Processing and release of human proinsulin-cleavage products into culture media by different engineered non-endocrine cells: a specific assessment by capillary electrophoresis

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Abstract

The aim of this study was to compare the metabolic pathway to mature insulin through the intermediate forms (32–33 split, 65–66 split, des31,32 and des64,65) in human or murine cells engineered for the release of wild-type human proinsulin and in a genetically mutated one, in the search for a new approach for an insulin-dependent diabetes mellitus cure by gene therapy.

Primary human fibroblasts, myoblasts and stabilized cell lines (HepG2 and NIH3T3) were transduced either with a retroviral vector coding for wild-type proinsulin or for a genetically mutated one, carrying cleavage sites sensitive to furin. The pattern of all the proinsulin cleavage products released into the cell culture supernatants was analyzed by capillary electrophoresis.

All the cells transduced with the wild-type gene released intact proinsulin. HepG2 released a considerable

amount of 65–66 split and des64,65, while primary myoblasts released all the intermediate forms and a limited amount of mature insulin. All the cells transduced with a furin-sensitive proinsulin gene released a higher amount of mature insulin (23–59% conversion yield) than the cells expressing wild-type proinsulin, whereas the total insulin was nearly constant. Only primary cells released all the cleavage products.

Screening a wide variety of non-endocrine cells has revealed a large difference in the processing and release of immature and mature insulin forms, pointing to human hepatic cells as the most efficacious. Capillary electrophoresis provided on-line and in a single run a complete overview of the proinsulin metabolic pathway in different cells.

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Introduction

In the search for a gene therapy-based cure for diabetes (Kolodka *et al.* 1995, Levine 1997), many different non-endocrine cells have been genetically modified by introduction of the human pre-proinsulin gene (HPI), thus obtaining synthesis of the prohormone (Groskreutz *et al.* 1994). In non-endocrine cells, furin, a Golgi-anchored enzyme (Groskreutz *et al.* 1994, Vollenweider *et al.* 1995) whose concentration is cell line specific, exerts a proteolytic activity like PC2 and PC3 (endocrine cell-restricted endoproteases) recognizing a cleavage site composed of the tetra-basic amino acid sequence Arg⁻⁴-X⁻³-Lys/Arg⁻²-Arg⁻¹-X⁺¹. As a following step, the human proinsulin (hPI) cDNA was modified, inserting the furin-cleavable motifs at the B chain/C-peptide and C-peptide/A chain junctions (Falqui *et al.* 1999). The furin-cleavable hPI

gene (FurHPI) permanently expressed a double-mutated protein more easily cleavable by furin into mature insulin at a conversion rate which correlates well with the furin expression (Yanagita *et al.* 1993). In the secretory granules of pancreatic beta cells hPI is processed to mature human insulin (hI) through the formation of four intermediate forms (des31,32; 32–33 split; des64,65 and 65–66 split) (Fig. 1) (Rhodes & Halban 1987, Vollenweider *et al.* 1992, Halban 1994, Hutton 1994, Kahn & Halban 1997) whose accumulation can vary in relation to pathological conditions (Sizonenko *et al.* 1993, Ward *et al.* 1987, Yoshioka *et al.* 1988, Mykkanen *et al.* 1997).

In order to verify the proinsulin metabolism in non-endocrine cells transduced both with the wild-type human proinsulin gene (HPI) and with modified HPI, a specific method able to quantify all the conversion products is necessary. RIA gives the 'total immunoreactive insulin'

with the modified gene constructs. To reach this goal hPI, hI and the split and des intermediate forms released into the cell supernatants were purified by solid phase extraction and directly quantified by capillary zone electrophoresis (CZE) coupled with UV absorbance (Arcelloni *et al.* 1998).

Materials and Methods

Standard proteins

hI was purchased from Boehringer (Mannheim, Germany) and the lyophilized powder reconstituted with bi-distilled water to a final concentration of 1.6 mmol/l. hPI, des31,32, des64,65, 32–33 split and 65–66 split were kindly donated by Eli Lilly and Company (Indianapolis, IN, USA) and reconstituted with water to a final concentration of 20 µmol/l. Aliquots were stored at –20 °C.

Retroviral vector and cell transduction

The insertion of the EcoR1/Hga1343 bp fragment of the wild-type hPI into the EcoR1/Hpa1 sites of retroviral vector L-X-SFCM, derived from the Moloney murine leukemia virus backbone, was described previously (Falqui *et al.* 1999). For cell marking and selection, the L-X-SFCM vector coding for the truncated human low-affinity nerve growth factor receptor (Δ LNGFR), was employed (Mavilio *et al.* 1994). Subsequently, two mutations were introduced by PCR technique into the wild hPI cDNA, to obtain the permanent synthesis of a proinsulin molecule with modified cleavage sites (Falqui *et al.* 1999). The modified hPI cDNA (FurHPI) was then inserted into the LX-SFCM retroviral vector. The generation of infective supernatants with high titers of viral particles was obtained in packaging cells, as previously described (Mavilio *et al.* 1994, Falqui *et al.* 1999). Infective supernatants were harvested from confluent packaging cells and overlaid for 24 h onto target cells in the presence of polybrene (8 µg/ml). After 1 week, the percentage of retrovirally transduced cells was evaluated by flow cytometry for *de novo* surface expression of Δ LNGFR.

Cell culture and media collections

Primary human fibroblasts (PHF) and HepG2 were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Life Technologies, Paisley, Scotland) supplemented with 10% (v/v) fetal calf serum (FCS) (Sigma, St Louis, MO, USA), whereas murine fibroblasts (NIH3T3) were cultured in DMEM supplemented with 10% (v/v) newborn calf serum and primary rat hepatocytes (PRH) in Medium 199 (GIBCO) supplemented with growth factors. Primary human myoblasts (PHM) were

cultured in RPMI medium containing 20% FCS. For CZE analysis, culture media were harvested after 24 or 48 h of incubation. Cells were counted and hPI, hI and intermediates accumulation in culture media was expressed as pmol/24 h per 10^6 Δ LNGFR-positive cells.

CZE

All reagents were of analytical grade. Diethylenetriamine (DETA), N-[tris(hydroxymethyl)methyl]-3-aminopropane-sulfonic acid (TAPS), trifluoroacetic acid and acetonitrile were from Fluka (Buchs, Switzerland). The analyses were carried out with slight modifications of the previously published procedure (Arcelloni *et al.* 1998) to allow resolution of all the proinsulin cleavage products. A P/ACE 5010 equipped with UV detector and computer controlled (Beckman, Palo Alto, CA, USA) was used. All the proteins were separated in an uncoated fused silica capillary (50 µm i.d. × 27 cm total, 20 cm to the detector) eluted with a running buffer composed of TAPS 100 mmol/l, pH 11.5, DETA 17.8 mmol/l and methanol (45:45:10 v/v/v), at a constant voltage of +9 kV (about 18 µA). The detector was set at 200 nm and the capillary thermostated at 20 °C.

Purification procedure

Cell supernatants (20–30 ml) were purified as described (Arcelloni *et al.* 1998). Briefly, after a solid-phase extraction with Sep-Pak Vac 6 ml, C₁₈ 500 mg cartridges (Waters, Milford, MA, USA), the fraction containing the proteins of interest was concentrated under vacuum and ultrafiltered (Centricon-50, 50 000 M_r cut-off; Amicon Division, Beverly, MA, USA). Aliquots of the ultrafiltrates were injected (5–10 s, corresponding to 9.8–19.5 nl) at pressure mode 3.4×10^{-3} MPa.

hI and hPI routine assay

RIA Determination of IRI was carried out by RIA (Insulin RIA Kit; Incstar Corporation, Stillwater, MN, USA). The standard curve concentration ranged from 0 to 600 pmol/l. ¹²⁵I-insulin, antibody and anti-insulin serum were incubated overnight at 4 °C. Precipitating complex was added, vortexed, centrifuged and the supernatant discarded. Radioiodine in the precipitates was counted for 60 s using a gamma scintillation counter. The polyclonal antibodies used cross-react 100% with bovine and porcine insulin and 30–80% with proinsulin-like molecules.

hI IRMA The determination of mature insulin was done with a BI-INSULIN IRMA kit (Sanofi-Diagnostic Pasteur, Paris, France), based on a monoclonal antibody. The analytical range was 1.4–3000 pmol/l and the

Figure 2 Representative electropherogram of hI, hPI and hPI conversion products (about 4 μ M each protein) injected for 5 s (about 50 fmol of each protein) in an uncoated fused silica capillary under the electrophoretic conditions described in Materials and Methods.

cross-reactivity was 100% with porcine and bovine insulin and 65–66 split or des64,65 proinsulin, while <0.0001% with hPI and 32–33 split and des31,32.

ELISA Two non-isotopic microplate assays (Dako, Glostrup, Denmark) for total hPI and intact hPI (IhPI) proinsulin were used. The first assay detects unprocessed hPI and cross-reacts (65–99%) with all intermediate forms of hPI (32–33 split, des31,32; 65–66 split and des64,65) while the IhPI assay detects the unprocessed molecule along with 65–66 split and des64,65. Antibodies used in the kits give a cross-reactivity of 0.1% with insulin and C-peptide. The standard analytical range was 0–100 pmol/l and the detection limit <0.2 pmol/l for both (as provided by the manufacturer).

Results

Validation of CZE analysis showed a calculated recovery rate for each protein from solid-phase extraction of $85 \pm 14\%$ (s.d.) and a sensitivity limit of the CZE analysis of 2 fmol injected, corresponding to 0.5 nmol/l in the supernatant. The response was linear in the 0.1–10 μ mol/l range corresponding to 2–200 nmol/l in the supernatants. With respect to the already published method (Arcelloni

et al. 1998), the separation has now been optimized to include also 32–33 split. In Fig. 2 is reported the electrophoretic separation of the six standard proteins. Figure 3 shows the electropherograms obtained by analysis of the supernatants from non-transfected PHM cells, PHM-HPI and PHM-FurHPI cells. The abundant peak at 6.4 min (just before hI) is unrelated to cell metabolism, being present in all the fresh culture media employed for cells growth (data not shown). By adding increasing amounts of standard hI to wild-type supernatant, however, its resolution from the big peak migrating just before was never hindered. The major peaks at 7.4, 7.8 and 10.0 min, together with numerous other minor peaks were present in both transfected cells but not in the non-transfected lines. Although their identification is not available yet, they are probably related to proinsulin production by these cells and can be also used for monitoring cell transfection.

The identification of the (pro)insulin-related proteins within the rather complex electrophoretic profiles was accomplished by an extensive post-analytical review with the System Gold software (Beckman version 8.1). First a comparison with a non-transfected supernatant spiked with the pure standard and extracted as the unknown samples was done (external calibration). The good reproducibility of the migration time (Mt) and the presence of

Figure 3 Representative electropherograms of non-transduced PHM (A), PHM-HPI (B) and PHM-FurHPI (C) supernatants. Samples were injected for 10 s and the peaks of hPI, 32–33 split, des31,32, 65–66 split, des64,65 and hI were identified by enrichment of aliquots of the samples with the pure standard proteins. The peak having Mt 6.4 min was present in all the profiles (A, B, C) and is related to a culture medium component. The other major peaks (Mt 7.4, 7.8 and 10 min) found only in both the transfected cells and not in the wild-type ones, may be related to cell transfection and hPI production.

some important peaks taken as ‘references’ for peak assignment ensured the accuracy of the procedure. The identification of the separated components, however, was always confirmed by co-injection of the samples with one pure standard at a time. The increase of only one peak with respect to the surrounding ones was always well evidenced and confirmed the previous assignment. No peaks at the Mt of the proteins under investigation were found in the CZE profile of the non-transfected cells.

(Pro)insulin metabolism in transduced cell supernatants

hPI was produced by all the HPI cell lines investigated, at a rate ranging from 0.6 ± 0.2 (s.d) to 1.9 ± 1.2 pmol/24 h per 10^6 cells, while mature insulin (as expected) was never identified in these cells, except for a small amount (less than 1.0% of the total (pro)insulin-related proteins) in PHM-HPI cells (B in Fig. 3; Table 1). Looking at the sum of all the (pro)insulin-related proteins, PHM-HPI displayed the highest production efficiency. The metabolism of HepG2 cells seems to be more prone toward maturation products like 65–66 split and des64,65 (35 and 46% respectively) while in PHF culture media the major components were intact hPI and des31,32 (50 and 35%

respectively) and NIH3T3 released a high amount (68% of all the (pro)insulin-related proteins) of 32–33 split. When cells were genetically modified to express the FurHPI, mature hI was found to accumulate in the culture media of all the FurHPI cell lines at a rate ranging from 0.6 ± 0.1 pmol/24 h per 10^6 cells in PRH and NIH3T3 cells to 2.4 ± 0.3 pmol/24 h per 10^6 cells in the HepG2 (Table 1). The conversion of intermediates to hI was decreased in the order HepG2>NIH3T3>PRH>PHF>PHM (59, 46, 32, 26 and 23% respectively). PHF and PHM showed in the media the presence of all the intermediate forms of hPI to hI maturation (C in Fig. 3), while in HepG2 cells only accumulation of des64,65 (34%) was found. Conversely, PRH released into the supernatant only a large amount of des31,32 (68%) in addition to mature hI (32%). Traces of unprocessed hPI were observed in all cells except PRH (Table 1).

Immunological assays

In non-transduced cells, RIA and hI IRMA values were always under the detection limit (data not shown). Comparison of results obtained by CZE with RIA and hI IRMA determinations are reported in Table 2. The sum of

Table 1 CE analysis of supernatants of retrovirally transduced cells. Values are means ± s.d. pmol/24 h per 10⁶ cells of three different cell incubations. Each purified sample was injected into the CZE five times

	hPI	32–33split	des31,32	65–66split	des64,65	hI	Total
HPI cells							
PHM	1.9 ± 1.2	0.6 ± 0.1	1.1 ± 0.8	0.5 ± 0.3	1.6 ± 0.1	0.05	5.8 ± 1.0
PHF	1.8 ± 0.2	0.2 ± 0.1	1.6 ± 0.6	n.d.	n.d.	n.d.	3.6 ± 1.2
NIH3T3	0.6 ± 0.2	1.3 ± 0.9	n.d.	n.d.	n.d.	n.d.	1.9 ± 0.9
HepG2	0.6 ± 0.3	n.d.	n.d.	1.3 ± 0.1	1.7 ± 0.6	n.d.	3.7 ± 0.9
FurHPI cells							
PHM	1.3 ± 0.1	0.6 ± 0.1	1.9 ± 0.1	0.6 ± 0.4	1.1 ± 0.2	1.6 ± 0.5	6.8 ± 1.0
PHF	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.9 ± 0.6	1.2 ± 0.1	1.2 ± 0.3	4.6 ± 1.0
PRH	n.d.	n.d.	1.3 ± 0.5	n.d.	n.d.	0.6 ± 0.1	1.9 ± 0.7
NIH3T3	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	n.d.	n.d.	0.6 ± 0.1	1.3 ± 0.2
HepG2	0.2 ± 0.02	n.d.	n.d.	n.d.	1.4 ± 0.3	2.4 ± 0.3	4.1 ± 1.3

n.d. under the detection limit.

all (pro)insulin-related proteins calculated by CZE was in good agreement with the RIA quantification, while IRMA value appeared overestimated with respect to the mature hI quantification by CZE.

Some of the samples were tested also with two ELISA kits developed for total and intact hPI determination on human plasma. The tests carried out directly on the cell culture media yielded an hPI overestimation of 130–600% (data not shown). This was corrected neither by 1:8000 dilution with a human serum pool, nor by purification of the supernatant or by the procedure described for CZE.

Discussion

The gene therapy approach to insulin-dependent diabetes mellitus requires the development of a retroviral vector-based system able to induce the permanent production, in

a regulated way, of mature insulin in endocrine (Clark *et al.* 1997) or non-endocrine (Falqui *et al.* 1999) cell lines. To evaluate the metabolic activity of transfected cells, however, an accurate method able to detect the residual hPI, mature hI and all the other related cleavage products is necessary. The determination of hI and intact hPI (Deberg *et al.* 1998, Houssa *et al.* 1998) without interference from the intermediates is now possible thanks to the availability of RIA and ELISA tests for plasma or serum based on the use of specific monoclonal antibodies. However, the quantification of each single split- or des- form remains still laborious. Some groups successfully employ HPLC to separate hPI, hI and the intermediate forms and RIA to quantify the proteins in the collected fractions (Nagi *et al.* 1990, Vollenweider *et al.* 1992, Ostrega *et al.* 1995). Alternatively, cells are labeled with [³H]leucine, [³⁵S]cysteine or [³⁵S]methionine, immunoprecipitated with anti-insulin antibodies and the radioactivity associated with each peak evaluated directly on the collected

Table 2 Comparison between CZE, RIA and IRMA quantification of total (pro)insulin related-proteins and mature hI. Values are means ± s.d. pmol/24 h 10⁶ cells of three determinations

	Total (pro)insulin-related proteins		Mature hI	
	RIA	CZE	IRMA	CZE
HPI cells				
PHM	6.0 ± 1.5	5.8 ± 1.0	1.3	0.05
PHF	2.3 ± 0.7	3.6 ± 1.2	n.d.	n.d.
NIH3T3	2.2 ± 1.6	1.9 ± 0.9	n.d.	n.d.
HepG2	3.8 ± 1.3	3.7 ± 1.6	n.a.	n.d.
FurHPI cells				
PHM	4.1 ± 1.2	6.8 ± 1.0	2.3 ± 0.2	1.6 ± 0.5
PHF	5.0 ± 2.3	4.6 ± 1.0	3.0 ± 0.7	1.2 ± 0.1
PRH	1.3 ± 0.2	1.9 ± 0.7	0.8 ± 0.3	0.6 ± 0.1
NIH3T3	1.5 ± 0.2	1.3 ± 0.2	1.0 ± 0.3	0.6 ± 0.1
HepG2	4.5 ± 1.4	4.1 ± 1.3	4.2 ± 0.2	2.4 ± 0.3

n.d. under the detection limit.
n.a. quantification not available.

fractions (Neerrman-Arbez *et al.* 1993, Sizonenko *et al.* 1993, Kaufmann *et al.* 1997). Although these remain the only methods providing the required sensitivity for the determination in plasma of all the proinsulin intermediates, the approach is based on two or more steps and on the use of labeled compounds. Recently, an isotope dilution MS (ID-MS) method was proposed for insulin analysis in human plasma (Stocklin *et al.* 1997) and affinity CE-based methods employing fluorescence detection were optimized to monitor insulin secretion from islets of Langerhans (Tao & Kennedy 1997, Tao *et al.* 1998). However, both ID-MS and affinity CE methods are specific only for hI but not for the intermediate forms.

CZE has been successfully employed to detect the hPI to mature hI processing in the culture medium of engineered PHFs (Arcelloni *et al.* 1998, Falqui *et al.* 1999). In this paper the method has been improved to allow the direct quantification of hPI and of four intermediate forms, including the 32–33 split, which was previously lacking in our standard mixture. Quantification of single intermediates by RIA is not available, in fact, and only their sum can be evaluated as the RIA – IRMA difference. Cross-reactivity of 65–66 split and des64,65 in the IRMA assay on plasma is of minor importance because in the beta cells' secretory pathway these intermediates are negligible. Otherwise, use of an IRMA assay may lead to an overestimated hI quantification when dealing with non-endocrine cells having an hPI cleavage metabolism different from that of beta cells, or when other factors (insulin-like growth factor, degradation products) may interfere. The hPI quantification by ELISA on cell supernatant failed to provide reliable results, probably because of a matrix effect generated by the culture media components or by the reagents employed in the solid-phase extraction. To minimize the source of misreading, a more specific sample pre-treatment (precipitation step) is probably necessary (Kjems *et al.* 1993).

The first finding of this study with respect to our previous investigation (Falqui *et al.* 1999) is that although the gene construct inserted into the cells was the same, the hPI conversion pathway appeared different from the primary to the stabilized cell lines, maybe due to the combined action of endogenous endoproteases (Vollenweider *et al.* 1992) or modified enzyme-to-substrate ratio. The quali-quantitative pattern of the products released into the medium by transfected cells may vary in relation to the gene constructs and constitutive or co-transfected PC3, PC2, furin (Vollenweider *et al.* 1992, 1995, Yanagita *et al.* 1993, Groskreutz *et al.* 1994, Falqui *et al.* 1999) or SPC4 (subtilisin-related proprotein convertases) conversion enzymes (Smeekens *et al.* 1992), and a spontaneous production of hPI derivatives was noted also after subcutaneous administration of hPI *in vivo* (Peavy *et al.* 1985).

A very low amount of mature hI was found only in the supernatant of PHM-HPI which comprised also all the

intermediate forms and showed the highest production rate on the sum of (pro)insulin related proteins. The 32–33 split and/or des31,32 deriving from furin cleavage of wild-type hPI at the B chain/C-peptide junction (Hutton 1994) were detected in different percentages among the tested cells. Intermediates 65–66 split and/or des64,65, known to have an increase in receptor binding and biological potency *in vitro* and *in vivo* as compared with intact hPI (Peavy *et al.* 1985), were released only by PHM-HPI and HepG2-HPI, maybe by the co-action of another widely expressed enzyme (PACE4) (Hutton 1994). The expression system used here did not fully reproduce the conditions within normal beta cells because of the lack of the specific endoproteases and of the regulated secretory pathway, but the processing conditions occurring in non-endocrine transfected cells could favor the action of furin, which has a neutral pH optimum instead of the more acid one required for PC2 and PC3 action (Smeekens *et al.* 1992).

The efficacy of double-mutated hPI gene transduction and of the prohormone processing was confirmed by the high amount of mature hI released into the supernatant by all the transfected cells. In addition to mature hI, all the four intermediates together with the precursor hPI were found in PHM and PHF and the conversion rate to hI (23–29% respectively) was similar to that of PRH (32%) but lower than in the other tested cells (46–59%). In HepG2 cells, hI maturation occurred the most efficient way (59%), probably related to the highest constitutive furin content (Smeekens *et al.* 1992, Yanagita *et al.* 1993), although the unprocessed hPI was still present and the distribution (percentage) of the processing intermediates was different with respect to that found in the primary cells. However, an incomplete post-translational processing of hPI to mature hI was also observed in engineered insulinoma cell lines when proinsulin was expressed at very high levels (Clark *et al.* 1997).

On the basis of the results obtained we can now better explain the previously demonstrated efficacy of the *in vivo* transplantation of primary human cells (Falqui *et al.* 1999). Moreover, we showed that some of the tested cell lines, although unable to quantitatively process hPI to mature hI, released a considerable amount of 65–66 split and/or des64,65, whose activity should be taken into account for a possible therapeutic utilization. These cells seem to be a promising model to develop a regulated insulin-release machinery, being highly expressing lines with efficient maturation of hPI into hI and all the processing intermediates, showing low proliferation and *in vitro* and *in vivo* stability of their phenotypic characteristics. Future clinical application of engineered cells requires a regulated release of insulin. Since non-endocrine cells do not possess the mechanisms for regulated (exocytotic) secretion, a conceivable approach is to regulate the level of hormone synthesis by expressing the gene under the transcriptional control

of glucose-inducible regulatory sequences (Lu *et al.* 1998). Alternatively, an inducible release of insulin from engineered cells has been recently obtained by engineering a fusion protein, FKBP12-insulin, that accumulates as aggregates in the endoplasmic reticulum. Rapid release of insulin can be stimulated by a drug that induces protein desegregation (Rivera *et al.* 2000).

In conclusion, the method proposed proved appropriate for the screening of the metabolic products of human and rodent cells engineered for the non-regulated constitutive released of mature insulin.

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