O-Glycosylation of G-protein-coupled receptor, octopus rhodopsin
Direct analysis by FAB mass spectrometry

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Abstract In addition to the *N*-glycan that is evidently conserved in G-protein-coupled receptors (GPCRs), *O*-glycans in the N-terminus of GPCRs have been suggested. Using a combination of enzymatic and manual Edman degradation in conjunction with G-protein coupled receptor mass spectrometry, the structure and sites of *O*-glycans in octopus rhodopsin are determined. Two *N*-acetylgalactosamine residues are *O*-linked to Thr4 and Thr5 in the N-terminus of octopus rhodopsin. Further, we found chicken iodopsin, but not bovine rhodopsin, contains *N*-acetylgalactosamine. This is the first direct evidence to determine the structure and sites of *O*-glycans in GPCRs.

Key words: *O*-Glycosylation; G-protein-coupled receptor; Octopus rhodopsin; Visual pigment; Post-translational modification

1. Introduction

G-protein-coupled receptor (GPCR) constitutes a large superfamily of membrane proteins that transduce a wide variety of extracellular signals such as light, odorant molecules, neurotransmitters and hormones, by activating G-protein on the interior surface of the plasma membrane on these receptors [1]. Rhodopsin, visual pigment, is an example par excellence of the receptors that couple to the G-protein in the signal transduction. Upon illumination, the 11-*cis* retinal, the chromophore of rhodopsin, isomerizes to the all-*trans* form and drives the protein through a series of transient photointermediates. One of the intermediates interacts with the G-protein, resulting in the electrical excitation of a photoreceptor cell [2,3].

The members of the GPCR exhibit considerable sequence similarity and form a common topological structure consisting of seven α-helical segments spanning the lipid bilayer [4]. In addition, many GPCRs share common post-translational modifications such as fatty acid acylation, phosphorylation and glycosylation. Both vertebrate and invertebrate rhodopsins are palmitylated at the conserved cysteine residues in the C-terminus [5,6]. The fatty acid modification of the receptors is expected to form the fourth loop on the cytoplasmic side, which may contribute to regulate the interaction between the receptor and G-protein.

The GPCRs are phosphorylated after receptor activation by stimulus. It was shown that both vertebrate and invertebrate rhodopsins are phosphorylated upon illumination at Ser or Thr residues in the C-terminus [7–9]. Phosphorylation of the receptor has been proposed to be involved in signal termination.

The third post-translational modification for GPCRs is glycosylation. One or two potential *N*-glycosylation sites are included in the N-terminus of visual pigments and actually glycosylated [10,11]. An *N*-glycan at Asn8 of the N-terminus of octopus rhodopsin whose structure has recently been elucidated by us [12] has a quite unique structure as compared with those of other mammalian [10,11,13] and frog rhodopsins [14]. Another glycosylation event of membrane and secretory glycoproteins is *O*-glycosylation at particular Ser or Thr residues. *O*-Glycosylation of G-protein coupling receptor has gone unnoticed until very recently, although the existence of *O*-glycans in two GPCRs, V2 vasopressin receptor [15] and δ-opioid receptor [16] has been demonstrated by metabolic labeling and enzymatic deglycosylation in heterologous expression systems. Since most of the GPCRs are expressed at very low levels in natural tissues and cells, these approaches address a number of fundamental questions related to both structural and functional features of these proteins. However, the conclusion of the site and structure of the *O*-glycans by this approach was based upon indirect evidence. The lack of certainty in the analysis prompted us to perform a substantial analysis of the *O*-glycans of octopus rhodopsin.

In this study, we describe the successful isolation of N-terminal decapeptide of octopus rhodopsin and determination of the structure and sites of *O*-glycans by mass spectrometry. In

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Abbreviations: APMSF, 4-(aminophenyl) methanesulfonyl fluoride; Con A, concanavalin A; DTT, dithiothreitol; FAB, fast atom bombardment; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; GPCR, G-protein-coupled receptor; HPLC, high performance liquid chromatography; Man, mannose; MES, 2-(*N*-morpholino) ethanesulfonic acid; PAD, pulsed amperometric detection; RP, reversed phase; TFA, trifluoroacetic acid

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addition to octopus rhodopsin, chicken iodopsin, but not bovine rhodopsin, contain *N*-acetylgalactosamine. We suggest that a cluster of Ser/Thr in the upstream of a single *N*-glycosylation site is a candidate for *O*-glycosylation site.

2. Materials and methods

2.1. Purification of microvillar membranes and preparation of N-terminal decapeptide of rhodopsin

Microvillar membranes of octopus retina (*Octopus dofleini*) were isolated by sucrose flotation as described previously [17]. The microvillar membranes were repeatedly washed with buffer A (400 mM KCl, 10 mM $MgCl_2$, 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) (pH 6.5), 1 mM dithiothreitol (DTT), 20 μM 4-(aminophenyl) methanesulfonyl fluoride (APMSF)) and then with buffer B (10 mM $MgCl_2$, 10 mM MES (pH 6.5), 1 mM DTT, 20 μM APMSF). Bovine rhodopsin was prepared as previously described [18]. The membranes were digested with 100 nM *Achromobacter* protease I (Wako Pure Chemical Ind.) in 50 mM Tris–HCl buffer (pH 9.0), containing 0.1% SDS, 5 mM EDTA (ethylenediaminetetraacetic acid) and 5% acetonitrile at 37°C for 48 h. After centrifugation (at 51 000×*g* for 10 min at 4°C), the supernatant of the digest was applied to a concanavalin A (Con A)-Sepharose (Amersham Pharmacia Biotech) column equilibrated with buffer C (10 mM MOPS (3-(*N*-morpholino) propanesulfonic acid) (pH 7.4), 500 mM NaCl, 0.2% sucrose monolaurate, 1 mM $MnCl_2$, 1 mM $CaCl_2$). After extensive washing of the column with buffer C, glycopeptides bound to the column were eluted with buffer C containing 250 mM α-methyl mannopyranoside. The glycopeptide thus obtained was fractionated by reversed phase (RP) high performance liquid chromatography (HPLC) using a RESOURCE RPC column (Amersham Pharmacia Biotech) (6.4×30 mm). The gradient elution was performed at a flow rate of 1.0 ml/min with solvents A (0.05% trifluoroacetic acid (TFA) in water) and B (0.05% TFA in acetonitrile) as follows: 0–15 min, 0% B; 15–35 min, 0–100% B; 35–37.5 min, 100% B; then to 45 min, 0% B. The N-terminal peptide composed of 25 amino acids was isolated and then digested with 0.2 μM thermolysin (Seikagaku Corp.) in 50 mM Tris–HCl (pH 8.0) and 2 mM $CaCl_2$ at 37°C for 24 h. The digest was subjected to the RP-HPLC in a similar way as described above, except that elution was performed with 100% solvent A from 0 to 17.5 min and with a linearly increasing volume of 0–40% of solvent B from 17.5 to 40 min.

2.2. Sugar composition analysis

Glycopeptide was heated in 0.5 ml of 4 N TFA at 100°C for 3 h, and freed from acid by repeated evaporation with water. The hydrolysate was analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (PAD) using a Bio-LC system (Dionex Co., CA, USA) equipped with a CarboPac PA-1 column (4×250 mm) as described [19]. A Power Chrom system (AD Instruments, Tokyo, Japan) connected with a personal computer monitored PAD response.

2.3. Amino acid composition and sequence analyses

Conventional amino acid sequencing of the peptides was performed by a protein sequencer (Model 473A, Perkin-Elmer Applied Biosystem Inc., CA, USA). The amino acid composition of the peptides was determined by an amino acid analyzer (L8500, Hitachi, Tokyo, Japan) after hydrolysis with gas-phase HCl at 110°C for 24 h. Manual Edman degradation for the peptides was performed according to Tarr's method [20].

2.4. Mass spectrometry

Positive-ion fast atom bombardment (FAB) mass spectra were obtained using a JMS-HX/HX110A four-sector tandem mass spectrometer (Jeol, Tokyo, Japan), equipped with a FAB ion source. The mass spectra were acquired in the range of *m/z* 2000–3400. Mass calibration was performed using a mixture of KI and CsI (1:2 w/w). Solutions of analytes were placed on a stainless steel probe tip and mixed with liquid matrix (DTT/dithioerythritol, 5/1 (w/w)). The sample was ionized by bombardment with xenon atoms accelerated at a potential of 6 kV in the ion source. All other experimental details have been described previously [21].

3. Results

Octopus rhodopsin was first digested with *Achromobacter* protease [22]. The resulting glycopeptides were recovered in the fraction bound to a Con A-Sepharose column and further fractionated by RP column chromatography. The chromatogram showed two main peaks (data not shown). Both of the peptides were composed of 25 amino acid residues and identified as N-terminal peptide of rhodopsin with a sequence of VESTT...WAK on the basis of amino acid composition analysis [23]. The data also revealed that not only *N*-acetyl-D-glucosamine (GlcNAc) but also *N*-acetyl-D-galactosamine (GalNAc) residues are included in the peptides, suggesting that the peptides are *O*-glycosylated.

The mass analysis showed that the molecular mass of these peptides was 5142.7 and 5184.9 Da (data not shown). The difference in mass of the peptides (42 Da) was suggested due to *N*-acetylation of one of the peptides. Since molecular masses of the peptides are more than that of the known structure (the peptide 3023.4 Da and the *N*-glycan ($Gal_2Man_3GlcNAc_3Fuc$) 1566.4 Da), it is expected that the peptides contained extra sugars, in accordance with the results from amino acid composition analysis.

To identify the extra sugars the smaller peptide (5142.7 Da) was further digested with thermolysin. The digest was subjected to RP-HPLC (data not shown) and a decapeptide with amino acid sequence VESTTLVNQT was recovered. Sugar composition analysis revealed that the decapeptide contains galactose (Gal), GlcNAc, mannose (Man), fucose (Fuc)

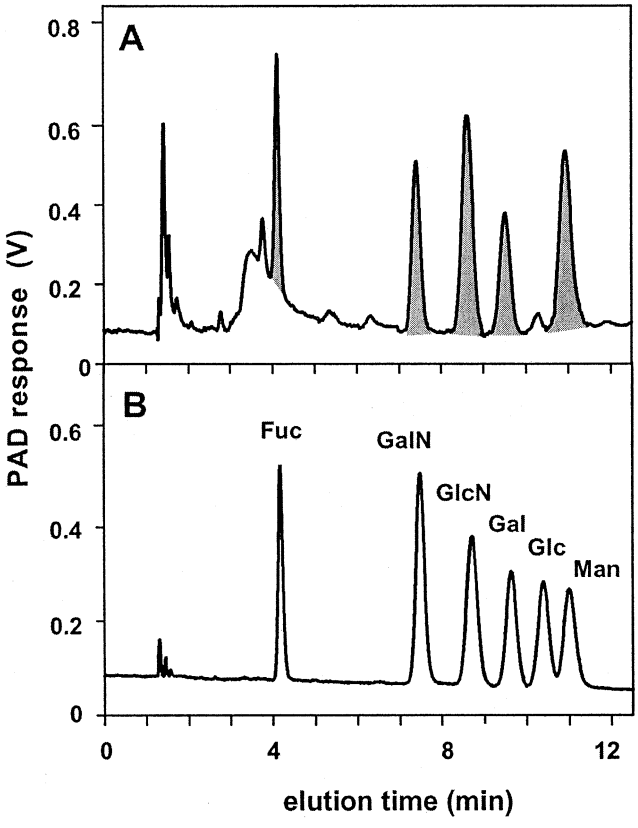

Fig. 1. Sugar composition analysis of the decapeptide of octopus rhodopsin. (A) Acid hydrolysate of the decapeptide. (B) A mixture of 1 nmol each of Fuc, galactosamine (GalN), glucosamine (GlcN), Gal, glucose (Glc), and Man.

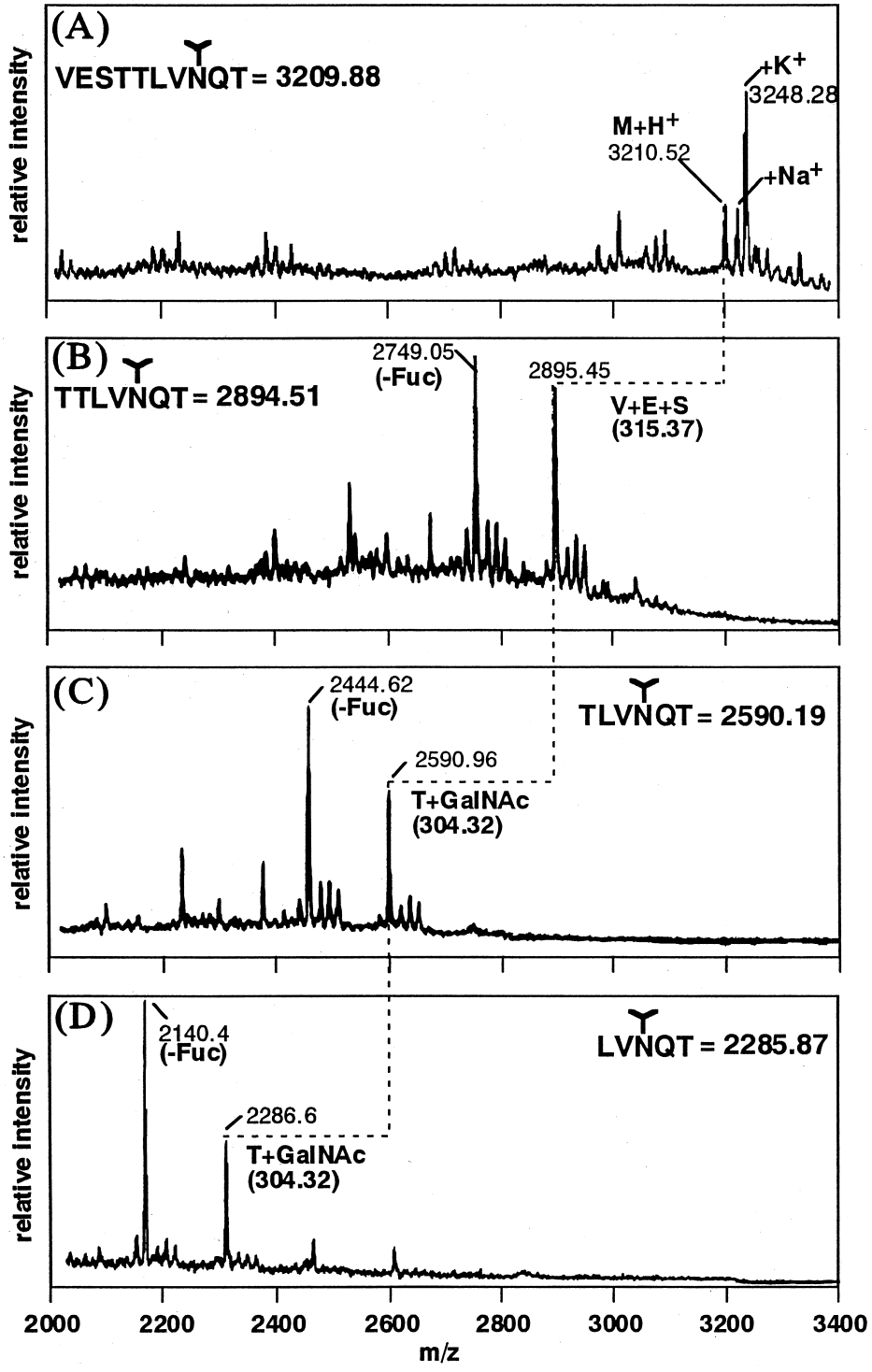

Fig. 2. FAB mass spectrometry spectra of manual Edman degradation products of the decapeptide. The decapeptide before Edman degradation (A). The peptides obtained after the third (B), fourth (C) and fifth (D) cycles of Edman degradation of the decapeptide.

and GalNAc residues in a molar ratio of 2:3:3:2:2 (Fig. 1). Considering our previous analysis indicating that the *N*-glycan is composed of Gal, GlcNAc, Man, and Fuc residues in a molar ratio 2:3:3:1 [11], it is calculated that the glycopeptide contains extra two GalNAc and one Fuc residues except for sugar residues included in the *N*-glycan. These results were confirmed by mass analysis; the FAB mass spectrum of the peptide gave ion signals at *m/z* 3210.5 ($[M+H]^+$) and 3248.3 ($[M+K]^+$), which correspond to the sum of the peptide portion (1091.1 Da) and sugars ($Hex_5HexNAc_5Fuc_2$, 2118.7 Da) (Fig. 2A). Thus, the results suggest the presence of *O*-glycans in the peptide. In order to determine the site to which these sugars (two GalNAc and one Fuc) attach, the peptide was subjected to manual Edman degradation. The mass spectrum of the peptide obtained after the third cycle of degradation showed that the tripeptide sequence VES (315.4 Da) was re-

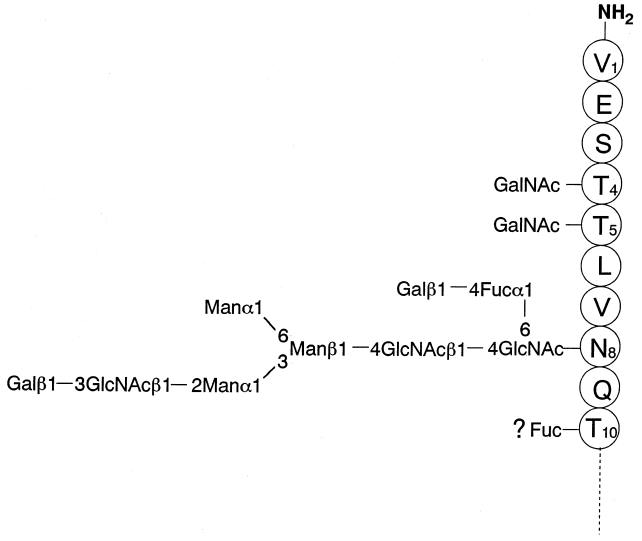

Fig. 3. Proposed structure of the N-terminus of octopus rhodopsin. The structure of the *N*-glycan is based on the previous study [11].

moved (Fig. 2B), indicating that Ser3 is not glycosylated. However, the mass spectra of the peptides after the fourth and fifth cycles of degradation showed that Thr and HexNAc were removed at each cycle (Fig. 2C,D), indicating that both Thr4 and Thr5 are *O*-glycosylated. There was an extra signal which is smaller by 146 Da (corresponding to Fuc) than the largest ion at each cycle of Edman degradation. This might be due to the acid labile nature of α-fucosyl linkages, since the degradation process includes acid hydrolysis by TFA. The mass spectrum (Fig. 2D) showing the two strong ion signals with *m/z* 2286.6 and 2140.4 (devoid of a Fuc residue) suggests that the extra Fuc residue is actually included in the residual pentapeptide, LVNQT, with the larger mass. Its location is suspected to be at the C-terminal Thr residue on the basis of the structure of *N*-glycan. Actually, the Thr residue was not released from the decapeptide by carboxypeptidases P and Y. However, more direct evidence will be needed to address the location of the Fuc residue.

Next, we asked whether *O*-glycosylation in the N-terminus is specific to octopus rhodopsin in visual pigments. Since *O*-glycosylation occurs at a cluster of Thr residues in the N-terminus of octopus rhodopsin, the clustered Thr/Ser residues in the GPCRs are suggested to be potential *O*-glycosylation sites. Two visual pigments were used to prove this hypothesis. One is chicken iodopsin containing a cluster of Thr residues in the N-terminus, and the other is bovine rhodopsin containing Ser and Thr residues in its N-terminus which are not clustered. Composition analysis showed that the content of GalNAc residue per molecule is 2.2 (octopus rhodopsin), 2.8 (chicken iodopsin) and 0.1 (bovine rhodopsin), respectively. These results suggest that *O*-glycan is preserved in iodopsin as well as in octopus rhodopsin, but not in bovine rhodopsin.

4. Discussion

Accumulating evidence suggests important roles of sugar moieties of glycoproteins in the signal transduction processes as shown in the cases of follicle stimulating hormone [24], granulocyte-macrophage colony stimulating factor [25], β2-adrenoceptor [26], and insulin receptor [27]. *N*-Glycosylation of GPCRs is well characterized for which a well defined consensus sequence, the tripeptide Asn-Xaa-Ser/Thr, has been determined. On the other hand, *O*-glycosylation of GPCRs has not been shown until very recently. This is partly because parameters that determine sites of *O*-glycan attachment to glycoproteins are poorly understood and no obvious consensus sequence motif has emerged [28].

In the last 2 years, *O*-glycosylation of two GPCRs, vasopressin receptor [15] and δ-opioid receptor [16] was demonstrated. Sadeghi and Birnbaumer [15] tried to identify *O*-glycosylation sites of V2 vasopressin receptor by alanine scanning mutagenesis, and suggested that the clustered amino acids (Ser5, Thr6, Thr7, Ser8), Ser15 and Ser18 located in the upstream of a single *N*-glycosylation site are likely to be sites for *O*-glycosylation. However, the exact identification of the *O*-glycosylation sites was uncertain, because the disappearance of a site opened the availability of others to transferases. Petaja-Repo et al. [16] suggested in the study on the synthesis and maturation of the human δ-opioid receptor that the M_r 45 kDa precursor form of the receptor is converted in the Golgi to the M_r 55 kDa mature form, and that this conversion involves processing of two high Man type *N*-glycans as well as addition of *O*-glycans containing *N*-acetylgalactosamine, Gal, and sialic acid. However, they did not determine the structure and sites of *O*-glycans.

In the present study, we successfully isolated the N-terminal

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H.sapiens            MA-QQWSLQRLAGRHPQDSYEDSTQSSIFTYTNSNSTRGPFEGPNYHIAPRWV
M.fascicularis LW    MA-QQWSLQRLAGRHPQDSYEDSTQSSIFTYTNSNSTRGPFEGPNYHIAPRWV
M.fascicularis MW    MA-QQWSLQRLAGRHPQDSYEDSTQSSIFTYTNSNSTRGPFEGPNYHIAPRWV
X.laevis             MA-SQLNEAIFAARRRNDD-DDTTRSSVFTYTNSNNTRGPFEGPNYHIAPRWV
B.taurus             MA-HAWGPQRLAGGQPQANFEESTQGSIFTYTNSNSTRDPFEGPNYHIAPRWV
F.catus              MT-QRWGPQRLAGGQPHAGLEDSTRASIFTYTNSNATRGPFEGPNYHIAPRWV
P.vitulina           MA-QTWGLQRLADGRPQPGYEDSTQASIFTYTNSNATRGPFEGPNYHIAPRWV
G.gallus             MA--AW-EAAFAARRRHEE-EDTTRDSVFTYTNSNNTRGPFEGPNYHIAPRWV
T.guttata            MATGVWDGAVFAARRRHDD-EDTTRDSIFTYTNSNNTRGPFEGPNYHIAPRWV
D.copressiceps       MA-EEWGKQSFAARRYH---EDSTRGSAFAYTNSNNTRDPFEGPNYHIAPRWI
C.auratus            MA-EQWGDAIFAARRRG---DETTRESMFVYTNSNNTRDPFEGPNYHIAPRWV
D.rerio              MA-EHWGDAIYAARRKG---DETTREAMFTYTNSNNTKDPFEGPNYHIAPRWV
M.zebra              MA-EEWGKQSFAARRYH---EDSTRGSAFAYTNSNNTRDPFEGPNYHIAPRWI
O.niloticus          MA-EEWGKQSFAARRYH---EDTTRGSAFTYTNSNNTRDPFEGPNYHIAPRWV
L.fuelleborni        MA-EEWGKQSFAARRYH---EDSTRGSAFAYTNSNNTRDPFEGPNYHIAPRWI
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Fig. 4. Amino acid sequences of five mammalian (*Homo sapiens* Z68193; *Macaca fascicularis* long-wave AF158968; *M. fascicularis* middle-wave AF158975; *Bos taurus* AF280398; *Felis catus* AF132040; *Phoca vitulina* AF110495), one amphibian (*Xenopus laevis* U90895), two avian (*Gallus gallus* X57490; *Taeniopygia guttata* AF222333), and six teleost red opsins (*Dimidiochromis compressiceps* AF247131; *Carassius auratus* L11867; *Danio rerio* AF109371; *Metriaclima zebra* AF247126; *Oreochromis niloticus* AF247128; *Labeotropheus fuelleborni* AF247127) are compared. Dashes indicate gaps introduced in the sequence to optimize the alignment. Clustered Thr/Ser residues are framed. The potential N-glycosylation sites are framed and shaded.

decapeptide of octopus rhodopsin and determined the structure and sites of *O*-glycans in the peptide by mass spectrometry. The results show that GalNAc residues are attached to a cluster of Thr (Thr4 and Thr5) which are located in the upstream of a single *N*-glycosylation site in the N-terminus of octopus rhodopsin (Fig. 3). On the other hand, we found no significant GalNAc in bovine rhodopsin which contains two Ser and two Thr at separate sites in its N-terminus. Therefore, we assumed that the clustered Ser/Thr residues, if present in the upstream of a single *N*-glycosylation site of other rhodopsin or related proteins, might be *O*-glycosylated. We looked for the similar sequence as potential *O*-glycosylation sites with the help of a database of visual pigments. Vertebrate red pigments were the case, that is, four pigments out of the 15 red pigments include two clusters of Ser/Thr and 10 pigments include a cluster of Ser/Thr in the upstream of a single potential *N*-glycosylation site in their N-terminus, respectively (Fig. 4). Most of the color pigments are hard to isolate due to their low abundance and instability in detergent, but a chicken red pigment called iodopsin is an exception [29]. Therefore, we determined the sugar and amino acid composition of chicken iodopsin, and found that it contains 2.8 residues of GalNAc per molecule. In addition to a cluster of Thr, N-terminus of chicken iodopsin contains two Ser and two Thr residues at discrete sites in the upstream of the *N*-glycosylation site and it is possible that either of them is also *O*-glycosylated.

It was shown that removal of *N*-glycan from bovine rhodopsin [30] and the β-adrenergic receptor [26] has lost their ability to couple to G-proteins. It was also shown that *N*-glycosylation is required for normal expression of the receptors at the cell surface [31,32]. On the other hand, there are some reports showing opposing results [33–35]. Importance of *O*-glycosylation has been suggested in some of cell surface receptors. For example, contribution of *O*-glycans/*O*-glycosylated domains to the expression of binding activities of prostaglandin receptors [36], to stability of the very low density lipoprotein receptor [37], or to apical sorting of neurotrophin receptors [38] has so far been reported. However, functional studies on *O*-glycans of GPCRs are limited, because their existence has recently been found. Sadeghi et al. [15] showed that neither the level of expression of the receptor nor its function is altered by eliminating the *O*-glycosylation sites as well as an *N*-glycosylation site by alanine scanning mutagenesis. Petaja-Repo et al. [16] showed that less than 50% of the precursor of human δ-opioid receptor is processed to a fully *O*- and *N*-glycosylated mature form on the cell surface, and the turnover rate for the mature form (15 h) is substantially slower than those for partially glycosylated intermediates (99–134 min) or unglycosylated form (35 min). They also suggested that the internal precursors with faster half-lives are destined to a degradation pathway. The function of *O*-glycans of octopus rhodopsin is not elucidated at present. Further efforts are required to understand the function of *O*-glycans of a large family of GPCRs which might contribute to maturation, transport and cell surface expression. When the expression system for invertebrate rhodopsin will be established, we will undertake to pursue the role of *O*-glycosylation of octopus rhodopsin.

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