Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 282-290

ISOLATION AND FUNCTIONAL EXPRESSION OF HUMAN PANCREATIC PEPTIDYLGLYCINE α-AMIDATING MONOOXYGENASE

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Received October 3, 1994

Summary: Pancreastatin (PST) is processed from chromogranin A and the C-terminal amide of the peptide is an absolute requirement for biological activities. Human pancreatic carcinoma cells QGP-1 which produce both chromogranin A and PST were used to isolate cDNAs encoding two forms of peptidylglycine α-amidating monooxygenase (PAM). The two forms are a full length bifunctional enzyme and a variant lacking the transmembrane domain-coding region. When the cDNAs of these two forms were expressed in COS-7 cells, cells transfected with the predicted soluble form released into the culture medium a very much higher amidating activity which converts human chromogranin A-(273-302) to PST-29. The optimal pH for amidating activity was 5.4 and Cu^{2+}, ascorbate and catalase were required as cofactors for the both forms of PAM. Km values for the membrane-bound and the soluble forms of PAM were 15.7 ± 3.1 μM and 12.4 ± 1.6 μM, respectively. These results demonstrate that both forms of PAM can function in the posttranslational processing of chromogranin A to PST in the environment of a secretory vesicle.

Pancreastatin (PST) that is produced by posttranslational processing from chromogranin A has been shown to inhibit endocrine (1) and exocrine pancreatic secretions (2) and the C-terminal amide of the peptide is an absolute requirement for both biological activities (3). The chromogranin A precursor contains a motif with a glycine C-terminal to the PST. The only enzyme known to convert a peptidyl C-terminal glycine to an amide is

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Abbreviations: PAM, peptidylglycine α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase; PAL, peptidyl-α-hydroxyglycine α-amidating lyase; PST, pancreastatin; MES, 2-morpholinoethanesulfonic acid.

0006-291X/94 $5.00

peptidylglycine α-amidating monooxygenase (PAM). PAM consists of two enzymatic activities, peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α- amidating lyase (PAL) which are encoded by separable domains of the PAM precursor. Various splice variants of PAM mRNA have been found in rat (4,5). Functional expression of human PAM cDNA encoding both PHM and PAL domains has not been demonstrated. The existence of another different form of amidating enzyme has been suggested because immunoreactivity for PAM was not detected in pancreatic polypeptide cells and some cells of the small intestine which are known to produce C-terminally amidated peptides (6,7). The aim of this study was to identify the amidating enzymes relating to production of biologically active PST, therefore we isolated cDNA clones of amidating enzyme from a human pancreatic islet cell tumor cell line (QGP-1) already known to produce both the precursor chromogranin A and the processed amidated PST (8).

METHODS AND MATERIALS

cDNA Cloning and Sequencing: A human cDNA fragment containing an N-terminal region of human PAM (9) was prepared by RT-PCR from QGP-1 cells to obtain a probe for screening. Primers used in the PCR reaction were: 5'-TTCCGAAGCCCACTTTCTGTC-3' and 5'-CATGAAATGTCAGAATTC-ACCAC-3', corresponding to nucleotides 141-161 and 723-745 of human PAM cDNA (Fig. 1), respectively. The PCR product was ligated to pT7Blue vector and the sequence of the PCR product was confirmed by sequencing by the Dye Deoxy Terminator method (Applied Biosystems Inc. CA). A fragment generated by digestion with *Eco* RI and *Bam* HI was radiolabeled with [α-^{32}P]dCTP by priming with random hexamers and used as a hybridization probe to screen a λZAP QGP-1 cDNA library constructed as described previously (10). Fifteen clones obtained after the second screening were cultured with helper phage M13KO7, and insert cDNAs were obtained in pBluescript SK(-) plasmid vector by the automatic excision process. The cDNA inserts were characterized by restriction endonuclease mapping. Two clones (PAM-3 and PAM-15) were chosen for the construction of expression plasmids.

Construction and Transfection of Expression Plasmids: *Nco* I site for the digestion was constructed by point mutation of A^{79} to C in cDNAs of the two clones by the method of Kunkel (11). The mutant cDNAs were digested with *Nco* I and *Hind* III and inserted into the pSG5 vector using a *Bgl* II linker. The resulting plasmids were designated as PAM-3-pSG5 and PAM-15-pSG5, respectively. The cDNA sequences were determined using various synthetic primers by the Dye Deoxy Terminator method. Each plasmid (20 μg) was transfected into COS-7 cells (10 x 10^6 cells) using an electroporation apparatus (Gene Pulser, Bio-Rad) (12) and the transfected cells (2.8 x 10^6 cells/dish) were cultured in dishes containing 5 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Analysis of Expressed Proteins: After 48 h transient expression, transfected cells were labeled with [^{35}S]methionine for 6 h. Labeled cells were extracted in the presence of protease inhibitors as described previously(13). Cell lysate and culture medium were immunoprecipitated with two anti-PAM

antisera (CC and PAL 2) (6) raised against peptides from the PHM and PAL regions, respectively, of the PAM molecule (Fig. 4b). Immunoprecipitates were analyzed by SDS-PAGE/fluorography (13).

Cell Extracts and Enzyme Assay: After 48 h transient expression, conditioned medium was collected, concentrated 10-fold in Centricon-30 (Amicon MA) and used for enzyme activity. Soluble and membrane-bound fractions from cells were prepared using Na(N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) without and with 1% Triton-X-100, respectively, according to the method of Milgram et. al. (14). Soluble and membrane fractions were assayed for amidating activity. Aliquots (20 µl) of the enzyme solutions were incubated in MES buffer containing 0.1 mM ascorbate, 5 µM $CuSO_4$ and 100 µg/ml catalase with 0.49 µM C-terminally glycine-extended human PST-29 (PST-29-Gly) (Peninsula Lab. CA) in a final volume of 130 µl at 37 °C for 3 ~ 5 h and the reaction was stopped by the addition of 100 µl of PST radioimmunoassay (RIA) buffer containing 25 mM EDTA. Kinetic experiments were performed with varying concentrations of PST-29-Gly (0.49-60 µM). Km was derived using Hanes-Woolf plot. The amount of amidated product was measured by an RIA specific for C-terminally amidated human PST (13). The anti-human PST antiserum (R711) is specific for the C-terminal glycine amide of human PST-29 and cross-reactivity with PST-29-Gly was less than 0.001%. Variations in the reaction mixture above were made to measure dependence on ascorbate, catalase, and copper and to obtain the pH-dependence. For characterization of amidating activities, membrane-bound fraction from PAM-3 expressing cells and culture medium from PAM-15 expressing cells were used.

PCR Analysis of Region B Splicing: RNA was isolated and purified using RNA zol™B (Biotex Laboratories, TX). cDNA prepared from RNA using a First-stand cDNA synthesis kit (Pharmacia LKB, Sweden) was amplified by PCR at 35 cycles using primers: 5'-CTGAAGATGGGACTGTGTACA-3' (sense strand) and 5'-TCAAAAGTGTAAATGGGCAAATCT-3' (antisense strand), separated by electrophoresis on an agarose gel, and transferred to a nylon membrane and then hybridized with a probe (TCCTGGACCTCAATGCCAG, 2665a) end-labelled with [γ-^{32}P] ATP. The membrane was washed twice with 2 x SSC and 0.5% SDS at 25 °C for 30 min and twice with 0.1 x SSC and 0.5% SDS at 25 °C for 30 min, autoradiographed and followed to densitometoric analysis.

RESULTS

We have identified and sequenced two human PAM clones from QGP-1 cells derived from a human pancreatic tumor. PAM-3 encoded the transmembrane domain-coding sequence (Ba region) and PAM-15 lacked the Ba region and there were no base substitutions or deletions in the overlapping sequences of the two cDNAs (Fig. 1). cDNAs of PAM-3 and PAM-15 coded for 972 and 905 amino acid residues, respectively. As shown in Fig. 2, when the PAM-15-pSG5 lacking the Ba region was transfected to COS-7 cells, a 110 kDa protein was detected as the major component in the conditioned medium by immunoprecipitations using antisera directed to both the PHM and PAL domains of PAM (Fig. 2A). A faint immunoreactive band at 46 kDa was detected by the antiserum for PHM domain upon longer exposure in the conditioned media of COS-7 cells transfected with PAM-3-

Fig. 1. cDNA sequences of clone PAM-15 and PAM-3 and deduced amino acid sequences. Region Ba is inserted at position marked by ▼ in PAM-3.

pSG5, but no immunoreactive protein corresponding to the full length form was detected. In lysates of COS-7 cells transfected with PAM-3-pSG5 and PAM-15-pSG5, major immunoreactivities with the both antisera were

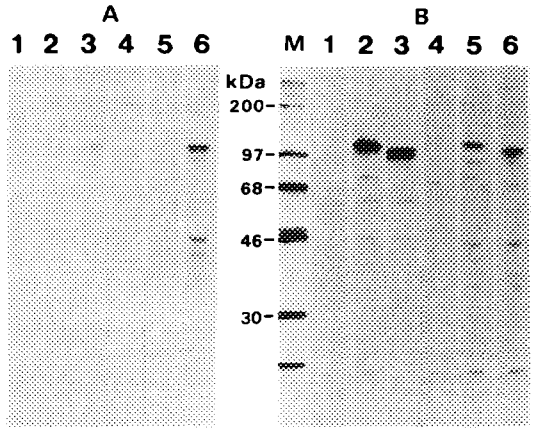


Fig. 2. SDS-PAGE of PAM produced by cells transfected with PAM cDNA. COS-7 cells transfected with PAM-3-pSG5 (lanes 2, 5) or PAM-15-pSG5 (lanes 3,6) and without transfection (lanes 1,4) were labeled with [^{35}S]methionine for 6 hrs. Culture media (A) and cell lysates (B) were immunoprecipitated with anti-PHM (lanes 1,2,3) and anti-PAL (4,5,6) antisera. Immunoprecipitates were analyzed by SDS-PAGE (10%)/fluorography. M: molecular size markers.

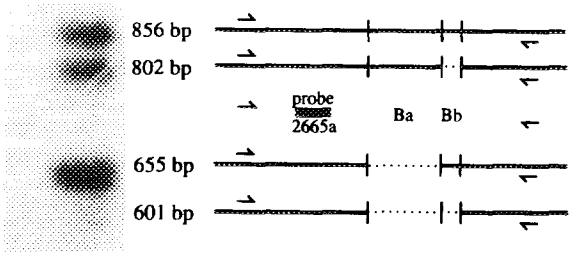
detected at 120- and 110- kDa for PAM-3 and 110- and 100- kDa for PAM-15 (Fig. 2B), respectively. No similarly sized immunoreactive bands were observed in lysates of untransfected COS-7 cells (Fig. 2B) and COS-7 cells transfected with another plasmid such as human chromogranin A-pSG5 (data not shown).

Membrane fractions and conditioned media of COS-7 cells transfected with expression plasmids carrying PAM-3 and PAM-15 cDNA exhibited higher amidating activities than those of control cells (Table 1). Cells transfected with PAM-15-pSG5 which lacks the transmembrane-domain sequence released a much higher activity for conversion of PST-29-Gly to

Table 1. Amidating activity in transfected COS-7 cells

Plasmid	α- Amidating activity (fmol/h/10^5 cells)		
	Membrane bound	Soluble	Conditioned medium
None	N.D.	14 ± 2	288 ± 60
PAM-3/pSG5	451 ± 8	24 ± 1	1215 ± 55
PAM-15/pSG5	31 ± 5	21 ± 4	20254 ± 882

Amidating activity is expressed as fmol of PST-29 produced from chromogranin A (273-302) (PST-29-^{30}Gly) (63 pmol). n=5.



(Southern blot shown above with probe 2665a, optical density quantitated by densitometry)

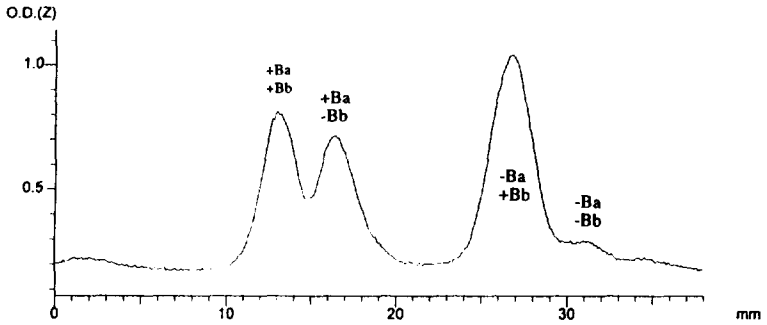


Fig. 3. Southern blot analysis of region B splicing in QGP-1 cells. Arrows denote sequence sites of sense and antisense primers used for amplification of cDNA by PCR; Ba, region Ba; Bb, region Bb; bp, base pairs.

PST-29 into culture medium than cells transfected with PAM-3-pSG5 of membrane-bound form.

The relative mRNA levels of forms having and missing Ba region in QGP-1 cells were examined in comparison with analysis of exon Bb referred by Eipper et. al (5). As shown in Fig. 3, the form missing Ba region was major mRNA species in QGP-1 cells, although there was also some of the form missing the short Bb exon.

The amidating activities of the membrane-bound (PAM-3) and secretory (PAM-15) forms of PAM were decreased by the absence of either copper, catalase or ascorbate. The optimal pH of the amidation was 5.4 for the both forms of PAM. Km values for PAM-3 and PAM-15 were 15.7 ± 3.1 μM and 12.4 ± 1.6 μM, respectively.

DISCUSSION

Two types of cDNA, human PAM-3 and PAM-15 were identified in a human pancreatic carcinoma cell. PAM-15 lacking transmembrane sequence is a human version of clone 203 found in the rat pituitary by Kato et al. (4) as summarized in Fig. 4a. This form has not been found in human

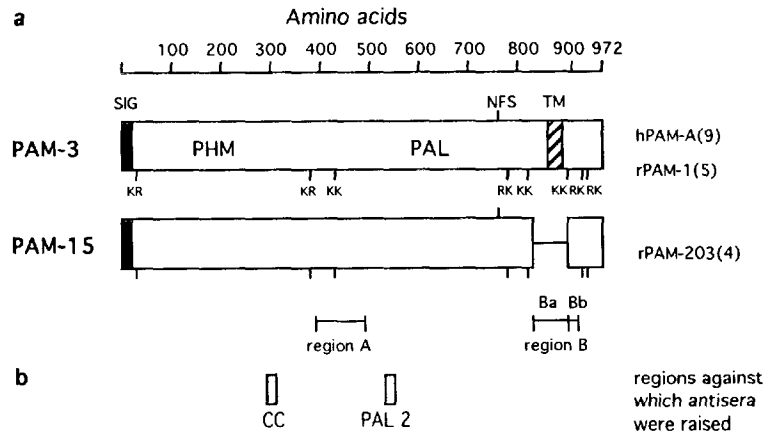


Fig. 4. a) Schematic representation of PAM cDNA clones identified in human pancreatic carcinoma (QGP-1) cells. SIG, signal sequence. TM, transmembrane domain. Paired basic amino acid residues representing potential cleavage sites (K=Lys, R=Arg) and N-glycosylation site (N=Asn, F=Phe, S=Ser) are indicated in single letter code. The forms corresponding to clones found in this study have been identified to right. b) Localization of the two regions against which antisera (CC and PAL 2) used for immunoprecipitations were raised.

and is neither of two forms (PAM A and PAM B) found from a human thyroid carcinoma by Glauder et al. (9). The PAM B found by Glauder et al. is a form lacking the region between the PHM and PAL domains of the PAM proenzyme and corresponds to rat PAM-2 found by Eipper et al. (5). The region is one which is called exon A by Eipper et al. (5) and is important in the endoproteolytic processing of the proenzyme PAM to PHM and PAL (14). PAM-3 corresponds to rat PAM-1 found by Eipper et al. (5) and was similar to the human PAM A (9). The human PAM A additionally encoded AAG at 5' and CAG at 3' sides of the region Ba shown in Fig. 1 and there were two base substitutions of nucleotide (nt) (G of nt 1801 in Fig. 1 is to A in nt 1009 of Glauder's and T of nt 2456 in Fig. 1 is to C in nt 2564 of Glauder's). By the substitution of nt 1801, ^{574}Gly in our PAM cDNA was ^{574}Glu in Glauder's. The substitution of nt 2456 did not replaced the amino acid.

It has been reported that exon Ba (exon 25) and exon A within the rat PAM gene account for most of the alternatively spliced forms of PAM mRNA and that soluble forms of rat PAM are produced when exon 24 is spliced to exon 26, skipping exon 25 entirely (15). The region Ba (201 bp) in Fig. 1 corresponds to what Eipper et al. calls exon Ba (204 bp) in rat PAM gene. Alternative splicing of exon Ba was preferred in human pancreatic carcinoma cells as observed in this study. Thus, the similar alternative

splicing between human and rat helps to confirm the similarity of the genomic organization of PAM which should exist between the both species.

Peptide α-amidation by PAM proceeds via a two-step mechanism catalyzed by the two domains PHM and PAL. It is known that the optimal pH of each enzyme activity is acidic (16) and that spontaneous conversion of the hydroxyglycine intermediate into α-amidated product occurs under alkaline conditions (17). The intravesicular pH is between 5 and 6 and the hydroxyglycine product of PHM is stable at this pH range (18). In this study, PAM-3 and PAM-15 which are consisting of PHM and PAL domains had optimal amidating activity at acidic pH. It has been demonstrated that PST (19) and PAM (20) localize in secretory granules of the pancreatic islet cells. This report confirms that two domains of PHM and PAL are both required for the amidation which is performed in secretory vesicles and demonstrates that the PAM expressed by QGP-1 pancreatic islet cells known to produce chromogranin A and PST operates effectively in the process carried out in secretory granules to produce bioactive PST from chromogranin A.

It has been recently reported that the cellular trafficking of integral membrane and soluble secretory granule-associated enzymes differs(21). cDNA clones of soluble and integral membrane forms of human PAM may be useful as a tool for further studies of delivery and targeting of proteins destined for secretory granules.

ACKNOWLEDGMENT

We thank Miss N. Iwamoto for technical assistance.

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