Volume 297, number 3, 302–305 FEBS 10692 February 1992

Immunological characterization of the endoproteases PC1 and PC2 in adrenal chromaffin granules and in the pituitary gland

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Received 16 December 1991

Specific antisera against synthetic fragments of the endoproteases, PC1 and PC2, were used to characterize these proteins. In one-dimensional immunoblots these antisera labelled components of 85 kDa for PC1 and of 70 kDa for PC2 in purified bovine chromaffin granules and anterior and posterior pituitary of ox and rat. In membranes of bovine chromaffin granules glycoprotein H was identified as the major PC2 immunoreactive spot. A major part of these endoproteases appeared membrane bound.

Endoprotease; PC1; PC2; Chromaffin granule; Adrenal medulla; Pituitary

1. INTRODUCTION

PC1 and PC2 are two endoproteases which split proteins at dibasic amino acid residues [1–3]. Convincing evidence has been presented that these proteases are prohormone convertases [4,5]. The messages coding for these proteins are found in neuroendocrine tissues where such prohormone processing takes place [2]. In bovine chromaffin granules amino acid sequences derived from both PC1 and PC2 were found in a membrane glycoprotein [6] which has previously been named glycoprotein H [7,8]. In the present study these proteases were identified in tissue extracts from pituitary and adrenal medulla by immunoblotting with antisera against synthetic peptides derived from their amino acid sequences.

2. MATERIALS AND METHODS

Membranes and soluble lysates of bovine chromaffin granules were obtained as previously described [9,10]. Large granule fractions (20,000 × *g* for 20 min) obtained from bovine anterior and posterior pituitary and total rat adrenal medulla were resuspended in 5 mM Tris–succinate buffer (pH 5.9) and separated into a soluble and membrane bound fraction [9,10]. Anterior and neurointermediate lobes of rat pituitary were dissolved directly in electrophoresis buffer.

Glycoproteins from soluble proteins of bovine chromaffin granules were isolated as described earlier [11]. One- and two-dimensional electrophoresis followed either by immunoblotting or concanavalin A (ConA) labelling was performed as described previously [11]. The antisera were raised in rabbits by immunization with synthetic peptides coupled to keyhole limpet hemocyanin via a cysteine residue. The peptides were synthesized with standard t-Boc Chemistry followed by HF cleavage and reversed phase HPLC purification. For PC1 an 18-mer corresponding to the C-terminal amino acids 709–726 [2] and for PC2 a 19-mer corresponding to the C-terminal amino acids 592–610 [2] were used. Protein content was measured by the Folin method [12].

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3. RESULTS

Immunolabelling with an antiserum against the PC1 peptide identified a major band at 85 kDa in chromaffin granule membranes (Fig. 1). In addition a faster moving band (70 kDa) was also clearly labelled. For the soluble proteins of bovine chromaffin granules (up to 200 μg) only two faster moving bands were weakly labelled. Analogous results were obtained for the membranes of total rat adrenal medulla. For this species we also detected an immunolabelled band of 85 kDa in the soluble proteins.

With the antiserum against PC2 we found a strongly labelled band (70 kDa) in the membranes of bovine chromaffin granules and an analogous but faintly labelled band in the soluble proteins. In rat adrenal medulla analogous bands were present, but in the soluble fraction a strongly reactive band of 40 kDa was labelled (see Fig. 2).

In two-dimensional gels the positions of PC1 and PC2 were identified by sequential labelling with ConA and the respective antisera (see Fig. 3). The PC1 antiserum labelled a minor ConA binding spot, which has not been previously characterized [8]. Immunostaining for PC2 exactly coincided with a glycoprotein previously named glycoprotein H [7].

When a ConA eluate from the soluble lysate of bovine chromaffin granules was subjected to the same procedure (compare [11]) the glycoprotein H present in

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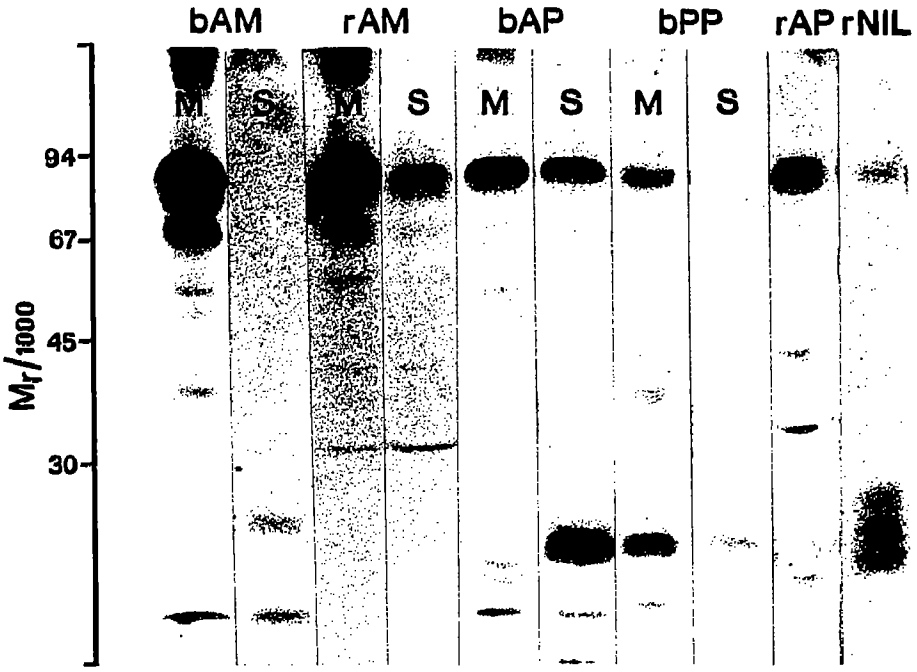

Fig. 1. Immunoblots with anti-PC1 antiserum. Tissues were subjected to one-dimensional SDS-electrophoresis followed by immunoblotting. Amounts of proteins were: bAM (bovine adrenal medulla:chromaffin granules), M (membrane bound) and S (soluble) 100 μg each; rAM (rat adrenal medulla), M and S, 150 μg each; bAP, bPP (bovine anterior and posterior pituitary), M and S, 200 μg each; rAP (rat anterior pituitary), 40 μg; rNIL (rat neurointermediate pituitary), 100 μg.

this extract could again be labelled with the PC2 antiserum (results not shown). In this extract a minor spot corresponding to the PC1 spot in the membranes could also be immunolabelled.

Anti-PC2 labelled corresponding bands in rat and bovine posterior and anterior pituitary with a much stronger reaction in the posterior lobe (Fig. 2). In bovine pituitary a considerable amount of PC2-reactive material is soluble. For PC1 in both rat and bovine pituitary an immunoreactive band was present at 85

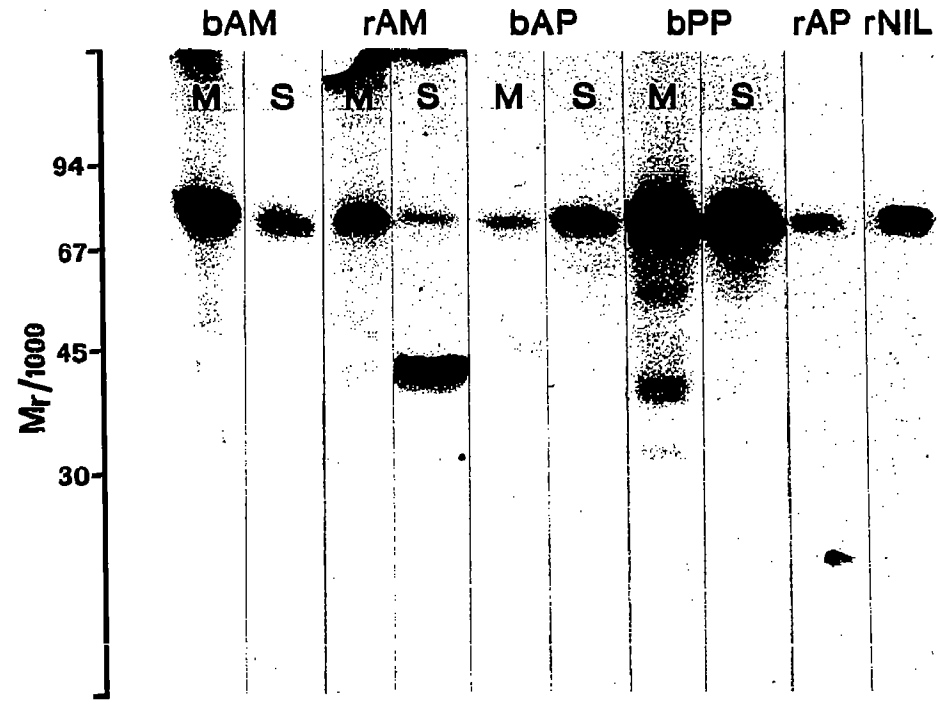

Fig. 2. Immunoblots with anti-PC2 antiserum. The results are presented as described in Fig. 1 (same amounts of protein).

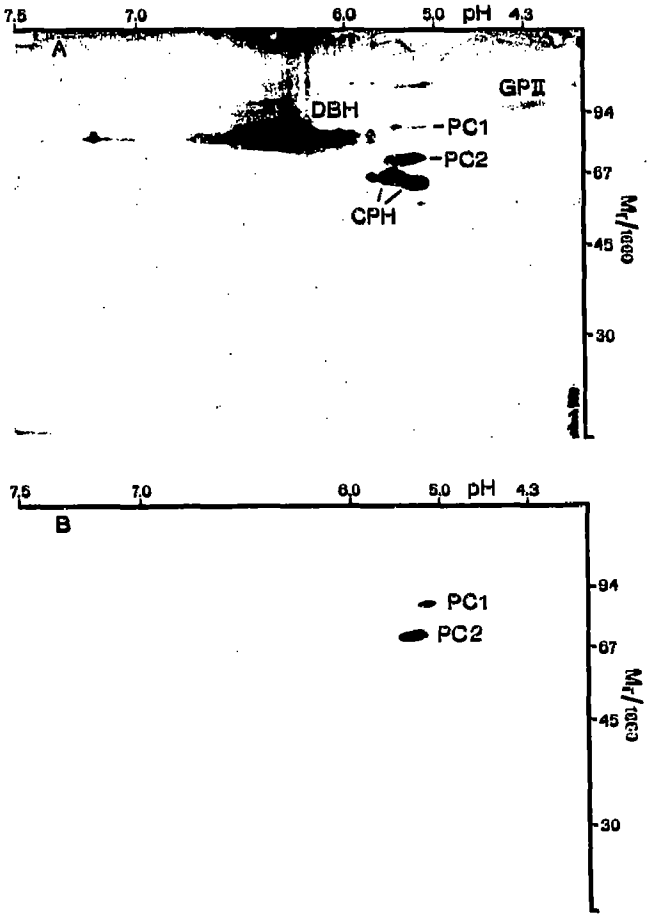

Fig. 3. Two-dimensional immunoblots with antisera against peptides present in PC1 and PC2. Membrane proteins of bovine chromaffin granules were subjected to two-dimensional electrophoresis. After transfer to nitrocellulose the proteins were first labelled with concanavalin A (A) and then immunolabelled (B). Immunolabelling was first performed with the antiserum against the PC1 peptide which labelled the spot marked PC1 shown in B, followed by labelling with the PC2 antiserum which labelled the spot marked PC2 in B.

kDa which corresponds to the band identified in chromaffin granules. However additional faster moving bands were apparently also immunostained with the PC1 antiserum. These bands were especially marked in the soluble fractions of bovine anterior pituitary and in rat total posterior pituitary.

4. DISCUSSION

This study establishes that antisera against PC1 and PC2 immunostain specific proteins in adrenal medulla as well as in posterior and anterior pituitary of rat and ox. In the membranes of bovine chromaffin granules the PC2-reactive protein is apparently identical to glycoprotein H, whereas PC1 is represented by a glycoprotein not previously identified. These results are only in partial agreement with data of Christie et al. [6]. These authors found amino acid sequences for both PC1 and PC2 in the glycoprotein H spot and concluded that this protein spot contained the mature forms of both enzymes. This interpretation was rather surprising since, as judged from the amino acid sequence [2], the molecular weights of these proteins differ by about 10,000 kDa. Our results now establish that PC1 is actually represented by a protein of larger molecular weight, probably the intact proprotein. In addition a minor PC1 component was present which had a molecular weight corresponding to glycoprotein H. Christie et al. [6] might have picked up this component since their N-terminal amino acid analysis indicated that this protein started at amino acid 84 from the N-terminal site. However, surprisingly, their analysis also indicated that the PC2 component present in glycoprotein H started at N-terminal amino acid 85 which would make it again by about 10,000 kDa smaller than PC1. Is it possible that the N-terminus of intact PC2 in the glycoprotein H spot was blocked and that the N-terminus actually determined was exposed by autodigestion of the molecule? In any case our results are best explained by PC1 and PC2 being present as intact molecules. Previously we identified carboxypeptidase H as the glycoproteins J and K [11] and, as shown by N-terminal analysis glycoprotein J, represents the complete amino acid sequence of this enzyme [13].

However, our present results also indicate that some endogenous proteolysis of PC1 and PC2 is occurring. Thus in rat adrenal medulla a significant additional PC2-reactive band is found in the soluble proteins. For PC1 there are also some additional bands of smaller molecular size present. Since our antibody reacts only with the C-terminal end, a larger N-terminal part of the molecule as formed by the removal of a smaller C-terminal part might also be present as a significant molecule, but was not picked up by our antiserum. In any case our results are consistent with endogenous proteolytic processing of PC1 and PC2, which agrees with the presence of several dibasic cleavage sites in the molecules [1–3]. Both endoproteases represent significant membrane components. This appears remarkable considering the difficulties encountered in discovering these enzymes. Only part of the endoproteases as shown here, and for carboxypeptidase as shown previously [11], is soluble. Thus these proteins belong to the group of proteins found in chromaffin granules which are partly membrane bound and partly soluble, and which include dopamine β-hydroxylase [14] and glycoprotein III [15]. Are these proteins sorted into chromaffin granules in a membrane bound form, where only limited amounts become soluble for acting on the secretory proteins? These proteins together with the soluble endoproteases might be secreted, whereas the membranes with the rest of the enzymes still being bound to them might be re-used for forming new vesicles [16]. At present this remains speculative. We are even not yet in a position to conclusively state how these proteins are

anchored in the membranes, possibly by an uncleaved signal peptide, as suggested for dopamine β-hydroxylase [17], or via its C-terminal, as proposed for the carboxypeptidase H [18].

Acknowledgements: This study was supported by the Fonds zur Förderung der wissenschaftlichen Forschung (Austria), by the Dr. Legerlotz-Stiftung and by fonds from the NIH (Grant NF 22697, R.H.A.).

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