Biosynthesis of Ependymins from Goldfish Brain*

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Ependymins \( \beta \) and \( \gamma \) constitute a novel family of secretory proteins in the extracellular fluid of goldfish brain. Here we demonstrate that at least two different transcripts exist in goldfish brain differing mainly in the length of their 3' noncoding regions but encoding very similar precursors for ependymins. Both precursors consist of 216 amino acid residues including two potential N-glycosylation sites. Prepro-ependymin-I is the main but not the only precursor of ependymin \( \beta \), whereas prepro-ependymin-II is preferentially processed to ependymin \( \gamma \).

This is in line with our results showing that both ependymins \( \beta \) and \( \gamma \) represent different glycoforms with very similar protein backbones. Additionally, we show that both ependymins share the same C-terminal ends indicating that ependymin \( \gamma \) is not a proteolysis product of ependymin \( \beta \). We also demonstrate that processing at three internal pairs of basic residues does not occur in either ependymin.

Originally, ependymins \( \beta \) and \( \gamma \) (\( M_r = 37,000 \) and \( 31,000 \), respectively) were discovered in goldfish brain due to their enhanced turnover rates after learning events (Shashoua, 1976); each class of ependymins consists of seven isoelectric variants (Shashoua, 1985).

Both ependymins share common characteristics. They aggregate to form dimers (Schmidt and Shashoua, 1981), and radioimmunoassay data showed complete cross-reactivity between the two ependymins. Furthermore, both molecules have very similar amino acid compositions (Schmidt and Shashoua, 1983). Therefore, in the past, the possibility that ependymin \( \beta \) is proteolytically processed to ependymin \( \gamma \) in the brain extracellular fluid was discussed (Schmidt and Shashoua, 1983).

Ependymins are also recognized by the monoclonal antibody HNK-1 (Shashoua et al., 1986). This antibody is known to bind to a complex carbohydrate epitope including a terminal 3-sulfoglucuronyl residue (Chou et al., 1985). This epitope is characteristic of calcium-independent cell adhesion molecules (N-CAM, L1, MAG etc.), and a general role in cell-cell interaction has been proposed (Kruse et al., 1984).

In the absence of calcium ions, ependymins can polymerize to form insoluble aggregates (Shashoua, 1985 and 1988). Two experiments using antibodies have been performed to establish a physiological role of ependymins. Intracerebroventricular injection of antisera in goldfish has been reported to block memory consolidation (Shashoua and Moore, 1978; Piront and Schmidt, 1988). Furthermore, infusion of anti-ependymin antibodies into the tectal ventricles blocks the sharpening of the regenerating retinotectal projection in goldfish (Schmidt and Shashoua, 1988).

Initially, these central nervous system specific glycoproteins were localized to the ependymal zone (Benowitz and Shashoua, 1977) and later on detected in high concentration as secretory proteins in the brain extracellular fluid (Shashoua, 1979; Schmidt and Lapp, 1987). It has also been reported that cultures of zona pellucida cells release ependymins into the medium (Majocha et al., 1982). Recently, increased labeling of ependymins after intracocular injection of \([\text{H}]\)proline has been found in the optic tectum during optic nerve regeneration (Thorndossen et al., 1988).

In the past, we presented a first molecular characterization of ependymins from goldfish brain (Königstorfer et al., 1989): Gas-phase sequencing of purified ependymins \( \beta \) and \( \gamma \) revealed that they share the same N-terminal sequence, although each sequence displays microheterogeneities at several positions. After cDNA cloning, we deduced the amino acid sequence of prepro-ependymin-I, which consists of a typical N-terminal cleavable signal sequence characteristic of secretory proteins, followed directly by the mature ependymin sequence. A computer search did not show significant homology to any other sequenced protein. In this publication, we show that both ependymins are glycosylation variants originating from a set of common precursors.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of cDNA Clones—Different cDNA libraries from goldfish brain were constructed by inserting double-stranded cDNA into the PstI site of pUC8 or pUC18 (Yanisch-Perron et al., 1985) using methods similar to those described previously (Hoffmann, 1988). First strand synthesis was primed with oligo(dT) in the presence of 60 ng/ul actinomycin D (Krug and Berger, 1987), and for second strand synthesis we used RNase H and DNA polymerase but no DNA ligase (Gubler, 1987). GC-homopolymeric tailing was performed as described earlier (Rowekamp and Firtel, 1980), the double-stranded cDNA being made blunt-ended by Klenow polymerase. Alternatively, PstI-linker d(pGCTGCAGC) was added followed by ligation into the PstI cut vector. Screening of cDNA libraries with synthetic oligonucleotides has been described elsewhere (Singer-Sam et al., 1983) with the exception that Hybond-N membranes (Amer sham) were used. DNA sequences were obtained by a combination of the Maxam-Gilbert protocol (Maxam and Gilbert, 1980) using a modified G+A reaction (Cooke et al., 1981) and the dideoxy method (Sanger et al., 1977) on double-stranded templates. DNA sequencing data were computerized using programs purchased from Sisware (Salzburg). Methods used for Northern blot analysis have been described previously (Hoffmann and Franz, 1984).

Southern Analysis—Portions of 30 \( \mu \)g of genomic DNA from a single goldfish prepared according to Sisware (1987) were digested with various restriction enzymes, separated on a 0.7% agarose gel, and transferred in alkaline to a Zeta-probe membrane (Bio-Rad) as recommended by the supplier. For hybridization, we used restriction fragments of ependymin clones labeled with \( ^{32} \)P by random priming.
**Fig. 1. Nucleotide sequence and translation of the open reading frame of the ependymin-II transcript, as derived from the inserts of cDNA clones pGF16.3.6.5 and pGF16.15.6.5.** The insert of cDNA clone pGF4.1 contains part of this sequence (position 27 to 462). Restriction sites and polyadenylation signals are underlined. The cleavage site for signal peptidase is indicated by an arrow. Also underlined are potential N-glycosylation sites and pairs of basic residues. Cysteine residues in the mature product are marked by stars.

(Amersham). Hybridization conditions were 50% formamide, 1% SDS, 4 x SSC at 42 °C. Washings were performed under stringent conditions in 0.1 x SSC, 0.1% SDS at 65 °C.

**Isolation of Ependymins and Production of Antisera—Ependymins were isolated from goldfish brain extracellular fluid (Carassius auratus) and enriched by chromatography on concanavalin A-Sepharose (Amersham) as reported previously (Shashoua, 1988). As a final purification step, we used elution after gel electrophoresis. For the production of antisera, a mixture of ependymins β and γ were injected into rabbits. In order to obtain specific antisera against the C-terminal end of ependymins, we coupled the synthetic peptide CEGVA-FEEAPDDHSFDDLFHD (PEP-1) through the cysteine of the peptide to keyhole limpet hemocyanin with N-hydroxysuccinimide ester as the coupling reagent as described (Doolittle, 1986). Peptide PEP-1 was kindly provided by Dr. W. Risau (Martinsried).

Rabbits were immunized by subcutaneous injection of about 3-500 μg of antigen in complete Freund's adjuvant (1:1) on day 0 and in incomplete Freund's adjuvant (1:1) on days 21 and 35 and were bled a week after the last boost.

**Glycopeptidase F Treatment—**After boiling for 3 min, 100 ng of gel-purified ependymin β or γ were treated with 4 units of glycopeptidase F (Boehringer) in 50 mM disodium hydrogen phosphate at pH 7 as recommended by the suppliers (final volume: 150 μl). After precipitation with 4 volumes of acetone at -20 °C, proteins were analyzed by Western blotting (see below) using different anti-ependymin antisera.

**Western Blots—**Methods used for Western blot analysis have also been reported previously. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred onto nitrocellulose (Towbin et al., 1979), and staining was performed as described (Hawkes et al., 1982) after blocking unoccupied protein binding sites with Tween 20 (Battiger et al., 1982).

**RESULTS**

cDNA Cloning—Screening of an oligo(dT)-primed cDNA library from goldfish brain with radioactively labeled oligo-nucleotide EP4 (Königstorfer et al., 1989) revealed positive clones encoding ependymin sequences (pGF4.1 and pGF6.5). Rescreening of further clones from different libraries resulted in the detection of clones pGF16.3.6.5 and pGF16.15.6.5. Fig. 1 represents the sequence of the derived mRNA encoding propro-ependymin-II. Fig. 2 displays the hydropathic profiles (Kyte and Doolittle, 1982) of both ependymin precursors. At each N-terminal end, a hydrophobic sequence typical of secretory proteins can be recognized. There are notable differences mainly in two specific regions (about positions 80 and 125-160).

As outlined in Table 1, a comparison of the amino acid compositions predicted from the cDNA sequences (see Fig. 6) and the previously published amino acid compositions of ependymins β and γ shows good correspondence, with exception of the oxidation-sensitive amino acid residues and glycine, which is present in the buffer system.

**Northern Analysis**—Fig. 3 represents the hybridization pattern of poly(A)-RNA from goldfish brain with an ependymin probe. A double band of approximately 0.8 kilobase pairs was detected indicating at least two different transcripts.

**Southern Analysis**—Fig. 4 displays a Southern analysis of genomic DNA from a single goldfish with an ependymin probe. Since in every restriction digest (including BamHI in lane d), two bands can be recognized, we assume that the goldfish contains at least two ependymin genes. This is in agreement with the Northern analysis and the results obtained from cDNA cloning.

**Glycopeptidase F Treatment—**As shown in Fig. 5, both ependymins β and γ (lanes a and d), as well as their deglycosylated products (lanes b and c), were recognized by an antisera raised against the synthetic peptide PEP-1. This peptide represents the C-terminal end of propro-ependymins I and II, starting with cysteine-175 as deduced from the cDNA sequence (see Fig. 6).
TABLE I
Percent amino acid compositions of ependymins

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Ependymin-β</th>
<th>Ependymin-γ</th>
<th>Ependymin-I</th>
<th>Ependymin-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparagine or aspartic acid</td>
<td>8.9</td>
<td>9.2</td>
<td>8.7</td>
<td>9.2</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.8</td>
<td>6.3</td>
<td>5.6</td>
<td>5.1</td>
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<tr>
<td>Serine</td>
<td>8.9</td>
<td>9.2</td>
<td>10.3</td>
<td>11.3</td>
</tr>
<tr>
<td>Glutamine or glutamic acid</td>
<td>13.1</td>
<td>12.5</td>
<td>11.8</td>
<td>10.8</td>
</tr>
<tr>
<td>Proline</td>
<td>5.5</td>
<td>6.3</td>
<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.0</td>
<td>10.8</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Alanine</td>
<td>6.2</td>
<td>6.3</td>
<td>4.6</td>
<td>5.1</td>
</tr>
<tr>
<td>Valine</td>
<td>5.8</td>
<td>5.8</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.0</td>
<td>0.8</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.7</td>
<td>0.4</td>
<td>2.6</td>
<td>1.5</td>
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<tr>
<td>Isoleucine</td>
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<td>3.3</td>
<td>2.6</td>
<td>3.1</td>
</tr>
<tr>
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<td>7.5</td>
<td>6.2</td>
<td>7.7</td>
</tr>
<tr>
<td>Tyrosine</td>
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<td>2.5</td>
<td>3.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>5.4</td>
<td>7.2</td>
<td>6.7</td>
</tr>
<tr>
<td>Ornithine</td>
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<td>0.8</td>
<td>.</td>
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<tr>
<td>Lysine</td>
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<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Histidine</td>
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<td>3.6</td>
<td>7.2</td>
<td>5.6</td>
</tr>
<tr>
<td>Arginine</td>
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<td>3.3</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND</td>
<td>ND</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Fig. 3. Northern analysis. The hybridization of the radioactively labeled fragment of the insert from cDNA clone pGF4.1 (see Fig. 1) with poly(A) RNA from goldfish brain is shown. As size marker, a RNA ladder was used (purchased from Bethesda Research Laboratories).

Treatment of ependymins β and γ with glycopeptidase F resulted in a shift of both ependymin bands into a single band of approximately 24,000 (lanes b, f, and g in Fig. 5).

DISCUSSION

cDNA Cloning of Different Transcripts: Structure of Precursors—cDNA cloning revealed at least two different transcripts for ependymins. Both transcripts differ mainly by point mutations distributed throughout the sequence (about 92% homology), the length of their coding regions being identical (encoding 216 amino acid residues), whereas the 3’-noncoding region of transcript I (Konigstorfer et al., 1989) is 122 nucleotides shorter than that of transcript II (Fig. 1) due to the use of a different polyadenylation signal (Proudfoot and Brownlee, 1976). This result was also apparent by Northern analysis, which shows a double band of about 0.8 kilobase pairs (Fig. 3).

The ependymin precursors encoded by the two transcripts differ only by point mutations. Remarkable is the nonrandom distribution of these microheterogeneities; they accumulate within two regions between positions 3 and 66 and 119 and 153 (see Fig. 6). The M of prepro-ependymin-II is 24,064. It is reduced in the mature product (without the carbohydrate chains) to 21,757 (for comparison, M of ependymin-I: 21,960).

In contrast to transcript I encoding the major variant of β-ependymins (Konigstorfer et al., 1989), the amino acid sequence deduced from transcript II delineates a variant typical of γ-ependymins (Aspβ, Ile18). The deduced serine at position 9 was not detectable by microsequencing probably due to the fact that sequence II represents a minority species within the ependymin family. Since both sequences deduced so far represent Hisβ variants, we have to predict at least a third transcript encoding a Gluβ variant which was previously found by protein sequencing (Konigstorfer et al., 1989).

Both variants (I and II) of ependymin precursors (Fig. 6) contain two potential N-glycosylation sites. Since ependymins exist in two different forms, one could argue that γ-ependymins correlate with single-glycosylated and β-ependymins represent double-glycosylated polypeptide chains. Alternatively, partial trimming and modification of one N-linked
carbohydrate chain could account for the difference observed between ependymin \( \beta \) and ependymin \( \gamma \). Indeed, this all is in line with out results of the sugar analysis by glycopeptidase F.

Ependymins \( \beta \) and \( \gamma \) Are Glycosylation Variants—As indicated in Fig. 5, ependymins \( \beta \) and \( \gamma \) mainly differ by their N-linked carbohydrate chains but share very similar protein backbones. This again fits well with results showing that both ependymins share the same N-terminal sequences (Konigstorfer et al., 1989), and that they are recognized by the same antibodies and have similar amino acid compositions (Schmidt and Shashoua, 1983). The occurrence of different glycoforms has been reported repeatedly. In analogy to Thy-1 showing cell-specific glycosylation superimposed by site specificity of three N-glycosylation sites (Parekh et al., 1987), similar mechanisms could be postulated for ependymins. In order to establish a logical nomenclature of these two glycoform states (\( \beta \) and \( \gamma \)), we now rename them to ependymin (37k) and ependymin (31k), respectively.

Additionally, the results presented in Fig. 5 (lanes a–d) clearly demonstrate that both glycoforms, epd(37k) and epd(31k), must exhibit identical C-terminal structures. Therefore, the previous hypothesis that epd(31k) might result from processing via proteolytic cleavage of epd(37k) in the extracellular fluid of goldfish brain (Schmidt and Shashoua, 1983) can be eliminated. Only deglycosylation of epd(37k) might account for conversion to epd(31k).

Since both ependymins are recognized by the antibody raised against the C-terminal end deduced from cDNA cloning, we also can conclude that ependymins are not processed at three internal pairs of basic residues.

The challenge will now be to correlate these unique proteins with a precise molecular function. Based on findings presented in the introduction and on recent experiments indicating that ependymins can serve as substrates for axon outgrowth,\(^5\) one could assume that these unique proteins might have a function during regeneration.

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\(^5\) C. Stürmer, personal communication.

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