Proinsulin processing in the rat insulinoma cell line INS after overexpression of the endoproteases PC2 or PC3 by recombinant adenovirus

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Proinsulin is converted to insulin by the two endoproteases PC2 and PC3. For complete conversion to insulin, cleavage must occur at both the B-chain/C-peptide and C-peptide/A-chain junctions of proinsulin. Studies *in vitro* have shown the specificity of PC3 for the B-chain/C-peptide junction and that of PC2 for the C-peptide/A-chain junction. In contrast, studies *in vivo* have suggested that the proinsulin cleavage substrate specificity of these two endoproteases might be more complex. We have now used recombinant adenovirus to overexpress PC2 or PC3 in the rat insulinoma cell line INS. These cells convert proinsulin more slowly than primary pancreatic β -cells, possibly reflecting their lower levels of PC3. Infection of INS cells with the corresponding recombinant adenovirus led to 5–10-fold and 20–40-fold in-

creases in PC2 and PC3 expression respectively. Recombinant adenovirus is thus an effective tool for expressing proteins at high levels in slow-growing INS cells. Overexpression of either PC2 or PC3 in INS cells led to a striking acceleration of conversion of proinsulin to insulin and to a decreased accumulation of the conversion intermediate des-64.65-split proinsulin (cleaved only at the A-chain/C-peptide junction). There was no detectable accumulation of des-31.32-split proinsulin (cleaved only at the B-chain/C-peptide junction) after overexpression of either enzyme. Taken together, the data indicate that when expressed at high levels, both PC2 and PC3 seem to be able to cleave proinsulin at both its junctions *in vivo*.

INTRODUCTION

A large number of protein precursors are processed at basic amino acids [1]. The specific endoproteases performing such cleavage belong to the family of mammalian subtilisin-like serine proteases [1,2]. Two members of this family, PC2 and PC3, have been localized to a variety of endocrine and neural tissues [3–5] and have been shown to be involved in proteolytic processing of a variety of prohormones, including proinsulin, in the brain and many endocrine tissues. The relative level of expression of PC2 and PC3 varies in different cell types, which often results in differential processing of prohormones. Such differential processing does not apply to proinsulin, which can be converted only to insulin and C-peptide. Proinsulin does, however, contain two pairs of basic residues linking the insulin A- and B-chains to the C-peptide (connecting peptide) and both these junctions must be cleaved (and the resulting C-terminal basic residues trimmed off by carboxypeptidase H [6]) to produce mature, fully processed insulin and C-peptide. Given that insulin is much more active than either proinsulin or its conversion intermediates (proinsulin molecules cleaved at just one of their two junctions) [7] and that one conversion intermediate (des-31.32-split proinsulin) is found in unusually high amounts in the circulation of individuals with non-insulin-dependent diabetes (reviewed in [8]), understanding the enzymology of proinsulin conversion is of particular importance.

Both PC2 and PC3 have been implicated in proinsulin processing [9–11]. Studies *in vitro* have suggested that PC3 cleaves efficiently only at the B-chain/C-peptide junction and PC2 at the

C-peptide/A-chain junction [9–14]. We have suggested previously that proinsulin conversion *in vivo* is dependent on the structure and/or sequence of its cleavage sites [15–19] and on the relative levels of expression or activity of the two endoproteases [20]. In this latter context we have thus shown that cells of the rat insulinoma line INS [21] convert proinsulin to insulin more slowly than native β -cells [20], possibly owing to a lower ratio of PC3:PC2 in INS cells than in pancreatic β -cells [20,22], suggesting that PC3 is a dominant force in proinsulin conversion [20].

Expressing proteins in cells that grow slowly in culture is problematic with conventional transfection techniques. We have now taken advantage of recombinant adenovirus [23] to examine the impact on proinsulin conversion of overexpressing PC2 or PC3 in the slowly growing INS cells. The results indicate that these enzymes are less fastidious than previously supposed in terms of proinsulin junction specificity.

MATERIALS AND METHODS

Materials

Chemicals were from Fluka (Buchs, Switzerland) or Sigma (St. Louis, MO, U.S.A.), and [3 H]leucine (80 Ci/mmol) was from ARC (St. Louis, MO, U.S.A.). Rabbit antiserum against PC3, recognizing the N-terminus of the mature enzyme, was kindly provided by Dr. Iris Lindberg (New Orleans, LA, U.S.A.). The antiserum against the C-terminal part of PC2 was generously given by Dr. Chris Rhodes (Boston, MA, U.S.A.). The anti-actin

Abbreviations used: Ad-, contained in adenovirus; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; moi, multiplicity of infection; pfu, plaque-forming units; X-Gal, 5-bromo-4-chloroindol-3-yl- β -D-galactopyranoside.

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antibody was provided by Dr. G. Gabbiani (Geneva Switzerland). The 293 cells and the recombinant adenovirus expressing β -galactosidase were gifts from Dr. Newgard (Dallas, TX, U.S.A.). The human PC2 cDNA was from Dr. Steiner (Chicago, IL, U.S.A.), and the mouse PC3 cDNA was from Dr. Nakayama (Tsukuba, Japan).

Construction of recombinant replication-defective adenovirus

Recombinant adenovirus was constructed by homologous recombination of the plasmid pJM17 [24], containing a modified adenovirus genome, and the plasmid pACCMV-hPC2 or pACCMV-mPC3 (where CMV stands for cytomegalovirus). The plasmids pACCMV-hPC2 and pACCMV-mPC3 were constructed by inserting hPC2 or mPC3 cDNA into the *Sma*I site of the polylinker of pACCMV.pLpA [23,25] downstream of a CMV promoter and upstream of an SV40 polyadenylation site. Nearly confluent 50 cm² dishes of 293 cells were co-transfected with pJM17 and pACCMV-hPC2 or pACCMV-mPC3 by a modified calcium phosphate precipitation method. After 2–3 weeks, lysis could be observed in all dishes. The medium was collected and used to infect fresh 293 cells. A clonal recombinant virus was selected as follows: a serial dilution of the second lysis was prepared and used to infect 20 cm² dishes of 293 cells. One day after infection, the dishes were overlaid with 0.7% agar in Dulbecco's modified Eagle's medium/10% (v/v) fetal calf serum (DMEM/FCS) and cultured for 7–10 days until plaques could be observed. Ten plaques were selected and used to reinfect 20 cm² dishes of 293 cells. Once the cells had lysed, the medium was subjected to PCR analysis with a 5' primer derived from the CMV promoter, and a 3' primer from the SV40 polyadenylation signal, from plasmid pACCMV.pLpA, to verify the correct insertion of the PC2 or PC3 cDNA. The selected clone was then amplified and purified on a CsCl gradient. The purified virus preparation was titrated as follows: 20 cm² dishes of 293 confluent cells were infected with a serial dilution of the virus preparation and overlaid with 5 ml of 0.7% agar as described above. When the plaques had reached a visible size, 2 ml of 0.7% agar (in DMEM/FCS) containing 0.018% Neutral Red was overlaid on the first agar layer. After a few hours of incubation, the plaques appeared as clear spots on a red background. Typically a titre of approx. 10⁹ plaque-forming units (pfu)/ml was obtained after a 1 h infection and approx. 10¹¹ pfu/ml after a 24 h infection. Despite the relative ease of seeing plaques when Neutral Red is added to agar, values for pfu are still by nature only indicative of the true number of viral particles in any given preparation. Because these pfu values were used to calculate the multiplicity of infection (moi) for each series of experiments, the given moi must itself be taken as only a rough indication of viral titre.

Infection of INS cells with recombinant adenovirus

Nearly confluent INS cells were incubated with recombinant adenovirus for 1 h at moi values of 0.2–100 in DMEM/FCS. The cells were then rinsed and incubated for 24 h at 37 °C in DMEM/FCS.

Analysis of overexpression of PC2 or PC3 by Western blots

The level of overexpression of PC2 or PC3 was analysed by infecting INS cells with increasing moi values from 0.2 to 100. After 24 h the cells were extracted, the proteins in the extracts were separated on SDS/7–10% (w/v) gels and transferred to a nitrocellulose membrane as described [22]. Immunodetection was performed with an enhanced chemiluminescence detection procedure from Amersham (Amersham, Bucks., U.K.). Antibody

dilutions, incubations and densitometric measurements were performed as described previously [22].

Pulse-chase analysis of proinsulin conversion

Dishes (10 cm²) of subconfluent INS cells were infected with Ad-PC2 or Ad-PC3 as described above. One day after infection, the cells were labelled in Krebs buffer containing 0.3% glucose and 33 μ Ci/ml [³H]leucine for 10 min and then chased in Krebs buffer containing 0.03% glucose and 1 mM non-radioactive leucine for up to 120 min. The medium was then collected and the cells were extracted and subjected to HPLC analysis as described previously [20]. With cells infected with Ad-PC2 it was found that radioactive peaks unrelated to (pro)insulin co-eluted with such peptides from HPLC. It was therefore necessary to immunoprecipitate extracts of such cells with anti-insulin serum as described in detail previously [26].

Detection of *LacZ* gene expression

INS cells were infected with Ad- β Gal as described above. One day after infection the cells were washed and then fixed with 0.5% glutaraldehyde and stained with 5-bromo-4-chloroindol-3-yl- β -D-galactopyranoside (X-Gal).

RESULTS AND DISCUSSION

Two enzymes have been proposed as being responsible for converting proinsulin to mature insulin [9]. Studies *in vitro* examining the cleavage of human proinsulin by semi-purified enzymes have suggested that PC3 cleaves very specifically at the B-chain/C-peptide junction leading to 32.33-split proinsulin [11], whereas PC2 cleaves at the C-peptide/A-chain junction yielding 65.66-split proinsulin [10] and further seems to display a net preference for des-31.32-split proinsulin rather than the intact prohormone [13]. Comparisons of the kinetics of proinsulin conversion with the relative levels of PC2 and PC3 in a variety of cell types have, however, suggested that PC3 might also be implicated in cleavage at the C-peptide/A-chain junction *in vivo* [19,20,27]. It is thus still not clear whether PC2 and PC3 have strict substrate specificity for proinsulin cleavage at physiological levels of expression and whether the specificity is maintained when these endoproteases are overexpressed, as with conversion of pro-opiomelanocortin [28] or proglucagon [29].

INS cells are an interesting model for studying proinsulin processing, with slow conversion attributed to a high ratio of PC2:PC3 [20]. However, these cells proliferate only slowly, with a doubling time of about 100 h, which limits their suitability for stable expression of transfected proteins. We have taken advantage of the recombinant adenovirus expression system [23] for overexpressing PC2 and PC3 in these cells.

Infection of INS cells with Ad-PC2 or Ad-PC3

PC2 and PC3 were overexpressed in INS cells by using a defective recombinant adenovirus, containing PC2 (Ad-PC2) or PC3 (Ad-PC3) cDNA, driven by the CMV promoter. The level of overexpression could be modulated by infecting the cells at a different moi (Figure 1). Toxic effects were observed at high moi values in INS cells, consisting of morphological changes and cell detachment from culture dishes. There was also a loss of radioactive products, which seemed to be due to the intracellular degradation of proinsulin or (pro)insulin-related products (results not shown). To study proinsulin conversion it was therefore important to avoid very high moi values while allowing for appropriate overexpression of the enzymes. For this purpose the

Figure 1 Overexpression of PC2 or PC3 in INS cells

Nearly confluent INS cells were infected with recombinant adenovirus Ad-PC2 (right panel) or Ad-PC3 (left panel) at increasing moi values, and Western blots were performed with antibodies against PC2 (right panel) or PC3 (left panel) as described in the Materials and methods section. For PC2, the 0 moi lane was exposed to film for twice as long as the other lanes.

Figure 2 HPLC analysis of (pro)insulin-like products after 10 min label and 30 min chase in INS cells 24 h after infection with Ad- β -Gal (a), Ad-PC2 (b) or Ad-PC3 (c)

The cells were infected at the following moi values: Ad-PC2, 1; Ad-PC3, 29; Ad- β -Gal, 10. Cell extracts were injected into the HPLC column without (Ad- β -Gal and Ad-PC3) or with (Ad-PC2) prior immunoprecipitation with anti-insulin serum, as described in the Materials and methods section. Radioactivity in fractions eluting as insulin has not been corrected for the relative numbers of leucine residues in insulin (6 residues) compared with proinsulin (11 residues), thereby underestimating the true extent of conversion. The elution times of insulin I (1), insulin II (2), proinsulin I and II (3), des-64.65-split-proinsulin I (4) and des-64.65-split-proinsulin II (5) for each representative profile are indicated by arrows.

cells were infected at increasing moi values and the level of expression of PC2 or PC3 was determined by Western blots. Moi values of approx. 1 for Ad-PC2 and 29 for Ad-PC3 were shown to be appropriate, leading to a 5–10-fold overexpression of PC2 and a 20–40-fold overexpression of PC3 (Figure 1). At these

levels of infection, none of the above-mentioned toxic effects of the virus could be observed. There is, however, some evidence that even at very low moi values more subtle effects occur (see below), but we believe these to be tolerable in view of the important advantages offered by this DNA delivery system.

Characterization of overexpressed PC2 and PC3

In uninfected native INS cells, both the 75 and 67 kDa forms of PC2 are observed by Western blots [20]. Identical forms of PC2 were observed in INS cells overexpressing PC2, and there was no increase in their ratio with increasing titres of infection up to a moi of 30. In cells overexpressing PC3, the enzyme was present as the major 66 kDa mature form. At very high levels of expression, the 87 and 84 kDa pro-forms appeared as relatively minor bands (Figure 1). From these observations we conclude that the post-translational modifications of the *trans*-proteins, including the conversion of the pro-form to the mature form of overexpressed PC2 or PC3, are similar to or identical with those of the endogenous endoproteases occurring in native cells.

Effects of overexpression of PC3 on the kinetics of conversion of proinsulin

INS cells were pulse-labelled with [3 H]leucine and chased for up to 120 min to analyse the conversion of newly synthesized proinsulin. The cells were extracted and (pro)insulin-related products were quantified directly by HPLC. INS cells, like their primary rat β -cell counterparts, express the two non-allelic rat insulin genes. Our HPLC system was able to separate insulin I and II and des-64.65-split proinsulin I and II, but not proinsulin I and II (Figure 2). Knowing the ratio of expression of proinsulin I and II in INS cells [20], and given that these are the only peptides of interest that could not be separated, it was possible to calculate the proportion of proinsulin I and II in the proinsulin peak at any given time of chase.

In non-infected INS cells, proinsulin I and II were converted to insulin with a significant, transient accumulation of des-64.65-split proinsulin, whereas no des-31.32-split proinsulin I or II could be observed at any time, as reported previously [20].

As a control for the use of recombinant adenovirus to overexpress proteins in INS cells, the cells were infected with a recombinant adenovirus expressing β -galactosidase (Ad- β Gal). Cells expressing β -galactosidase could be detected by their colour after incubation with the substrate X-Gal, and most were seen to express the foreign protein even at low moi values (results not

Table 1 Times at which 50% of total (pro)insulin-related radioactivity appeared in the form of proinsulin or insulin

Subconfluent INS cells were either not infected (—) or infected with Ad- β Gal, Ad-PC3 or Ad-PC2 at the following moi values: Ad-PC2, 1; Ad-PC3, 29; Ad- β Gal, 2–17. They were then labelled for 10 min with [3 H]leucine and chased for up to 120 min; the cells were extracted and analysed as described in the legend to Figure 3. The radioactivities eluting from HPLC as proinsulin, des-64.65-split-proinsulin and insulin are expressed as a percentage of the sum of these three insulin-related products at each chase time (see the legend to Figure 3). The results show the times at which proinsulin or insulin represented 50% of total (pro)insulin-related products and are presented as the means $\pm$ S.E.M. for three independent experiments.

	Time (min)			
	(—)	Ad- β Gal	Ad-PC3	Ad-PC2
Proinsulin I	41 $\pm$ 2	47 $\pm$ 1	23 $\pm$ 1	19 $\pm$ 1
Insulin I	63 $\pm$ 7	60 $\pm$ 2	24 $\pm$ 2	23 $\pm$ 2
Proinsulin II	50 $\pm$ 4	52 $\pm$ 1	38 $\pm$ 1	20 $\pm$ 2
Insulin II	88 $\pm$ 5	75 $\pm$ 3	41 $\pm$ 1	29 $\pm$ 5

shown). A slight variation in proinsulin conversion was observed in these cells compared with native INS cells (Table 1). Proinsulin I disappeared at a slower rate, whereas insulin I appeared at approximately the same rate as in non-infected cells, reflecting a lower accumulation of des-64.65-split proinsulin I. Similar results were obtained for proinsulin II conversion (Table 1). We do not yet know whether this slight difference in proinsulin conversion was due to overexpression of β -galactosidase itself or to the expression of viral proteins. Although the variation in proinsulin conversion was small, we elected to use Ad- β Gal-infected INS cells as a control for all further experiments. A representative HPLC profile of such cells extracted at 30 min of chase is shown in Figure 2a, and the kinetics of disappearance of proinsulin I and of appearance of des-64.65-split proinsulin I and insulin I in Figure 3.

In cells infected with Ad-PC3 and shown to overexpress PC3 protein by approx. 20–40-fold (Figure 1), proinsulin conversion was accelerated strikingly. Even by 30 min of chase (Figure 2c) there had been extensive conversion to fully processed insulin. Detailed analysis of the kinetics of conversion revealed a more rapid disappearance of labelled proinsulin, less accumulation of des-64.65-split proinsulin and a more rapid appearance of labelled insulin throughout the course of the 120 min chase than in control (Ad- β Gal infected) cells (Figure 3). The acceleration of conversion by PC3 was comparable for proinsulin I and II (Table 1).

We had expected a more rapid rate of conversion of proinsulin in the face of higher levels of expression of PC3 in INS cells, given that we had previously attributed slow conversion in these cells to their unusually low level of PC3 [20]. It was nevertheless surprising that overexpression of PC3 did not lead to generation of detectable amounts of des-31.32-split proinsulin during the chase, given the previously documented specificity of this enzyme for the B-chain/C-peptide junction [11]. It seems that cleavage at the B-chain/C-peptide junction of proinsulin I and II is the rate-limiting step in conversion in INS cells regardless of the absolute level of PC3 (or PC2; see below). It is nevertheless important to note that the percentage of radioactivity in the form of des-64.65-split proinsulin was considerably less in INS cells overexpressing PC3 (Figure 3). This observation, taken together with the dramatic acceleration of proinsulin conversion in INS cells overexpressing PC3, indicates that this endoprotease can cleave at both junctions of proinsulin. There is evidence from

Figure 3 Time course of the disappearance of proinsulin I (a) and of the appearance of des-64.65-split proinsulin I (b) and insulin I (c) in INS cells infected with Ad- β Gal, Ad-PC2 or Ad-PC3

Subconfluent INS cells were infected with Ad- β Gal (●), Ad-PC2 (▲), or Ad-PC3 (□) at moi values as indicated in the legend to Figure 2, then pulse-labelled, extracted and analysed as described in the legend to Figure 2. Radioactivity eluting as insulin was corrected by a factor of 11/6 to account for the 11 residues of leucine in proinsulin compared with 6 in insulin. The radioactivities in proinsulin, des-64.65-split proinsulin and insulin were then expressed as percentages of the sum of the three. The results are presented as means $\pm$ S. E. M. for three independent experiments.

other cell types to support this contention. Thus in AtT20 cells, in which PC3 is expressed at exceptionally high levels in the face of vanishingly small, even undetectable, levels of PC2 [3,30,31], proinsulin is converted quite rapidly [18,26]. Furthermore when PC3 is expressed in the absence of any PC2 in COS cells, again proinsulin is converted to insulin [19,27]. Interestingly, however, in both these situations, des-31.32-split proinsulin is the major conversion intermediate.

Effects of overexpression of PC2 on the kinetics of conversion of proinsulin

In Ad-PC2 infected cells, because additional non-(pro)insulin-related radioactive products were detected, cell extracts had to be immunoprecipitated with an anti-insulin antibody before being subjected to HPLC analysis. Overexpression of PC2 was, if anything, more effective in accelerating proinsulin conversion than that of PC3 (Figures 2 and 3 and Table 1). Again, smaller amounts of des-64.65-split proinsulin were generated during the chase (Figure 3b). Interestingly, the acceleration by overexpression of PC2 of the conversion of proinsulin II was more pronounced than that of proinsulin I. The end result was that the kinetics of conversion of the two proinsulins were similar in INS cells overexpressing PC2, in contrast with control cells in which proinsulin I was converted more rapidly than proinsulin II. By using similar reasoning to that above for PC3, it is concluded that PC2 can cleave proinsulin at both its junctions, albeit with

a preference for the C-peptide/A-chain junction. It is further reasoned that slow cleavage at the B-chain/C-peptide junction of proinsulin II compared with proinsulin I (attributed to absence of a P4 basic residue, four residues N-terminal to the site of cleavage, at this junction in proinsulin II [15]) is essentially overcome in the face of high levels of PC2. It is relevant to note here that in COS cells, PC2 is able to convert rat proinsulin II and a mutant human proinsulin (with a P4 basic residue at the C-peptide/A-chain junction), although less efficiently than PC3 [19].

In conclusion, recombinant adenovirus offers a powerful and versatile means of expressing proteins in INS cells. It is, however, important to relate the impact of the expression of a desired foreign protein to possible effects of expressing unwanted viral proteins, and therefore to use an moi high enough for the desired level of expression of the *trans*-protein but low enough to restrict untoward toxic effects of viral proteins to an acceptable minimum. When PC2 and PC3 are overexpressed in this way in INS cells, proinsulin conversion is markedly accelerated to rates exceeding those seen in primary β -cells [15]. The results suggest that, at least when overexpressed, both enzymes are able to cleave proinsulin at both its junctions.

We thank Dr. Yves Rouillé and Ms. Jocelyne Kaufmann for useful discussions and for help in writing this paper. This work was supported by grant number 31-40839-94 from the Swiss National Science Fund.

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