Discussion

In traditional studies, most bioactive peptides have been identified on the basis of certain biological activities, and then their receptors have been determined. However, in this study we started with the isolation of a receptor gene, and subsequently utilized it to identify its ligand. Although our approach is opposite to the traditional one, it would be able to apply widely to identify various types of bioactive molecules, including peptides, as the ligands for orphan 7TMRs. One thousands or more kinds of 7TMR genes reportedly may exist in the entire human genome (Wilson *et al.*, 1998). Although many of these 7TMR genes would correspond to homologues of sensory olfactory receptors, which are predicted to exist in considerable number in the genome (Buck and Axel, 1991), the rest would code for as yet unknown receptors which may play important roles in the regulation of physiological functions. To reveal the biological functions and physiological significance of these receptors, it is necessary to identify their endogenous ligands.

To identify an endogenous ligand of APJ, we first established CHO cells expressing APJ. These cells were then utilized for the detection of the APJ ligand. By utilizing a Cytosensor, we could detect changes in extracellular proton excretion from the cells expressing APJ induced by the interaction of APJ and its ligand (McConnel *et al.*, 1992). We thereby identified the endogenous ligand for APJ, apelin, from animal tissue extracts. Apelin is the first example that a novel peptide was identified by using a Cytosensor. Our study demonstrated that Cytosensor is a powerful tool to search for the ligands of orphan 7TMRs.

We could not find the identical sequence to purified apelin in any

known peptides and proteins in the database. However, we found a mouse expressed sequence tag (EST) which showed significant homology with the sequence. we utilized this EST sequence information to isolate human and bovine cDNAs encoding the apelin sequences. Each cDNA thus isolated encoded a preproprotein with 77 amino acid residues (Tatemoto *et al.*, 1998). The N-terminal portion contained a signal peptide sequence, and the apelin sequence was found in the C-terminal region (Tatemoto *et al.*, 1998). We synthesized the C-terminal peptide with 36 amino acid residues presumably corresponding to the isolated endogenous bovine apelin structure. When this synthetic peptide, apelin-36, was subjected to the microphysiometric assay, I found that it was capable of promoting the acidification rates in CHO-A10 cells, but a rather high concentration (10 nM) was required to obtain the maximum response (Fig. 3). To be interested, the activity of [pGlu]apelin-13, the short C-terminal fragments of preproapelin, exhibited higher activity than apelin-36 (Fig. 3). These results suggest that the C-terminal structure with fewer than 13 residues is responsible for the receptor binding and biological activities of apelin.

I isolated a rat *apj* cDNA and found that the primary structure of APJ was highly conserved between human and rat (87.2% in amino acid identity) (Fig. 1). The amino acid sequence of mature apelin peptide (e.g., apelin-36) also highly conserved among human, bovine, rat, and mouse (i.e., 86 to 100% amino acid identity) (Habata *et al.*, 1999), suggesting that the structures of APJ and apelin have been highly conserved in evolution. Cloning of the rat *apj* cDNA enabled us to study the precise distribution of *apj* mRNA in rat tissues. The highest level of rat *apj* mRNA expression was detected in the lung by quantitative RT-PCR (Fig. 2). In our recent study, we also detected the very high level of *apelin* mRNA as well as bioactive apelin in the lung

(Habata *et al.*, 1999), which strongly suggests that APJ and its ligand play a crucial role in the pulmonary system in rats. The significant levels of *apj* mRNA were detected in the heart, adipose, ovary, femur and costal cartilage in rats (Fig. 2). Since *apelin* mRNA has been also detected in these tissues (Habata *et al.*, 1999), apelin might have regulatory functions widely in the circulation, lipid metabolism, and bone formation. In the human tissues, a high level of *apj* mRNA has been reportedly detected in the spleen (Edinger *et al.*, 1998), however, the expression of *apj* mRNA was low level in the rat spleen. In addition, the expression of APJ was low level in the human lung. These results might reflect the functional differences of APJ between the two species. It has been demonstrated that human *apj* mRNA is highly expressed in the corpus callosum and spinal cord, and the expression pattern of *apj* and *dopamine D4 receptor* are quite similar in the brain, though *apj* mRNA is more abundantly expressed than that of *dopamine D4 receptor* (Matsumoto *et al.*, 1996). In this study, I also detected rat *apj* mRNA in a wide variety of regions in the brain as well as spinal cord (Fig. 2). It has also been reported that human *apj* mRNA is detected in neuron-like NT2 cells differentiated by retinoic acid (Edinger *et al.*, 1998). Taken together with these reports and my results, APJ and apelin are supposed to play important roles in the nervous tissues. RT-PCR analyses in this study indicated that the expression of *apj* mRNA in the infant rat was higher than that in the adults (Fig. 2). These results were well consistent with the previous report that *apj* mRNA is highly expressed in early developmental stage of the rat brain (O'Dowd *et al.*, 1993). Since the expression level of rat *apelin* mRNA was also high in infants (Habata *et al.*, 1999), APJ and its ligand was expected to have regulatory functions in the process of development. We found that the extremely high level of *apelin* mRNA was expressed in the mammary gland

in pregnant and lactating rats and bioactive apelin was abundantly secreted in the milk (Habata *et al.*, 1999). Since the relatively higher amount of *apj* mRNA was expressed in the digestive organs in infants (Fig. 2), apelin may be served tropic and functional supports for neonates through milk.

Apelin preproprotein contained many basic amino acid residues which were possible proteolytic cleavage sites (Tatemoto *et al.*, 1998; Habata *et al.*, 1999). Our western blot and chromatographic analyses indicated that the endogenous apelin molecules were very heterologous (Habata *et al.*, 1999). [pGlu]apelin-13 has been presumed to be nearly the shortest form of apelin. In this paper, I attempted to reveal functional differences in heterogeneous apelin molecules, and found that the pattern of the accelerated extracellular acidification of CHO-A10 cells was quite different between [pGlu]apelin-13 and apelin-36. The elevated acidification rates induced by apelin-36 at higher concentrations were kept even after removal of the ligand, however, [pGlu]apelin-13 did not elicit sustained elevation of the acidification rates. I believe this is the first report demonstrating that the different sizes of a peptidic ligand cause distinct patterns in the extracellular acidification in cells expressing its receptors. Somatostatin also exists in multiple forms (i.e., somatostatin-14, somatostatin-25, and somatostatin-28) (Bohlen *et al.*, 1980). I compared somatostatin-14 and somatostatin-28 in the microphysiometric assay with somatostatin receptor type 2-expressing CHO cells, but I could not detect obvious difference between them (data not shown).

In the assay using a Cytosensor, changes in cellular acidification rates are thought to reflect all of the cellular events, and the inhibitors for signal transduction pathways are useful tools to distinguish specific pathways. In this study, I confirmed the coupling of APJ to Gi by the treatment with PTX.

The PTX treatment abolished the responses induced by both [pGlu]apelin-13 and apelin-36 (Fig. 4), suggesting that signal transductions induced by both peptides to APJ are similarly via the activation of Gi. NHE is the major regulator of intracellular pH in mammalian cells, and it excretes proton as a consequence of cellular metabolism, such as glycolysis and respiration (Neve *et al.*, 1995). The promotion of acidification caused by both [pGlu]apelin-13 and apelin-36 was significantly inhibited by the treatment with MIA, a specific inhibitor for NHE (Fig. 5). These results suggest that acidification induced by both [pGlu]apelin-13 and apelin-36 was via NHE. Although the pattern of the induced extracellular acidification differed between [pGlu]apelin-13 and apelin-36, the signal transduction pathways used seemed to be almost the same.

In my receptor binding assays, [^{125}I] [pGlu65, Nle75, Tyr77]apelin-13 specifically bound to CHO-A10 cells with high affinity (Fig. 6), and [pGlu]apelin-13 and apelin-36 competitively inhibited its binding (Fig. 7). The IC_{50} of these two peptides almost paralleled the EC_{50} of them in the cAMP production-inhibitory assay (Habata *et al.*, 1999). The specific binding of apelin to APJ with high affinity undoubtedly demonstrates that apelin is an endogenous ligand for APJ. In addition, the binding experiments using the cells treated with [pGlu]apelin-13 and apelin-36 gave us a cue to explain the differences between [pGlu]apelin-13 and apelin-36 in the induction of the extracellular acidification. [pGlu]apelin-13 bound to APJ was supposed to rapidly dissociate and thus effectively substituted with [^{125}I] [pGlu65, Nle75, Tyr77]apelin-13 on CHO-A10 cells treated with [pGlu]apelin-13 (Fig. 8A). In contrast, the labeled ligand scarcely bound to CHO-A10 cells treated with apelin-36, suggesting that apelin-36 bound to APJ hardly dissociate even after washing (Fig. 8B). The temporal and sustained patterns in the

acidification response might reflect differences in the dissociation from APJ between [pGlu]apelin-13 and of apelin-36. In my preliminary experiments, the binding of the labeled apelin to CHO-A10 cells treated with apelin-36 was significantly recovered after treating the cells with acid (data not shown), suggesting that the decrease of the labeled apelin binding by the treatment with apelin-36 is caused neither by desensitization nor by internalization of APJ. In the dose-response relationships in the microphysiometic assay, [pGlu]apelin-13 has been proven to be much more potent than apelin-36 (Tatemoto *et al.*, 1998). Although the precise comparison of association rate constants by utilizing the corresponding radioligands of [pGlu]apelin-13 and apelin-36 are required, I imagine that the dose-response relationships of these two peptides in the microphysiometric assay might rather reflect the rate of association of the peptides.

In this study, I demonstrated that apelin showed a chemotactic activity to CHO-A10 cells (Fig. 9). [pGlu]apelin-13 was more potent in the chemotactic assay than apelin-36, suggesting that the potency of the chemotactic activity in apelin also depends on the association rates to APJ. My results indicate that the N-terminal portion of apelin is very important to modulate the interaction with APJ and the biological activity, although the core structure of apelin is situated in the C-terminal portion. Although further studies are required to confirm whether apelin physiologically acts as a chemotactic factor on cells expressing originally APJ, a kind of chemokine receptor, CXCR4 is demonstrated to be involved in celebellar development as well as haematopoiesis (Zou *et al.*, 1998). I found that APJ and its ligand were also expressed in neonatal tissues, suggesting the functional importance of APJ and apelin in cell migration and patterning in the development.

The identification of endogenous ligand peptide, apelin, for APJ

enabled us to characterize the function of APJ. Our recent study revealed that apelin partially suppressed cytokine production from mouse spleen cells in response to T cell receptor/CD3 cross-linking (Habata *et al.*, 1999). In addition, it was reported that intravenous injection of apelin caused immediate lowering of both systolic and diastolic blood pressures, and intraperitoneal injection induced an increase in drinking behabior (Lee *et al.*, 2000). I believe that further studies on APJ and apelin will give us new insights into unknown mechanisms in physiological regulation through APJ-apelin system. In addition, I hope that these studies will help of novel drug discovery in the future.