for 30 minutes. After the undissociated materials were discarded, the cell suspension was collected and centrifuged for 5 minutes at 160 $\times g$. Some of the cells were washed twice and suspended in phosphate-buffered saline and immediately used for immunization, and the remainder were stored in liquid nitrogen for further immunization. Eight-week-old BALB/c mice were injected intraperitoneally with 2×10^6 cells every 3 weeks. The spleen cells of an immunized mouse were fused with X63Ag8.653 mouse myeloma cells, using 50% polyethylene glycol 1500 (BDH Chemicals, England, UK) on the third day after the final immunization (Kohler and Milstein, 1975). The fused cells were cultured in RPMI medium supplemented with 15% FCS, 10% Boehringer Mannheim-condimed H1 (Boehringer, Germany), and hypoxanthine aminopterine thymidine (Flow Company, Scotland, UK) on 96-well plates. An indirect immunofluorescence method with frozen sections of bovine tissue as described below was used for screening the supernatants of growing hybridomas. The positive hybridomas were cloned twice and were injected intraperitoneally into mice previously treated with pristane (2,6,10,14-tetramethylpentadecane; Tokyo Kasei Company, Tokyo, Japan). IgG was purified from ascitic fluid with Affi-Gel protein A (Bio-Rad, Hercules, CA). Ig isotype was determined using an isotype-specific antibody for mouse mAbs (Serotec Ltd., Oxford, United Kingdom).

Indirect Immunofluorescence Staining

The tissue was embedded in Tissue-Tek OCT compound (Tissue-Tec; Miles Scientific, Naperville, Illinois) and snap-frozen in liquid nitrogen. Frozen sections were sliced to a thickness of 8 μm with a cryostat microtome (Microm HM 500M; Carl Zeiss, Jena, Germany) and immediately air-dried on Neoplene-coated (Nisshin EM, Tokyo, Japan) glass slides. The sections were fixed in acetone at $-20°$ C for 5 minutes. The slides of sections were incubated with 42C mAb (5 μg/ml, diluted in culture medium) or anti-TNP mouse mAb (IgG 2a, 5 μg/ml, for negative control) (Tsujimura et al, 1990) for 30 minutes at room temperature. After washing in PBS, they were incubated with fluorescein isothiocyanate-conjugated second antibody (DAKO Japan, Kyoto, Japan; dilution 1:50) for 40 minutes at room temperature in the dark. The slides were then washed, mounted with Perma Fluor Aqueous Mounting Medium (Immunon, Pittsburgh, Pennsylvania), and examined under a fluorescence microscope (Nikon, Tokyo, Japan). For high resolution, immunofluorescence was observed and recorded with a confocal laser scanning microscopy with an argon-ion laser (Carl Zeiss Microscope System LSM 510). A 488-nm laser light was used for excitation; emitted light was collected through a FT510 dichroic beam splitter and through a 515-nm–long pass filter. A 63× water-immersion objective with a 1.2 numerical aperture was used, affording total magnification of ×630. Images of 1024 × 1024 pixels were used.

Purification of 42C Antigen (Affinity Chromatography and SDS-PAGE)

The ciliary processes (0.5 g) were homogenized with Polytron (Kinematica AG, Luzern, Switzerland) in 5 ml of ice-cold 40 mM phosphate buffer (pH 7.3) containing 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40 (Iwai Chemicals, Tokyo, Japan), and protease inhibitors including 1 mM p-amidinophenyl methanesulphonyl fluoride hydrochloride and 10 μg/ml leupeptin and pepstatin (Peptide Institute, Osaka, Japan). After centrifugation (10,000 $\times g$, 30 minutes, 4° C), the concentration of Nonidet P-40 in the lysate was reduced by dilution to 0.3%. The effluent was passed through the column of Affi-gel 10 (Bio-Rad) that was conjugated with anti-TNP mouse IgG 2a mAb (2 mg IgG/ml gel). The lysate was then incubated with 0.2 ml of Affi-gel 10 that was conjugated with 42C mAb (2 mg IgG/ml gel) at 4° C for 3 hours. After sufficient washing with the buffer, the antigen was eluted with 0.5 M NH4OH containing 0.1% Nonidet P-40. The eluate was dried in vacuo at room temperature. The samples were dissolved in lysis buffer with 0.1 M dithiothreitol (Wako Pure Chemical Industries Ltd.) and were separated with 12% SDS-PAGE. Proteins in the gel were visualized by the silver stain kit (Wako Pure Chemical Industries Ltd.).

Partial Amino Acid Sequencing of the 44-kDa Protein Purified from Bovine Ciliary Body

Bovine ciliary processes (3 g) were homogenized with Polytron in 30 ml of ice-cold 40 mM phosphate buffer containing 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, and 1 mM p-amidinophenyl methanesulphonyl fluoride hydrochloride, as described above. After absorbing nonspecific binding with anti-TNP mAb-conjugated gel, the lysate was incubated with 1 ml of Affi-gel 10 conjugated with 49C mAb at 4° C for 3 hours. After the gel was washed, the antigenic molecules were eluted as above. The purified antigen was dissolved in lysis buffer for SDS-PAGE. After the electrophoresis, the proteins in the polyacrylamide gel were transblotted onto PVDF membrane (Millipore Corporation, Bedford, MA) in Tris/Boric acid buffer including 0.03% SDS and 10% methanol. The protein on PVDF membrane was stained with Coomassie blue (Nacalai Tesque, Kyoto, Japan), and the 44.0-kDa protein band was obtained, then N-terminal amino

acid sequences were analyzed. In some experiments, the proteins in the polyacrylamide gel were stained with Coomassie blue, and then the 44.0-kDa band was treated with lysylendopeptidase. The fragments released from the 44.0-kDa protein were collected and separated by reversed-phase HPLC for amino acid sequencing. N-terminal amino acid sequence of each polypeptide was analyzed (sequencing was performed by the APRO Life Science Institute, Tokushima, Japan). The SWISS-PROT databases were used in the analysis of amino acid sequence homology.

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