Deletion of peptide amidation enzymatic activity leads to edema and embryonic lethality in the mouse

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Abstract

Peptidylglycine alpha-amidating monooxygenase (PAM) catalyzes the COOH-terminal amidation of peptide hormones. We previously had found high expression of PAM in several regions of the developing rodent. To determine the function of PAM during mouse embryogenesis, we produced a null mutant of the PAM gene. Homozygous mutants die in utero between e14.5 and e15.5 with severe edema that is likely due to cardiovascular deficits. These defects include thinning of the aorta and carotid arteries and are very similar to those of the recently characterized adrenomedullin (AM) gene KO despite the presence of elevated immunoreactive AM in PAM KO embryos. No peptide amidation activity was detected in PAM mutant embryos, and there was no moderation of the AM-like phenotype that could be expected if any alternative peptide amidation mechanism exists in the mouse. Despite the proposed contribution of amidated peptides to neuronal cell proliferation, no alteration in neuroblast proliferation was observed in homozygous mutant embryos prior to lethality. Mice heterozygous for the mutant PAM allele develop normally and express wildtype levels of several amidated peptides despite having one half the wildtype levels of PAM activity and PAM protein. Nonetheless, both an increase in adiposity and a mild glucose intolerance developed in aged (>10 months) heterozygous mice compared to littermate controls. Ablation of PAM thus demonstrates an essential function for this gene during mouse development, while alterations in PAM activity in the adult may underlie more subtle physiologic effects.

Keywords: Peptide amidation; Knockout mouse; PAM; Edema; Cardiovascular; Adrenomedullin; PACAP; Glucose tolerance test

Introduction

The formation of biologically active peptide hormones is a multistep process beginning with the synthesis of large inactive pro-proteins. These precursors are first proteolytically cleaved at dibasic consensus sequences by a family of prohormone convertases (PCs) (reviewed in Steiner, 1998; Seidah and Chretien, 1999). The COOH-terminal basic residues exposed by these cleavages are then removed by carboxypeptidases (CPs). If a glycine residue is exposed during this process, it can serve as a substrate for the amidation reaction. This final posttranslational processing step is catalyzed by peptidylglycine alpha-amidating monooxygenase (PAM). In mammals, amidation activity is thought to be encoded by a single gene product containing two enzymatic domains, peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL), that act sequentially to amitate peptides (Prigge et al., 2000).

Peptide amidation is often required to confer full biological activity to a peptide because the presence of an amide group can significantly increase the affinity of the peptide for its receptor and can also extend peptide half-life (Cuttitta, 1993; Merkler, 1994), possibly by stabilizing its secondary structure (In et al., 2001). PAM substrates are numerous and include neuroendocrine hormones and neurotransmitters such as gonadotropin-releasing hormone (GnRH), adrenomedullin (AM), neuropeptide Y (NPY), and cholecystokinin (CCK) (Bradbury and Smyth, 1987; Eipper et al., 1992; Merkler, 1994).
PAM catalyzes the amidation of all Gly-extended peptides, with kinetic parameters determined by the penultimate residue. Amided peptides are abundant in both vertebrates and invertebrates such as *Cnidarians* (Grimmelikhuijzen et al., 1996), *Aplysia* (Kaldany et al., 1985) and *Drosophila* (Nassel, 1999; Taghert, 1999). Over 90% of neuropeptides in *Drosophila* are predicted to be amidated (Kolhekar et al., 1997b), while at least half of all biologically active peptides in mammals are amidated (Eipper et al., 1992). Amided peptides contribute to processes as diverse as glucose homeostasis (Drucker, 2002), skeletal development (Karaplis et al., 1994), weight regulation (Cupples, 2002; Gundlach, 2002), cardiovascular (CV) function (Eto and Samson, 2001), cell proliferation and cell survival (Hansel et al., 2001a,b). Several amidated peptides are present during development including adrenomedullin (AM) (Montuenga et al., 1997), parathyroid hormone-related peptide (PTHrp) (Lee et al., 1995) and pituitary adenylate cyclase-activating polypeptide (PACAP) (Shuto et al., 1996), suggesting that these amidated peptides have particularly important functions during embryogenesis as well as in the adult. PAM expression is widespread in the adult CNS and is highest in the hypothalamus, hippocampus and pituitary (Braas et al., 1989; Schafer et al., 1992). PAM is also expressed in several peripheral tissues including the heart, lungs, adrenal gland and pancreas (Braas et al., 1992). In general, the expression pattern of PAM in both the adult and developing embryo corresponds well with the expression patterns of amidated substrates. However, PAM has not been detected in the PP cells of the endocrine pancreas (Martinez et al., 1993a) or the epithelial cells of the small intestine (Martinez et al., 1993b) where amidated peptides have been found. These findings raised the possibility that an alternative amidation mechanism may exist.

In the developing rodent, there is early and widespread expression of PAM (Zhang et al., 1997) implicating PAM (and thus amidated peptides) in important roles in embryogenesis. The highest expression of PAM in the embryo proper begins in the heart by e9. High expression of PAM in the heart continues throughout embryogenesis, suggesting that PAM has an important role in embryonic cardiac development and function. PAM is also highly expressed in the ventricular zone regions and mantle layers of multiple CNS regions as early as e12.5 where it could potentially regulate neuronal cell proliferation and differentiation. PAM is expressed at moderate levels in peripheral structures such as the gut and in developing tooth and limb buds. Taken together, these expression patterns implicate PAM in multiple developmental processes. Lastly, expression of PAM in uterine cells as early as e6 suggests that PAM could function in normal pregnancy. A mutation of the PHM gene in *Drosophila* results in lethality at midgestational stages (Kolhekar et al., 1997b). However, since PHM and PAL enzymatic activities are encoded by separate genes in *Drosophila* (Kolhekar et al., 1997b; Jiang et al., 2000) and at least two copies of PAL have been found in its genome (Han et al., 2004). *Drosophila* is likely not a good model to predict PAM function in vertebrates.

Inhibitors of PAM have been used to examine tissue-specific functions of PAM in the adult (Mueller et al., 1993; Ogonowski et al., 1997), but lack of specificity and efficacy has limited their utility. Mouse models of copper deficiency (Grimes et al., 1997; Kuo et al., 2001; Steveson et al., 2003), which have reduced amounts of PAM enzymatic activity, exhibit severe developmental phenotypes. However, many enzymes in addition to PAM require copper to function, and these mice would be expected to have multiple defects unrelated to any decrease in PAM activity. The early ontogeny of PAM, and the discrete developmental expression patterns of PAM mRNA transcripts, implicates PAM in multiple functions during embryogenesis. We therefore made a null mutant of the PAM gene via traditional gene targeting methods to determine the function of PAM during development. We have also tested for the presence of alternative peptide amidating mechanisms in PAM null mutant mice.

**Materials and methods**

**Gene targeting**

PAM knockout (KO) mice were produced by conventional gene targeting methods. The targeting vector was constructed in two sequential subcloning steps (Fig. 1). First, a 4.5 kb XbaI fragment 5’ of exon 2 (Ouafik et al., 1992) was subcloned into an XbaI-digested pBluescript-based vector (obtained from Dr. S. Potter, University of Cincinnati) containing both a neomycin resistance cassette (neo') and the Herpes Simplex Virus thymidine kinase (HSV-TK) gene. The resulting vector was subsequently digested with ClaI (blunted), and a 3 kb EcoRI fragment (blunted) 3’ of exon 3 was subcloned into this site. Linearized targeting vector (15 μg) was electroporated into AB2.2 ES cells (Lexicon Genetics, Texas). Targeted ES cells were identified by genomic Southern blotting using a probe external to the targeting vector (Fig. 1B) and were microinjected into C57Bl6/J e13.5 blastocysts to generate chimeric mice.

**PAM activity assays**

Embryos were collected from timed heterozygous matings at e12.5, divided into heads and trunks, and frozen on dry ice. Placental tissue was saved for use in genomic Southern blotting. For analysis of adult mice, dissected tissue was frozen on dry ice and stored at ~80°C until use. All tissue was homogenized in 10 volumes of buffer containing NaTES–mannitol–Triton X-100 (TMT) (pH 7.4) and protease inhibitors, as previously described (Kolhekar et al., 1997a). Protein concentrations were determined using BCA reagent (Pierce). Samples were diluted 10-fold in PHM diluent and incubated for 1 h in the presence of [125I]-Acetyl-Tyr-Val-Gly (for PAL assays) (Kolhekar et al., 1997a). Uncharged amidated product was extracted with ethyl acetate and counted. Data were converted to picomoles per microgram of protein per hour (pmol/μg protein/h) and analyzed with unpaired t tests.

**Western blot analysis**

Tissue was homogenized in 10 volumes of buffer containing TMT and protease inhibitors, as described for PAM activity assays. Samples were denatured with Laemmli sample buffer and electrophoresed on 4–15% polyacrylamide gradient gels. Following electroblotting onto PVDF membranes, membranes were blocked with 5% non-fat milk and incubated with primary antibody to PAM (Ab 629; 1:200-1:2000) for crude serum and 1:200 for affinity-purified antibody) as previously described (Ciccotosto et al., 2000). Ab 629 recognizes Exon A (exon 16) of the PAM gene and recognizes full-length PAM-1 and the monofunctional PHM and PAL products derived from it. Previous in situ hybridization studies using a probe that is specific
for this exon have shown that transcripts containing exon A are abundant at e14.5 in the heart (Zhang et al., 1997) and in adult tissue (Braas et al., 1992).

Histological analysis of embryos

Embryos were collected from timed heterozygous matings and immediately submerged in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and stored at 4°C until being embedded in paraffin wax (Paraplast). Placental tissue was collected for use in genomic Southern blotting. Transverse sections of embryos were cut with a microtome (8–10 μm thick).

BrdU immunohistochemistry

Pregnant females were injected with 50 μg/g BrdU (Sigma) and sacrificed 30 min later. Embryos were then prepared as described for histology. Sections were treated with 1 mg/ml trypsin (Sigma) dissolved in deionized dH₂O for 20 min at 37°C followed by 2 N HCl for 30 min and then blocked with 10% horse serum/0.1% Tween-20/1× PBS. Sections were then incubated with a sheep polyclonal BrdU antibody diluted 1:1000 (RDI, Inc. Flanders, NJ) in 5% horse serum/0.1% Tween-20/1× PBS. The next day, sections were incubated with biotinylated secondary antibody, treated with ABC reagent (Vector Laboratories) and visualized with DAB (Sigma). It should be noted that only cells actively synthesizing DNA during the 30-min treatment period would be labeled with BrdU. Hence, the rate of cell division can be compared between genotypes using this method.

BrdU immunohistochemistry

Cells from several CNS regions were counted from the ventricle to the end of the ventricular zone (defined as the end of BrdU-labeled cells). The length along the ventricle that was counted in each section was identical for wildtype and mutants (approximately 200 μm), allowing for their comparison. The labeling index (LI) was the ratio of BrdU-positive cells over the total number of cells counted multiplied by 100. Data were analyzed using unpaired t tests.

Determination of fasting blood glucose levels and adiposity

Blood glucose levels were determined after a 6-h fast in 2-month- and 10-month-old male heterozygous and wildtype mice. Blood was collected from the tail vein, and glucose levels were determined using the Accu-chek Advantage glucometer (Roche). Blood glucose levels were measured in milligrams per deciliter (mg/dL) and analyzed with an unpaired t test.

Adiposity was determined as the combined weight of three white adipose tissue pads (bilateral reproductive, inguinal and retroperitoneal) expressed as a percentage of body weight ± SEM. Adiposity was measured in male 10-month-old heterozygous and littermate control mice.

Glucose tolerance test

Experimentally naive 2-month- and 10-month-old male heterozygous and wildtype mice were fasted for 6 h and then given an i.p. injection of 2 mg/g body weight of d-glucose (Dextrose, Sigma Chemicals). Blood glucose measurements were taken as described above at 15, 30, 60 and 120 min post-injection. Data were analyzed with an unpaired t test for each time point.

Radioimmunoassays (RIAs)

To compare the levels of amidated peptide in adult and 3-day-old (p3) PAM heterozygous mice to that of age-matched wildtype controls, we performed RIA analysis on pituitary and hypothalamic samples from each age. Tissue was homogenized in 10% acetic acid/0.5 mg/ml bovine serum albumin (BSA)/0.3 mg/ml PMSF, lyophilized and resuspended in 50 mM
NaPO₄/0.1% Triton-X-0.3 mg/ml PMSF. Two-fold serial dilutions of each sample were incubated overnight at 4°C with trace amounts of iodinated peptide and the appropriate corresponding antibodies: amide-specific alpha melanocyte-stimulating hormone (α-MSH) Ab Wanda (1:50,000), amide-specific joining peptide (JP) Ab Jamie (1:2000) (Eipper et al., 1986), adrenocorticotropic hormone (ACTH) COOH-terminus specific Ab Kathy (1:2000) (Schnabel et al., 1989; Bruzzaniti et al., 1999; Ciccotosto et al., 1999), CCK-8 amide-specific Ab 8007 (1:400,000) (generous gift from Dr. Jens Rehfeld, University of Copenhagen) or NPY mid-region Ab JH3 (1:20,000) (Hansel et al., 2001a). The JH3 antibody recognizes both amidated and non-amidated forms of NPY. Bound and free peptides were separated by precipitation with goat anti-rabbit secondary antibody, the supernatant was discarded, and the amount of 125I in the pellet was counted. Synthetic ACTH (1–39) (Ciba Pharmaceutical Co., Summit, NJ), α-MSH-NH₂ (Bachem), D-Tyr-joining peptide (12–18)-NH₂ (Vega Biotechnologies, Tucson, AZ), CCK-8-NH₂ (Peninsula) and NPY (Bachem) were used to generate standard curves. Assays for total mouse AM were performed with a kit from Phoenix Pharmaceuticals (Belmont, CA). PACAP and AM assays were also performed using peptides and antisera from Bachem (Torrance, CA). Data were analyzed using unpaired t tests.

ACTH (1–39) is the immediate precursor to α-MSH and corticotropin-like intermediate lobe peptide (CLIP) and served as an internal control for protein concentration in each pituitary sample. The amount of total NPY was determined in each hypothalamic sample and served as a control for variations in protein levels. Furthermore, all samples from both the pituitary and hypothalamus were run on polyacrylamide gels, transferred to PVDF membranes and then stained with Coomassie blue to verify the relative protein levels and integrity of each sample.

Results

Gene targeting of PAM

Approximately 17% of ES clones screened were heterozygous for the mutant PAM allele. Two clones were used for microinjection and were also screened by genomic Southern blotting using a 1.2 kb 32P-labeled neo probe. Only the predicted 17 kb mutant band was labeled, demonstrating that there was no random integration of the targeting vector. Four male chimeras were obtained and mated to C57Bl/6/J females. One targeted ES clone generated a chimera that was 100% germ-line transmitting and, as expected, transmitted the mutant PAM allele to 50% of its offspring. Heterozygotes born from this chimera were mated to obtain all mice analyzed. To complement analysis of the 3′ integration site that used an external probe for Southern analysis (Figs. 1B, C), the integrity of the 5′ genomic insertion site was also verified by genomic Southern blotting of BamHI digested DNA from wildtype, heterozygous and mutant embryos using a 304 bp probe from the region 5′ of the targeting vector (Fig. 1C). Only the predicted bands of 33 kb and 15 kb were obtained for the wildtype and mutant samples, respectively.

Production of PAM KOs

Analysis of mice derived from heterozygous matings revealed that no homozygous mutants were present at birth. Out of 355 offspring from heterozygous matings genotyped at 3–4 weeks of age, 123 (35%) were wildtype and 232 (65%) were heterozygous for the mutant PAM allele. Analysis of the genotypes of embryos collected from timed heterozygous matings demonstrated that PAM null mutants were present only at e14.5 and earlier (Table 1). There was no evidence to suggest that any of the PAM mutant embryos died before e14.5. At e14.5 and earlier, PAM mutants were present in normal Mendelian proportions. Moreover, there was no increase in the number of resorbed homozygous mutant embryos collected at this stage as compared to other genotypes (Table 1). It was therefore concluded that lethality occurred during a discrete developmental time period between e14.5 and e15.5.

Morphological and histological analysis of mutant PAM embryos

By e13.5, all homozygous mutant embryos collected (Table 1) had notable differences in the yolk sac vasculature as compared to wildtype or heterozygous littermate controls (Figs. 2A, B). In the homozygous mutants, there appeared to be both the absence of vessels and poorly formed vessels. Furthermore, the umbilical cord vein in the homozygous mutants always appeared constricted as compared to littermate controls (Figs. 2C, D). All embryos collected were subsequently dissected out of the yolk sac and subjected to careful inspection for gross abnormalities (Table 1). While the gross morphology of mutant PAM embryos appeared similar to wildtype controls at e11.5 and e12.5 and earlier, all e13.5 and e14.5 (Figs. 2G, J) mutant embryos had visible edema. Several embryos from e10.5, e11.5, e12.5, e13.5 and e14.5 were subsequently selected for histological analysis. One out of two e11.5 mutant embryos and all three e12.5 mutant embryos that were analyzed already had fluid accumulation near the aorta and rib primordia (Figs. 3A, B). Fluid accumulation was also present in all three e13.5 mutant embryos examined, and the amount of fluid had substantially increased (Figs. 3C, D). At e14.5, the fluid accumulation in mutant embryos was massive and was now also clearly seen in the pleural and peritoneal cavities. We were unable to find morphological abnormalities in aortic and venous structures of e11.5 mutant embryos, but we did observe that six out of nine e12.5, e13.5 and e14.5 homozygous mutant embryos had thinner arterial walls, with the thoracic and abdominal aortas and major arteries being relatively thinner as compared to wildtype controls (Figs. 4A, B). Also notable were the abnormal development of the umbilical cord and its vessels in the homozygous mutants (Figs. 4C, D).

Table 1

<table>
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<tr>
<th>Age</th>
<th>+/+</th>
<th>+/−</th>
<th>−/−</th>
<th>Total</th>
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<td>23 (2)</td>
<td>0 (10)</td>
<td>31</td>
</tr>
<tr>
<td>e14.5</td>
<td>13 (1)</td>
<td>35 (10)</td>
<td>21 (2)</td>
<td>69</td>
</tr>
<tr>
<td>e13.5</td>
<td>18 (2)</td>
<td>45 (9)</td>
<td>29 (5)</td>
<td>92</td>
</tr>
<tr>
<td>e12.5</td>
<td>45 (0)</td>
<td>64 (14)</td>
<td>49 (0)</td>
<td>158</td>
</tr>
<tr>
<td>e11.5</td>
<td>27 (1)</td>
<td>40 (7)</td>
<td>14 (2)</td>
<td>81</td>
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Shown are embryos obtained from timed heterozygous matings. No homozygous mutants have been obtained at e15.5 or later. At e14.5 and earlier, wildtypes, heterozygotes and mutants were detected in the expected Mendelian inheritance ratio of 1:2:1 (totals 103:184:113). Indicated in parenthesis is the number of resorbed embryos that were collected. Note that there were no disproportionate numbers of resorbed homozygous mutant embryos collected at e14.5, indicating that mutants die after this age. The numbers in the last column are the total number of viable embryos that were collected at each age.

a These embryos were already in the process of being resorbed.
abdominal as well as carotid arteries sharing this abnormality in all affected embryos (Figs. 3E–P). The severe edema in e13.5 and e14.5 homozygous mutant embryos often made analysis difficult. Several structures, including the aorta, lungs and liver, were often disfigured by the increasing fluid accumulation. We did, however, observe a decrease in the thickness of the epithelial cells of the developing bronchi in the lungs of e14.5 homozygous mutant embryos as compared to wildtype controls (Figs. 3Q–T) which is consistent with the relatively strong expression of PAM and AM that has been reported in these cells (Zhang et al., 1997; Garayoa et al., 2002). Additionally, we consistently observed an increased density of trabeculae carneae in the left ventricle of mutant PAM embryos from e12.5 through e14.5 (Fig. 4). This phenomenon was observed in two out of three e12.5 mutant embryos and in all three e13.5 and e14.5 homozygous mutant embryos when compared to the same number of wildtype controls at each stage.

Fig. 2. As shown in panels A and B, e13.5 PAM mutant embryos lack a well-developed yolk sac vasculature as compared to wildtype (not shown) or heterozygous littermate controls. The red arrow points to the umbilical cord vein in panels C and D. PAM mutant embryos develop severe edema by e14.5 (E–J). Although the gross morphology of e12.5 (E–G) PAM KOs appears similar to wildtype, by e14.5 (H–J), the edema is visible in all mutant embryos (J) and is severe. The arrow in panel J points to the skin.

Fig. 3. Histological analysis demonstrates edema as well as alterations in the vasculature and lung of mutant PAM embryos. Mutant PAM embryos have begun to accumulate fluid as early as e12.5 near the aorta and rib primordium (B). By e13.5, the amount of fluid has increased (D) and has extended into the subcutaneous region (denoted by red arrow). Low power images (A–D) were taken at 16× magnification. Also shown are higher-magnification pictures of the carotid artery (E, H, K, N), the thoracic aorta (F, I, L, O), and the abdominal aorta (G, J, M, P) in e13.5 (E–J) and e14.5 (K–P) wildtype and homozygous mutant embryos. Note that the arterial walls are thinner in homozygous mutant embryos (H–J, N–P). Shown in panels Q–T are low- and high-magnification pictures of the lungs of e14.5 wildtype and homozygous mutant embryos. The arrows in panels S and T point to the developing bronchi. Note that the columnar epithelial cells surrounding the bronchi are smaller in homozygous mutants. The scale bar in panels E–P and S–T is equal to 100 μm. (Abbreviations are a = accessory lobe of right lung, c = cranial lobe of right lung, DRG = dorsal root ganglion, l = left lung, m = middle lobe of right lung, RV = right ventricle.)
PAM activity in embryos

To determine whether there was any alternative amidation enzymatic activity, PHM and PAL activities were assayed in crude protein extracts of e12.5 embryos. No PHM or PAL activity above background was detected in PAM homozygous mutants, suggesting that the sole source of amidation activity detectable with this assay is the result of the PAM gene product (Figs. 5A, B). Furthermore, both PHM and PAL activity were one half the wildtype levels in both the heads and trunks of heterozygous embryos. These experiments demonstrate that these assays can successfully detect amidation activity in early mouse embryos and are consistent with the high and widespread expression of PAM by e12.5 in both the developing heart and brain (Zhang et al., 1997). Western blot analysis verified the absence of PAM protein in trunk extracts prepared from homozygous PAM KO embryos (Fig. 5C). Radioimmunoassays specific for amidated CCK-8 and amidated PACAP were not sensitive enough to detect expression of these peptides in wildtype embryos of this age, precluding their use to assess amidation in KO mice (not shown). Assays for total AM (amidated and non-amidated) did show a significant increase in levels of AM in the trunks of KO embryos (Fig. 5D), which we interpret as a possible compensatory response to the lack of amidated AM in PAM KO embryos.

BrdU immunohistochemistry

PACAP, an amidated peptide, is found in differentiated neurons throughout the CNS at least as early as e12 and is thought to be an inhibitor of mitosis in the developing cortex (Lu and DiCicco-Bloom, 1997; Suh et al., 2001). Cells located in the proliferative region adjacent to PACAP-positive cells express both PAM and the PACAP receptor (PAC 1), consistent with possible paracrine regulation of PACAP function by PAM (Dicicco-Bloom et al., 1998). We used BrdU to label cells undergoing DNA synthesis and found that there were no differences in the labeling index of e12.5 mutant PAM embryos as compared to littermate controls in the telencephalon (Fig. 6), diencephalon and metencephalon brain regions or in the spinal cord. Furthermore, the number of BrdU-positive cells and the total cell numbers in the brain and spinal cord of mutant embryos were not changed at this stage (not shown). Additionally, there were no differences in labeling indices in the telencephalon and diencephalon regions in e13.5 PAM mutant embryos as compared to wildtype controls. Taken together, the data suggest that neuronal cell proliferation is normal in the absence of PAM. The overall morphology and cellular structure of all brain regions analyzed in mutant PAM embryos appeared normal, suggesting that amidated peptides may not be required for CNS development at e13.5 or earlier in the mouse.

Characterization of PAM heterozygous mice

Mice heterozygous for the mutant PAM allele are viable and fertile and show no gross motor abnormalities. Analysis of peptide amidation activity in adult heterozygous mice showed that heterozygotes have approximately one half the levels of PHM (Fig. 7A) and PAL (Fig. 7B) activity, as well as about one half the PAM protein levels in the atria as compared to wildtype
controls (Fig. 7C). Heterozygous mutant mice also have one half the levels of PAM protein in both the cortex and pituitary (not shown). Taken together, these data are consistent with the hypothesis that the PAM gene encodes all peptide amidation enzymatic activity in the adult mouse and that no compensatory increase in expression occurs in heterozygous animals. Since several peptides involved in nociception are known to be amidated, we compared the response of PAM heterozygous mice to that of wildtype controls in the tail-flick assay and determined that heterozygous mice had similar tail-flick latencies in response to a radiant heat source as compared to wildtype controls (+/–4.7 s, n=10; +/-–4.4 s, n=8, P=0.60). Furthermore, there were no differences in the weights of male outbred PAM heterozygotes at 2 months (P=0.32) (Fig. 7D), 10 months (P=0.70) or 2 years of age (P=0.61) as compared to age-matched littermate controls. We also determined that there were no significant differences in the weights of female heterozygotes at 2 months, 7 months and 18 months after birth as compared to age-matched controls. In addition, the weights of congenic 129S6 PAM heterozygous and wildtype littermates were determined during the first postnatal months as well as at selected ages through 10 months; again, no difference was seen between genotypes. These data indicate that there are no alterations in total body weight in PAM heterozygous mice. However, 10-month-old 129S6 PAM heterozygous mice showed an increased adiposity when compared to age-matched littermate controls of similar total body weight (+/–7.6% ± 0.8, n=10; +/-+5.4% ± 1.5, n=11; P=0.05). These results indicate that there may be deficits in one or more amidated peptides involved in the regulation of adiposity.

Several amidated peptides modulate glucose metabolism. Therefore, we performed glucose tolerance tests on both 2-month- and 10-month-old (aged) PAM heterozygous and wildtype mice. There were no differences in blood glucose

Fig. 5. Amidation activity and PAM protein are undetectable in PAM mutant embryos, while total AM levels are upregulated. PAM activity in both the heads and trunks of e12.5 embryos was analyzed. No PHM (A) or PAL (B) activity was detected in PAM homozygous mutants. There was approximately 1/2 the activity level of both PHM and PAL in PAM heterozygous embryos as compared to wildtype controls, as would be expected if there were no compensatory upregulation of PAM in heterozygotes. Shown in panel C is the absence of any detectable PAM protein by Western blot analysis of the trunks of e14.5 homozygous mutant embryos using an antibody that detects the full-length PAM protein (Ab 629) which is normally abundant in wildtype embryos at this age. To verify that equal amounts of protein were loaded into each well, actin and γ-adaptin levels were also determined (n=4+/+, n=8+/+, n=7+/-). Below is the PAM cDNA structure showing the location of exon A. In panel D, assays for total AM using e14.5 trunks showed an increase in AM in the homozygous mutant mice (**P<0.01 compared to the pool of wildtype and heterozygous mice).

Fig. 6. Cell proliferation is unaltered in the CNS of e12.5 mutant PAM embryos. Shown in panels A and C are the results of immunohistochemical analysis on transverse sections (10 μm) of the telencephalon of e12.5 wildtype and PAM mutant embryos that were incubated with a BrdU polyclonal antibody (brown) and counterstained with methyl green. When the primary antibody was omitted, no staining was obtained (B, D). The average labeling indices for wildtype and homozygous mutants were 22.5% and 23.3%, respectively, in the telencephalon. The scale bar represents 100 μm in panels A–D. No significant differences were found in the labeling index in any of the CNS regions analyzed.
levels at either age after a 6-h fast (time 0 in Figs. 7E and F). While there were no significant differences in blood glucose levels at any time point after glucose challenge in 2-month-old PAM heterozygotes (Fig. 7E), aged PAM heterozygous mice had significantly elevated blood glucose levels at 60 min \((P = 0.006)\) and 120 min \((P = 0.02)\) post-injection (Fig. 7F). Similar results were obtained in PAM heterozygous mice on both C57Bl6/129SvEv outbred and 129S6 congeneric backgrounds.

To begin to determine if one half of the normal level of amidating enzyme activity is sufficient to amidate peptides to the wildtype level, we compared the amounts of amidated and non-amidated peptides by RIA analysis of pituitary and hypothalamic extracts from early postnatal and adult heterozygous and wildtype mice. There were no significant differences in the ratios of amidated \(\alpha\)-MSH to ACTH (1–39) \((P = 0.11)\) and amidated JP to ACTH (1–39) \((P = 0.13)\) in the pituitary of p3 heterozygous mice as compared to littermate controls or in the ratio of amidated CCK to NPY in the hypothalamus \((P = 0.18)\) (Fig. 7G). Although not significant, there was a noticeable trend for the ratios of the postnatal heterozygotes to be lower, suggesting that there may be a slight decrease in the production of amidated peptides in these younger mice. In the adult, there were no detectable differences in the ratios of amidated \(\alpha\)-MSH to ACTH (1–39) \((P = 0.11)\) and amidated JP to ACTH (1–39) \((P = 0.98)\) in the pituitary as compared to littermate controls or in the ratio of amidated CCK to NPY in the hypothalamus \((P = 0.78)\) (Fig. 7H).

**Discussion**

PAM is the sole source of peptide amidating enzymatic activity in the mouse

Early immunohistochemical studies raised the possibility that there might be an alternative peptide amidation mechanism since PP cells in the pancreas (Martinez et al., 1993a) and epithelial cells of the small intestine (Martinez et al., 1993b) contain amidated substrates but no detectable PAM. Our data indicate that the PAM gene is the sole source of peptide amidating activity in the mouse embryo since amidation activity was completely absent from homozygous mutants prior to death. This finding is consistent with the absence of an alternative amidation mechanism in *Drosophila*, where a loss of PHM activity results in embryonic lethality at midgestation (Kolhekar et al., 1997b). Both during embryonic development and as adults, PAM heterozygous mice have one half the amidation activity of wildtype mice. Thus, we conclude that PAM is almost certainly exclusively responsible for amidating all peptide hormones capable of serving as substrates in midgestational embryos. It is possible that a minor amidation enzyme could be upregulated in late gestational embryos and in the adult mouse in the complete absence of PAM. A conditional PAM mutant will be needed to test this possibility.
**PAM enzymatic activity is essential for normal mouse development**

We have found that deletion of PAM produces an embryonic lethal phenotype that appears to result from cardiovascular deficits. Furthermore, lethality occurs between e14.5 and e15.5 which corresponds to the time when the architecture of the fetal circulatory system is completed (Rugh, 1968). It is unlikely that PAM enzymatic activity is involved directly in the formation of the heart because cardiac muscle contraction (e8) and cardiac looping (e10) (Rugh, 1968) both occur well before the onset of edema by e12.5 (Fig. 2). Furthermore, we found no evidence for obvious histological defects during morphogenesis of the heart in PAM KO mice. However, the first detectable evidence of a PAM KO phenotype occurs at the onset of the developmental process of vasculature remodeling, a process whereby the rudimentary six aortic arches are remodeled to form the dorsal aorta, the common carotids and the pulmonary artery (Gilbert, 1991). Therefore, we believe that the phenotype of the PAM KO is most consistent with a severe CV functional deficit rather than a morphogenetic defect in heart formation.

There are several notable differences when one compares the PAM KO with mice lacking the transcription factors retinoid X receptor alpha (RXR-α) (Sucov et al., 1994) and notch 2 (McCright et al., 2001), two KO mouse strains exhibiting prenatal edema. First, the edema in these models is not as severe as that seen in mutant PAM embryos and suggests that the CV deficits in PAM mutant embryos are more extensive and distinct from these other mouse strains. Secondly, we see an increase in the density in trabeculae carneae in the left ventricles as early as e12.5 in homozygous PAM mutants (Fig. 4). This increase is similar to the hypertrophy seen in hypertensive states in both mouse and human (Chien et al., 1991; Oliver et al., 1997; Sugden and Clerk, 1998; Yang et al., 1999; Chien and Olson, 2002) and provides further evidence that the PAM mutant phenotype is due to a functional deficit in the CV system. In contrast, these other KO mice have thin ventricular walls most likely due to a lack of expansion of cardiac myocytes. Mutant PAM embryos, therefore, may be a novel model to study CV disease in the embryo.

At this point, we cannot entirely rule out the possibility that a disruption of salt balance or fluid excretion, in addition to CV deficits, is contributing to the edema seen in mutant PAM embryos. However, the adrenal glands and kidneys are still functionally rudimentary between e12.5 and e14.5 when mutant PAM embryos first begin to show edema and when they die, respectively, and thus are unlikely to contribute significantly to fluid homeostasis at midgestational ages. At early embryonic ages, the heart probably also functions as an endocrine organ excreting circulating hormones such as AM and atrial natriuretic factor (ANF) that can also affect both blood pressure and salt balance (Kitamura et al., 2002). Therefore, it is possible that the severe edema seen in PAM mutants could partially be due to hormonal deregulation of fluid homeostasis.

**The PAM KO phenotype is a phenocopy of adrenomedullin gene disruption**

Mouse KO models exist for several amidated peptides. However, only mice containing mutations in the adrenomedullin (AM) gene die as early in gestation as PAM mutants. The AM gene encodes two potent vasodilators, pro-amino terminal peptide (PAMP) and adrenomedullin (reviewed in Eto, 2001) that absolutely require amidation for binding to their receptors in vitro (Eguchi et al., 1994; Iwasaki et al., 1996). Two mouse KO models of AM lack both PAMP and AM peptides (Caron and Smithies, 2001; Shindo et al., 2001), and one lacks the AM peptide only leaving PAMP intact (Hay and Smith, 2001; Shimosawa et al., 2002). All three mutant strains are lethal by midgestation. Deletion of both peptides results in a slightly more severe phenotype than when AM only is deleted, indicating that both AM and PAMP have at least some non-redundant functions.

There are striking similarities between the phenotypes of PAM KO and AM KO mice. The time of lethality and the severity of the edema of the PAM KO are similar to that observed in the AM KO. Several other characteristics of the PAM KO including poorly developed yolk sac vasculature (Fig. 2), thinning of multiple arterial structures (Fig. 3) and increased trabeculation in the left ventricles (Fig. 4) are also similar to the phenotype of the AM gene KOs (Caron and Smithies, 2001; Shindo et al., 2001). We therefore believe that most, if not all, of the phenotype of the PAM mutant is due to a lack of amidated AM hormones. Assays for total AM in PAM KO embryos revealed increased levels of this immunoreactivity, perhaps reflecting a compensatory increase in basal AM expression and/or a lack of feedback inhibition. Importantly, no evidence was found for any moderation of the AM KO phenotype which might be expected if an alternate mechanism could amidate AM-derived peptides. Although we did observe edema in PAM embryos earlier than has been reported for the AM KO, a more detailed comparative analysis of the two KO strains will be needed to determine if the sole cause of the PAM KO phenotype is the lack of functional AM peptides. If there were differences, this would suggest that PAM function extends beyond that of being an indirect CV regulator by conferring activity to AM peptides. It can be speculated that, because mutant PAM embryos lack functional AM peptides, they are hypertensive. This eventually leads to the ventricular hypertrophy observed in PAM mutant embryos.

At this time, it is unclear if amidated AM peptides or PAM activity is required for CV function in late gestation and in adulthood to the extent that they are required during midgestational ages. By temporally restricting deletion of PAM, it may be possible to bypass the absolute requirement for PAM and AM peptides during midgestation. Furthermore, it would allow us to determine if PAM or other amidated peptides besides AM have potential CV regulatory functions.
Loss of functional PAM enzymatic activity does not disrupt neuronal cell proliferation

The high expression of PAM in the ventricular zone regions of the telencephalon, diencephalon, metencephalon and spinal cord, adjacent to regions where PACAP expression is also high, suggested that PAM expressed on the cell surface might regulate neuronal cell proliferation by activating non-amidated PACAP peptides secreted from adjacent post-mitotic cells (D'icco-Bloom et al., 1998). However, we found no difference in neuronal precursor proliferation in several CNS regions of mutant PAM embryos at e12.5 or e13.5 as compared to littermate controls. The data are the first to examine the rate of neurogenesis in an in vivo model where there is a specific loss of amidated PACAP27 and PACAP38, the two biologically active forms of PACAP (Sherwood et al., 2000). Our finding of a lack of effect on cell proliferation after deletion of PAM suggests that any effects of these amidated forms of PACAP on proliferation are minimal, at least at the midgestational ages examined. An alternative possibility is that the potential effect of PACAP on proliferation does not require amidation. PACAP KO mice have been generated (Hashimoto et al., 2001; Hamelink et al., 2002). Although the rate of cell proliferation has not been measured directly yet in these mice, these mice have several behavioral deficits including hyperactivity in an open field test, suggesting that there is abnormal brain function and/or structure. Because these mice lack both amidated and non-amidated forms of PACAP peptides, it cannot be determined which deleted forms of PACAP are contributing to the phenotype of this mouse strain. Our data suggest that either COOH-terminal glycine extended PACAP or other non-amidated factors may be essential for neuronal proliferation at midgestational ages.

A comparison of the phenotype of PAM KO mice and other processing enzymes

The phenotype of the PAM KO mouse raises several questions concerning neuropeptide processing. Since PAM is the last step in the biosynthetic pathway, it was expected that the phenotype of a PAM KO mouse might be no more severe than those of the prohormone convertase and carboxypeptidase KOs that function in the preceding steps of the processing pathway (Czyzyk et al., 2003). Of the endoproteases, only furin (Roebroek et al., 1998) and PACE4 (Constam and Robertson, 2000) deletions have resulted in embryonic lethality, with the furin KO having the most severe phenotype with death at e10.5 with heart and axial rotation defects. Embryonic expression of furin (Zheng et al., 1994; Roebroek et al., 1998) most closely mimics that of PAM (Zhang et al., 1997), with both its early onset and high expression in the heart and in several other peripheral structures. Since proAM is most likely cleaved by furin as evidenced by its furin consensus sequence (Ishimitsu et al., 1994), it is interesting to speculate that a temporally restricted KO of furin that could bypass the early embryonic requirement for nodal (Roebroek et al., 1998; Beck et al., 2002) might share a similar phenotype to that of both the AM and PAM KO mouse strains.

A naturally occurring point mutation in the gene for carboxypeptidase E (CPEfat/CPEfat) results in a loss of CPE function. Adult KO mice have severe obesity, hyperglycemia and infertility (Naggert et al., 1995; Fricker et al., 1996). A KO mouse for CPD, a carboxypeptidase (CP) believed to be a potential candidate for redundant CP function (Fricker and Leiter, 1999), has yet to be generated. However, a CPD KO mouse is unlikely to have a phenotype much more severe than that of CPEfat/CPEfat mice as the overall expression patterns are similar for the two CPs at least in adult neuroendocrine tissues (Dong et al., 1999). The question remains what is the CP that is responsible for cleaving basic residues at the COOH-terminus to expose the glycine substrate for PAM in the heart.

PAM heterozygous mice develop mild glucose intolerance in the absence of altered amidated peptide levels

Radioimmunological analysis in both the central nervous system and pituitary indicate no significant disruption in the extent of amidation of several substrates in adult PAM heterozygotes. For example, we found that the relative levels of amidated α-MSH and JP in the pituitary compared to unamidated forms are unaltered in adult mice. We also found that the levels of amidated CCK in the hypothalamus were unaltered in these mice. These data are consistent with previous in vitro work showing that decreased peptide amidation was seen only when PAM activity was reduced more than 2-fold (Mains et al., 1991). Under basal conditions, our data suggest that 50% of wildtype amidation activity is sufficient to amidadate most glycine-extended substrates in the mouse.

Several tests were used to assess alterations in processes that are normally influenced by amidated peptides in PAM heterozygous mice. Heterozygous mice did not differ in fasting blood glucose levels at any age tested, while aged heterozygous mice had significantly elevated blood glucose levels 60 and 120 min after an i.p. injection of D-glucose as compared to littermate controls (Fig. 7F). These data raise the possibility that there are regional and peptide-specific alterations in amidated peptide levels in adult PAM heterozygous mice that affect physiology, especially when the system is perturbed. For example, amidated glucagon-like peptide 1 (GLP-1) which normally functions to lower blood glucose levels (Scrocchi et al., 1996) may be functionally impaired in PAM heterozygotes. Our own observations support such peptide-specific changes as we found that amidated PACAP38 was significantly increased in the hypothalamus of adult heterozygotes (T.A. Czyzyk, unpublished observations) but that levels of amidated CCK8 were unchanged in the same region (Figs. 7G, H). Alternatively, this disruption of glucose homeostasis could be secondary to structural abnormalities such as increased adiposity that could lead to insulin insensitivity. This possibility is supported by the increased adiposity of aged PAM heterozygous mice. It is not clear why younger heterozygotes show no evidence of altered glucose homeostasis, but one possibility is that PAM heterozygous mice have general...
circulatory defects due to a chronic decrease in functional AM peptides that could eventually lead to a disruption of several organs including the pancreas with subsequent effects on body composition and metabolism.

Several factors could influence amidated peptide levels, including dietary copper and ascorbate (reviewed in Prigge et al., 2000). Probably more important is the amount of glycine-extended substrate relative to the amount of PAM enzyme. It would be expected that amidated peptide levels would only be reduced if PAM were the rate-limiting factor (that is, there is more substrate than enzyme activity). Although we found no detectable changes in several amidated peptide levels in adult mice, we did notice a decline in amidated peptide levels in early postnatal PAM heterozygous mice. There is much less PAM present at this time than in the adult and is consistent with our findings. Therefore, at earlier postnatal (and possibly embryonic stages), the levels of PAM enzymatic activity may be the rate-limiting factor in the biosynthesis of amidated peptides in heterozygous mice.

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References


