Chapter 3

Placental Leucine Aminopeptidase

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Abstract: Human pregnancy serum and placenta are known to have the ability to degrade oxytocin (OT), the most potent uterotonic peptide. Placental leucine aminopepetidase (P-LAP), which is also called cystine aminopeptidase, is the only membrane aminopeptidase known to open the N-terminal cystine loop of OT. The soluble form of P-LAP present in maternal serum is converted from the membrane-bound form in placenta by an enzyme with metalloprotease activity. The findings that P-LAP activity increases with gestation to counteract the increasing fetus-, placenta- or mother-derived OT and that P-LAP activity is reduced in the patients with preterm delivery suggest a possible role of P-LAP in controlling the uterine contraction. In placenta, P-LAP is expressed in differentiated trophoblasts, which is regulated by the AP-2 and Ikaros transcription factors. Unexpectedly, P-LAP is a homologue of rat insulin-regulated membrane aminopeptidase (IRAP). P-LAP is translocated from the cytosol to the plasma membrane by oxytocin stimulation in vascular endothelial cells and by vasopressin in renal cells, which is similar to the finding that insulin stimulates IRAP translocation in adipocytes. P-LAP has a wide tissue distribution besides placenta, indicating possible roles not related to pregnancy. Since P-LAP hydrolyzes several peptides including vasopressin and angiotensin III other than OT, natural substrates interacting with P-LAP should be considered to elucidate its roles in various pathophysiological processes. Characterization of recently established P-LAP-deficient mice would serve for further elucidation of P-LAP functions. Recently two enzymes significantly homologous to P-LAP have been cloned, which belong to one distinctive group with P-LAP. Therefore, we propose the oxytocinase subfamily of M1 aminopeptidases.

Key words: aminopeptidase, oxytocin, placenta, pregnancy, vasopressin

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1. INTRODUCTION

Oxytocin (OT) is a potent and specific peptide hormone that stimulates myometrial contraction during pregnancy. Synthesized by both mother and fetus (Chard, 1989) as well as by placenta (Lefebvre *et al.*, 1992) during pregnancy, OT plays an important role in the regulation of labor. While less attention has been devoted to OT degradation, local concentrations of OT in the feto-placental-maternal unit depend upon a balance between synthesis and degradation. Human placenta and maternal serum are known to contain the enzyme responsible for OT degradation (Page *et al.*, 1961; Mizutani *et al.*, 1976; Sakura *et al.*, 1981; Tsujimoto *et al.*, 1992). At least two types of peptidases metabolize OT; these are post-proline endopeptidase and placental leucine aminopeptidase (P-LAP), which is also called cystine aminopeptidase or cystinyl aminopeptidase (EC 3.4.11.3). P-LAP opens the N-terminal ring structure of OT and thus effectively destroys OT activity, but whether or not post-proline endopeptidase has similar potential remains unclear (Ferrier *et al.*, 1974; Mizutani *et al.*, 1985; Mitchell and Wong, 1995). Hence, P-LAP should be regarded as a true oxytocinase. P-LAP plays a critical role in regulating endocrine, paracrine or autocrine OT activity in the placenta leading to the maintenance of pregnancy via regulating oxytocin levels.

Contrary to the initial hypothesis that P-LAP is a placenta-specific enzyme, a widespread tissue distribution of P-LAP has now been established (Rogi *et al.* 1996; Nagasaka *et al.* 1997). In addition, cDNA cloning of P-LAP (Rogi *et al.* 1996) have demonstrated that this enzyme is a homologue of rat insulin-regulated membrane aminopeptidase (IRAP) (Keller *et al.* 1995) (see Chapter 4), which is present in glucose transporter isotype GLUT4 vesicles of rat adipocytes (Mastick *et al.* 1994; Kandror *et al.* 1994). Therefore, it is conceivable that P-LAP would also play roles in organs other than placenta. Actually, several lines of evidence have been provided to elucidate the association of P-LAP with various pathophysiological processes.

In this article, we review the biochemical characteristics of P-LAP, its tissue distribution and cellular localization, regulatory mechanisms of P-LAP gene expression, and possible physiological roles of P-LAP in placenta as well as in other organs.

2. SUBSTRATES AND INHIBITORS OF P-LAP

As it has been also called cystine aminopeptidase, P-LAP cleaves an N-terminal cysteine in a disulfide linkage with an internal cysteine (Sjöholm &

Yman, 1967). Since OT and vasopressin have this N-terminal ring structure, P-LAP preferentially hydrolyzes these peptides (Tsujimoto *et al.*, 1992; Matsumoto *et al.*, 2000). Up to now, P-LAP is regarded as the only membrane aminopeptidase that cleaves OT and vasopressin with an N-terminal cystine. However, P-LAP dose not hydrolyze all the hormones with this structure, such as endothelins and calcitonin (Matsumoto *et al.*, 2000). In addition to the preference of N-terminal half-cystine residue involved in a disulfide loop, P-LAP also releases an N-terminal neutral or basic, not acidic, amino acid of peptides. P-LAP, therefore, is able to hydrolyze angiotensin III, somatostatin, Lys-bradykinin, Met-enkephalin, dynorphin A, and neurokinin A (Tsujimoto *et al.*, 1992; Herbst *et al.*, 1997; Matsumoto *et al.*, 2000; Matsumoto *et al.*, 2001).

Various kinds of inhibitors are reported to inhibit P-LAP activity (Sakura *et al.*, 1981; Herbst *et al.*, 1997; Matsumoto *et al.*, 2000). Among the known aminopeptidase inhibitors, amastatin and leucinethiol are potent, but bestatin and puromycin are less competitive inhibitors. On chelating agents, 8-hydroxyquinoline and 1,10-phenanthroline inhibit P-LAP activity, while EDTA has no influence on the activity even at 1 mM. Divalent cations such as Zn^{2+}, Cu^{2+} and Cd^{2+} also serve as strong inhibitors.

3. P-LAP PROTEIN STRUCTURE AND GENE

We and others have cloned the cDNA encoding human P-LAP from human placental cDNA library (Rogi *et al.*, 1996; Laustsen *et al.*, 1997). The sequence comparison has demonstrated that P-LAP shares 87% identity with rat IRAP at the amino acid level (Keller *et al.*, 1995), which is present in glucose transporter isotype GLUT4 vesicles of rat adipocytes (Kandror *et al.*, 1994; Mastick *et al.*, 1994), indicating that P-LAP and IRAP are the human and rat homologue of the same protein. cDNA cloning and subsequent genome cloning have provided a wealth of evidence that helps us to elucidate possible roles of P-LAP.

3.1 P-LAP Protein Structure

The predicted P-LAP contains three domains; an N-terminal 108 amino acid cytoplasmic domain, a 23-amino acid transmembrane domain and an 893-amino acid extracellular domain (Figure 1). The cytoplasmic tail is thought to be involved in the precise subcellular localization and the intracellular traffic by the stimulants including insulin and oxytocin (Keller *et al.*, 1995; Nakamura *et al.*, 2000). A large extracellular domain contains the HE*XX*H consensus sequence of zinc–binding site with a second glutamic

acid residue 18 amino acids away, which constitutes the active site of metallopeptidases (Jongneel *et al.,* 1989; Wang and Cooper, 1993). The residues within the two motifs are essential for the enzyme activity (Laustsen *et al.*, 2001).

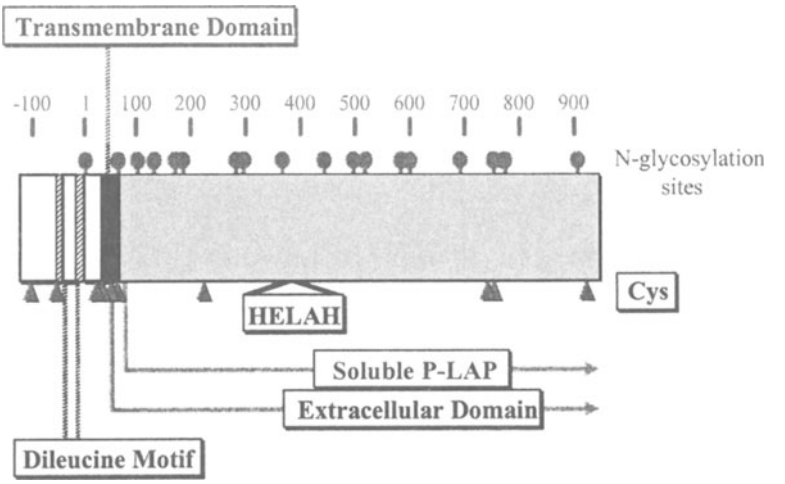

Figure 1. Domain structure of P-LAP. Black box represents a putative transmembrane domain. Positions of the consensus sequence of the zinc-binding site and the N-terminal of soluble P-LAP are also shown.

P-LAP is present as a soluble form in the maternal serum and also as a membrane-bound form in the placenta. P-LAP genome cloning has demonstrated that the presence of both membrane-bound and soluble forms is not due to the alternative splicing of mRNA (Horio *et al.*, 1999). It is conceivable that the soluble form of P-LAP derives from the membrane-bound from by post-translational proteolytic cleavage between Phe^{154} and Ala^{155}. Such type of conversion is also observed in angiotensin converting enzyme (ACE) and tumor necrosis factor-α (TNF-α), and the enzyme responsible for the proteolytic processing is generally termed secretase or sheddase (Hooper *et al.*, 1997). Although P-LAP secretase has not been definitively identified as yet, the secretase would be a metalloprotease, which recognizes the amino acid sequence of the cleavage site (Iwase *et al.*, 2001; Ofner and Hooper 2002). Success in P-LAP cDNA cloning led to the establishment of an expression system for the recombinant soluble form of P-LAP (Matsumoto *et al.*, 2000) as well as a one-step purification method for membrane-bound P-LAP from placenta using an immunoaffinity column (Nakanishi *et al.*, 2000).

3.2 P-LAP Gene Regulation

Human P-LAP genomic DNA has also been cloned (Horio *et al.*, 1999; Rasmussen *et al.*, 2000). The human P-LAP gene spans approximately 75 kb containing 18 exons and 17 introns. The gluzincin aminopeptidase motif, GAMEN-(31*X*)-HE*XX*H-(18X)-E, is encoded by exons 6 and 7, which is similar to aminopeptidase N and aminopeptidase A genes. The P-LAP gene is assigned to human chromosome 5q14.2-q15, with which no genetic disorders are known to be associated. In trophoblastic choriocarcinoma cells, the promoter region from –214 to –183 of P-LAP gene, to which activator protein-2 (AP-2) and Ikaros transcription factors bind, is critical for high promoter activity (Ito *et al.*, 2001; Ito *et al.*, 2002). AP-2 is now identified as a binding protein to the trophoblastic responsive element (TRE), which also regulates human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) expression (Johnson *et al.*, 1997; Richardson *et al.*, 2000). Ikaros, initially characterized as a lymphoid-restricted transcription factor (Georgopoulos *et al.*, 1994), functionally regulates P-LAP promoter activity in trophoblastic cells, which is the first to demonstrate that Ikaros is involved in gene regulation in cells other than hematopoietic cells (Ito *et al.*, 2002). AP-2 is the main activator, and Ikaros functions with AP-2 cooperatively for maximal expression of the human P-LAP gene.

4. EXPRESSION OF P-LAP IN PLACENTA AND OTHER TISSUES

Contrary to the initial concept that P-LAP expression would be restricted to the placenta, Northern blot analysis has demonstrated the wide tissue distribution of P-LAP mRNA in human (Rogi *et al.*, 1996) and rat (Keller *et al.*, 1995). Immunohistochemistry not only supports the finding but also determines the precise localization of P-LAP in each tissue, which would be helpful to address the potential functional roles of P-LAP. In addition, P-LAP location in some tissues varies during physiological processes such as pregnancy and development.

4.1 Expression of P-LAP in Placenta

During placental development, cytotrophoblasts differentiate and fuse to syncytiotrophoblasts, which requires the increase of intracellular cAMP levels. Immunohistochemical analysis of P-LAP has shown the predominant localization of P-LAP to syncytiotrophoblasts, but little or no positive staining in cytotrophoblasts throughout the gestation (Nagasaka *et al.*, 1997;

Yamahara *et al.*, 2000). In situ hybridization has also demonstrated that P-LAP mRNA is predominantly expressed in syncytiotrophoblast cells (Nomura *et al.*, 2002). Since consensus cAMP responsive elements have not been observed in the up-stream region of P-LAP, the increase in AP-2, especially AP-2α isoform among the AP-2 family members, could account for the differentiation-dependent expression of P-LAP in trophoblasts (Iwanaga *et al.*, 2003). Ultrastructurally, transmission immunoelectron microscopy reveals that P-LAP is expressed on the surface of apical microvilli of syncytiotrophoblast cells and, to a lesser extent, on the basal infoldings (Ito *et al.*, 2003). Since the surface of apical microvilli is a site of interaction between the mother and fetus, the predominant expression of P-LAP there suggests a possible involvement of P-LAP in cleaving peptide hormones in order to regulate their bioactivity, secretion of which increases with fetal growth.

4.2 Expression of P-LAP in Other Tissues

P-LAP has a wide, but not ubiquitous, tissue distribution. Heart and skeletal muscle express P-LAP mRNA as abundantly as placenta, while liver has little P-LAP mRNA (Keller *et al.*, 1995; Rogi *et al.*, 1996). According to an immunohistochemical study in human adult (Nagasaka *et al.*, 1997), P-LAP is present in epithelial cells of hepato-biliary, bronchial alveolar and renal tubular systems as well as gastrointestinal mucosal cells, sweat gland cells, islet cells of pancreas and neuronal cells of brain. In addition, in both adult and fetal tissues, P-LAP immunostaining is present in the endothelium of almost all kinds of vessels, from capillaries to large arteries.

The influences of pregnancy and development on P-LAP expression in mother and fetal tissues have been investigated in mice (Kobayashi *et al.*, 2003). In non-pregnant mice, strong P-LAP expression is noted in the pit epithelium of the stomach, cardiomyocytes of the heart, enterocytes in the intestine, bile canaliculi in the liver, islet of Langerhans, distal and collecting tubules in the kidney, and neuronal cells of brain. In pregnant mice, in contrast to tissues such as liver, kidney, ovary and brain that show no clear alterations, the pancreatic islet of Langerhans shows apparent changes in P-LAP staining pattern during pregnancy. Strong immunoreactivity is prominent only in the periphery of the islet in early gestation, but in middle gestation, P-LAP-reactive cells are observed both in the periphery and in the inner area of the islet, and then in late gestation, only the cells in the inner area, possibly insulin secreting B cells, show mosaic-like staining patterns. Since OT and vasopressin increase insulin secretion (Gao *et al.*, 1990; Richardson *et al.*, 1990), increase in P-LAP expression in B cells may

suggest that P-LAP regulates insulin secretion during gestation through degrading those peptides.

In fetal mice, P-LAP staining in the cytoplasm of megakaryocytes increases with their growth. P-LAP may regulate the bioactivity of peptides that are associated with the differentiation and maturation of megakaryocytes. Alternatively, P-LAP may serve as a cell-specific marker for megakaryocyte differentiation, as observed in various types of hematopoietic cells that express their own cell-surface aminopeptidases at unique stages of cell differentiation lineage (Kenny and O'Hare 1989; Shipp and Look 1993). Fetal islet of Langerhans shows weaker immunoreactivity and a smaller number of positive cells in the periphery even in late gestation.

5. PHYSIOLOGICAL FUNCTION OF P-LAP DURING PREGNANCY AND NON-PREGNANCY

In addition to the classical functions such as protein digestion in intestines, cell-surface peptidases generally have various physiological roles: metabolism of peptide hormones to regulate their bioactivity, modulation or markers of cell growth and differentiation, receptors for coronavirus (Yeager *et al.*, 1992), and the final trimming of antigen presentation (Saric *et al.*, 2002; Serwold *et al.*, 2002). The physiological functions of P-LAP, especially in organs other than the placenta, are not fully understood. To realize the functional roles of P-LAP, interactions with the substrate peptides, changes in activity and expression levels during various pathophysiological processes, and precise cellular and tissuelar location should be taken into account.

5.1 P-LAP Roles During Pregnancy

OT is the most potent uterotonic peptide hormone during pregnancy. Regulation of OT activity, therefore, is associated with the suppression and enhancement of labor pain as well as the onset of labor. As P-LAP has been identified as oxytocinase, P-LAP is known to have a suppressive role in controlling uterine contraction during pregnancy. P-LAP activities in maternal serum and placenta increase with gestational age to a maximum at near term (Mizutani *et al.*, 1976; Yamahara *et al.*, 2000). Since local concentrations of OT in the feto-placental-maternal unit, which is synthesized by both mother and fetus (Chard, 1989) as well as by placenta (Lefebvre *et al.*, 1992) increase during pregnancy, P-LAP seems to increase to balance between the synthesis and degradation. Therefore, P-LAP would prevent a premature onset of uterine contractions by degrading OT (Mizutani

& Tomoda, 1992), and when OT production overcomes the degradation, labor may occur (Mizutani *et al.*, 1982). In accordance with this, maternal serum P-LAP activities decrease in patients with spontaneous preterm delivery (Kozaki *et al.*, 2001). Interestingly, P-LAP in umbilical vascular endothelial cells translocates from the cytosol to the plasma membrane by OT stimulation, indicating the feedback regulation of OT by P-LAP (Nakamura *et al.*, 2000). Infection to the feto-maternal unit, chorioaminonitis, is associated with premature uterine contraction through the actions of prostaglandins as well as OT. A hypothesis that inflammatory cytokines including IL-6 and IL-1β may enhance OT uterotonic action via reducing P-LAP activity has proven to be unlikely (Ikoma *et al.*, 2003). IL-1β increases P-LAP activity and expression in trophoblastic cells, which requires de novo protein synthesis, suggesting that P-LAP may have a protective role from premature delivery, especially under mild infectious conditions.

Fetuses also produce the vasoactive peptide vasopressin during conditions leading to fetal acidemia such as pre-eclampsia, a hypertensive disorder peculiar to pregnancy. Since pre-eclampsia is immediately cured after the delivery, an increase in vasopressin release from the fetus seems to elevate blood pressure in pre-eclamptic patients. P-LAP activities in mild pre-eclamptic patients rather elevate, while P-LAP activities decrease below the normal range in the severe patients (Mizutani *et al.*, 1985). This finding may suggest that while P-LAP could counteract the increased vasopressin, pre-eclampsia is mild, but when P-LAP decreases, vasopressin augments its vasoconstrictive activity to induce severe hypertension.

Figure 2 illustrates the function of P-LAP as a barrier at the interface between mother and fetus. P-LAP maintains the homeostasis of pregnancy via degrading bioactive peptides such as oxytocin and vasopressin (Mizutani and Tomoda, 1996).

5.2 P-LAP Roles not Associated with Pregnancy

As mentioned above, P-LAP is expressed in a number of organs. P-LAP, therefore, is involved in various processes which are not limited during pregnancy.

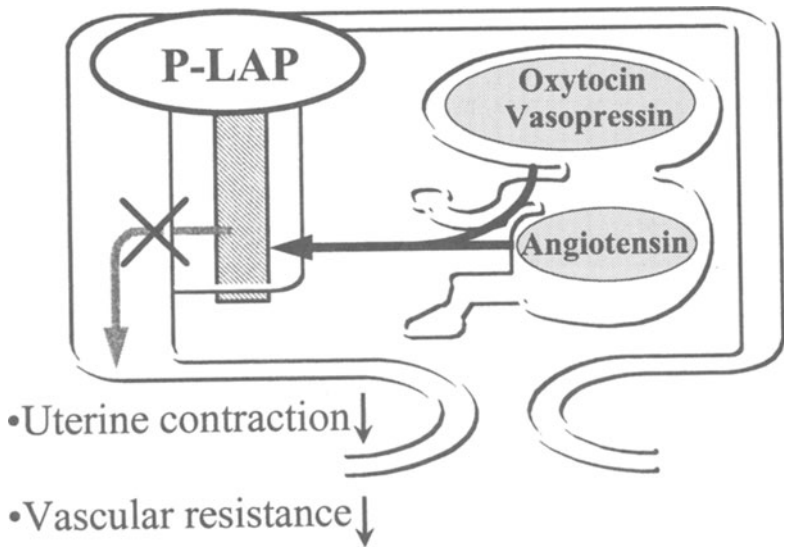

Figure 2. P-LAP functions at the interface between mother and fetus. P-LAP regulates uterine contraction and vasoconstriction via degrading peptide hormones in the placenta.

In adipocytes and skeletal muscle cells, IRAP, that is P-LAP, and GLUT4 co-localize and also translocate to the cell membrane in response to insulin through the same intracellular compartment (Kandror and Pilch, 1994; Ross *et al.*, 1996; Malide *et al.*, 1997; Sumitani *et al.*, 1997.) The finding that the cytosolic portion of IRAP containing a di-leucine sequence is required for dynamic retention in the endosomal recycling compartment (Shewan *et al.*, 2000; Johnson *et al.*, 2001) and affects GLUT4 translocation (Waters *et al.*, 1997) indicates a possible role of P-LAP in glucose homeostasis. However, P-LAP-deficient mice maintain glucose homeostasis, despite the dramatic decrease in GLUT4 expression (Keller *et al.*, 2002). Trafficking of P-LAP in response to insulin may merely mean that insulin up-regulates the cell surface peptidase activity and enhances the degradation of vasopressin in adipocytes (Herbst *et al.*, 1997).

In addition to OT and OT receptors, which are associated with ovulation and switching of endometrial glands from the proliferative to the secretory phase, human endometrial epithelial cells express P-LAP (Toda *et al.*, 2002). During the menstrual cycle, P-LAP is predominantly expressed around the time of ovulation, and after ovulation the membrane-bound P-LAP is released by apocrine secretion during the period of blastocyst implantation

and decreases to the time of menstruation. Thus P-LAP is thought to contribute to the ovulation as well as the onset of menstruation through degrading OT locally in the endometrium and myometrium.

In kidney, P-LAP is present in the distal tubules and collecting ducts. Interestingly, vasopressin stimulates the translocation of P-LAP to the basolateral plasma membrane in renal cells (Masuda *et al.*, 2003). Translocated P-LAP seems to facilitate the degradation of excessive vasopressin, suggesting a negative feedback system. It should be noted that although water-channel aquaporin-2 is also translocated by vasopressin (Nielsen *et al.*, 1995), the vesicles containing P-LAP would differ from those containing aquaporin-2.

P-LAP expression is observed in not only normal cells but also neoplastic cells such as endometrial adenocarcinoma. OT suppresses the growth of endometrial adenocarcinoma cells that express OT receptors (Cassoni *et al.*, 1997). In clinical specimens, P-LAP expression is correlated with potential malignancies, suggesting the possibility that tumors with abundant P-LAP grow rapidly due to the degradation of tumor suppressive factor OT (Suzuki *et al.*, 2003). Also in vitro, P-LAP overexpressing cells not only recover from OT-induced growth inhibition but also show a higher growth rate than parental cells without OT stimulation, which may be due to the degradation of autocrine OT (Suzuki *et al.*, 2003).

Recently P-LAP has been identified as an angiotensin IV receptor (Albiston *et al.*, 2001). Angiotensin IV binding to P-LAP in the brain facilitates learning and memory functions via several mechanisms, such as the inhibition of P-LAP activity leading to the prolongation of the half-life of neuropeptide substrates as well as the increase of glucose uptake (Albiston *et al.*, 2002).

6. CONCLUSION

Several lines of evidence have demonstrated that P-LAP is involved in various pathophysiological processes as well as the maintenance of pregnancy homeostasis. Although clinical application of P-LAP is now limited to monitoring the serum P-LAP activities for the management of placental dysfunction, threatened premature delivery and pre-eclampsia as well as the prediction of the onset of labor, progress in the molecular biological investigation of P-LAP open the way to novel therapeutic approaches. Stimulants that increase cAMP levels would suppress oxytocin-related uterine contraction during pregnancy via the enhancement of P-LAP by inducing trophoblast differentiation. Otherwise, administration of recombinant P-LAP would directly regulate substrate peptide actions. In

patients with endometrial cancers with high P-LAP activity, specific P-LAP inhibitors could serve as anti-proliferative agents. Nevertheless, preliminary analyses of P-LAP-deficient mice have shown no apparent changes deduced from abnormal peptide metabolism, such as preterm labor and reduced urinary volume. Further studies are required to investigate the roles of P-LAP in detail in the control of peptide metabolism in mice. Moreover, studies are also required to examine the presence of proteases that could compensate for the P-LAP activities, as well as to identify the natural substrates *in vivo*.

Recently two enzymes significantly homologous to P-LAP have been cloned: adipocyte-derived leucine aminopeptidase (A-LAP) (Hattori *et al.*, 1999), which has been also referred to as endoplasmic reticulum (ER)-aminopeptidase (ERAP)-1 (Saric *et al.*, 2002; Serwold *et al.*, 2002) (see Chapter 8), and Leukocyte-derived arginine aminopeptidase (L-RAP) (Tanioka *et al.*, 2003). A phylogenetic tree apparently indicates that P-LAP, A-LAP and L-RAP belong to one distinctive group. Therefore, we propose the oxytocinase subfamily of M1 aminopeptidases.

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