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ONTOGENETIC EXPRESSION OF PEPTIDYL-GLYCINE α-AMIDATING MONOOXYGENASE m RNA IN THE RAT PANCREAS

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Summary: Qualitative and quantitative expression of m.RNA coding for Peptidyl-Glycine α–Amidating Monooxygenase (PAM) in the developing rat pancreas was investigated by Northern and dot blot hybridization, with a bovine PAM c.DNA probe (0.7kb fragment). A specific hybridization signal was evidenced for a 3.7 kb m.RNA species. Measurement of PAM m. RNA rate during the rat pancreas ontogenesis revealed a biphasic profile which appeared corelated with that of gastrin and TRH m.RNA respectively. On the other hand, streptozotocin-treatment resulted in a 50% decrease of PAM m.RNA levels.

The biosynthesis of regulatory peptides in endocrine cells, is currently accepted to be achieved from a large precursor molecule processing through a series of post-translational modifications (e.g, phosphorylation, selective proteolytic cleavage, acetylation, amidation),(1).
In many instances, the mature peptides display a C-terminal α-amidated end which is required for their biological activity (2).
It is now well established that α-amidation is catalyzed by Peptidyl-Glycine α-Amidating Monooxygenase (PAM, [EC.1.14.17.3]), and occurs in the last step of peptide processing. The existence of this enzyme was first demonstrated in crude granules fractions from porcine pituitaries, and

Abbreviations: PAM, Peptidyl-Glycine α-Amidating Monooxygenase; TRH, Thyrotropin-Releasing Hormone: MOPS, 3-[N-Morpholino]propanesulfonic acid; SSC, Sodium-Sodium Citrate.

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shown to convert COOH-glycine extended precursor into carboxy α-amidated peptides (3). It has been studied extensively and shown to require copper, molecular oxygen and ascorbate (4).
PAM has been further purified from bovine neurointermediate pituitary (5), and recently its c.DNA has been cloned (6).
If the amidating enzyme has been mainly described in pituitary gland, it appears to have a wider tissue distribution. In rat pancreas, we have demonstrated that PAM activity exhibits an ontogenetic pattern which peaks at the third day of life (7). In addition, this differential expression seems to be correlated to the biosynthesis of TRH, as the tripeptide immunoreactivity culminates at the fourth postnatal day. Others had previously shown, that pancreatic gastrin is also subjected to neonatal changes (8).
In attempt to determine whether PAM activity is regulated at the pre- or the post-translational level we have studied the expression rate of PAM m.RNA in the developing rat pancreas.

MATERIAL AND METHODS

Animals
Sprague-Dawley rats from birth to adult stages were bred in our laboratory, while pregnant females were obtained from Janvier (Le Genest, France). Streptozotocin treatment was performed from birth to the day of sacrifice by daily i.p injection of the drug (100 µg/g in 100mM sodium citrate pH 4,6). Only pups with glycemia higher than 3 g/l were selected for m.RNA preparation.

m.RNA preparation
RNA were extracted in duplicate from pools of rat pancreas at different age, by the guanidine isothiocyanate method, and further purified by ultracentrifugation through a 5,7 M CsCl cushion (9). After a phenol-chloroform extraction, RNA were recovered by ethanol precipitation, and dissolved in 0,1% SDS. Total RNA amount was evaluated by absorbance at 260-280 nm, and selection of poly.A^+ m.RNA was carried out according to Aviv and Leder (11).

c.DNA probes
Bovine PAM and rat TRH c.DNA inserts (provided by B.A. Eipper and R.H. Goodman respectively) were excised from their respective vectors (Bluescript M13 and pUC 12) by Eco.R1 digestion. The probes were nick-translated with [$\alpha\ ^{32}P$] dCTP (3000 mCi/mmol) to a specific activity of $0,5\text{-}1.10^9$ dpm/µg.

Northern blot analysis
RNA were denaturated, fractionated on a 1% agarose gel containing 2,2 M formaldehylde buffered with 20 mM MOPS, 5 mM NaAcetate, 1mM EDTA and

electrotransferred onto Genescreen membrane (NEN Dupont) in 25 mM phosphate pH 6,5. Filters were then dried for 1 hr at 80°C before hybridization.
Blots were prehybridized under shaking at 42°C for 12 hrs. Prehybridization buffer was made of 40 mM phosphate pH 6.5, 1.6 X SSC, 4 X Denhardt's, 40% formamide, 0.2 µg/ml denaturated salmon sperm DNA, 0.08% SDS and 10% dextran sulfate. Hybridization was carried out for 20-24 hrs, by addition of the labelled probe in the same buffer (10^6 dpm/ml). Incubation temperature was 42°C and 55°C for PAM and TRH hybridization respectively.
Filters were subsequently washed twice with 2 X SSC, 0.1% SDS for 15 min (42°C), and with 0.1 X SSC, 0.1 % SDS for 20 min (42°C) before autoradiography.
PAM m.RNA molecular weight was estimated after staining of 28S and 18S ribosomal RNAs, and after hybridization of Hind.III digested λ.DNA (run on the same northern blot) with [^{32}P] nick-translated λ.DNA.

Dot blot analysis
RNA samples were diluted to appropriated concentrations, denatured and were spotted on Genescreen-plus membrane (NEN-Dupont) prealably and successively moistened with water and 20 X SSC using a manifold apparatus (Schleicher & Schuell). Filters were then washed twice with 0,5, M phosphate pH 6,5, and were allowed to dry overnight at room temperature.
PAM m.RNA hybridization conditions were the same as those described above for the northern blot analyses.
PAM m.RNA hybridization signal was corrected in terms of total polyA+ m.RNA signal. PolyA+ m.RNA measurement was directly performed on dot blots previously hybridized with PAM c.DNA probe. Briefly, dots were prehybridized for 12 hrs at 30°C in 6 X SSC and 10 X Denhardt's buffer containing 0.25µg/ml yeast t.RNA. Hybridization was performed in the same buffer for 20 hrs at 30°C after addition of 10^6 dpm/ml of [γ^{32}P] kinased 25-30mer oligo.dT. Dot blots were then successively washed twice at 30°C, with 2 X SSC, 25 mM phosphate pH 6,5 for 30 min and with 0.1 X SSC, 25 mM Phosphate buffer pH 6,5 for 30 min and were autoradiographed for 5-15 min.

Quantification of hybridization signal was evaluated on a area integrator equiped densitometor (Shimadzu). Results were expressed in arbitrary units: PAM m.RNA concentrations indexes were calculated as the density ratio of PAM signal to that of total polyA+.

RESULTS

In a preliminary experiment, PAM m.RNA detection was undertaken on northern blots of poly A+ m.RNA extracted from various bovine tissues and from rat fetal pancreas. As shown in fig.1A, inset, bovine PAM probe

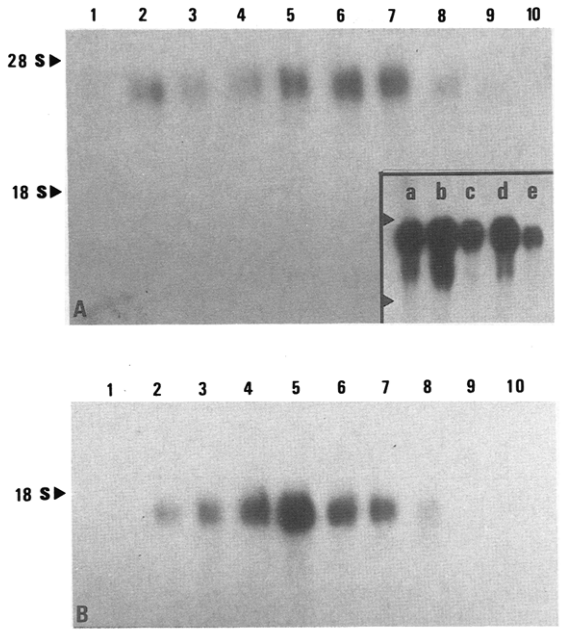
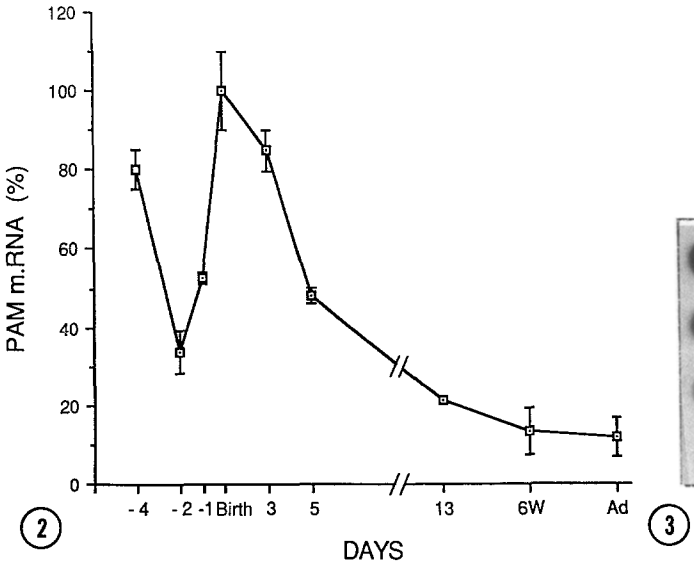


Fig.1: Northern blot analysis of PAM (panel.A) and TRH (panel.B) m.RNA at various time of the developement in the rat pancreas.
lanes 1, 2, 3, and 4, correspond respectively to fetus of 16, 18, 20, and 21 days; **lane 5** to birth; **lanes 6, 7 and 8** to 3-, 5-, and 13-day-old rats; **lane.9** to 6-week-old rat; **lane.10** to adult.
Inset: **a.** bovine anterior pituitary; **b.** bovine neurointermediate pituitary; **c.** bovine pancreas (3-month-old fetus); **d.** bovine pancreas (5-month-old fetus); **e.** rat pancreas (day 18th of gestation). Arrowheads indicate 28S and 18S r.RNA.

specifically hybridized to a single m.RNA species (approximately 3.7 kb long). Northern blot hybridization was then realized with the same probe on rat pancreatic poly.A+ m.RNA isolated at different ages (fig.1A). It appeared clearly that the hybridization signal varies from fetus to adult. These variations were more accurately measured after dot blot hybridization (fig.2). Results are expressed as a ratio of PAM m.RNA levels to total poly A+ m.RNA levels (arbitrary unit). PAM m.RNA levels evolutive profile was biphasic. PAM m.RNA were almost undetectable at day 16 of gestation, reached high level of expression at day 18, then decreased (day 20) and finally augmented to a maximum on day of birth. Thereafter, levels decreased towards barely measurable adult levels.
Interestingly, TRH m.RNA maximum expression coincided with the second PAM m.RNA peak on day of birth (fig.1B).
Streptozotocin-treatment results in a 50% decrease of PAM m.RNA levels on postnatal day 5 (fig.3). Similar results were obtained in experiments performed on postnatal day 3 (not shown).



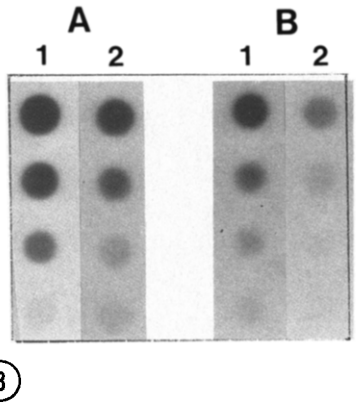


Fig.2: quantitative profile of PAM m.RNA levels during ontogenesis.
Results are expressed as the percent of PAM m.RNA levels obtained at birth. Vertical bars indicate the standard deviation of the data.
6W = 6 week-old rat, Ad = adult rat

Fig.3: effect of streptozotocin on PAM m.RNA levels in rat pancreas (dot blot analysis).
A: serial dilution of polyA+ m.RNA (extracted at birth), and hybridized either with the oligo.dT probe (1), or with the PAM probe (2).
B: PAM m.RNA hybridization on serial dilutions of poly.A+ m.RNA from (1) 5-day-old control rats, and (2) 5-day-old streptozotocin-treated rats.

DISCUSSION

The existence of specific m.RNA for PAM in the rat pancreas, clearly demonstrates that the synthesis of the enzyme is intrinsic to this organ. Furthermore, streptozotocin-treatment in neonates strongly suggests that one-half of PAM m.RNA is supplied by islet β cells. The other half may arise from others islet cells (e.g, gastrin and PP cells), and/or from the exocrine part of the pancreas. These data are supported from previous work of our group (7) indicating that subcellular distribution of pancreatic PAM enzymatic activity coincided with that of insulin and TRH. It was also shown in this study that PAM activity was depleted by 50% in pancreas from streptozotocin-treated rats. In addition, the enzyme has been evidenced in adult rat exocrine glands (parotid, submandibular, pancreas),(11). However, in pancreas, the levels reported were several order of magnitude lower than those we had measured in neonatal rat. Thus, the contribution from each part of the pancreas for PAM expression

is still questionable, and will benefit of in situ hybridization or immunohistochemical studies.

Of further interest, is the PAM m.RNA differential expression during ontogenesis. As it was already reported for other pancreatic hormones and exocrine enzymes (12), PAM m.RNA levels increase dramatically between the 16th and the 18th day of gestation, which coincides with the accumulation of insulin and zymogen granules (13). Since pancreatic PAM m.RNA ontogenetic profile is parallel to that of PAM activity previously described (7), our results suggest that the regulation of PAM expression takes place at the transcriptional level. Nevertheless, cofactors availability (such copper and/or ascorbate) may vary during ontogenesis and thus modulates PAM activity. Whether hormonal changes or nutritional status affect pancreatic PAM expression during the perinatal period remains to be clarified. Indeed it has already been shown that pituitary PAM activity and expression are down-regulated by glucocorticoids(6).

Interestingly, PAM activity seems to be coregulated with the expression of amidated peptides and their precursors. For instance, the PAM m.RNA peak on the 18th day of gestation in rat pancreas coincides with highest gastrin m.RNA levels as recently reported (14). In the same way, the second peak of PAM m.RNA (day of birth) corresponds to that of TRH. Therefore it may be that the mechanisms activating the expression of certain regulatory peptides genes also activate the genes coding for some processing enzymes involved in the post-translational maturation of these peptides precursors. On the other hand, factors modulating PAM activity (15) or PAM expression (6) in a given tissue, must logically affect α-amidated peptides production in this tissue.

In conclusion, our work clearly demonstrates the presence, in rat pancreas, of specific PAM m.RNA. It indicates that genes coding for the α-amidating enzyme and its potential substrates, are simultaneously expressed. Further studies will have to be performed to determine the regulatory elements which control PAM expression.

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