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Identification of a Ubiquitin-Like Protein in the Mammalian Vitreous Humor

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Abstract An 8 kDa ubiquitin-like peptide (ULP) was isolated by high performance liquid chromatography from the rabbit vitreous humor, and the N-terminal amino acid sequence of this peptide showed complete homology with ubiquitin. Western blot revealed the presence of free ULP in both the iris-ciliary (IC) complex and the aqueous humor extracts. In the IC complex, fluorescence and immunoelectron microscopy detected high concentrations of ULP in the posterior epithelial cells, suggesting this tissue as a possible source of ULP in the ocular fluids. Significantly, this is the first time that the presence of free ULP has been reported in mammalian extracellular fluids. Furthermore, we recently demonstrated that the 8 kDa fraction of vitreous humor containing ULP is a potent inhibitor of protein synthesis [Banerjee et al. (1992): J Cell Biochem 49:66–73]. These findings taken together suggest a novel biological role for ULP in the control of lens cell growth.

Key words: ubiquitin, vitreous humor, iris-ciliary complex, lens epithelial cells, eye

The vitreous humor (VH) is a unique tissue distinguished by an abundance of high molecular weight proteins, avascularity, the presence of macrophage-like hyalocytes, and transparency [Balazs and Denlinger, 1984]. It has intimate relations with the choroid, iris-ciliary body, lens, retina, and aqueous humor. Bito and DeRousseau [1980] postulated that the VH serves as a large metabolic pool for neighboring tissues and subserves their metabolic needs and/or disposes of their harmful metabolic products. Balazs and Delinger [1984] referred to the VH as a sink for the lens and retina, as it contains many degraded proteins of those tissues. Recent reports have assigned many other physiological and physical properties to this ocular fluid [Bito and DeRousseau, 1980; Banerjee et al., 1992]. Additionally, many physiologically important low molecular weight (LMW) proteins have also been detected in the vitreous humor [Banerjee et al., 1992]. Some of these LMW proteins are probably serum proteins, while others are secreted by adjacent ocular tissues or produced by hyalocytes. These LMW proteins are responsible for the antiangiogenic activity of normal bovine and human VH [Jacobson et al., 1984]. In a related study, Lisnayk [1988] isolated and characterized a 12 kDa protein from bovine VH with distinct antiangiogenic properties. Our laboratory has reported the presence of an 8 kDa VH protein with protein synthesis inhibitory activity [Banerjee et al., 1992]. In their investigations, Raymond and Jacobson [1982] isolated two growth inhibitors and two growth stimulators from the VH of fetal, newborn, young adult, and adult cows and determined that the molecular weight of one of these inhibitors to be 6 kDa.

Thus, it appears that LMW proteins of VH may play important roles in ocular physiology and pathology (3). In this investigation, we report the purification and characterization of an 8 kDa ubiquitin-like peptide isolated from rabbit vitreous humor and suggest the possible source of its synthesis.

MATERIALS AND METHODS

Isolation of Low Molecular Weight Proteins From Rabbit VH

Quick-frozen (in liquid nitrogen) whole eyeballs of mature (2 kg) rabbits were obtained from Pel-freez Biologicals (Rogers, AK). The eyeballs were thawed, and VH was removed by a posterior approach. Care was taken not to con-

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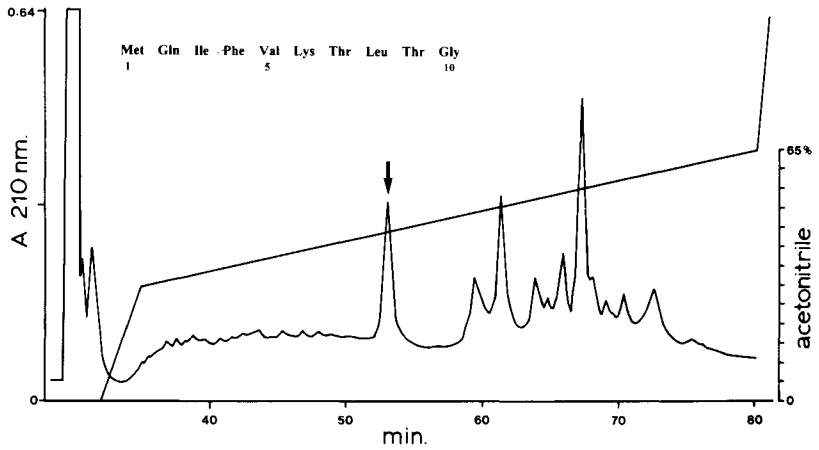

Fig. 1. A reverse phase HPLC fractionation of low molecular weight fractions of vitreal proteins. The protein peaks were monitored at 210 nm. The first peak, as indicated by the pointer, was isolated and microsequenced at its N-terminal. (The N-terminal sequence of the first ten amino acid residues is presented at the top of the graph.)

taminate the VH with other ocular fluids or tissues. Generally 400 intact eyeballs were used for each extraction. Thawed VH was centrifuged for 3 h at 100,000*g* in a Beckman ultracentrifuge. The supernatant which contains soluble proteins was centrifuged, first in an Amicon-10 concentrator to remove high molecular weight proteins (> 10 kDa) and then in an Amicon-3 concentrator to desalt the fraction and to remove proteins of 3 kDa and lower. Thus, the VH fractions of 3–10 kDa were retained for high performance liquid chromatography (HPLC) analysis.

HPLC of Low Molecular Weight VH Proteins

The low molecular weight VH was further purified by reverse phase HPLC. Approximately 300 µl low molecular weight VH was loaded onto a Zorbax protein plus column (4.6 cm × 25 mm; Dupont) and fractionated using a linear gradient formed by 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in 65% acetonitrile at a flow rate of 1 ml per minute. The eluant was monitored at 210 nm, and fractions were collected and lyophilized. The lyophilized proteins were submitted to vapor phase hydrolysis at 150° for 75 min in a picotag workstation (Waters Instruments). N-terminal amino acid microsequencing was obtained by automated Edman degradation on an Applied Biosystems gas phase sequencer (model 470) and a Nelson analytical chromatographic data system. The sequenced data was analyzed by Intelligenetics Protein Data Bank.

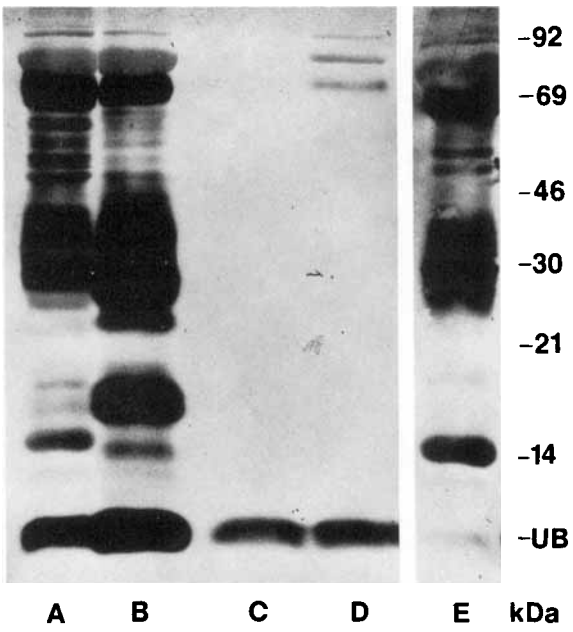

Fig. 2. Immunoblot of various ocular tissues and fluids using antiubiquitin antibodies. In each lane, 300 ug of total protein was loaded. *Lane A:* Immunoblot of aqueous humor proteins. *Lane B:* Immunoblot of total cell extract of iris-ciliary complex cells. *Lanes C, D:* Show immunoblots of total vitreous humor obtained from 2-month-old rabbit and 1-month-old rabbit, respectively. *Lane E:* Immunoblot of rabbit serum proteins. UB, ubiquitin as displayed by antiubiquitin antibody immunoblot.

Immunodetection of Ubiquitin in the Ocular Fluids and Tissues

Western blotting was used to determine the presence of ubiquitin in the rabbit aqueous humor (AH), vitreous humor, iris-ciliary complex (IC), and serum. The antiubiquitin polyclonal

antibody was obtained from the Sigma Biochemical Company. The AH, VH, IC, and serum were collected from the same rabbit. The aqueous humor was obtained by inserting a 26 gauge needle through the cornea near the limbus, and VH was removed from the posterior side of the eyeball near the entry of the optic nerve by aspiration with a 20 gauge needle. Both ocular fluids were transparent and were centrifuged at 10,000*g* for 3 h to remove any particulate matter. The rabbit serum was obtained by collecting blood in presence of anticlotting factor and then submitted to further centrifugation.

The presence of ULP in the IC was monitored by immunofluorescence microscopy. Freshly isolated rabbit IC was fixed in 0.1% glutaraldehyde *in cacodylate* buffer for 3 h on ice. The tissue was then repeatedly washed with cacodylate buffer, dehydrated by serial application of alcohol, and then embedded in hydrophilic resin (Lowicryl). The embedded tissues were sectioned to obtain both thick and thin sections. In the thick sections, immunofluorescence microscopy was used to locate specific tissue areas exhibiting the presence of ULP. Antiubiquitin antibody at a dilution of 40:1 was used as a primary antibody followed by fluorescent tagged IgG. The thin sections were exposed to antiubiquitin antibody as a primary antibody and then to 10 nm gold particles conjugated with IgG as secondary antibody. The immunogold labeled thin sections were visualized using a JEOL 100 CX electron microscope, operating at 60 kV.

RESULTS

Figure 1 shows the chromatograph of LMW proteins present in the VH as determined by HPLC fractionation. This fractionation procedure yielded ten major peaks. Peaks 2–10 overlapped, and immunoblot analysis indicated that some of the peaks contained fragmented α and B crystallins. Peak 1, which eluted at 43% acetonitrile concentration, was the focus of this investigation and therefore was collected and lypholized for further analysis. The N-terminal sequence of this fraction was determined by automated Edman degradation. The first amino acid could not be identified, but amino acids 2–10 were sequenced (Fig. 1) and showed a complete homology with ubiquitin (10,23). The first N-terminal amino acid of ubiquitin is methionine which is not detectable by our microsequencing technique. The peak 1 material was submitted to cyanogen bromide degradation followed by HPLC. The resulting chromatograph showed only one peak, confirming that the protein in peak 1, like ubiquitin, has no methionine except

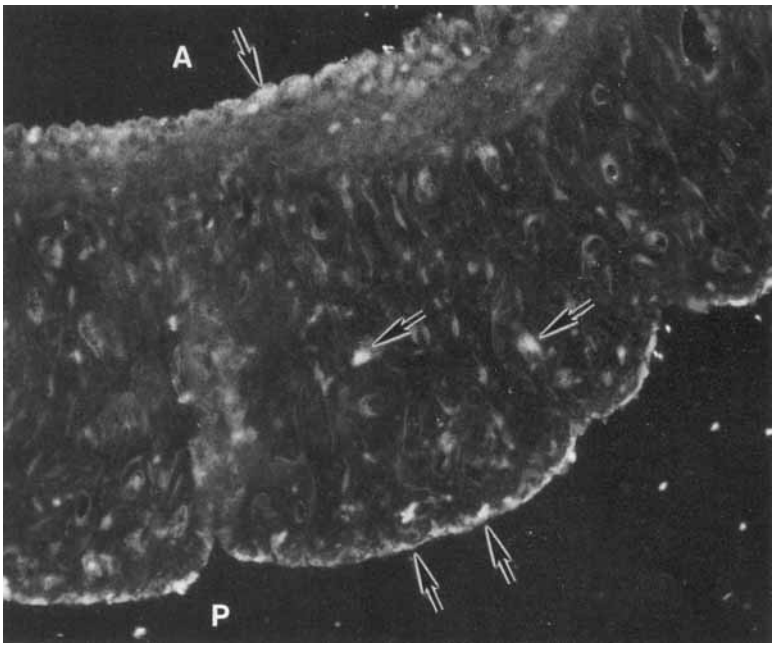

Fig. 3. Immunofluorescence pictures of iris-ciliary complex cells showing the presence of fluorescence in the posterior epithelial cells (*P*) and anterior epithelial cells (*A*). The *arrows* show blood vessels with abundant fluorescing antiubiquitin antibodies. ×250.

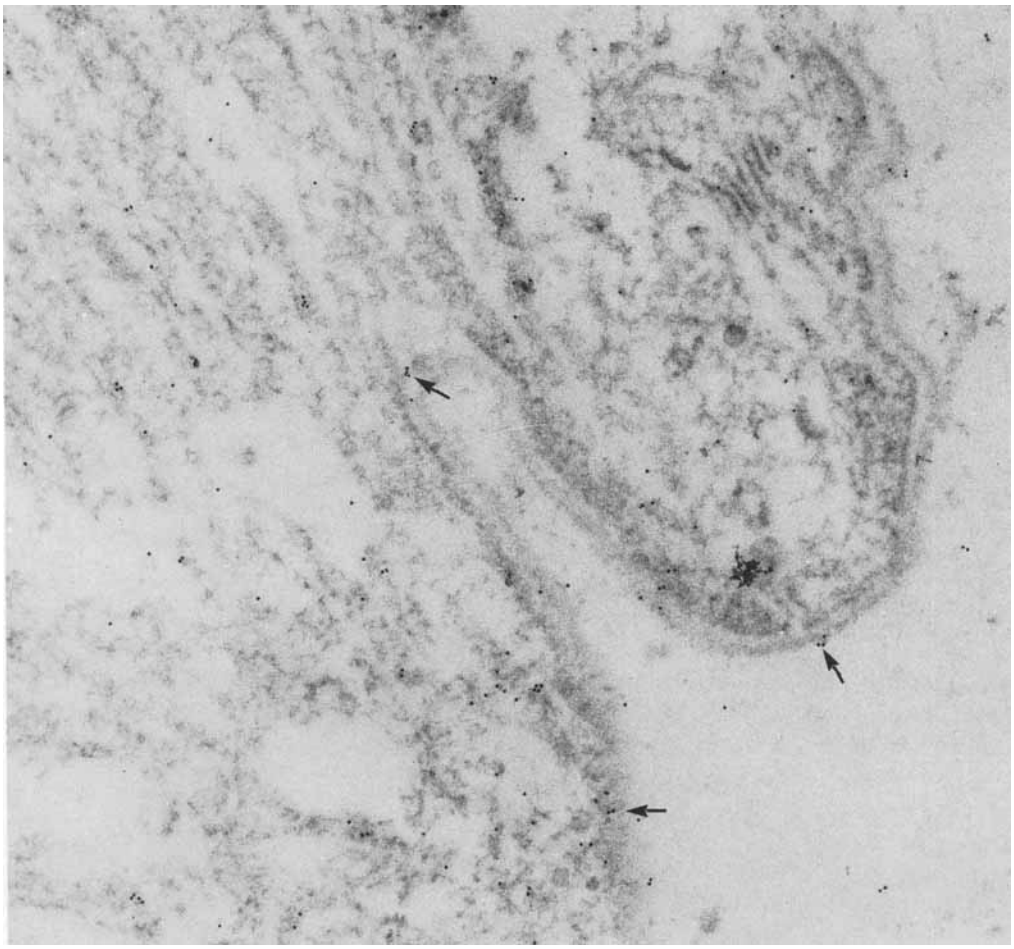

Fig. 4. Electron micrograph of the posterior iris-ciliary complex epithelial cells. This tissue was lightly fixed to produce integrity of the protein molecule; thus, the membrane and other ciliary structures do not show good contrast. *Arrows* indicate clusters of immunogold conjugated antiubiquitin antibody molecules. ×45,000.

in the N-terminus position, as indicated in the Figure 1 insert (data not presented).

Immunoblot analysis was also used to confirm the presence of free ULP in the VH. The data presented (Fig. 2) in this immunoblot clearly demonstrate the presence of ULP in VH, AH, and IC but not in the serum.

Figure 3 shows the thick sections of rabbit IC complex decorated with fluorescent conjugated antiubiquitin antibodies. It is apparent from this micrograph that the blood vessels of the IC complex and posterior epithelial cells contain abundant ULP, whereas the rest of the IC cells do not appear to contain detectable amounts of ULP. This micrograph clearly indicated the presence of ULP either conjugated or free in the posterior epithelial cells. Immunogold conjugated IgG was used to more precisely determine the location of ULP in these cells.

Figure 4 shows a thin section of a posterior ciliary epithelial cell. This cell was treated with antiubiquitin as a primary antibody and IgG complexed gold particle as a secondary antibody. The electron micrograph clearly reveals the presence of gold particles in the folds of posterior ciliary epithelial cells.

DISCUSSION

Ubiquitin is a multifunctional, cellular protein [Rechsteiner, 1987] first reported by Goldstein et al. [1975], which is present in most eukaryotic cells and has been implicated in the proteolysis of cellular proteins [Rechsteiner, 1987; Jentsch et al., 1991]. In addition, ubiquitination has also been shown to participate in chromatin structure, gene expression, repair, and organelle biogenesis [Mayer et al., 1991]. Scotting et al. [1991] demonstrated extensive

protein ubiquitination in the ocular lens, myotome, and notochord of the chicken embryo and correlated this process with cellular differentiation, suggesting a putative role of ubiquitination in the control of cell growth.

This investigation ascertains, for the first time, the presence of a ubiquitin-like protein (ULP) in ocular fluids. The presence of ULP in both VH and AH could be explained by the fact that these substances naturally mix in the posterior chamber of the eye. The immunoblot analysis clearly demonstrated the presence of ULP in the AH, VH, and IC but not in the serum. These data suggest that the ULP in VH has an ocular origin. The IC complex is intimately related to the lens and had been shown to affect lens *metabolism* [Owers and Duncan, 1979; Rothstein et al., 1972]. The ciliary body cells are responsible for aqueous humor production. Our immunofluorescent studies clearly show the presence of ULP, predominantly localized in the cellular folds. It has also been reported that the AH of the inflamed eye shows considerable lysosomal activity, possibly due to enzymes originating in the IC complex [Phylactos, 1991; Araki et al., 1993]. Therefore, it could be postulated that the IC complex is also the source of ULP. Moreover, the presence of antiubiquitin conjugated gold beads in the ciliary body folds suggests it to be a likely source of ULP in the AH and VH.

The mammalian lens begins a growth process during embyonic development which continues throughout its entire life span. This continuous growth is accomplished by the accretion of cell fibers at the periphery of the lens. However, the optical requirements of the lens and constraints imposed by the architectural integrity of the eye require that lens growth be strictly controlled by an as of yet unknown mechanism. Previous reports from several laboratories have indicated that the 6–12 kDa VH proteins participate in many growth control processes [Banerjee et al., 1992; Jacobson et al., 1984; Lisnyak, 1988; Raymond and Jacobson, 1982]. We reported that the chromatographic fraction containing the 8 kDa ULP has significant protein synthesis inhibitory activity in the lens. We also reported that this 8 kDa protein has the ability to inhibit the epithelial cells of the organ cultured mammalian lens from synthesizing DNA and from undergoing mitosis [Banerjee et al., 1992]. This evidence suggests that the ULP in the ocular fluids may be a key component in a mechanism which regulates lens cell growth. Therefore, our determination of the source of ULP should help to further define its physiological role in the mammalian lens.

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