Amphioxus homologs of Go-coupled rhodopsin and peropsin having 11-*cis*- and all-*trans*-retinals as their chromophores

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Abstract Because of low contents in the native organs and failure of the expression in cultured cells, the chromophore configurations of the pigments in Go-coupled opsin and peropsin groups in the opsin family are unknown. Here we have succeeded in expression of the amphioxus homologs of these groups in HEK293s cells and found that they can be regenerated with 11-*cis*- and all-*trans*-retinals, respectively. Light isomerized the chromophores of these opsins into the all-*trans* and 11-*cis* forms, respectively. The results strongly suggest that the physiological function of peropsin would be a retinal photoisomerase, while 11-*cis* configuration is necessary for the Go-coupled opsin groups.

Key words: Opsin; Chromophore; Amphioxus; Retinal; Peropsin; Phylogenetic tree

1. Introduction

Visual pigments and their related retinal proteins are 35–55 kDa membrane proteins that consist of a single polypeptide 'opsin' and a chromophore retinal. The opsin contains seven transmembrane α-helical domains connected by three extracellular and three cytoplasmic loops, which are the typical structural motif of G protein-coupled receptors (GPCRs). The retinal is bound to a lysine residue in the seventh helix of opsin through a Schiff base linkage. After cloning of the bovine rhodopsin cDNA in 1983 [1,2], more than 200 opsin cDNAs have been cloned and they have been classified into five groups in the molecular phylogenetic tree that was constructed on the basis of amino acid sequence similarity.

Some of them act as light-activated GPCRs in the photoreceptor cells; that is, vertebrate opsins couple with the Gt-type G protein (transducin) [3–5], while opsins in rhabdomeric photoreceptor cells of arthropods and cephalopods couple with the Gq-type G protein [6–8]. Our recent investigations showed that the opsin present in the scallop ciliary photoreceptor cells coupled with the Go-type G protein [9,10]. In addition to these three opsin groups, two different groups, which are distinct from each other in the molecular phylogenetic tree, have been reported. One contains squid retinochrome and mammalian RGR (retinal G protein-coupled receptor), both of which act as the retinal photoisomerase [11–14], and the other contains peropsin which is expressed in the retinal pigment epithelium of mammals, although its function is not clear yet [15].

The chromophore configurations of the pigments in three out of the five groups, that is, the groups of vertebrate Gt-coupled opsin, invertebrate Gq-coupled opsin and retinal photoisomerase, have been determined to be 11-*cis*, 11-*cis*, and all-*trans* forms, respectively [16–20]. The role of 11-*cis*-retinal present in the Gt- and Gq-coupled opsins has been extensively investigated and it was revealed that 11-*cis*-retinal acts as an inverse agonist and light fulfills a role to convert the inverse agonist into an agonist all-*trans*-retinal to form a state that activates retinal G proteins [21,22]. In contrast, retinochrome and RGR contain an all-*trans*-retinal as a native chromophore and act as a photoisomerase to produce 11-*cis*-retinal upon the absorption of light [11–14]. These results strongly suggest that there is a close relationship between the chromophore configuration and the physiological function of opsin. Thus it is important to determine the chromophore configuration of the opsin family to get insight into the physiological function of these pigments. However, because of low contents in the native organs and failure of the expression in cultured cells, the chromophore configuration of the pigments in the other two groups, the Go-coupled opsin and the peropsin groups, are still unknown.

Recently, six opsin genes (*Amphi*op1–*Amphi*op6) have been isolated from amphioxus, *Branchiostoma belcheri* and their sequences have been deposited into the DDBJ database (Miyata, 2002). The phylogenetic analysis revealed that each of them is closely related to Gq-coupled opsin, Gt-coupled opsin (encephalopsin), Go-coupled opsin and peropsin (Fig. 1). Thus we have tried to express the amphioxus opsin genes, especially the genes belonging to the Go-coupled opsin and peropsin groups to determine their chromophore configurations and physiological function. Current findings clearly show that two amphioxus opsins, which respectively belong to the Go-coupled opsin and peropsin groups, can be successfully expressed in HEK293s cells and they have 11-*cis*- and all-*trans*-retinals as their chromophores. Based on these results, the relationship between chromophore configuration and the functional property of opsin will be discussed.

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Abbreviations: Gt, transducin; Gq, Gq-type G protein; Go, Go-type G protein; GPCR, G protein-coupled receptor; HPLC, high-performance liquid chromatography

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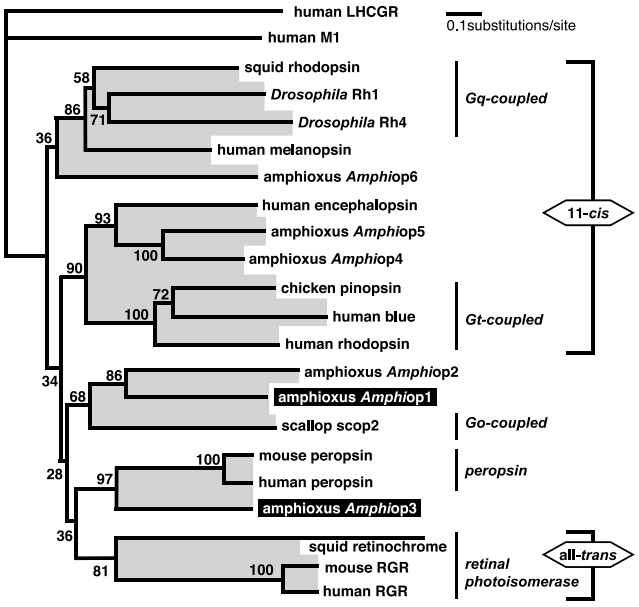

Fig. 1. Phylogenetic tree of the opsin family. The tree was inferred by the neighbor-joining method using two other GPCRs, human muscarinic acetylcholine receptor and human LHCG receptor as an outgroup. The number at each branch node represents the bootstrap probability. The phylogenetically distinct clusters (subgroups) are shaded and their names are shown on the right hand side of the tree. The classification by the isomeric form of chromophore is also indicated at the right end. The branch length is proportional to the number of accumulated amino acid substitutions. *Amphi*op1 and *Amphi*op3 can be successfully expressed in the cultured cells in this work (see text). Note that for some of the opsins belonging to the Gt-coupled opsin and the Gq-coupled opsin groups, the isomeric form of the chromophore has not yet been identified.

2. Materials and methods

2.1. Sequence data

Accession numbers of sequence data from DDBJ are as follows: human LHCGR (X84753); human M1 (Y00508); squid rhodopsin (X70498); *Drosophila* Rh1 (K02315); *Drosophila* Rh4 (M17730); human melanopsin (AF147788); amphioxus *Amphi*op6 (AB050611); human encephalopsin (AF140242); amphioxus *Amphi*op5 (AB050609); amphioxus *Amphi*op4 (AB050608); chicken pinopsin (U15762); human blue (M13299); human rhodopsin (U49742); amphioxus *Amphi*op1 (AB050606); amphioxus *Amphi*op2 (AB050607); scallop scop2 (AB006455); human peropsin (AF012270); mouse peropsin (AF012271); amphioxus *Amphi*op3 (AB050610); squid retinochrome (X57143); mouse RGR (AF076930); human RGR (U15790).

2.2. Alignment and phylogenetic tree inference

Optimal alignment of the amino acid sequences of opsin was obtained by the method developed by Katoh et al. [23], which is basically an extended version of the progressive approach of Feng and Doolittle [24] by improving the calculation procedure of dynamic programming [25] together with manual inspections. On the basis of the alignment described above, the number k_{aa} of amino acid substitutions per site or evolutionary distance was calculated by the simple Poisson correction as $k_{aa} = -\ln(1-K_{aa})$ [26]. The evolutionary distance was applied to phylogenetic inference by the neighbor-joining method [27]. Bootstrap analysis was carried out by the method of Felsenstein [28].

2.3. Expression and purification of amphioxus opsins

The cDNAs that encode opsins from amphioxus, *B. belcheri*, were the kind gift of Prof. T. Miyata at Kyoto University. The coding region of *Amphi*op1, 2 and 3 cDNA were isolated by PCR being tagged by the monoclonal antibody Rho 1D4 epitope sequence (ETSQVAPA) [29]. The tagged cDNA was inserted between a *Hin*dIII site and an *Eco*RI site of a plasmid vector SRα [30]. The vector was transfected into HEK293s cells by the calcium phosphate method according to the previous report [31]. The transfected cells were harvested for 2 days and collected by centrifugation. After addition of a mixture of 11-*cis*- and all-*trans*-retinals, *Amphi*ops were extracted with 1% dodecylmaltoside (DM) in HEPES buffer (pH 6.5) containing 140 mM NaCl and 3 mM $MgCl_2$ (buffer A), bound to 1D4-agarose, washed with 0.02% DM in buffer A and eluted with buffer A containing 0.02% DM and 0.1 mg/ml C-terminal peptide of bovine rhodopsin.

2.4. Western blot analysis

The collected HEK293s membrane was subjected to SDS–PAGE and then transferred to a polyvinylidene difluoride membrane. The transferred proteins were subjected to Western blot analysis by the monoclonal antibody Rho 1D4. Immunoreactivity was detected by the ABC method and visualized with horseradish peroxidase–diaminobenzidine reaction.

2.5. Spectroscopic and HPLC analysis

Absorption spectra were recorded at 0°C with a Shimadzu UV2400 spectrophotometer. High-performance liquid chromatography (HPLC) was carried out using a Shimadzu LC-7A interfaced with CR-5A. A 0.1 ml sample was mixed with 50 μl 1 M hydroxylamine (NH_2OH) and 0.3 ml of cold 90% methanol to convert retinal chromophore into retinal oxime and the retinal oxime was extracted with 0.6 ml *n*-hexane [32,33]. The extract was then injected into a YMC-Pack SIL column (particle size 3 μm, diameter 150×6.0 mm) and eluted with *n*-hexane containing 15% ethyl acetate and 0.15% ethanol at a flow rate of 1.0 ml/min while being monitored by the absorbance at 360 nm.

3. Results and discussion

Among the six amphioxus opsin genes, *Amphi*op1 and 2 belong to the Go-coupled opsin group and *Amphi*op3 belongs to the peropsin groups. Thus we have tried to express *Amphi*op1, 2 and 3 genes in HEK293s cells to determine their chromophore configuration. Western blot analysis clearly showed that *Amphi*op1 and 3 can be expressed in the cultured HEK293s cells. Like bovine rhodopsin, *Amphi*op1 exhibits multiple bands (Fig. 2, lane 1) probably due to heterogeneous glycosylation. On the other hand, *Amphi*op3 exhibits a compact band (Fig. 2, lane 3) as the squid retinochrome does. A

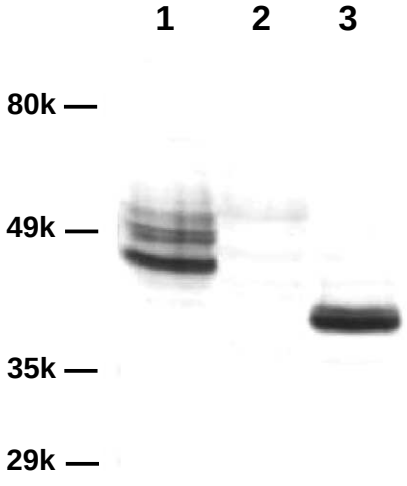

Fig. 2. Western blot analysis of *Amphi*op1 (lane 1), *Amphi*op2 (lane 2) and *Amphi*op3 (lane 3) expressed in HEK293s cells by the monoclonal antibody Rho 1D4. The molecular masses of protein size standards are indicated on the left. The expected molecular weights of *Amphi*op1, 2 and 3 from their amino acid sequences are 44, 58 and 41 kDa respectively.

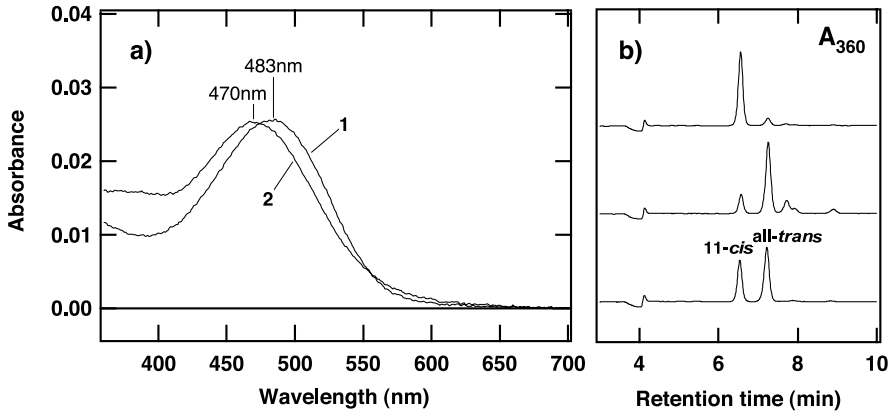

Fig. 3. Absorption spectra of *Amphi*op1 expressed in HEK293s cells (a) and the HPLC patterns of retinal oximes (b). Absorption spectra and the HPLC patterns were measured before (a, curve 1 and b, top trace) and after irradiation at $\lambda > 520$ nm for 2 min (a, curve 2 and b, middle trace). The HPLC pattern of retinal oximes extracted from a mixture of irradiated and non-irradiated bovine rhodopsin in equal amounts is indicated as a reference (b, bottom trace). The absorption maxima of the original pigment and its photoproduct are shown in panel a. Note that ε of 11-*cis*-retinal is 0.6-fold that of all-*trans*-retinal.

faint band derived from *Amphi*op2 was observed in lane 2, suggesting that the expression level of *Amphi*op2 was too low to analyze its biochemical and biophysical properties. The molecular weights of *Amphi*op1, 2 and 3 were consistent with the expected values, 44, 58 and 41 kDa respectively, that were estimated from their amino acid sequences.

*Amphi*op1 exhibited an absorption maximum at 483 nm (curve 1 in Fig. 3a), when it was regenerated with a mixture of 11-*cis*- and all-*trans*-retinals and purified by means of immuno-affinity column chromatography. Irradiation with orange light (> 520 nm) caused a shift in the absorption maximum to 470 nm with an increase in absorbance around 380 nm (curve 2 in Fig. 3a). The chromophore configuration of *Amphi*op1 and its photoproduct were analyzed by HPLC to be 11-*cis* and all-*trans* forms, respectively (Fig. 3b). These results clearly showed that *Amphi*op1 is a typical visual pigment in the sense that it contains 11-*cis*-retinal. This characteristic, together with the phylogenetic relationship (Fig. 1), strongly suggests that this pigment would activate the Go-type G protein after *cis–trans* isomerization of the chromophore.

The purified *Amphi*op3 showed an absorption maximum at 490 nm (curve 1 in Fig. 4a). When irradiated with orange light, the maximum shifted to about 475 nm with a decrease in absorbance (curve 2 in Fig. 4a). The chromophore configuration of *Amphi*op3 was determined to be an all-*trans* form by HPLC analysis (upper trace in Fig. 4b). Irradiation causes a change of about 70% of the original all-*trans* form into the 11-*cis* form (middle trace in Fig. 4b), indicating that light causes the *trans–cis* isomerization of the chromophore. This profile in configurational change of the chromophore is almost identical to that of the squid retinochrome [12]. Therefore, we concluded that a pigment in the peropsin groups has an all-*trans*-retinal as its chromophore. The clustering of the peropsin group with the retinal photoisomerase group in the phylogenetic tree is reasonable because they have chromophores of the same configuration.

These results clearly showed that in addition to the photo-

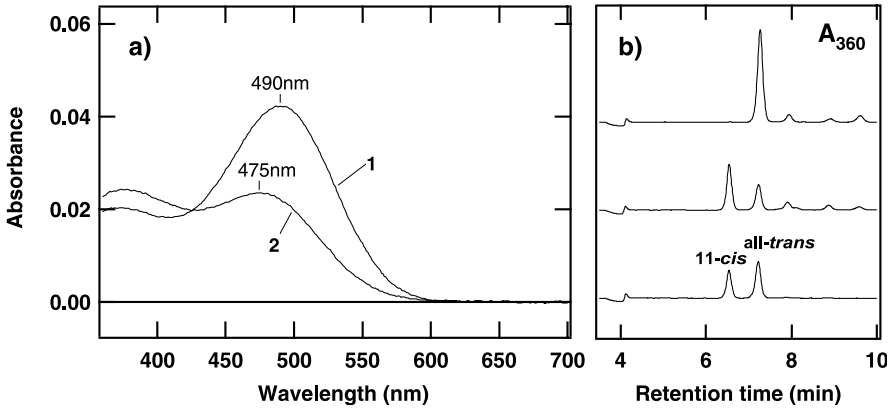

Fig. 4. Absorption spectra of *Amphi*op3 expressed in HEK293s cells (a) and the HPLC patterns of retinal oximes (b). Absorption spectra and the HPLC patterns were measured before (a, curve 1 and b, top trace) and after irradiation at $\lambda > 520$ nm for 2 min (a, curve 2 and b, middle trace). The reference for the HPLC patterns is indicated at the bottom in panel b (see legend of Fig. 3). The absorption maxima of the original pigment and its photoproduct are shown in panel a. It should be noted that the extinction coefficient of 11-*cis*-retinal is 0.6-fold that of all-*trans*-retinal.

isomerase group that includes retinochrome and RGR, there is another group of opsins, the peropsin group, which involves the opsin having an all-*trans*-retinal as a chromophore. Because of the same chromophore configuration, the physiological function of peropsin would be similar to those of retinochrome and RGR, that is, a retinal photoisomerase. However, peropsin exhibits several motifs that are highly conserved in the opsins that function as GPCRs. For example, peropsin and *Amphi*op3 have an 'NPXXY' motif in the seventh helix, while this motif was changed to FPLLI and NAINY in retinochrome and RGR, respectively. Therefore, another intriguing possibility is that peropsin is a GPCR in which all-*trans*-retinal does not act as an agonist.

It is likely that the amino acid residues which constitute the retinal binding pocket would determine what kind of retinal isomer can be accommodated. Thus we tried to deduce the amino acid positions in the primary structure of opsin, where the amino acid residue would select the binding of the isomeric form of the retinal chromophore. According to the crystal structure of bovine rhodopsin, the chromophore binding site is constituted of 30 amino acid residues [34]. If one can assume that the three-dimensional structures of opsins in various groups are similar, the amino acid positions where all of the RGR, retinochrome, peropsin and *Amphi*op3 have the same amino acid residues that are different from those of rhodopsins would be candidates to select binding of the isomeric form of the retinal chromophore. However, no amino acid positions meet this criterion, suggesting that the selectivity of the isomeric form of the retinal chromophore could originate from global structural changes such as the difference in relative position between seven transmembrane helical domains. Alternatively, the binding site of the all-*trans* chromophore could be different in position from that of the 11-*cis* chromophore. This idea comes from the fact that the β-ionone ring region of the retinal chromophore in rhodopsin flips over upon *cis–trans* photoisomerization of the chromophore, thereby resulting in a different position of the β-ionone ring in the protein moieties between rhodopsin and its later intermediates [35]. Thus a detailed analysis using chimeric mutants between the pigments having all-*trans* and 11-*cis* chromophores is necessary for furthering our understanding of the difference in selectivity of the isomeric form of the chromophore.

In the present study, we successfully expressed the pigments belonging to groups other than the Gt-coupled opsin group. To our knowledge, this is the first report that the invertebrate 'rhodopsin' can be expressed in cultured cells. These investigations allow us to comparatively investigate the mechanism of G protein subtype selectivity in the opsin family.

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