Laeverin/Aminopeptidase Q, a Novel Bestatin-sensitive Leucine Aminopeptidase Belonging to the M1 Family of Aminopeptidases*

Received for publication, March 28, 2007, and in revised form, May 16, 2007. Published, JBC Papers in Press, May 24, 2007, DOI 10.1074/jbc.M702650200

Masato Maruyama1, Akira Hattori1, Yoshikuni Goto1, Masamichi Ueda1, Michiyuki Maeda1, Hiroshi Fujiwara1, and Masafumi Tsujimoto1

From the 1Laboratory of Cellular Biochemistry, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, the 2Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, the 3Redox Bioscience Inc., Sakyo-ku, Kyoto 606-8507, and the 4Department of Gynecology and Obstetrics, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

Laeverin/aminopeptidase Q (APQ) is a cell surface protein specifically expressed on human embryo-derived extravillous trophoblasts that invades the uterus during placentation. The cDNA cloning of Laeverin/APQ revealed that the sequence encodes a protein with 990 amino acid residues, and Laeverin/APQ contains the HEXXHX18E gluzincin motif, which is characteristic of the M1 family of aminopeptidases, although the exopeptidase motif of the family, GAMEN, is uniquely substituted for the HAMEN sequence. In this study, we expressed a recombinant human Laeverin/APQ using a baculovirus expression system, purified to homogeneity, and characterized its enzymatic properties. It was found that Laeverin/APQ had a broad substrate specificity toward synthetic substrate, although it showed a preference for Leu-4-methylcoumaryl-7-amide. Searching natural substrates, we found that Laeverin/APQ was able to cleave the N-terminal amino acid of several peptides such as angiotensin III, kisseptin-10, and endokinin C, which are abundantly expressed in the placenta. In contrast to the case with other M1 aminopeptidases, bestatin inhibited the aminopeptidase activity of Laeverin/APQ much more effectively than other known aminopeptidase inhibitors. These results indicate that Laeverin/APQ is a novel bestatin-sensitive leucine aminopeptidase and suggest that the enzyme plays important roles in human placentation by regulating biological activity of key peptides at the embryo-maternal interface.

Aminopeptidases hydrolyze N-terminal amino acid of proteins or peptide substrates. Among them, the M1 family of zinc aminopeptidases (gluzincin) shares the consensus GAMEN and HEXXHX18E motifs essential for enzymatic activity and consists of 11 enzymes in human beings (1, 2). It is now becoming obvious that the M1 aminopeptidases are involved in many physiological events and are important for the maintenance of homeostasis. For instance, placental leucine aminopeptidase (P-LAP)2/oxytocinase plays a role in the progression of pregnancy by controlling the concentration of uterotonic and vasoactive hormones such as oxytocin and vasopressin to prevent premature delivery and pre-eclampsia (3). P-LAP is also referred to as insulin-regulated aminopeptidase, because it colocalizes with insulin-responsive glucose transporter 4 in the same vesicle in adipocyte and muscle cells and is translocated to plasma membrane by insulin stimulation, suggesting it has roles in the pathogenesis of diabetes (4). Recently, P-LAP/insulin-regulated aminopeptidase was also shown to be a specific receptor of angiotensin IV, further suggesting its significance in memory retention and retrieval (5). By searching databases for proteins homologous to P-LAP, we have cloned two novel aminopeptidases localized in the endoplasmic reticulum, adipocty-derived leucine aminopeptidase/endoplasmic reticulum aminopeptidase-1, and leukocyte-derived arginine aminopeptidase/endoplasmic reticulum aminopeptidase-2 (6, 7). Subsequent studies indicated these to be final processing enzymes that trim precursors to antigen peptides presented to major histocompatibility complex class I molecules (8, 9). Adipocty-derived leucine aminopeptidase is also reported to be involved in the regulation of angiogenesis, the shedding of cytokine receptor, and blood pressure (10–14). In addition, several physiological and/or pathological functions in the brain, including the regulation of blood pressure and apoptosis, are assigned to M1 aminopeptidases such as aminopeptidase A (APA), aminopeptidase N (APN), thyrotropin-releasing hormone-degrading enzyme, and puromycin-sensitive aminopeptidase. Because of the pathological significance of M1 family aminopeptidases, it is important to characterize their enzymatic properties in detail (15–20).

Laeverin was originally identified as a cell surface CHL2 antigen expressed in human extravillous trophoblasts (EVTs) (21). It was shown that the CHL2 antigen was specifically expressed in the outer layer of the chorion laeve in the human fetal membrane and on the migrating human EVT in the maternal decid-ual tissues but not on fetal amniotic epithelial cells and mater-
nal decidual cells by immunohistochemistry. The cDNA cloning predicted that the CHL2 antigen is a type II membrane-spanning protein and has the zinc binding motif, HEXXHX$_{18}$E motif and HAMEN sequence similar to the GAMEN motif, suggesting that the protein is a novel gluzincin M1 family of aminopeptidases. This novel protein was named Laeverin after its restricted expression in chorion laeve, and it is expected that its enzymatic action will be important for the EVT function during pregnancy. Another group (22) has predicted the existence of a novel aminopeptidase from the human Laeverin gene by using a genomic search and proposed to name it aminopeptidase Q (APQ). However, the enzymatic activity of Laeverin/APQ still remains to be explored because of its limited availability.

In this study, we established a large scale production system of recombinant protein of human Laeverin/APQ. The availability of purified protein made it possible to characterize its structure and enzymatic properties in detail. This is the first report describing the biochemical and enzymatic properties of the novel M1 aminopeptidase, Laeverin/APQ.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of a Soluble Form of Human Laeverin/APQ in a Baculovirus System—**cDNA encoding the full-length human Laeverin/APQ (1–990), with the His$_6$ tag at its C terminus, was generated by PCR. The amplified fragment was cloned into the EcoRI-Xhol site of pFastBac1 vector (Invitrogen). Then resultant plasmid was introduced into DH10Bac cells to produce recombinant bacmid DNA containing the Laeverin/APQ cDNA. Next, SF9 insect cells were transfected with the bacmid DNA using Cellfectin reagent (Invitrogen), and after 72-h incubation, recombinant baculoviruses were harvested. For the expression of recombinant human Laeverin/APQ, SF9 cells (2.0 x 10$^6$/ml) infected with the recombinant baculovirus (multiplicity of infection = ~1–3) were cultured for 72 h in 3 liters of SFM-900 III medium (Invitrogen) at 27 °C supplied with 8.0 ppm of O$_2$ (Cellmaster-1700, Wakenyaku, Kyoto, Japan).

The conditioned medium containing the soluble form of Laeverin/APQ (sLaeverin/APQ) was collected by centrifugation, and then applied to a hydroxyapatite column (2.5 x 10 cm, Nacalai Tesque, Kyoto, Japan) equilibrated in 50 mM Tris/HCl buffer (pH 7.5) and then applied to a hydroxyapatite column (2.5 x 10 cm, Nacalai Tesque, Kyoto, Japan) equilibrated in 50 mM Tris/HCl buffer (pH 7.5) and eluted with 100 mM sodium phosphate buffer (pH 7.5). The eluate were applied to a chelating-Sepharose (GE Healthcare Bio-Science, Piscataway, NJ) column (1.0 x 10 cm) preloaded with Ni$^{2+}$ and then eluted with 200 mM imidazole. The active fractions were collected, concentrated, and subjected to further characterization.

**De-glycosylation of sLaeverin/APQ—**After the heat denature (100 °C, 5 min), purified sLaeverin/APQ (100 µg/ml) was incubated with peptide-N-glycosidase F (30 µg/ml, New England Biolabs, Beverly, MA) in 50 mM sodium phosphate buffer (pH 7.5) at 37 °C for 2 h.

**Measurement of Aminopeptidase Activity of Laeverin/APQ—**The aminopeptidase activity of recombinant human sLaeverin/APQ was determined with various fluorogenic substrates, amiacoyl-4-methylocoumaryl-7-amides (aminoacyl-MCAs). The reaction mixture containing various concentrations of aminoclyl-MCA and the enzyme in 0.5 ml of 25 mM Tris/HCl buffer (pH 7.0) was incubated at 37 °C for 10 min. The amount of 7-amino-4-methylcoumarin released was measured by spectrophotofluorometry (F-2000, Hitachi) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The kinetic parameters were calculated from Lineweaver-Burk plots. The results are represented by $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values. All measurements were performed in triplicate.

**Cleavage of Peptide Hormones by sLaeverin/APQ—**Peptide hormones (25 µM, Peptide Institute, Osaka, Japan) were incubated with the enzyme (2 µg/ml) at 37 °C in 25 mM Tris/HCl buffer (pH 7.0). The reaction was terminated by adding of 2.5% (v/v) formic acid. The reactants and products were separated on a reversed-phase column, COSMOSIL (4.6 x 250 mm, Nacalai Tesque) using an automated HPLC system (AT-10, Shimadzu, Kyoto, Japan). Peptides generated from angiotensins, kallidin, and endokinin C were isocratically eluted with the following buffers at a flow rate of 0.5 ml/min: for peptides from angiotensins or kallidin, 19% acetonitrile containing 0.086% trifluoroacetic acid; for peptides from endokinin C, 30% acetonitrile containing 0.084% trifluoroacetic acid. Peptides generated from dynorphin A$_{1-8}$ were loaded onto the column equilibrated in 10% acetonitrile containing 0.088% trifluoroacetic acid and eluted with a linear gradient of 10% acetonitrile containing 0.088% trifluoroacetic acid to 30% acetonitrile containing 0.084% trifluoroacetic acid in 20 min at a flow rate of 0.5 ml/min, and peptides from kispeptin-10 were eluted with a linear gradient of 20% acetonitrile containing 0.086% trifluoroacetic acid to 40% acetonitrile containing 0.082% trifluoroacetic acid in 20 min at a flow rate of 0.5 ml/min. The molecular masses of peptides were determined by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry with a REFLEX mass spectrometer (Bruker-Franzen Analytik) using a-cyano-4-hydroxycinnamic acid as the matrix.

**Gel Filtration Chromatography—**The molecular mass of recombinant human Laeverin/APQ was measured by gel filtration chromatography. The sample was loaded onto a TSK G-3000SW (7.5 x 300 mm) ODS column (TOSOH, Tokyo, Japan) and eluted with 50 mM Tris/HCl buffer (pH7.5) containing 100 mM Na$_2$SO$_4$ at flow rate of 0.5 ml/min.

**Materials—**Asp$, \text{Gln}$, Glu$, \text{Gly}$, Ile$, \text{Val}$, and S-benzyl-Cys-MCAs were purchased from BACHEM AG (Bubendorf, Switzerland). Ala-, Arg-, Leu-, Lys-, Met-, and Phe-MCAs were from Pepide Institute (Osaka, Japan). Bestatin was obtained from Nacalai Tesque, and p-(4-amidinophenyl)methanesulfonyl fluoride was from Wako Pure Chemical Industries (Tokyo, Japan). Dynorphin A(1–8), amastatin, and E64 were purchased from Sigma, and actinonin was from LKT laboratories (St. Paul, MN). Leupeptin, pepstatin, and all peptide hormones except for dynorphin A$_{1-8}$ were from the Peptide Institute.

**RESULTS**

**Production of a Recombinant Human Laeverin/APQ—**To examine the enzymatic properties of Laeverin/APQ in detail, we tried to produce a recombinant human Laeverin/APQ using a baculovirus expression system. To facilitate purification, we introduced the His$_6$ tag at its C terminus as described previously in the production of recombinant human APA. Although...
Characterization of Human Laeverin/APQ

A. SDS-PAGE of purified sLaeverin/APQ. SDS-PAGE (6% gel) of sLaeverin/APQ was performed under the reducing and non-reducing conditions and then stained with Coomassie Brilliant Blue. B. monitoring of de-glycosylation of sLaeverin/APQ treated with peptide:N-glycosidase F (PNGase F). Purified sLaeverin/APQ (2 μg) was incubated with peptide:N-glycosidase F at 37 °C for 1 h. After SDS-PAGE (6% gel), the gel was stained with Coomassie Brilliant Blue.

Because homodimer formation is a characteristic feature of the membrane-bound M1 family of aminopeptidases (23–25), we measured the molecular weight of sLaeverin/APQ by gel-filtration column chromatography. The purified enzyme was eluted between ferritin (440 kDa) and catalase (232 kDa) (data not shown). These results suggest that sLaeverin/APQ is a non-sulfi de-linked homodimeric protein.

Characterization of Aminopeptidase Activity of Laeverin/APQ—We then measured the relative hydrolytic activity of sLaeverin/APQ toward various synthetic substrates, aminoacyl-MCAs (Fig. 2). Among the substrates tested, Leu-MCA was hydrolyzed by sLaeverin/APQ most efficiently, followed by Met-, Arg-, and Lys-MCA. Although showing lower activity, the hydrolysis of Phe-, S-benzyl-Cys-, Ala-, and Gln-MCA by sLaeverin/APQ was also observed, indicating a broad substrate specificity of the enzyme. The optimal pH was determined to be 7.0 using Leu-MCA as a substrate (data not shown).

We next performed kinetic studies on the aminopeptidase activities of sLaeverin/APQ toward several synthetic substrates (Table 1). The calculated $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values from a Lineweaver-Burk plot of sLaeverin/APQ for the most efficient substrate Leu-MCA were 78.2 ± 5.5 μM, 14.4 ± 0.6 s⁻¹, and 184 ± 4.6 μM⁻¹ s⁻¹, respectively. The catalytic efficiency ($k_{cat}/K_m$) of sLaeverin/APQ for Met-MCA was 23% of the value for Leu-MCA due to a significantly lower $k_{cat}$ value. In contrast, the $K_m$ values of sLaeverin/APQ for Lys- and Arg-MCA (41.5 ± 5.1 μM and 34.9 ± 1.7 μM) indicated higher affinity to these substrates than Leu-MCA. However, because the turnover numbers of the enzyme for both basic substrates were much less than that for Leu-MCA, the hydrolytic efficiencies for these substrates were calculated to be less than that for Leu-MCA. A similar inversion phenomenon in the kinetics parameters between neutral residues and basic residues was observed in the kinetic parameters of APN, which shows the highest homology (~36%) with Laeverin/APQ among M1 family members (26).

We next examined the effects of various divalent cations on the aminopeptidase activity of sLaeverin/APQ (data not shown). We found that Zn²⁺, an effective inhibitor of several M1 aminopeptidases, suppressed the Leu-MCA hydrolytic activity effectively in a dose-dependent manner ($IC_{50} = 6.6 ± 1.1 μM$). Cu²⁺ ($IC_{50} = 19.6 ± 3.6 μM$), Co²⁺ ($IC_{50} = 84.9 ± 12.2 μM$), and Ni²⁺ ($IC_{50} = 28.2 ± 3.7 μM$) also showed moderate inhibition.
inhibitory effects on the enzymatic activity of sLaeverin/APQ, which is consistent with the other M1 aminopeptidases so far tested. In our previous work, Ca²⁺ was shown to modulate the enzymatic activity of APA (24). However, no significant effect of Ca²⁺ on the enzymatic activity of sLaeverin/APQ was observed up to 3 mM.

The effects of various known protease inhibitors on the enzymatic activity of sLaeverin/APQ are shown in Fig. 3. It was obvious that bestatin, a competitive aminopeptidase inhibitor, was the most potent inhibitor of sLaeverin/APQ ($K_i = 0.96 \pm 0.33 \mu M$). The specific APN inhibitors phebestin and actinonin ($K_i = 3.02 \pm 0.61 \mu M$ and $259 \pm 30.7 \mu M$, respectively) and the specific inhibitor of APA bestatin ($K_i = 34.5 \pm 10.0 \mu M$) were less active. As in the case with other M1 aminopeptidases, 1,10-phenanthroline, a metal chelator, inhibited sLaeverin/APQ activity at 0.01–1 mM. Although leupeptin (a serine protease inhibitor) and pepstatin (an aspartic protease inhibitor) suppressed sLaeverin/APQ activity significantly only at high concentration (1 mM), E64 (a cysteine protease inhibitor) and $p$- (4-aminophenyl)methanesulfon- nyl fluoride (a serine protease inhibitor) had only small inhibitory effects at 1 mM. Taken together, these data revealed that Laeverin/APQ is indeed a novel M1 aminopeptidase having enzymatic properties characteristic to the M1 family (7, 13, 23, 24).

Cleavage of Natural Peptides by Laeverin/APQ—We then searched for sLaeverin/APQ-mediated degradation of natural peptide hormones to estimate the physiological roles of the enzyme. To identify possible substrates, we examined the competition of 18 human natural peptides with Leu-MCA by measuring its hydrolysis (Table 2). As for angiotensins, which are often good substrates of M1 aminopeptidases, Ang III and IV inhibited the hydrolytic activity of sLaeverin/APQ efficiently, but Ang II was less effective. Bradykinin and substance P showed moderate inhibitory activities. Furthermore, strong inhibition of the sLaeverin/APQ-mediated Leu-MCA hydrolysis was observed by kallidin (Lys-bradykinin), endokin C, dynorphin A$_{1–8}$, and kisspeptin-10. In contrast, nine other peptides (Met-enkephalin, endokinin D, oxytocin, and delta sleep-inducing peptide) were less inhibitory at 100 μM (<50%).

Based on our experience (24), peptides that show >50% inhibition of the hydrolysis of the fluorogenic substrate at 100 μM in this assay could be assumed to be substrates of the enzyme. Therefore, we examined the cleavage of candidate peptides by detecting degraded peptides after separating on reverse-phase HPLC. As shown in Fig. 4A, although Ang II slightly inhibited the Leu-MCA hydrolytic activity of sLaeverin/APQ at 100 μM, no significant degradation of the hormone was observed up to 6 h, indicating that Ang II is not a substrate of sLaeverin/APQ. On the other hand, sLaeverin/APQ-mediated cleavage of Ang III (Fig. 4B, peak a) to Ang IV (Fig. 4B, peak b) was clearly detected within 10 min (Fig. 4B). However, further degradation did not occur after longer incubation (6 h). Therefore, we examined whether or not Ang IV can be a substrate of sLaeverin/APQ. As expected from the Ang III hydrolysis profile, the degradation of Ang IV by sLaeverin/APQ was barely detectable (Fig. 4C), suggesting that Ang IV was not a substrate of the enzyme. Ang IV was shown to inhibit APA (24). We therefore tested whether or not Ang IV is an inhibitor of Laeverin/APQ and found that it is a competitive inhibitor of Laeverin/APQ with a $K_i$ value of 8.96 ± 1.07 μM (data not shown).

Because sLaeverin/APQ showed a moderate preference for basic amino acid residues toward synthetic substrates (Fig. 2), we next examined the degradation of several candidate peptides having basic amino acids at the N terminus (i.e. kallidin (Lys-bradykinin), endokin C, bradykinin, and substance P). As shown in Fig. 5A, kallidin was a good substrate, and release of the N-terminal lysine residue from kallidin (Lys-bradykinin) was detected within 3 min in this assay. Consequently, kallidin

**Characterization of Human Laeverin/APQ**

**TABLE 2**

| Peptide Sequence | Leu-MCA hydrolysis |
|------------------|---------------------|
| Ang II           | DRVYIHFP            | 68.1 ± 2.7 |
| Ang III          | RVYIHFP             | 11.9 ± 1.5 |
| Ang IV           | VYIHFP              | 18.0 ± 0.6 |
| Bradykinin       | RPPGFSPFR           | 37.8 ± 1.3 |
| Kallidin (Lys-bradykinin) | KRPPGFSPFR      | 2.8 ± 0.3 |
| Substance P      | RDPKQQQGLM-NH$_2$   | 15.0 ± 0.8 |
| Neurokinin A     | HKTDSFVGLM-NH$_2$   | 71.6 ± 2.9 |
| Neurokinin B     | DMHDFVGLM-NH$_2$    | 55.4 ± 0.7 |
| Endokin C        | KKYALEHHFQGLL-NH$_2$| 2.5 ± 0.7 |
| Endokin D        | VGYALEHHFQGLL-NH$_2$| 62.5 ± 6.0 |
| Met-enkephalin   | YGFPM               | 62.5 ± 10.5 |
| Dynorphin A$_{1–8}$ | YGGLRRI         | 3.9 ± 0.6 |
| Arg-vasopressin  | CYQNCPRG-NH$_2$     | 85.6 ± 5.9 |
| Oxytocin         | CYQNCFLG-NH$_2$     | 86.2 ± 2.0 |
| Parathyroid hormone-(69–84) | EADKADVNLVTFAKSQ | 910.1 ± 2.2 |
| Cholecystokinin-8 | DYMWMDF-NH$_2$    | 73.2 ± 0.6 |
| Kisspeptin-10    | YNNISPGRLF-NH$_2$   | 3.4 ± 0.1 |
| Delta sleep-inducing peptide | WAGGDASQ | 88.4 ± 10.1 |

$^a$The values are mean ± S.D. (n = 3).

---

**FIGURE 3. Effects of various protease inhibitors on the enzymatic activity of sLaeverin/APQ.** Purified sLaeverin/APQ (1 μg/ml) was incubated with 25 μM Leu-MCA with various protease inhibitors. The hydrolytic activity toward Leu-MCA measured in the absence of inhibitor was taken as 100%. ●, bestatin; ▲, pepstatin; ●, bestatin; ▲, phebestin; ○, actinonin; △, leupeptin; □, pepstatin; △, p-(4-aminophenyl)methanesulfonyl fluoride; □, E64; and ○, 1,10-phenanthroline.

**FIGURE 4. Competitive effects of various peptide hormones on Leu-MCA hydrolytic activity of sLaeverin/APQ.** Purified sLaeverin/APQ (2 μg/ml) was incubated with 25 μM Leu-MCA at 37 °C for 10 min in the presence of each peptide (100 μM). The hydrolytic activity of Laeverin/APQ toward Leu-MCA measured in the absence of peptide was taken as 100%.

---

**TABLE 2**

**Competitive effects of various peptide hormones on Leu-MCA hydrolytic activity of sLaeverin/APQ.** Purified sLaeverin/APQ (2 μg/ml) was incubated with 25 μM Leu-MCA at 37 °C for 10 min in the presence of each peptide (100 μM). The hydrolytic activity of Laeverin/APQ toward Leu-MCA measured in the absence of peptide was taken as 100%.
(Fig. 5A, peak a) was converted to bradykinin (Fig. 5A, peak b) completely within 30 min. As in the case with kallidin, rapid release of the N-terminal lysine residue from endokinin C and generation of de-[Lys]endokinin C (36%) (Fig. 5B, peak b) were also detected within 10 min. Of note, the transient accumulation of de-[Lys]endokinin C was observed at 30 min (Fig. 5B, peak b), and degradation to de-[Lys-Lys]endokinin C (Fig. 5B, peak c) seemed to be much slower than the first cleavage, suggesting that the second lysine residue is a poorer substrate of Laeverin/APQ than the first one. It is plausible that subsite interactions are required for the enzymatic action of Laeverin/APQ.

It has been reported that M1 aminopeptidases are not able to release an N-terminal amino acid residue adjacent to a proline residue (7, 13, 23, 24). Therefore we examined the susceptibilities of bradykinin and substance P, which both have Arg-Pro sequence at N termini to Laeverin/APQ. We found that, although both peptides bind to Laeverin/APQ, they were not cleaved by the enzyme at all and acted as competitive inhibitors ($K_i = 57.8 \pm 11.0$ and $4.63 \pm 0.49 \mu M$, respectively) (data not shown).

Because Laeverin/APQ also showed a moderate preference to Phe-MCA, we next examined the cleavage of kisspeptin-10 and dynorphin A$_{1-8}$, of which the N-terminal amino acid residues are tyrosine (aromatic amino acid). Fig. 6A shows the hydrolytic profile of kisspeptin-10, which is abundant in the placenta and suppresses the cell motility strongly by binding to a G-protein-coupled receptor, GPR54 (27). Release of the N-terminal tyrosine residue of kisspeptin-10 was detected within 3 min, and $\sim$30% of kisspeptin-10 was degraded to de-[Tyr]kisspeptin-10 (Fig. 6A, peak b) within 1 h. Fig. 6B shows the degradation profile of dynorphin A$_{1-8}$ by the enzyme. The release of N-terminal tyrosine residue of the hormone was also rapid and detected within 3 min. Although $\sim$95% of the hormone was inactivated to de-[Tyr]- and de-[Tyr-Gly]dynorphin A$_{1-8}$ after 30-min incubation, the generation of further degraded products, de-[Tyr-Gly-Gly]dynorphin A$_{1-8}$ (Fig. 6B, peak c) was found to be marginal. When we examined the cleavage of Met-enkephalin, which shares the N-terminal 4 amino acid sequence with dynorphin A$_{1-8}$, no degradation of Met-enkephalin was found, in accordance with its less competitive effect on Leu-MCA hydrolytic activity (data not shown).

We then examined the effect of chain length of substrates on the susceptibility to sLaeverin/APQ (Fig. 6C). We compared the degradation rate of C-terminal-extended dynorphins, dynorphin A$_{1-13}$ and dynorphin A$_{1-17}$. As shown in figure, sLaeverin/APQ cleaved 8-mer dynorphin A with highest efficiency, followed by 17-mer and 13-mer, suggesting that chain length is one of the determinants of the aminopeptidase activity of the enzyme, which was also shown in the case with adipocyte-derived leucine aminopeptidase-mediated processing of antigenic peptides (28). Taken together, our data indicate that Laeverin/APQ can indeed modulate the biological activities of several peptides by acting as an aminopeptidase.

**DISCUSSION**

Laeverin/APQ was originally isolated as a specific antigen expressed on EVTs in the human placenta (21). The cDNA
Characterization of Human Laeverin/APQ

encoding human Laeverin/APQ contains two conserved motifs, H(G)AMEN and HEXXH\(_{18}\)E sequences, predicting that it is a novel enzyme belonging to the M1 family of zinc aminopeptidases. However, its enzymatic properties have remained elusive. In this study, we have established a baculovirus expression system to produce a recombinant Laeverin/APQ, purified it to homogeneity, and characterized its enzymatic properties for the first time.

We infected recombinant baculovirus having the cDNA encoding the full length of Laeverin/APQ on SF9 cells. Unexpectedly, however, we detected the majority of the expressed Laeverin/APQ in the culture medium, but not in cell lysate. The N-terminal sequence of purified sLaeverin/APQ was shown to be homogenous and starts from Lys\(^{65}\). It is plausible that sLaeverin/APQ is processed by some sheddase on the plasma membrane, but not in cell lysate. The shedded form of Laeverin/APQ contains two conserved cysteine residues Cys\(^{40}\) and Cys\(^{43}\) of human APA located in the vicinity of the transmembrane domain, suggesting its role in the formation of an intermolecular disulfide bond. However, the role of this cysteine residue of Laeverin/APQ should be examined in a future study using site-directed mutagenesis.

In this report, we characterized the physicochemical and enzymatic properties of the recombinant human soluble form of Laeverin/APQ. The formation of a homodimer is one of the characteristic features of the membrane-bound M1 family of aminopeptidases (23–25). Judging from the molecular weight calculated from SDS-PAGE and gel filtration chromatography, sLaeverin/APQ also forms a homodimer without an intermolecular disulfide linkage. In contrast, Laeverin/APQ expressed in HeLa S3 cells was detected at >300 kDa in the non-reduced condition on SDS-PAGE, but as ~160 kDa in the reduced condition, indicating that the membrane-bound full-length Laeverin/APQ is a homodimeric protein with intermolecular disulfide bond(s) (data not shown). It was shown that the membrane-bound form of native APA forms a homodimer with the disulfide linkage and that a cysteine residue Cys\(^{43}\) of human APA located in the vicinity of the transmembrane domain is responsible for the formation of the disulfide bond between each subunit (34, 35). As for human Laeverin/APQ, Cys\(^{40}\) is also located in the vicinity of the transmembrane domain, suggesting its role in the formation of an intermolecular disulfide bond. However, the role of this cysteine residue of Laeverin/APQ should be examined in a future study using site-directed mutagenesis. It was also revealed that the C-terminal domain consisting of ~400 amino acid residues acts as an intramolecular chaperone and plays important roles in the dimer formation of the M1 aminopeptidases of not only disulfide-linked enzyme (APA) but also non-covalently linked enzymes (P-LAP and APN) (36). Because the C-terminal domain of Laeverin/APQ shares some similarity with other members of the M1 family, it is reasonable to speculate that the domain also acts as a chaperone and maintains the structure of the substrate pocket properly in the absence of an intermolecular disulfide bond, and sLaeverin/APQ retains the principal enzymatic properties of native Laeverin/APQ.

In this report, we characterized the enzymatic properties of Laeverin/APQ for the first time and found that the substrate specificity of the enzyme is rather broad. The favored order of sLaeverin/APQ for aminoacyl-MCA is shown to be Leu-, Met-, Arg-, Lys-, Phe-, and S-benzyl-Cys-MCA. It was also found that sLaeverin/APQ sequentially removes a variety of amino acid residues from the N terminus of oligopeptides in accordance with its broad substrate specificity toward synthetic substrates. Although the substrate specificities of APN, P-LAP, and APA (in Ca\(^{2+}\)-free condition), which are highly expressed in the human placenta, were also found to be broad (23, 24, 26), they explored to understand the pathophysiological functions of Laeverin/APQ in the near future.

We characterized the physicochemical and enzymatic properties of the recombinant human soluble form of Laeverin/APQ. The formation of a homodimer is one of the characteristic features of the membrane-bound M1 family of aminopeptidases (23–25). Judging from the molecular weight calculated from SDS-PAGE and gel filtration chromatography, sLaeverin/APQ also forms a homodimer without an intermolecular disulfide linkage. In contrast, Laeverin/APQ expressed in HeLa S3 cells was detected at >300 kDa in the non-reduced condition on SDS-PAGE, but as ~160 kDa in the reduced condition, indicating that the membrane-bound full-length Laeverin/APQ is a homodimeric protein with intermolecular disulfide bond(s) (data not shown). It was shown that the membrane-bound form of native APA forms a homodimer with the disulfide linkage and that a cysteine residue Cys\(^{43}\) of human APA located in the vicinity of the transmembrane domain is responsible for the formation of the disulfide bond between each subunit (34, 35). As for human Laeverin/APQ, Cys\(^{40}\) is also located in the vicinity of the transmembrane domain, suggesting its role in the formation of an intermolecular disulfide bond. However, the role of this cysteine residue of Laeverin/APQ should be examined in a future study using site-directed mutagenesis. It was also revealed that the C-terminal domain consisting of ~400 amino acid residues acts as an intramolecular chaperone and plays important roles in the dimer formation of the M1 aminopeptidases of not only disulfide-linked enzyme (APA) but also non-covalently linked enzymes (P-LAP and APN) (36). Because the C-terminal domain of Laeverin/APQ shares some similarity with other members of the M1 family, it is reasonable to speculate that the domain also acts as a chaperone and maintains the structure of the substrate pocket properly in the absence of an intermolecular disulfide bond, and sLaeverin/APQ retains the principal enzymatic properties of native Laeverin/APQ.
are clearly distinct from each other. Moreover, although P-LAP efficiently degrades Met-enkephalin and dynorphin A_{1-8} but not dynorphin A_{1-17} and APN degrades all dynorphin-related peptides (37, 38), sLaeverin/APQ cleaves dynorphin A_{1-8} efficiently and dynorphin A_{1-17} moderately but not Met-enkephalin, indicating the distinguishable preference of substrate chain length between the enzymes. These results suggested that each aminopeptidase plays distinct roles in the metabolism of a variety of bioactive peptides in the placenta.

For most of the M1 aminopeptidases, amastatin more efficiently inhibits the enzymatic action than bestatin. In contrast, bestatin inhibited the aminopeptidase activity of Laeverin/APQ ($K_i = 0.96 \pm 0.33 \mu M$) much more effectively than an APA inhibitor, amastatin ($K_i = 34.5 \pm 10.0 \mu M$), and the APN inhibitors actinonin ($K_i = 259 \pm 30.7 \mu M$) and pebestin ($K_i = 3.02 \pm 0.61 \mu M$). It has been suggested that the GAMEN motif is essential for the exopeptidase activity of M1 aminopeptidases for interacting with the N terminus of substrates (39–41). It was also suggested that bestatin interacts with the GAMEN motif from the crystal structure of human leukotriene A_{4} hydrolase (42). In Laeverin/APQ, a glycine residue comprising the GAMEN motif is substituted by a histidine residue (i.e. HAMEN sequence). Hence, it might be possible that His^{379} of Laeverin/APQ plays an important role in the interaction with bestatin and contributes to the distinct feature of this enzyme. Further studies are required to elucidate this possibility.

We searched for sLaeverin/APQ-mediated degradation of natural hormones to estimate the pathophysiological role of Laeverin/APQ and found that Ang III, kallidin, kisspeptin-10, and endokinin C were susceptible to the enzyme. It is well known that angiotensins and kallidin play important roles in the regulation of blood pressure. It is also reported that the renin-angiotensin system and kallikrein-kininogen-kinin system are active in the placenta (43, 44). Furthermore, their signal-transducing receptors are also expressed in the placenta, strongly suggesting that these placenta-derived hormones play roles in the development and/or function of the placenta such as the cell migration of invasive trophoblasts and angiogenesis (43, 44). It is noteworthy here that both Ang IV and bradykinin are products of the enzymatic action of Laeverin/APQ and inhibit its activity, suggesting the existence of a negative feed-
back mechanism of the enzyme. Because both peptides are vasodilative, the inhibition of enzymatic activity may have some physiological relevance in the regulation of blood pressure.

Kisspeptin-54 is a C-terminally amidated peptide with 54 amino acid residues and is referred to as metastin after its suppressive activity on metastasis of cancer cells (27). Several fragments proteolytically generated from kisspeptin-54, -14 (14-mer), -13 (13-mer), and -10 (10-mer) are also detected in the human placenta and conditioned medium of first trimester human trophoblasts (45, 46). It was shown that kisspeptin-10 bound to its specific receptor, GPR54, with higher affinity than kisspeptin-54 (~8-fold) and suppressed the cell migration of GPR54-transfected Chinese hamster ovary cells and human trophoblasts more strongly than kisspeptin-54 (26). On the other hand, 9-mer peptide generated by releasing the tyrosine residue from the N terminus of kisspeptin-10 is significantly less active than kisspeptins. Taking all these findings together, it is tempting to speculate that Laeverin/APQ may be involved in the regulation of kisspeptin-10 activity.

Endokinin C is a recently discovered tachykinin and was found to be highly expressed in the adrenal gland, placenta, uterus, and cerebellum (47, 48). Although some hemodynamic effects of endokinin C such as arterial blood pressure and mesenteric vasoconstriction have been identified, its function in the placenta still remains to be explored. We have to await a future study exploring the function of endokinin C to comprehend the biological relevance of this unique enzyme.

In our initial characterization, the expression of Laeverin/APQ was restricted to EVTs in the placenta (21). But we recently found that Laeverin/APQ is also expressed in the brain.3 Because several peptides shown to be potential substrates of Laeverin/APQ are also detected in the brain, it is possible to speculate that Laeverin/APQ plays roles in brain function by hydrolyzing brain peptides such as dynorphins, kisspeptins-10, and Ang III as shown in this study. In addition, it was recently reported that the mRNA level of Laeverin/APQ was markedly increased in Epstein-Barr virus-established lymphoblastoid B cell lines and synovial tissues from patients with rheumatoid arthritis (49). Considering our results, Laeverin/APQ converts kallidin to bradykinin and may enhance inflammatory response by producing bradykinin in synovial fluid of patients. Further work is required to elucidate the pathophysiological function of Laeverin/APQ.

In this study, we established a large scale production system for a recombinant human Laeverin/APQ and characterized its physicochemical and enzymatic properties. We observed aminopeptidase activity of Laeverin/APQ for the first time. Among the M1 aminopeptidases, Laeverin/APQ uniquely showed higher sensitivity to bestatin, which may be due to its characteristic HAMEN sequence. We also identified several placenta-derived peptides as possible substrates of Laeverin/APQ. These enzymatic properties of Laeverin/APQ suggest it has important roles in human placenta by regulating the biological activity of key peptides at the embryo-maternal interface. Our results are the first step in elucidating the pathophysiological function of this unique enzyme.

Acknowledgment—We are grateful for Dr. Naoshi Dohmae of Advanced Development and Supporting Center, RIKEN, for the N-terminal amino acid sequence analysis.

REFERENCES

1. Hooper, N. M. (1994) FEBS Lett. 31, 1–6
2. Tsujimoto, M., and Hattori, A. (2005) Biochim. Biophys. Acta 1751, 9–18
3. Nomura, S., Ito, T., Yamamoto, E., Sumigama, S., Iwase, A., Okada, M., Shibata, K., Ando, H., Ino, K., Kikikawa, F., and Mizutani, S. (2005) Biochim. Biophys. Acta 1751, 19–25
4. Keller, S. R., Scott, H. M., Mastick, C. C., Aebersold, R., and Lienhard, G. E. (1995) J. Biol. Chem. 270, 23612–23618
5. Albiston, A. L., McDowell, S. G., Matsacou, D., Sim, P., Clune, E., Mustafa, T., Lee, J., Mendelssohn, F. A., Simpson, R. I., Connolly, L. M., and Chai, S. Y. Y. (2001) J. Biol. Chem. 276, 48623–48626
6. Hattori, A., Matsumoto, H., Mizutani, S., and Tsujimoto, M. (1999) J. Biochem. (Tokyo) 125, 931–938
7. Tanioka, T., Hattori, A., Masuda, S., Nomura, Y., Nakayama, H., Mizutani, S., and Tsujimoto, M. (2003) J. Biol. Chem. 278, 32275–32283
8. Saric, T., Chang, S.-C., Hattori, A., York, I. A., Markant, S., Rock, K. L., Tsujimoto, M., and Goldberg, A. L. (2002) Nat. Immunol. 3, 1169–1176
9. Saveanu, L., Carroll, O., Lindo, V., Del Val, M., Lopez, D., Lepelletier, Y., Greer, F., Schomburg, L., Fruci, D., Niedermann, G., and van Endert, P. M. (2005) Nat. Immunol. 6, 689–697
10. Miyashita, H., Yamazaki, T., Akada, T., Niizeki, O., Ogawa, M., Nishikawa, S., and Sato, Y. (2002) Blood 99, 3241–3249
11. Cui, X., Hawari, F., Alsasat, S., Lawrence, M., Combs, C. A., Geng, W., Rouhani, F. N., Miskinis, D., and Levine, S. J. (2002) J. Clin. Invest. 110, 515–526
12. Cui, X., Rouhani, F. N., Hawari, F., and Levine, S. J. (2003) J. Biol. Chem. 278, 28677–28685
13. Hattori, A., Kitatani, K., Matsumoto, H., Miyazawa, S., Rogi, T., Tsuorgu, N., Mizutani, S., Natori, Y., and Tsujimoto, M. (2000) J. Biochem. (Tokyo) 128, 755–762
14. Yamamoto, N., Nakayama, J., Yamakawa-Kobayashi, K., Hamaguchi, H., Miyazaki, R., and Arinami, T. (2002) Hum. Mutat. 19, 251–257
15. Zini, S., Fournier-Zaluski, M. C., Chavel, E., Roques, B. P., Corvol, P., and Llorens-Cortes, C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11968–11973
16. Reaux, A., Fournier-Zaluski, M. C., David, C., Zini, S., Roques, B. P., Corvol, P., and Llorens-Cortes, C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13415–13420
17. Schomburg, L., Turwitt, S., Prescher, G., Lohmann, D., Horsthemke, B., and Bauer, K. (1999) Eur. J. Biochem. 265, 415–422
18. Constand, D. B., Tobler, A. R., Rensing-Ehl, A., Kemler, I., Hersh, L. B., and Fontana, A. (1995) J. Biol. Chem. 270, 26931–26939
19. Tobler, A. R., Constand, D. B., Schmitt-Graff, A., Malipiero, U., Schlappbach, R., and Fontana, A. (1997) J. Neurochem. 68, 889–897
20. Osada, T., Ikiyama, S., Akagi, K., Kikikawa, F., Yamaguchi, K., Kato, C., Takeuchi, T., and Koga, A. (1997) J. Neurosci. 15, 6068–6078
21. Fujiwara, H., Higuchi, T., Yamada, S., Hirano, T., Sato, Y., Nishiyama, Y., Yoshio, H., Tsutsumi, K., Ueda, M., Maeda, M., and Fuji, S. (2004) Biochim. Biophys. Res. Commun. 313, 962–968
22. Puente, X. S., Sanchez, L. M., Overall, C. M., and Lopez-Otin, C. (2003) Nat. Rev. Genet. 4, 544–558
23. Matsumoto, H., Rogi, T., Yamashiro, K., Sursuoka, N., Hattori, A., Takio, K., Mizutani, S., and Tsujimoto, M. (2000) Eur. J. Biochem. 267, 46–52
24. Goto, Y., Hattori, A., Ishii, Y., Mizutani, S., and Tsujimoto, M. (2006) J. Biol. Chem. 281, 23503–23513
25. Danielsen, E. M. (1990) Biochemistry 29, 305–308
26. Turner, A. J. (2004) in Handbook of Proteolytic Enzymes, 2nd Ed. (Barret, A. J., Rawlings, N. D., and Woessner, J. F., eds) pp. 289–294, Academic
Characterization of Human Laeverin/APQ

Press Inc., San Diego, CA

27. Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., Terao, Y., Kumano, S., Takatsu, Y., Masuda, Y., Ishibashi, Y., Watanabe, T., Asada, M., Yamada, T., Suenaga, M., Kitada, C., Usuki, S., Kurokawa, T., Onda, H., Nishimura, O., and Fujino, M. (2001) *Nature* **31**, 613–617

28. Chang, S. C., Momburg, F., Bhutani, N., and Goldberg, A. L. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **22**, 17107–17112

29. Ito, N., Nomura, S., Iwase, A., Ito, T., Kikkawa, F., Tsujimoto, M., Ishiura, S., and Mizutani, S. (2004) *Biochem. Biophys. Res. Commun.* **314**, 1008–1013

30. Yamahara, N., Nomura, S., Suzuki, T., Itakura, A., Ito, M., Okamoto, T., Tsujimoto, M., Nakazato, H., and Mizutani, S. (2000) *Life Sci.* **66**, 1401–1410

31. Okuyama, T., Ishiura, S., Nojima, M., Tsukahara, T., Yanagida, M., and Sugita, H. (1991) *Clin. Chim. Acta* **196**, 207–215

32. Watanabe, Y., Iwaki-Egawa, S., Mizukoshi, H., and Fujimoto, Y. (1995) *Biol. Chem. Hoppe-Seyler* **376**, 397–400

33. Rogi, T., Tsujimoto, M., Nakazato, H., Mizutani, S., and Tomoda, Y. (1996) *J. Biol. Chem.* **271**, 56–61

34. Wu, Q., Li, L., Cooper, M. D., Pierres, M., and Gorvel, J. P. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 676–680

35. Ofner, L. D., and Hooper, N. M. (2002) *Biochem. J.* **362**, 191–197

36. Rozenfeld, R., Muller, L., El Messari, S., and Llorens-Cortes, C. (2004) *J. Biol. Chem.* **279**, 43285–43295

37. Matsumoto, H., Nagasaka, T., Hattori, A., Rogi, T., Tsuruoka, N., Mizutani, S., and Tsujimoto, M. (2001) *Eur. J. Biochem.* **268**, 3259–3266

38. Safavi, A., and Hersh, L. B. (1995) *J. Neurochem.* **65**, 389–395

39. Vazeux, G., Iturriot, X., Corvol, P., and Llorens-Cortes, C. (1998) *Biochem. J.* **334**, 407–413

40. Luciani, N., Marie-Claire, C., Ruffet, E., Beaumont, A., Roques, B. P., and Fournie-Zaluski, M. C. (1998) *Biochemistry* **37**, 686–692

41. Laustsen, P. G., Vang, S., and Haeggstrom, J. Z. (2001) *Eur. J. Biochem.* **268**, 98–104

42. Thunnissen, M. M., Nordlund, P., and Haeggstrom, J. Z. (2001) *Nature* **8**, 131–135

43. Xia, Y., Wen, H. Y., and Kellems, R. E. (2002) *J. Biol. Chem.* **277**, 24601–24608

44. Vonnahme, K. A., Fernando, S. C., Ross, J. W., Ashworth, M. D., DeSilva, U., Malayer, J. R., and Geisert, R. D. (2004) *Biol. Reprod.* **70**, 132–138

45. Kotani, M., Detheux, M., Vandenbogaerde, A., Communi, D., Vanderwiden, J. M., Le Poul, E., Brezillon, S., Tyldesley, R., Suarez-Huerta, N., Vandeput, F., Blanpain, C., Schifflmann, S. N., Vassart, G., and Parmentier, M. (2001) *J. Biol. Chem.* **276**, 34631–34636

46. Bilban, M., Ghaffari-Tabrizi, N., Hintermann, E., Bauer, S., Molzer, S., Zoratti, C., Malli, R., Sharabi, A., Hiden, U., Graier, W., Knoffel, M., Andreae, F., Wagner, O., Quaranta, V., and Desoye, G. (2004) *J. Cell Sci.* **117**, 1319–1328

47. Page, N. M., Bell, N. J., Gardiner, S. M., Manyonda, I. T., Brayley, K. J., Strange, P. G., and Lowry, P. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 6245–6250

48. Page, N. M. (2004) *Cell. Mol. Life Sci.* **61**, 1652–1663

49. Haas, C. S., Creighton, C. J., Pi, X., Maine, I., Koch, A. E., Haines, G. K., Ling, S., Chinnaian, A. M., and Holoshitz, J. (2006) *Arthritis Rheum.* **54**, 2047–2060
Laeverin/Aminopeptidase Q, a Novel Bestatin-sensitive Leucine Aminopeptidase Belonging to the M1 Family of Aminopeptidases
Masato Maruyama, Akira Hattori, Yoshikuni Goto, Masamichi Ueda, Michiyuki Maeda, Hiroshi Fujiwara and Masafumi Tsujimoto
Masato Maruyama, Akira Hattori, Yoshikuni Goto, Masamichi Ueda, Michiyuki Maeda, Hiroshi Fujiwara and Masafumi Tsujimoto

Access the most updated version of this article at doi: 10.1074/jbc.M702650200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 18 of which can be accessed free at http://www.jbc.org/content/282/28/20088.full.html#ref-list-1