Introduction

Intracellular signal transmission plays a very important role in the regulation of homeostasis, growth, development, reproduction in multicellular organisms. This transmission is frequently mediated by specific molecules such as growth factors, hormones, and neurotransmitters/neuromodulators. These molecules exert their effects on cells by binding to specific receptors (Himmelweit, 1960). The receptors activated by their binding evokes intracellular signal transduction following various cellular events including the secretion of biologically active substances, induction of gene expressions, activation of metabolism, influx/efflux of ions, and actuation of cell cycles. Although intracellular signal transmission appears to consist of very complicated processes, an initial key step is simply to bind certain molecules to their specific receptors, which triggers the cascades of following cellular events. A variety of molecules are used as mediators for intercellular signal transmission, and bioactive peptide is one of the representative molecules. It binds to receptors as a specific ligand and transmits a signal to cells expressing the receptors.

Most of receptors for peptidic ligands belong to seven-transmembrane-domain receptors (7TMRs) which make up a huge receptor family, and they have common structural features spanning the cell membrane seven times (Strosberg, 1991; Probst *et al.*, 1992). Since 7TMRs are coupled to G protein, which is a heterotrimer consisting of α, β, and γ subunits as machinery to transduce intracellular signals, they are also called G protein-coupled receptors (Strosberg, 1991; Probst *et al.*, 1992). The binding of peptidic ligands to 7TMRs activates the G proteins which transmit signals to

intracellular effector molecules. To know the precise action of the bioactive peptides, it is very important to know the properties of their receptors. In traditional studies, the functional characteristics of the receptors for bioactive molecules have been studied utilizing animal tissues or cell lines endogenously expressing their receptors. For example, the binding properties of the receptors were analyzed with membrane preparations obtained from animal tissues; however, the content of the receptor molecules in the tissue sample was often insufficient to use in experiments. In addition, a variety of receptors are expressed in the tissues, which makes it difficult to analyze precisely the specific interaction between the ligand and receptor of interest. The progress of gene engineering has enabled us to isolate cDNAs encoding receptor proteins. To date, I can produce cells expressing receptors which I aim, by introducing cDNAs into appropriate cells. This technique has greatly aided to perform the pharmacological characterization of receptors. Various strategies have been so far conducted to isolate cDNAs encoding receptors: on the basis of the functions (i.e., binding and signal transduction), structural similarity, and the amino acid sequences of purified receptor proteins.

On the other hand, the recent progress in cDNA and genome DNA analyses has brought the discovery of numerous genes encoding 7TMR-like proteins. Most of them show low degree of sequence homology with 7TMRs for known bioactive molecules. As their ligands are unidentified, they are referred to as 'orphan' 7TMRs (Stadel *et al.*, 1997). Some 7TMRs and their ligands play crucial roles in the regulation of physiological phenomena, so that the identification of the endogenous ligands for orphan 7TMRs is essential to understand regulatory mechanisms for various physiological phenomena. In addition, many medical drugs currently on the market have been designed to act on 7TMRs (Stadel *et al.*, 1997; Wilson *et al.*, 1998). It

can thus be said that the identification of the ligands for orphan 7TMRs is very important in respect to both basic and applied science. For the first time, the utility of orphan 7TMRs was demonstrated in the identification of nociceptin/orphanin FQ (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). In this case, however, the orphan 7TMR, ORL1/LC132, had been known to belong to the opioid receptor family, since it showed a significant homology with opioid receptors so far known. In addition, the signal transduction pathway, that is, the inhibition of cAMP production, was readily predicted because the opiates specifically acted on the cells expressing this receptor.

We have recently established a strategy which can be widely applicable to the identification of endogenous peptidic ligands for many orphan 7TMRs (Hinuma *et al.*, 1998; Tatemoto *et al.*, 1998). Employing this method, we succeeded in identifying the endogenous ligand of the orphan 7TMR, hGR3. The ligand peptide identified for hGR3 was found to show a specific prolactin-release-promoting activity in primary cultured rat anterior pituitary cells and in the pituitary tumor cell line RC-4B/C, and so named 'prolactin-releasing peptide' (PrRP) (Hinuma *et al.*, 1998).

APJ is a member of 7TMR family, which was originally isolated from human genomic DNA by polymerase chain reaction (PCR) (O'Dowd *et al.*, 1993). *Apj* gene encodes a protein consisting of 380 amino acid residues, showing about 30% amino acid sequence identity to angiotensin II receptor overall. Despite the significant structural relationships between angiotensin II receptor and APJ, angiotensin II did not interact with APJ when expressed in fibroblasts (O'Dowd *et al.*, 1993) and Chinese hamster ovary (CHO) cells (Tatemoto *et al.*, 1998). On the other hand, APJ reportedly acts as a coreceptor with CD4 in the process of human immunodeficiency virus type 1

(HIV-1) infection (Choe *et al.*, 1998; Edinger *et al.*, 1998; Farzan *et al.*, 1998). Although APJ does not show overall homology with chemokine receptors, such as CCR5, CCR3, and CXCR4, which also acts as coreceptors in HIV infection, APJ shows sequence similarity to them in N-terminal region (i.e., tyrosines and acidic amino acids) which is believed to be important for the interaction with HIV.

To identify an endogenous ligand for APJ, I first established CHO cells expressing APJ (CHO-A10). These cells were then utilized for the detection of a ligand which could interact to APJ. To detect a signal transduction caused by the interaction of a APJ ligand and CHO-A10 cells, we used a Cytosensor which was capable of measuring the increase in extracellular acidification rate as a consequence of the stimulation of intracellular signaling pathways (Tatemoto *et al.*, 1998). Among the samples prepared from a variety of tissues, extract prepared from bovine stomach was found to elicit evident increase in the acidification rate (Tatemoto *et al.*, 1998). In the extracts, the activities were detected mainly in the fractions containing peptides with a molecular weight in the range of 3,000-5,000 daltons. We thus decided to use bovine stomach as the starting material, and succeeded in purifying a ligand peptide. We named it 'apelin' after APJ endogenous ligand. On the basis of the information on the purified peptide sequence, cDNAs encoding apelin was then isolated. Preproapelins deduced from cDNA sequence consisted of 77 amino acid residues, and apelin was encoded in the C-terminal regions. Synthetic apelin peptides (TABLE) were capable of specifically promoting the extracellular acidification rate in CHO-A10 cells, indicating that apelin is the endogenous ligand for APJ. In this paper, I report the molecular and functional characteristics of APJ by utilizing the identified endogenous ligand, apelin.