A cDNA encoding a novel heptahelical receptor from the prothoracic glands of the silkworm, *Bombyx mori* was cloned and sequenced during screening of a prothoracicotropic hormone (PTTH) receptor. Orthologs of this receptor are found not only in insects, but also in the vertebrates. In *B. mori*, ubiquitous expression of the mRNA was observed in the larva. Also, a higher expression level in the prothoracic glands was observed before molting and metamorphosis and was impaired after pupal molting. But, further analysis is required to confirm whether this receptor cDNA encodes the PTTH receptor.

Key words: *Bombyx mori*; heptahelical receptor; molecular cloning; prothoracic glands; prothoracicotropic hormone (PTTH) receptor

Prothoracicotropic hormone (PTTH) is a peptide hormone playing a crucial role in insect postembryonic development by stimulating the prothoracic glands to induce molting hormone (ecdysone) secretion. Since PTTH regulates precisely timed molting and metamorphosis, it is important for normal growth and development in insects. Characterization and identification of the PTTH molecule from the silkmoth, *Bombyx mori*, has contributed to progress in physiological studies of the mechanisms of insect molting and metamorphosis. On the other hand, the molecular mechanisms involving the ecdysteroidogenesis in the prothoracic glands from PTTH stimulation to ecdysone production remain to be investigated. We aimed at molecular identification of the PTTH receptor from *Bombyx mori*. Since *Bombyx* PTTH exhibits no homologous molecules in the database, we could not obtain clues as to the PTTH receptor. Thus PCR cloning methodologies based on the known sequences are not suitable for PTTH receptor cloning. Hence we utilized the mammalian expression cloning methodology, for which no information on the receptor was necessary. In the present study, we came across a cDNA encoding a novel type of heptahelical putative receptor from a cDNA library derived from *Bombyx* prothoracic gland mRNA.

For the purpose of molecular identification of the PTTH receptor, we performed an expression cloning procedure using a mammalian cell line, COS-7 cells. Since the prothoracic gland is a strong candidate organ expressing the PTTH receptor, we constructed a cDNA library from 1,000 pairs of the *Bombyx* prothoracic glands of last instar *Bombyx* larvae. The extracted mRNA was reverse-transcribed followed by introduced into a mammalian expression vector, pME18S, which has an SRα promoter driving in a large T antigen expressing vertebrate cell lines, such as COS-7 cells. The resulting cDNA library contained approximately 3.0 × 10^6 independent clones and was inserted more than 95% of cDNAs. The screening procedure for a cDNA encoding the PTTH receptor was based on ligand-receptor affinity, as Seed and Aruffo have been described. Practically, we incubated the biologically active recombinant *Bombyx* PTTH and COS-7 cells, which were transfected with a cDNA library derived from *Bombyx* prothoracic glands. The complex of ligand and receptor was fixed covalently by a bifunctional chemical cross linker, BS3 (50 μg/ml PBS) (Pierce Biotechnology, Rockford, IL). COS-7 cells possessing ligand-receptor complex were selected by polyclonal rabbit antibody raised against recombinant *Bombyx* PTTH followed by magnetic beads-conjugated anti-rabbit IgG (Applied Biosystems, Foster City, CA). The cDNAs extracted from the selected positive cells were reintroduced into *E. coli* for amplification. This amplified cDNA was retransfected into COS-7 cells, and then we repeated the cycle of the screening procedure until some concentrated cDNA appeared on the agarose
During the course of this screening, a cDNA sized approximately 1.3 kb was concentrated, then the insert cDNA was sequenced with an Autosequencer 310 (Applied Biosystems, Foster City, CA) (GenBank Accession no., DQ228724) (Fig. 1A). The cDNA had an open-reading frame encoding a protein composed of 331 amino acid residues. Hydrophobicity analysis revealed that this deduced protein had several hydrophobic regions, which were predicted to be transmembrane domains.

**Fig. 1.** cDNA Sequence of Pgdr and the Deduced Amino Acid Sequence. A, Nucleotide and deduced amino acid sequences of Bombyx Pgdr. Seven putative transmembrane spanning domains are shaded in gray and labeled TM1-7. An asterisk indicates a putative N-glycosylation site (consensus N-X-T). The sequence is registered with GenBank, accession no. DQ228724. B, The hydropathy plot of PGDR was calculated by the Kyte and Doolittle method with averaging over a window of 15 residues. Bars represent the putative transmembrane domains, calculated with the SOSUI program, which is at [http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html](http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). C, Specific binding of $^{125}$I-PTTH to PGDR expressing COS-7 cells. COS-7 cells transiently expressing PGDR were incubated with $^{125}$I-PTTH ($10^{-8}$ M) in the presence of various concentrations of non-labeled PTTH (closed circles and solid line). As a control, BSA was incubated for competition against $^{125}$I-PTTH binding to PGDR (triangles and dotted line).
membrane regions (Fig. 1B). The SOSUI program, software calculating transmembrane regions from amino acid sequences (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html), implied that this protein has seven transmembrane regions, possibly spanning the plasma membrane. This prediction program also suggested translocation of the protein to the plasma membrane, indicating that the protein is a typical receptor protein. Hereafter, we designate this novel receptor prothoracic gland-derived receptor (Pgdr).

Next we confirmed the binding activity of PGDR using radio-labeled iodinated Bombyx PTTH (125I-PTTH). 125I-PTTH was prepared by the reaction of Iodogen (Pierce Biotechnology, Rockford, IL) methodology by mixing recombinant Bombyx PTTH and Iodine-125 (NEN/C212 Life Science Products, Boston, MA) in an Iodogen-coated tube. COS-7 cells, transiently expressing PGDR, were incubated with 125I-PTTH. In the presence of non-labeled recombinant Bombyx PTTH in the incubation mixture, the level of 125I-PTTH binding to COS-7 cells transiently expressing PGDR decreased. By contrast, a mixture of BSA as a control did not influence the binding between 125I-PTTH and PGDR, indicating that PGDR expressing COS-7 cells exhibited Bombyx PTTH-specific binding (Fig. 1C).

In the GenBank database, this protein did not show similarity to the known heptahelical receptors. Hence we therefore concluded that it encodes a novel type of receptor. Interestingly, the cDNA of PGDR (Pdgr) has orthologs not only in arthropods but also in vertebrates (Fig. 2). The database indicated homologous cDNAs in the nematode Caenorhabditis elegans and the mosquito Anopheles gambiae, which show 46% and 58% of amino acid identity with the deduced amino acid sequence of Bombyx PGDR, respectively. It is also interesting that the fruitfly, Drosophila melanogaster, has two orthologs of this receptor, which are 53% and 48% of identity. In mouse and human, the homologous genes showed 46% and 47% of identity, respectively. These results indicate that this receptor subfamily is preserved over the animal kingdom.

To confirm the expression of Pgdr mRNA, we performed Northern blot analysis. When we utilized Bombyx larvae of the 5th instar day-5, we observed Pgdr mRNA expression in various organs (Fig. 3A). Pgdr mRNA was expressed significantly in brain, prothoracic glands, salivary glands, gut, ovary, and testis, while the mRNA was expressed at a low expression level in silk gland, fat body, and hemocyte.

Next, we analyzed the expression levels of Pgdr mRNA in the prothoracic glands during growth within the 5th instar (Fig. 3B). As the larvae grew, the expression level of Pgdr decreased gradually. In day-1 and day-2 of the wandering period, when larvae start preparation for pupation, the expression level of Pgdr greatly increased. Just after pupation, in turn, the Pgdr expression level decreased. This result indicates that expression of PGDR might be involved in ecdysteroid regulation, and consequently in insect molting and metamorphosis.

In the present study, we cloned a cDNA encoding a novel receptor by screening targeting for the Bombyx PTTH receptor. It is a strong candidate for cDNA encoding Bombyx PTTH receptor. However, to confirm whether this Pdgr cDNA encodes the Bombyx PTTH receptor, further analysis, such as investigation of transduction of the second messenger via PGDR in response to Bombyx PTTH, is required. In addition, it is noteworthy that the mouse homolog of this receptor has been identified by the differential cloning methodology.
as the growth hormone-induced transcript in the mouse adipocyte. In terms of growth regulation, these evolutionarily preserved receptor proteins should be investigated.

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