Volume 245, number 1,2, 70–74 FEB 06895 March 1989

Stable and transient expression of mouse submaxillary gland renin cDNA in AtT20 cells: proteolytic processing and secretory pathways

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Received 24 December 1988

Apart from kidney, where renin synthesis takes place in all mammals, the submaxillary gland (SMG) of most mouse strains constitutes an important source of an isoenzyme, renin-2, that is highly homologous to renal renin, but unglycosylated [(1982) Nature 298, 90–92]. This unique phenotype is due to the presence of an extra copy of the renin gene. A puzzling observation is that (pro)renin-2 cannot be detected in the kidney of these animals, although both mRNAs accumulate at similar levels [(1985) Proc. Natl. Acad. Sci. USA 82, 6196–6200]. In order to investigate whether (pro)renin-2 expression is detectable in mouse heterologous cell lines we transfected the renin-2 cDNA into AtT20 (pituitary corticotrope) and BTG9A (hepatoma) cells. Stable clones expressing renin were obtained in both cases. BTG9A cells secreted only prorenin while AtT20 cells secreted prorenin and active renin. In addition, in AtT20 cells the secretion of active renin was stimulated by 8-Br cAMP. Our results show that unglycosylated (pro)renin-2 can be expressed and secreted in two murine cell lines. Moreover, it is correctly processed to active renin and secreted upon stimulation in AtT20 cells.

Renin; Glycosylation, N-linked; Gene expression; Precursor processing; (AtT20 cell, Hepatoma)

1. INTRODUCTION

Renin catalyses the rate-limiting step in the regulation of blood pressure and saline balance. It is synthesized as an inactive precursor, prorenin, and converted to active renin by proteolytic cleavage following two basic residues [1]. In all species, renin gene expression is highest in the juxtaglomerular cells (JGC) of the kidney. However, in most mouse strains, a high level of renin expression is also observed in the submaxillary gland (SMG). These mice bear an additional copy of the renin gene (*Ren*2), the product of which is an unglycosylated thermosensitive isoenzyme (remin-2), 96% homologous to the glycosylated and thermostable renal renin (renin-1) [3,4]. The predominance of renin-2 in SMG is perfectly correlated with the greater efficiency of *Ren*2 promoter in this tissue. In contrast, in the kidney *Ren*1 and *Ren*2 mRNAs are present at the same level but only the protein product of *Ren*1 is detectable [2,3]. Specific regulation of translation or RNA stability seems unlikely, since non-coding 5′- and 3′-sequences are highly homologous in both mRNAs [1,5]. Therefore, it is probable that the absence of renin-2 isoenzyme in kidney is due to a posttranslational event: (pro)renin could be rapidly degraded in some intracellular compartment or after secretion. Interestingly, this same protein is detected in large amounts, and hence is not degraded, in the SMG.

We have undertaken the transfection of renin-2 cDNA into heterologous cell lines, since SMG- or

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Abbreviations: SMG, submaxillary gland; JGC, juxtaglomerular cells; AI, angiotensin I; POMC, proopiomelanocortin; MMTV, mouse mammary tumor virus; RSV, rous sarcoma virus

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JGC-derived cell lines are not available. The mouse pituitary corticotrope cell line AtT20 is suitable for this study, since it has the ability to carry out the posttranslational processing of its endogenous precursor proopiomelanocortin (POMC) and also other foreign precursors [6,7]. In addition, the two secretory pathways which have been described for JGC [8] also exist in AtT20 cells: a constitutive pathway where precursors are rapidly secreted, the other being of the regulated type in which precursor processing takes place and secretion of granule content can be stimulated by secretagogues. Finally, we have also used a constitutive secreting cell line, BTG9A, derived from a mouse hepatoma [9].

Here, we report results of transfection of both cell lines with renin-2 cDNA. We have analysed in detail the kinetics of secretion and maturation of prorenin in AtT20 cells.

2. MATERIALS AND METHODS

BTG9A and AtT20/D16v cells were grown in RPMI and DMEM media, respectively, supplemented with 10% fetal calf serum (FCS). Gene transfer was performed by the calcium phosphate coprecipitation method [9]. 48 h after transfection selective medium was added and stably transformed clones were isolated after 3–7 weeks of selection.

cDNA cloning of SMG preprorenin has been described [3]. The 5′ poly(G) tail was deleted by in vitro mutagenesis and the cDNA fragment was introduced into the *Sma*I site of pMSG (Pharmacia) under the control of MMTV promoter (pMSG.Rn14, fig.1a). This plasmid was used to transfect BTG9A cells and selection was performed through EcoGPT (*E. coli* guanosine phosphoribosyltransferase) in standard HAT (hypoxanthine, aminopterin, thymidine) medium [9]. In the case of AtT20 cells EcoGPT is unsuitable for selection [10], therefore *hph* gene (hygromycin B phosphotransferase; *Pvu*II fragment from plasmid pAG452.2 [11]) was introduced into the *Nde*I site of pMSG.Rn14 (pMH.Rn14, fig.1b). Selective medium contained 150 μg/ml of hygromycin B (Boehringer). Preprorenin cDNA was also exchanged with the *Cat* gene in pRSVCAT [12], (pRSV.Rn14, fig.1c). This construction was transfected into AtT20 cells and transient expression was assayed 65 h after transfection.

Renin activity was determined by radioimmunoassay (RIA) of angiotensin I (AI) generated after 1 h at 37°C in the presence of excess substrate from plasma of nephrectomised rats [13]. Total renin activity (prorenin plus active renin) was determined after trypsin activation [14]. Prorenin values were obtained from the difference between activities before and after trypsin treatment. Intracellular ACTH immunoreactivity was determined by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5 N acetic acid cell extracts and elution of 1 mm long gel slices in RIA buffer [15]. RNA preparation and Northern blotting have been previously described [16,17].

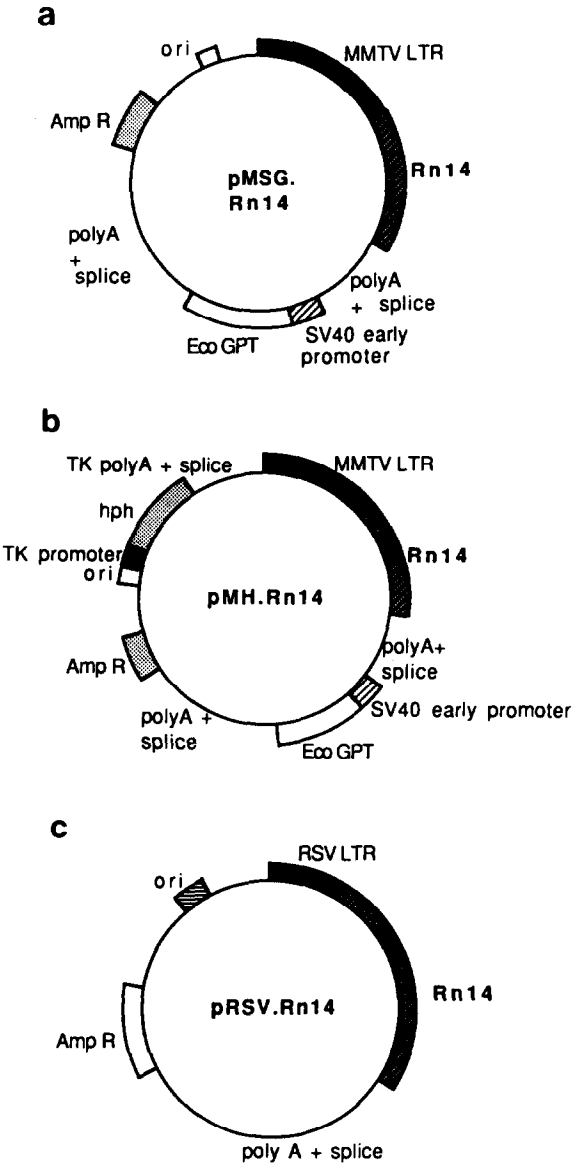

Fig.1. Diagram of preprorenin expression plasmids. Rn14, preprorenin-2 cDNA; MMTV LTR, MMTV long terminal repeat; polyA + splice, SV40 small t antigen splice site and polyadenylation site; Eco GPT, *E. coli* guanosine phosphoribosyl transferase; Amp R, ampicillin resistance gene; TK promoter and TK polyA + splice, herpes simplex virus timidine kinase promoter, polyadenylation and splice sites; hph, hygromycin B phosphotransferase.

3. RESULTS AND DISCUSSION

Two renin expression vectors, pMSG.Rn14 and pMH.Rn14 (fig.1a,b), were used to transfect BTG

and AtT20 cells, respectively. After selection, stable clones expressing renin were obtained for both cell types. Different clones were screened for total renin activity in the supernatant (after trypsin pretreatment). Two clones, BT.MSG.Rn14 and At.MH.Rn14 were selected for further studies, since their level of expression was among the highest tested.

Fig.2 shows Northern blot analysis of renin expression in both control and renin-expressing cells. RNA levels are approx. 500-times lower than those of SMG from male mice (estimated by dot blot; not shown). The size of the RNA corresponds to the expected 1400 bp of preprorenin RNA + 800 bp of SV40 sequences. Control cells do not produce detectable levels of renin mRNA.

BT.MSG.Rn14 cells produced only prorenin (after 24 h induction with 1 μM dexamethasone; not shown). In contrast, At.MH.Rn14 cells secreted both prorenin and renin (fig.3). Dexamethasone was not used to stimulate MMTV promoter in AtT20 cells because it inhibits secretion in these cells [18]. The kinetics of prorenin and renin secretion were studied in detail in At.MH.Rn14 cells. Cells were incubated in the presence or absence of the secretagogue 8-Br cAMP. Fig.3 shows basal and stimulated renin (a) or prorenin

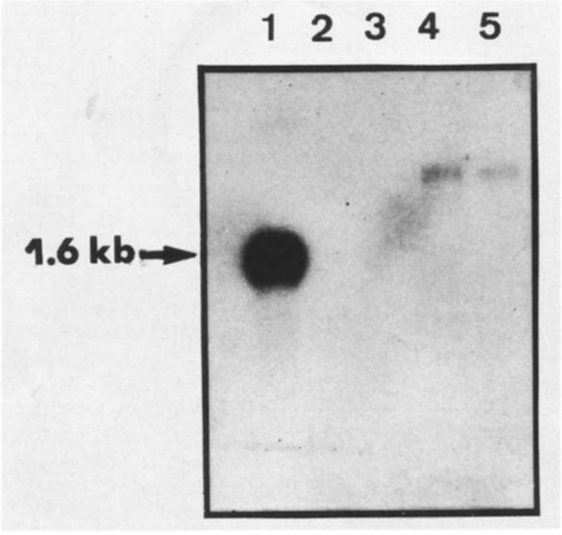

Fig. 2. Northern blot analysis of control and transfected cells total RNA. 15 μg total RNA from BTG9A cells (lane 2), AtT20 cells (lane 3), BT.MSG.Rn14 cells (lane 4), At.MH.Rn14 cells (lane 5) and 2 μg total male SMG mouse (lane 1) were probed with ^{32}P-labeled preprorenin cDNA.

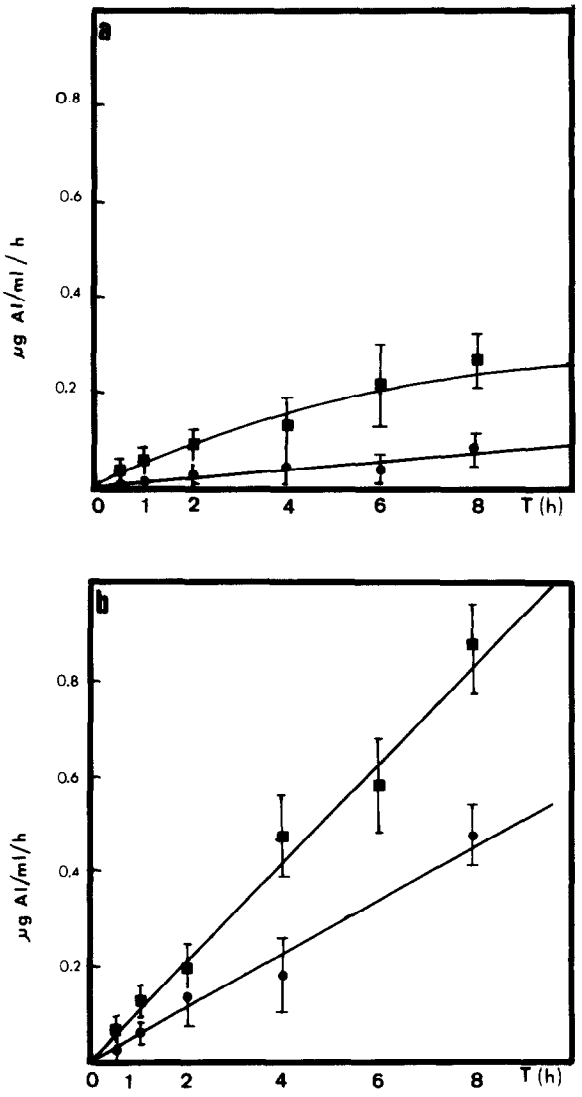

Fig. 3. Kinetics of prorenin and renin secretion of At.MH.Rn14 cells. Cells were cultured up to 80% confluence in 6-well plates. The medium was replaced at time = 0 by fresh medium with or without 5 mM 8-Br cAMP. Aliquots were taken at different times and assayed for renin activity (a) or total renin activity (after trypsin treatment). Prorenin values (b) were calculated by subtraction of renin activities from total renin activity. Renin activity is expressed as μg/ml of AI generated after 1 h incubation at 37°C with renin substrate. Values represent means ± SD of three different experiments, each assayed in duplicate.

(b) accumulation in the culture medium. In the presence of secretagogue there is an increase in secretion rate of active renin; interestingly, there is also a significant rise in precursor secretion. This elevation appears to arise from the regulated

secretory granule pool, since 8-Br cAMP is known not to affect constitutive secretion.

Previous work has pointed out that human renin is completely processed in the regulated secretory pool of AtT20 cells [19,20]. A possible explanation for this difference was that we had used a variant AtT20 cell line with diminished processing capacity. Fig.4a shows that the parental cell line has a normal processing capacity, since the majority of the intracellular POMC-like immunoreactivity corresponds to ACTH and glycosylated ACTH (peaks 3,4). However, for clone At.MH.Rn14 these peaks appear less significant with respect to POMC peaks 1,2 (fig.4b). Therefore, the reduced prorenin maturation observed for this clone correlates with diminution of endogenous precursor processing. This cannot be explained by competition of prorenin with the endogenous precursor POMC as has been described for proneuropeptide Y [7], since renin expression in these cells is many orders of magnitude lower than that of endogenous POMC (estimated by cell labeling, immunoprecipitation and PAGE; not shown). It seems probable that a cellular variation occurred during subcloning, involving a general decrease in maturation capacity. This result emphasizes the need to ascertain the processing capacity of each stable clone.

The possibility could still not be ruled out that the incomplete maturation observed was due to some specific feature of prorenin-2 as a substrate of AtT20-processing enzyme(s) (i.e. the absence of glycosylation, an amino acid mutation neighbour-

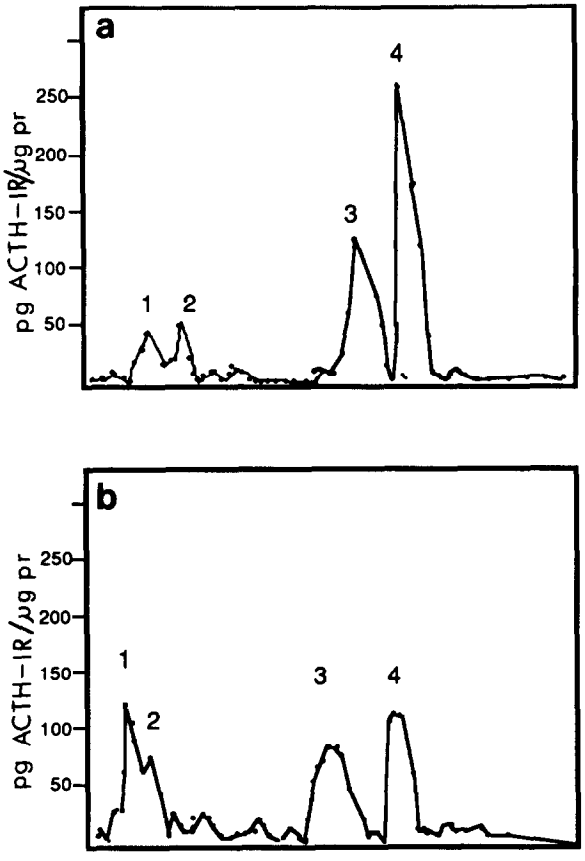

Fig. 4. ACTH immunoreactivity of AtT20 intracellular extracts. Acetic acid (5 N) extracts from AtT20 (a) or At.MH.Rn14 (b) cells were analysed by 15% SDS-PAGE, 1 mm long gel slices were eluted and ACTH immunoreactivity (IR) was determined. Peaks: 1,2, glycosylated and unglycosylated POMC; 3,4, glycosylated and unglycosylated ACTH, respectively.

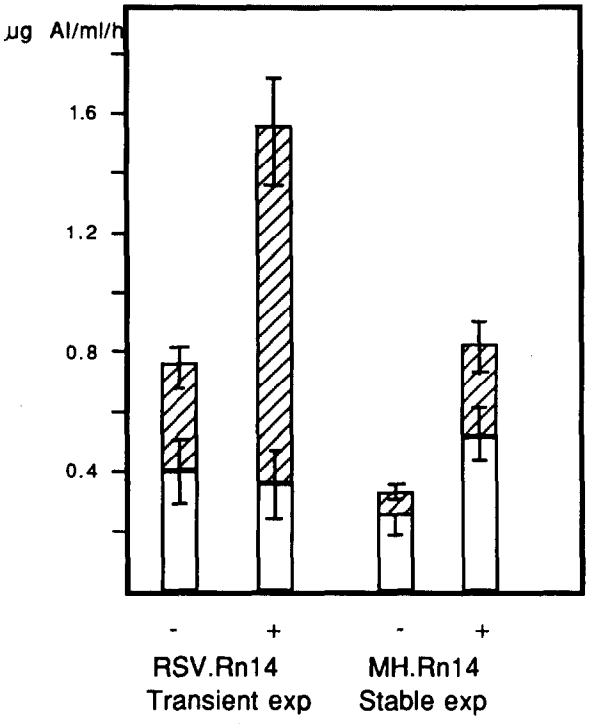

Fig. 5. Transient expression of prorenin and renin from pRSV.Rn14 transfected AtT20 cells. 65 h after transfection with pRSV.Rn14, media were replaced by fresh medium with or without 5 mM 8-Br cAMP. At.MH.Rn14 cells were also plated at the same density and media replaced ± 8-Br cAMP. Media were collected after 4 h and assayed for renin activity (hatched bars) or total renin activity (after trypsin treatment). Prorenin values (empty bars) were calculated by subtraction of renin activities from total renin activity. Renin activity is expressed as µg/ml of AI generated after 1 h incubation at 37°C with renin substrate. Values represent means ± SD of five different experiments, each assayed in duplicate.

ing the processing site, etc.). In order to obtain results independent of subcloning variations, we have analysed transient renin expression. For this purpose we have transfected the AtT20 cells with preprorenin-2 cDNA under the control of RSV promoter (pRSV.Rn14, fig.1c), which is much stronger than MMTV promoter in these cells [10]. Fig.5 shows that as much prorenin as active renin was secreted from these cells under non-stimulating conditions. In the presence of secretagogue only active renin release was stimulated, indicating the complete maturation of prorenin in the 8-Br cAMP responsive secretory pool. Therefore, renin-2 apparently behaves similarly to human renin in AtT20 cells [19,20].

The present data demonstrate that in AtT20 cells, mouse prorenin-2 is appropriately stored, processed and released upon stimulation with 8-Br cAMP. This indicates that in these cells unglycosylated renin-2 is stable and not (or not completely) proteolytically degraded. Moreover, in the constitutive secreting hepatoma cell line prorenin-2 is also correctly released, although exclusively in the precursor state. The apparent lack of (pro)renin-2 in JGC of two renin gene-bearing mice could therefore be due to some cell-specific factor(s) which would affect intracellular (pro)renin-2 stability.

Acknowledgements: We thank Drs Marc Dreyfus, Isabelle Chupin and Gordon Langsley for critical reading of the manuscript. We gratefully acknowledge Dr Jean-Louis Guenet for help in the preparation of renin substrate. The text was skilfully typed by Mme Catherine Gachet. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (INSERM 861023), the Fondation pour la Recherche Médicale Française and the Institut Pasteur de Paris.

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