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Protein expression changes in a cell system of beta-cell maturation reflect an acquired sensitivity to IL-1β

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Abstract

Aim/hypothesis. Type 1 diabetes mellitus (T1DM) is caused by specific destruction of the pancreatic beta cells in the islets of Langerhans. Increased sensitivity to cytokines, in particular to interleukin-1β (IL-1β) seems to be an acquired trait during beta-cell maturation. In response to cytokines both protective and deleterious mechanisms are induced in beta cells, and when the deleterious prevail, T1DM develops. The aims of this study were to identify perturbation in protein patterns (PiPP) associated with beta-cell maturation, and compare these changes to previous analyses of IL-1β exposed rat islets. For this purpose, proteome analyses were carried out using a cell-line, which matures from a glucagon-producing pre-beta-cell phenotype (NHI-glu) to an insulin-producing beta-cell phenotype (NHI-ins). We have previously shown that this maturation is accompanied by acquired sensitivity to the toxic effects of IL-1β.

Methods. 2D-gel electrophoresis was used to separate the proteins and MALDI-MS and database searches were performed to identify the proteins.
Results. During beta-cell maturation 135 protein spots out of 2239 detectable changed expression levels. Of these, 74 were down-regulated, 44 up-regulated, 16 were suppressed and 1 was expressed de novo. Using MALDI-MS, positive identification was obtained for 93 out of the 135 protein-spots revealing 97 different proteins. Of these, 22 proteins were in common with changes identified in previous proteome analysis of perturbation in protein pattern in IL-1β exposed rat islets. Several of the proteins were present in more than one spot suggesting post-translational modification.
Conclusion/interpretation. Several proteins and protein modifications were identified that could be critically involved in beta-cell maturation, insulin-gene expression and the acquired IL-1β sensitivity. [Diabetologia (2004) 47:62–74]

Keywords Beta-cell maturation · IL-1β · proteome analysis · mass spectrometry · pathogenesis

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Abbreviations: T1DM, Type 1 diabetes mellitus; PiPP, perturbation in protein pattern; NO, nitric oxide; iNOS, inducible nitric oxide synthase; 2D-GE, 2 dimensional gel electrophoresis; IEF, isoelectric focusing; NEPHGE, non-equilibrium pH-gradient electrophoresis; WF, Wistar Furth; BB, Bio Breeding; PDX-1, pancreatic duodenum homeobox 1; ASS, argininosuccinate synthetase; HSP, heat shock protein; Picot, PKC-interacting cousin of thioredoxin; JNK, Jun N-terminal kinase; VDAC, voltage-dependent anion channel; GST, glutathione-S-transferase; Mw, molecular weight; pI, isoelectric point.

Autoimmune Type 1 diabetes mellitus (T1DM) is caused by specific destruction of the insulin-producing beta cells in the islets of Langerhans in the pancreas [1]. During this process the islets are infiltrated with macrophages and lymphocytes, and these cells release a mixture of cytokines, such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) to which beta cells are particularly sensitive [2]. Cytokines have been shown to induce different free radicals [3] including nitric oxide (NO), catalyzed by inducible nitric oxide synthase (iNOS), as described in the Copenhagen Model of beta-cell destruction in T1DM [4].

During development of the pancreas, all four endocrine cell-types (α, β, δ and pp cells) are believed to arise from the same stem cell [5] and this specialization is dependent upon activation of different transcription factors [6, 7, 8, 9]. Since sensitivity to cytokines, free radicals and toxic chemicals is exquisite for the beta cell, this could reflect an acquired trait during the maturation of stem cells into mature insulin producing beta cells. We therefore hypothesize, that PiPP leading to susceptibility to IL-1β-damage is a consequence of beta-cell maturation. Here we address this hypothesis by using a glucagon producing pre-beta-cell cell line (NHI-glu), which following in vivo passage as a subcutaneous tumour in a rat, was re-established as an insulin producing beta-cell line (NHI-ins) [10, 11]. Our previous analyses of these two phenotypes showed that this maturation process was accompanied by an acquired sensitivity to the toxic effects of IL-1β [12]. Hence, the aim of our study was to characterize by proteome analysis [13, 14, 15] changes in protein expression accompanying the final maturation from the glucagon positive and IL-1β resistant pre-beta-cell phenotype to the insulin positive and IL-1β sensitive beta-cell phenotype and to relate these data to our previous analysis of PiPP in IL-1β exposed rat islets. [14, 16].

Materials and methods

Cell culture. The NHI-cell system [12] is based on the subclone NHI-glu derived from the glucagon-producing MSL-G2 culture [17]. Following in vivo passage by transplantation into syngeneic NEDH rats, the NHI-glu cells mature into insulinomas [10, 18]. The insulinomas re-established in vitro have an insulin-producing phenotype (NHI-ins) for prolonged periods of time [19], closely resembling beta cells with respect to the mRNA expression profile [20].

The two NHI-phenotypes were cultured in RPMI 1640 Glutamax (GibcoBRL Life Technologies, Paisley, Scotland, UK) supplemented with 10% FCS (GibcoBRL Life Technologies) and 1% penicillin/streptomycin (GibcoBRL Life Technologies) at 37°C in a 5% CO_2 atmosphere in four independent experiments. For metabolic labelling 2×10^5 cells/well were set up in 24-well plate (Costar, Cambridge, Mass., USA) containing 3 ml media. To prepare a large amount of protein for preparative gels and MS, cells were cultured in 50 ml tissue-culture flasks (10^7 cells/bottle) for 4 days.

Cell labelling. Cells used for 2D-GE analysis were cultured for 44 h and then washed twice in HBSS and labelled for 4 h in 250 μl/well methionine-free Dulbecco's modified Eagle's medium (DMEM) [21] with 10% dialyzed NHS and 18.5 MBq/ml [^{35}S]-methionine (SJ 204 Amersham International, Amersham, UK). After labelling the cells were washed twice in HBSS and lysed with 100 μl lysis buffer (8.5 mol/l urea, 2% nonidet P-40, 5% 2-mercaptoethanol and 2% carrier ampholytes, pH range 7–9) and stored at −80°C.

Determination of [35S]-methionine incorporation. [^{35}S]-methionine incorporation was quantitated in duplicate by adding 10 μl BSA (0.2 μg/ml in H_2O) as a protein-carrier to 5 μl of each sample diluted 1:10 with lysis buffer, followed by 0.5 ml of 10% TCA. This was left to precipitate for 30 min at 4°C before being filtered through 0.25 μm HAWP filters (Millipore) presoaked in BSA-saturated 10% TCA. The filters were washed twice with 1 ml BSA-saturated 10% TCA, once with ethanol, dried and placed into scintillation liquid for counting.

2D-gel electrophoresis. The procedure was as previously described [22]. Briefly, first-dimensional gels contained 4% acrylamide, 0.25% bisacrylamide and carrier ampholytes. An equal number of counts (10^6 cpm) of each sample were applied to the gels. The samples were analyzed on both isoelectric focusing (IEF; pH 3.5–7.0) and non-equilibrium pH-gradient electrophoresis (NEPHGE; pH 6.5–10.5) gels. Second-dimension gels contained 12.5% acrylamide and 0.063% bisacrylamide run overnight at 20°C. After electrophoresis, gels were fixed with Amplify (Amersham) before being dried. The gels were exposed to a PhosphoImage screen and the protein-pattern was scanned on a PhosphorImager (ADC 70 AGFA, Belgium). The resulting approximately 8 MB images were converted to 16-bit tiff images for image analysis.

Computer-assisted analysis. Computer analysis was carried out using the BioImage program 2D-Analyzer (version 6.1; Genomic Solutions, Ann Arbor, Mich., USA). The computer program assists in the matching of the spots among the eight independent gels, four from each phenotype, into a composite image followed by manual editing (Fig. 1). All protein spots that were marked were then re-evaluated to ensure that the boundaries assigned to the protein spots were correct and that no matching errors between the gels had been made. Significant changes in percent integrated optical density (IOD) between individual spots in the NHI-glu compared with the NHI-ins phenotype were determined using a double-sided non-paired Student's t-test comparing four independent experiments from each phenotype. Changes with a p value less than 0.01 were accepted as being statistically significant.

Protein characterization. For preparative purpose, a pool of 2×10^7 cells were prepared and separated on 2D-gels as described above. For localization of the spots, a sample of 10% of the cells was radioactively labelled to be used as a tracer as previously described. We ran six gels of each cell type, and loaded each with 200 μg of non-labelled protein and 4×10^6 cpm/gel of [^{35}S]-methionine labelled protein per gel.

Protein identification using matrix assisted laser desorption/ionization mass spectrometric (MALDI-MS) peptide mass mapping. Protein spots of interest were obtained by cutting them out of the dried gel using a clean scalpel. All 135 selected spots could technically be cut out of the preparative-gels for protein identification. The proteins were enzymatically digested in the gel as described [23] with minor modifications [24]. In short, the excised gel plugs were washed in 50 mmol/l NH_4HCO_3/acetonitrile (60/40) and dried by vacuum centrifugation. Digestion buffer (50 mmol/l NH_4HCO_3) containing endoproteinase lys-C (8 ng/μl, Calbiochem, San Diego, Calif., USA) was added to the dry gel pieces and incubated on ice for 1 h for re-swelling. After removing the supernatant, an additional 20–40 μl digestion buffer was added, and the digestion was continued at 37°C for 4–18 h. The peptides were desalted and concentrated prior to MALDI-MS as previously described [15]. The peptides were deposited directly onto the MALDI target by elution with matrix solution [α-cyano-4-hydroxy cinnamic acid in 70% acetonitrile/29.9% water/0.1% TFA (20 g/l)]. MALDI-MS analysis was carried out using a Voyager STR instrument (PerSeptive BioSystems, Framingham, Mass.,

2D-gels of the NHI-glu phenotype
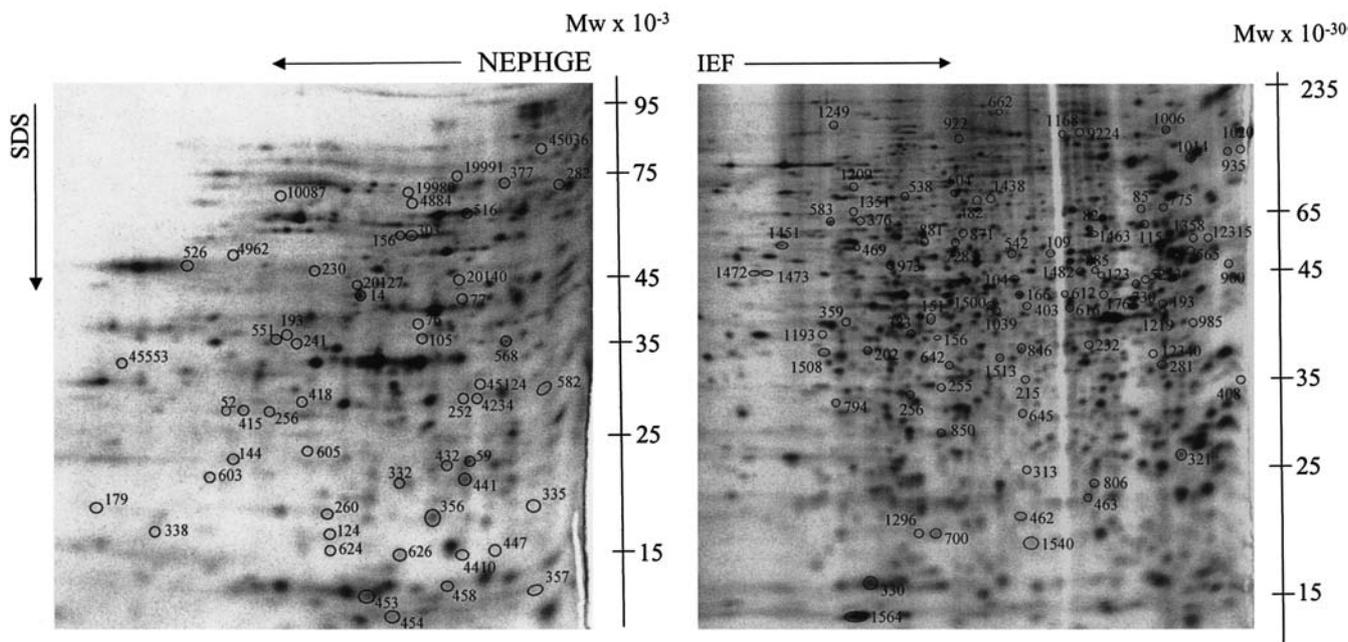
Fig. 1. Sections of the NEPHGE and the IEF 2D-gels of the NHI-glu phenotype covering the spots of changed expression level. The spot number refers to the match number assigned by the Bio Image computer program and used throughout the manuscript

USA) equipped with delayed extraction. Spectra were obtained in reflector positive mode using an accelerating voltage of 20 kV. Protein identification was done by searching the peptide mass maps in a comprehensive non-redundant protein sequence database (nrdb, European Bioinformatics Institute, Hinxton, Cambridge, UK) using the ProFound software (http://129.85.19.192/profound_bin/WebProFound.exe) with a mass accuracy of below 50 ppm [25]. Both first and second pass searches were carried out and the validation of the individual protein identifications was carried out [25]. In short, the Z-score described in the ProFound software was used as the primary evaluation of a positive identification, supported by information on sequence coverage, induced oxidation of methionine residues and terminal sequence tags, For identifications with a Z-score between 1.2 and 2.0 a careful evaluation including information on protein molecular weight together with the above-mentioned parameters were taken into account.

Determination of Mw and pI. Determination of the Mw and pI of all the spots were done by cutting 17 spots with high density form the NEPHGE 2D-gels and 24 spots of the IEF 2D-gels. Identification of the spots was carried out by MS and the Mw and pI values, from the identified proteins, were used as markers in the Bioimage Program to calculate the Mw and pI values for all the spots in the gel. Theoretical pI and Mw were calculated for all the identified proteins using the 'Compute pI/Mw tool' at the ExPASy Molecular Biology Server (http://www.expasy.ch/tools/pi_tool.html) taking into account any post-translational cleavage known.

Results

Expressions of 135 (53 spots from the NEPHGE 2D-gels and 82 spots from the IEF 2D-gels) out of 2239 reproducibly detectable spots were significantly (*p*<0.01) changed when samples of NHI-glu and NHI-ins were compared by 2D-GE. A total of 97 different proteins were unambiguously identified in 94 out of the 135 spots by mass spectrometry (a success rate of 70%, Table 1). The remaining 41 spots (Table 2) yielded either good mass spectra but no matches in the databases (17 spots), weak spectrum but no protein identification (19 spots) or no spectra at all (5 spots).

Our data show that at least 37% of the proteins identified might be post-translationally modified, since they were either found in more than one spot on the 2D-gel (22 proteins) or their observed and calculated theoretical Mw and/or pI were in discordance (19 proteins, see below). A total of five proteins were overlapping between these two groups. We found that 15 proteins were present in two spots each (ATP synthase α chain, γ-enolase, pyruvate kinase M1 isozyme, flag structure-specific endonuclease, ezrin, T-complex protein 1 ε and γ, protein disulfide isomerase, Hsc70-ps1, alpha-2-macroglobulin receptor-associated protein, dihydropyrimidase-related protein 2, voltage-dependent anion-selective channel protein 1, sorting nexin 6, septin-like protein and Erp 44). Furthermore six proteins were present in three spots each (fructose-bisphosphate aldolase A, T-complex protein 1 ζ, probable protein disulfide isomerase ER-60, P60 protein, voltage-dependent anion-selective channel protein 2 and 26S protease regulatory subunit). Finally, one protein was present in four spots (78 KDa glucose regulated protein). Major inconsistencies were found between observed

Table 1. Known and putative functions of identified proteins during maturation from the pre-beta to the beta-cell phenotype. Spots on the NEPHGE side of the 2D-gels have prefix "N" and spots on the IEF side have prefix "T". Each protein is listed according to its major known function. The percent IOD of the spots from the NHI-glu phenotype is given, and the IOD ratios are given relative to the expression level in the NHI-glu phenotype. Thus, values below 1 represent proteins that are down-regulated and values above 1 represent proteins that are up-regulated after beta-cell maturation. Furthermore proteins that are suppressed or undetectable after beta-cell maturation are indicated by "supp". Theoretical pI and Mw values were calculated using the 'Compute pI/Mw tool' at the ExPASy Molecular Biology Server, and the observed pI and Mw values are obtained directly from the gel. The expression data are based on the matching and comparisons of gels from four independent experiments for each phenotype

Gel match No.	Protein	Function	Database Acc #	% IOD in the NHI-glu phenotype	Ratio (NHI-ins/ NHI-glu)	Mw	Theo Mw	pI	Theo degradpI
Energy transduction and redox potentials									
N 14	Isocitrate dehydrogenase (#)	Energy generation	P48735	0.31	0.7	45.0	50.9	8.6	8.9
N 77*	Citrate synthase	Energy generation	O75390	0.10	0.2	44.6	51.7	7.4	8.1
N 377*, 516	ATP synthase α chain (#, ¤)	Energy generation	P15999	0.02, 0.07	5.1,2.2	70.4, 63.3	58.8	6.7, 8.2	9.2
N 20140	Fumarate hydratase	Energy generation	P14408	0.03	supp	48.2	54.5	7.5	9.1
I 1513	Isocitrate dehydrogenase	Energy generation	Q28480	0.01	1.9	38.6	37.6	5.9	6.0
I 616	Creatine kinase, B chain (#)	Energy generation	P07335	0.13	0.5	45.0	42.7	5.5	5.3
Glycolytic enzymes									
N 76	Phosphoglycerate kinase (¤)	Glycolysis	P16617	0.02	0.3	41.0	44.4	8.0	7.5
N 124, 193, 241	Fructose-bisphosphate aldolase A (¤)	Glycolysis	P05065	0.24, 0.08, 0.07	0.3, 0.3, 0.3	16.8, 37.3, 37.0	39.2	7.2, 8.8, 8.7	8.4
N 568	Glyceraldehyde-3 phophate-dehydrogenase (¤,#)	Glycolysis	P04797	0.13	2.1	37.5	35.7	7.4	8.4
I 166*	Enolase α (¤)	Glycolysis	P04764	0.07	1.5	50.0	47.0	5.6	6.2
I 193*, 1219*	Enolase γ	Glycolysis	P07323	0.12, 0.18	0.6, 0.6	42.3, 41.1	47.0	4.9, 4.9	5.0
I 1472*, 1473*	Pyruvate kinase, M1 isozyme (¤)	Glycolysis	P11980	0.03, 0.02	4.8, 3.8	51.3, 51.8	57.7	6.7, 6.6	6.7
Protein synthesis (incl. DNA/RNA processing, synthesis, transcription, nucleosides synthesis and amino acid metabolism), chaperones and protein folding									
N 357*	40S ribosomal protein S18	Protein synthesis	P25232	0.12	2.6	9.1	17.7	5.6	11.0
N 4410*	60S ribosomal protein L26	Protein synthesis	P12746	0.25	supp	13.5	17.3	6.7	10.6
N 335*	60S ribosomal protein L10	Protein synthesis	Q29195	0.24	0.5	17.3	24.5	6.1	10.1
I 166*	Histidyl-tRNA synthetase	Protein synthesis	Q61035	0.06	1.5	50.0	57.4	5.6	5.7
I 255*	60S Acidic ribisomal protein P0	Protein synthesis	P19945	0.05	0.3	35.2	34.2	6.1	5.9
I 12315*	Translation initiation factor 5	Protein synthesis	Q07205	0.02	supp	51.1	49.0	4.9	5.4
N 105	Heterogeneous nuclear ribonucleoprotein A3	RNA-processing	P51991	0.12	0.3	38.6	39.7	7.9	8.7
N 551	Heterogeneous nuclear ribonucleoprotein A2/B1(#)	RNA-processing	P22626	0.20	0.4	37.3	37.4	8.8	9.0
I 1014*	Heterogeneous nuclear ribonuclearprotein R	RNA-processing	BAB27533	0.08	1.5	115.7	67.2	4.8	8.9
N 377*	Heterogeneous nuclear ribonuclearprotein L	RNA-processing	XP_133298	0.02	5.1	70.4	60.1	6.9	6.7
N 230*	EIF-2-γ Y	RNA-processing	Q9Z0N2	0.08	0.6	40.3	51.0	8.8	8.8
N 357*	RNA polymerase II transscriptional coact. P15	Transcription	Q63396	0.12	2.6	9.1	13.7	5.6	9.7

Table 1. (continued)

Gel match No.	Protein	Function	Database Acc #	% IOD in the NHI-glu phenotype	Ratio (NHI-ins/ NHI-glu)	Mw	Theo Mw	pI	Theo degradpI
I 330*	Nucleoside diphophate kinase B	Nucleoside synthesis	P19804	0.90	0.5	15.9	17.3	6.0	6.9
I 775*	Heterogeneous nuclear ribonucleoprotein K	RNA synthesis	Q07244	0.02	1.6	63.9	51.0	5.0	5.4
I 846*	Zinc finger protein 26	RNA synthesis	P10076	0.06	2.6	39.7	48.9	5.7	9.3
N 230*, 20127*	Flag structure-specific endonuc.	DNA synthesis	AAF81265	0.12, 0.05	0.6, supp	40.3, 47.1	42.6	8.8, 8.6	8.8
I 383*	Isovaleryl-CoA dehydrogenase	Leucin metabolism	P12007	0.04	0.6	41.9	46.4	6.2	8.0
N 77*	Argininosuccinate synthase	Arginine metabolism and NO	P00966	0.1	0.2	44.6	46.4	7.4	8.4
N 19991	Caldesmon (¤)	actin/myosin cytoskeleton	NP_149131	0.06	supp	75.6	93.3	7.5	5.6
I 612	Keratin, type II cytoskeletal 8	actin/myosin cytoskeleton	Q10758	0.02	1.5	48.9	53.9	5.5	5.8
I 604, 1438	Ezrin	actin/myosin cytoskeleton	P26040	0.09, 0.03	0.5, 8.2	75.9, 76.0	69.2	6.0, 5.8	5.8
I 330*	Cofilin, non-muscle isoform	Muscle contra	P45592	0.9	0.5	15.9	18.5	6.0	8.2
I 662	Nonmuscle myosin heavy chain-B	Muscle contra	AAF61445	0.02	3.0	229.0	229.0	5.5	5.5
N 454*	Destrin	Muscle contra	JE0223	0.60	0.7	13.9	18.5	7.2	8.1
N 156, 303	T-complex protein 1, η subunit	Chaperone/folding	Q99832	0.05, 0.07	2.5, 1.9	59.5, 59.4	59.4	8.2, 8.1	7.6
I 109*	T-complex protein 1, θ subunit	Chaperone/folding	P42932	0.02	1.5	59.3	59.6	5.5	5.4
I 469*, 1472*, 1473*	T-complex protein, ζ subunit (¤)	Chaperone/folding	P80317	0.06, 0.03, 0.02	0.7, 4.8, 3.8	63.0, 51.3, 51.8	58.0	6.4, 6.7, 6.6	6.6
I 728*, 881*	T-complex protein 1, γ subunit (¤)	Chaperone/folding	P49368	0.01, 0.08	2.6, 0.6	63.1, 63.0	60.3	6.0, 6.1	6.2
N 453	Peptidyl-prolyl cis-trans isomerase A	Protein folding	P10111	0.33	1.6	13.9	17.7	7.3	8.4
I 109*, 542, 973*	Probable protein disulfide isomerase ER-60 (¤,#)	Chaperone	P11598	0.02, 0.03, 0.08	1.5, 0.8, 1.4	59.3, 58.5, 58.2	56.6	5.5, 5.7, 6.3	5.9
I 565, 12315*	Protein disulfide isomerase (#)	Chaperone	P04785	0.11, 0.02	0.6, supp	69.7, 51.1	57.0	4.9, 4.9	4.8
I 151	ERJ3 protein	Chaperone	Q9UBS4	0.06	0.5	46.3	40.5	6.2	5.8
I 82, 85*	Hsc 70-ps1	Chaperone	CAA49670	0.11, 0.02	0.6, 4.0	65.1, 67.6	70.9	5.4, 5.1	5.4
I 1463*	Hsc 71 (¤)	Chaperone	P08109	0.02	16.7	64.8	70.9	5.4	5.4
I 85*, 775*, 846*, 1358	78 Kda glucose-related protein (¤,#)	Chaperone	P06761	0.003, 0.02, 0.06, 0.05	4.0, 1.6, 2.6, 0.5	67.6, 63.9, 39.7, 85.4	72.3	5.1, 5.0, 5.7, 4.9	5.1
I 469*, 881*, N 282	P60 protein	Chaperone	O35814	0.01, 0.06, 0.08, 0.01	2.6, 0.7, 0.6, supp	63.0, 63.0, 69.5	62.6	6.4, 6.1, 6.1	6.4
I 730	Hsc 70-interacting protein	Chaperone	P50503	0.01	2.9	45.5	41.3	5.1	5.3
I 935	Endoplasmin	Chaperone	P08113	0.23	0.4	168.3	92.5	4.6	4.7

Table 1. (continued)

Gel match No.	Protein	Function	Database Acc #	% IOD in the NHI-glu phenotype	Ratio (NHI-ins/ NHI-glu)	Mw	Theo Mw	pI	Theo degradpI
I 1463*	Mortalin (GRP75) (¤)	Chaperone	P48721	0.02	16.7	64.8	73.9	5.4	6.0
Signal transduction, regulation, differentiation and apoptosis									
I 232	PKCq-interacting protein PICOT	Signal transduction	Q9JLZ2	0.06	0.5	38.2	31.4	5.4	5.4
I 313	Growth factor receptor bound protein 2	Signalling, cell regulation	NP_110473	0.01	3.8	24.0	25.2	5.7	5.9
I 202,1193	α-2-macroglobulin receptor-associated protein	Receptor signalling	Q99068	0.13, 0.03	0.7, 0.4	41.8, 44.9	41.7	6.3, 6.5	6.9
N 59*	PAX 1	Differentiation	P15863	0.40	0.7	23.9	24.4	7.0	6.6
I 1728*, 871*	Dihydropyrimidase related protein-2 (CRMP-2) (#, ¤)	Nerve cell diff. and signal transduction	P47942	0.01, 0.03	2.6, 1.6	63.1, 64.8	62.3	6.0, 6.0	6.0
I 973*	Lamin C2	Apoptosis	CAA67641	0.08	1.4	58.2	52.5	6.3	6.2
I 1351	Lamin C	Apoptosis	S04333	0.31	0.4	73.0	74.3	6.3	6.5
I 1154	Lamin B1 (¤)	Apoptosis	AAB09600	0.01	2.6	61.4	66.5	5.1	5.2
N 252, 4234*	Voltage-dependent anion channel protein 1 (#)	Apoptosis	Q60932	0.13, 0.05	0.3, supp	29.4, 29.5	32.3	7.4, 7.3	8.6
N 582, 4234*, 45124	Voltage-dependent anion channel protein 2	Apoptosis	P81155	0.10, 0.05, 0.05	4.3, supp, supp	32.7, 29.5, 30.1	31.7	4.9, 7.3, 7.1	7.4
Cellular defence									
N 441	Glutathione S-transferase P (class π)	Cellular defence	P46424	0.52	0.5	21.2	23.5	7.0	8.1
N 605	Glutathione S-transferase YB1 (class μ)	Cellular defence	P04905	0.07	0.3	24.3	25.9	8.0	8.4
Cellular transport									
I 885	Importin α	Glucose transport	Q9Z0N9	0.001	2.2	52.0	57.8	5.3	5.4
N 59*	RAN	Nucleocytoplasmic transport	P17080	0.40	0.7	23.9	37.8	7.0	9.4
N 332	RAS-related protein RAB-5C	Protein transport	P51148	0.16	0.5	19.8	23.6	7.4	8.9
I 376	N-ethylmaleimide sensitive factor	Vesicular transport	Q9QUL6	0.02	0.6	70.9	82.7	6.3	6.6
I 408	Clathrin light chain	Vesicular transport	AAA40891	0.01	5.2	32.9	25.1	4.4	4.6
I 462	Cytidylate kinase	Phosphate transport	P30085	0.23	0.6	19.5	22.2	5.7	5.4
I 463	RAS-related protein RAB-11B	Vesicular transport	P46638	0.14	1.5	21.1	24.5	5.3	5.6
I 583	Vesicular-fusion protein NSF	Vesicular transport	P46460	0.08	0.7	72.8	82.6	6.4	6.5
I 871*	Coatomer delta subunit (bovin, human) (¤)	Vesicular transport	P53619	0.01	1.6	64.8	57.2	6.0	5.9
I 922	Kinesin heavy chain	Organelle transport	P33176	0.01	2.4	162.3	109.9	5.7	6.1

Table 1. (continued)

Gel match No.	Protein	Function	Database Acc #	% IOD in the NHI-glu phenotype	Ratio (NHI-ins/NHI-glu)	Mw	Theo Mw	pI	Theo degradpI
I 1014*	Amphiphysin-like protein	Vesicular transport	O08839	0.08	1.5	115.7	64.5	4.8	5.0
I 256	Pyridoxal kinase	Transferase (e.g. methyl transport)	O35331	0.13	0.7	34.7	34.9	6.2	6.3
I 1564	Fatty acid-binding protein, epidermal	Fatty acid transport	P55053	0.7	2.1	12.8	15.1	6.0	5.6
I 1039*, 1500*	Sorting Nexin 6	Intracellular transport	Q9UNH7	0.09, 0.03	0.6, 2.8	46.6, 47.2	46.6	5.8, 5.8	5.8
Other functions									
N 19980, 45036	Septin-like protein	Cell division/ Chromo. alignment	Q9QZR6	0.03,0.06	supp, supp	69.2, 77.2	63.8	8.1, 6.4	8.7
I 156	Septin 2	Cell division/ Chromo. alignment	NP_476489	0.02	0.1	42.8	41.5	6.2	6.1
I 5223	Dynactin, 50 Kda isoform	Cell division/ Chromo. alignment	Q13561	0.03	supp	44.9	44.8	5.1	5.1
I 1403, 1039*, 1500*	26S protease regulatory subunit 7	Degradation	Q63347	0.03, 0.09, 0.03	1.8, 0.6, 2.8	46.7, 46.6, 47.2	48.6	5.6, 5.8, 5.8	5.6
N 454*	Complement component C4	Degradation	Q29439	0.60	0.7	13.9	14.5	7.2	5.3
I 850	Proteasome component C2	Degradation	P18420	0.22	0.6	29.4	29.5	6.1	6.1
N 335*	Polyubiquitin	Degradation	Q63654	0.24	0.5	17.3	11.2	6.1	5.4
N 4410*	Ubiquitin-conjucating enzyme E2	Degradation	O76069	0.25	supp	13.3	20.9	6.7	7.6
I 383*	Ubiquitin fusion degradation protein 1 homolog	Degradation	P70362	0.04	0.6	42.9	34.5	6.2	7.0
I 482	Neurolysin	Degradation	P42676	0.004	2.7	73.9	80.3	5.9	6.0
I 1508	Arginase 1	Arginine degradation	P07824	0.11	0.3	42.7	35.0	6.5	6.8
I 215	Serine/threonine protein phosphatase PP1-beta	Many functions	P37140	0.06	0.1	35.9	37.2	5.8	5.8
I 1209	MAP2 RNA trans-acting protein MARTA1	Nerve cell development	NP_598286	0.03	0.4	82.5	74.2	6.3	6.4
I 1255*	Transaldolase	Pentose pathway	Q93092	0.05	0.3	35.2	37.4	6.1	6.6
N 20127*	Hypothetical 44.7 protein	Similar to GTP-binding protein	CAB66481	0.05	supp	40.3	44.7	8.6	7.6
I 1482	Vitamin D-binding	Bone growth	P04276	0.07	1.5	53.5	53.5	5.4	5.7
I 1451*	Fibrinogen gamma-α-chain	Blood-coagulation	P02679	0.07	1.8	62.4	49.5	6.6	5.6
I 1451*	Apolipoprotein A-I	Blood-coagulation, calcium binding	P02647	0.07	1.8	62.4	30.8	6.6	5.6
I 123	Similar to leucine rich repeat interac. protein 2	Unknown	AAH14761	0.03	0.4	50.6	47.1	5.4	5.5
I 1219*, 193*	Erp 44	Unknown	NP_083848	0.18, 0.12	0.6, 0.6	41.1, 42.3	47.0	4.9, 4.9	5.1

* Spots containing two proteins (*column 1*)

¤ Proteins previously shown also to change expression level after IL-1β exposure of Wistar Furth rat islets [14]

Proteins shown also to change expression level after IL-1β exposure of BB rat islets [16]

Table 2. Unidentified protein spots with statistically significant changed expression level after beta-cell maturation are shown. The percent IOD of the spots in the NHI-glu phenotype are given and the IOD ratios are given relative to the expression level in the NHI-glu phenotype. Furthermore, proteins that are suppressed or undetectable after beta-cell maturation are indicated by "supp". One protein only expressed in the beta-cell phenotype is indicated by "de novo". Observed pI and Mw values obtained directly from the gel are given

Gel match No.	MS results	% IOD in the NHI-glu phenotype	Ratio (NHI-ins/NHI-glu)	Mw	pI
N 52	Weak spectrum no id	0.02	1.4	28.4	8.6
N 144	Weak spectrum no id	0.08	2.0	23.4	8.1
N 179	Weak spectrum no id	0.11	0.3	20.8	8.9
N 256	Weak spectrum no id	0.20	0.4	28.5	8.4
N 260	Weak spectrum no id	0.06	1.8	18.6	7.4
N 338	Weak spectrum no id	0.24	0.3	18.6	7.8
N 356	Good spectrum	0.87	0.6	16.3	7.0
N 415	Good spectrum	0.19	0.5	28.3	8.5
N 418	Good spectrum	0.17	0.4	29.4	8.4
N 432	Good spectrum	0.38	0.5	22.5	7.2
N 447	Good spectrum	0.14	0.1	13.0	6.3
N 458	Good spectrum	0.49	0.4	13.9	6.6
N 526	Good spectrum	0.27	1.7	50.0	9.1
N 603	Weak spectrum no id	0.15	0.5	22.3	8.1
N 624	Weak spectrum no id	0.10	0.4	15.5	7.1
N 626	Weak spectrum no id	0.39	0.5	14.2	7.2
N 4884	Good spectrum	0.02	Supp	69.5	8.4
N 4962	Weak spectrum no id	0.10	Supp	49.5	9.0
N 10087	Weak spectrum no id	0.04	Supp	53.0	8.9
N 45553	Good spectrum	0.05	2.2	36.4	9.2
I 104	Good spectrum	0.21	0.8	53.4	5.6
I 176	Good spectrum	0.05	0.4	46.5	5.3
I 281	Weak spectrum no id	0.23	0.8	35.5	5.0
I 321	No spectrum	0.17	2.0	26.6	4.8
I 359	Weak spectrum no id	0.05	0.3	47.5	4.5
I 538	Good spectrum	0.03	0.6	76.9	6.1
I 642	Weak spectrum no id	0.04	0.5	38.6	6.1
I 645	No spectrum	0.07	0.7	31.2	5.8
I 700	Weak spectrum no id	0.21	0.5	19.5	6.0
I 794	Good spectrum	–	de novo	35.1	6.3
I 806	Weak spectrum no id	0.16	0.8	21.6	5.3
I 900	Weak spectrum no id	0.17	0.4	101.7	4.8
I 985	Good spectrum	0.06	0.6	48.9	4.8
I1006	Weak spectrum no id	0.13	0.5	149.5	4.9
I 1020	Good spectrum	0.15	1.3	222.5	4.5
I 1168	No spectrum	0.03	0.5	158.5	5.4
I 1249	No spectrum	0.05	0.3	160.9	6.1
I 1296	Good spectrum	0.03	0.3	19.5	6.0
I 1540	No spectrum	0.17	0.7	18.4	5.6
I 9224	Weak spectrum no id	0.01	supp	151.1	5.6
I 12340	Good spectrum	0.01	supp	36.5	5.1

and calculated theoretical Mw and/or pI, for 19 protein-spots (20%) (NEPHGE 59, 124, 332, 335, 357, 377, 454, 582, 4410, 19991, 20140, 45036 and IEF 330, 376, 846, 935, 922, 1014, 1451). These inconsistencies could reflect post-translational modifications.

Some spots (33 spots) contained two identified proteins (*Table 1): NEPHGE 59, 77, 230, 335, 357, 377, 454, 4234, 4410, 20127 and IEF 85, 109, 166, 193, 255, 330, 383, 469, 728, 775, 846, 871, 881, 973, 1014, 1039, 1219, 1451, 1463, 1472, 1473, 1500 and 12315. The identified proteins have been assigned in seven groups according to their major known or putative functions allowing easy comparison with previous publications on PiPP of IL-1β exposed rat islets [14, 16]: (i) energy transduction and redox potential, (ii) glycolytic enzymes, (iii) protein synthesis, chaperones and protein folding, (iv) signal transduction, regulation, differentiation and apoptosis, (v) cellular defence, (vi) cellular transport, (vii) other functions. The function and possible importance for beta-cell maturation and T1DM pathogenesis are discussed below for selected proteins, including the 22 proteins in common with our previous analysis of PiPP in IL-1β exposed rat islets [14, 16].

Discussion

Substantial research has been carried out to characterize the molecular mechanisms involved in the development of the pancreatic beta cells. It is generally believed that the endocrine, exocrine and ductal cell types are derived from endodermal cells [5]. Several studies have shown that the endocrine stem cell require several specific transcription factors to mature into single-hormone-expressing cells [6, 7, 8, 9]. The pancreatic duodenum homeobox 1 (PDX-1) has been shown to be important both for pancreas development [26] and subsequent maturation of the endocrine cells [27], whereas later in the mature islets PDX-1 expression is restricted to the insulin-producing beta cells [8]. Another transcription factor, Nkx-6.1, is important for maturation of the insulin-producing beta cells [8], whereas Brain-4 is important for maturation of the glucagon-producing α-cells [7]. Previous analysis of mRNA gene expression profiles by semi-quantitative multiplex RT-PCR of the two NHI-phenotypes used in this study, grown under the same conditions, showed that several genes associated with late stages of beta-cell maturation, such as PDX-1 were expressed in both phenotypes, whereas Nkx-6.1 was restricted to the beta-cell phenotype [20]. We showed that maturation into the beta-cell phenotype was accompanied by insulin gene expression and an acquired susceptibility to toxic effects of IL-1β, not seen in the pre-beta-cell NHI-glu phenotype [12]. Proteome analyses of the two phenotypes could therefore identify proteins and pathways important for beta-cell maturation and the acquired IL-1β sensitivity.

High resolution 2D-gel technology can efficiently separate thousands of proteins. Statistical analysis of the expression pattern of the NHI-glu and NHI-ins phenotypes allowed us to separate a small group of 135 protein spots, which were differentially expressed between these phenotypes. Subsequent MALDI-MS identified the majority of the proteins (70%) in these protein spots using available protein or translated nucleotide sequence databases. Compared to transcriptome analyses of mRNA expression, proteome analysis offers the possibility to quantitate changes in protein expression and to identify post-translational protein modifications, such as phosphorylation [15]. Post-translational modifications are often required for the functional activation of a protein or a protein complex [28], and indeed the importance of these modifications in the beta-cell maturation process is suggested by our finding that 37% of the identified proteins are present either in more than one spot, or exhibited modified mobility. Several of the other proteins might also be modified in more subtle ways. The nature of the post-translational modifications observed was not studied as part of this study. Thus, currently our data provide information on changes in protein expression profiles during beta-cell maturation. Future analyses

will allow identification of the precise mechanisms as well as the functional relevance responsible for these changes.

Due to the high sensitivity of the MALDI-MS technique, 33 spots were found to contain two proteins (34%). One study has described in detail how one spot is able to contain more than one protein [29]. In short, this could be due to overlapping protein bands or co-migration in polyacryamide gel bands due to SDS-PAGE limited resolving power or contamination with cytokeratins. This is important to note, since it is therefore presently not possible to say which protein in the protein spot in question actually changed expression level during the beta-cell maturation process. Future analysis using zoom gels covering narrow pH ranges with considerably higher resolution could resolve this [30]. Another limitation in proteome analysis is that only the proteins that are metabolically labelled with methionine under the present experimental conditions and are within the detectable pI and Mw ranges could be identified. Therefore proteins (e.g., pro-insulin and insulin) below a Mw of 12 kDa are not detected.

The protein spots that changed expression levels after maturation into the beta-cell phenotype are grouped in Table 1 according to their known or putative functions. Similar group assignments were recently used in the description of protein changes in rat islets exposed to IL-1β [14, 16]. It is beyond the scope of our study to discuss in detail the possible role of all identified proteins. Hence, only proteins previously shown to be influenced by IL-1β [14, 16], and/or relevant for beta-cell maturation or beta-cell destruction are discussed.

We have previously shown that IL-1β influences PiPP in islets of Langerhans [13, 14, 16]. PiPP could reflect up- or down-regulation of protein expression levels and/or gel-position shifts resulting from post-translatory modifications. Consequently, when in this paper the expression level of a protein is described as up- or down-regulated, this could reflect changes in the expression level of the native protein (including alterations in protein synthesis and degradation) or expression changes in a modified form of that protein. Therefore protein expression changes in common between beta-cell maturation and IL-1β exposed WF rat islets [14] and BB rat islet [16] (Table 1, ¤ and #, respectively) could reflect both native or modified forms of the protein. Of the 97 proteins of changed PiPP reflecting maturation into the beta-cell phenotype identified in this study, the expression pattern of 26 of these was previously shown to be influenced by IL-1β in rat islets. Of these, 16 were identified in IL-1β exposed WF rat islets [14] and 10 in IL-1β exposed BB rat islets [16], representing five of the common pathways (Table 1). Of these 26 proteins, only four (ATP synthase alpa chain, glyceraldehyde-3 phosphate-dehydrogenase, probable protein disulfide

isomerase ER-60 and 78 Kda glucose-regulated protein) were common in all three studies.

Due to the nature of the in vitro and in vivo metastasing cell-lines used in the present experiments, it cannot be excluded that proteins involved in cell-proliferation and cell-transformation might also be included in Table 1.

Energy transduction and redox potentials. Protein spots involved in the Citric acid cycle (such as isocitrate dehydrogenase, citrate synthase and fumarate hydratase) were down-regulated or suppressed after beta-cell maturation, suggesting decreased ATP production. In contrast ATP synthase α chain and a different form of isocitrate dehydrogenase were highly up-regulated. ATP synthase α chain, creatine kinase B and isocitrate dehydrogenase were also changed after IL-β exposure of rat islets [14, 16]. IL-1β has an inhibitory effect on the mitochondrial energy production in islets [31], and IL-1β exposure of rat islets showed down-regulation of several proteins involved in energy generation [14, 16].

Glycolytic enzymes. During beta-cell maturation six glycolytic enzymes changed expression level. Of these, five protein spots were also changed in rat islets after IL-1β exposure (¤, # Table 1). The functional relevance of the glycolytic pathway in insulin production still needs to be elucidated, since it has previously been shown that IL-1β does not affect the activity of any glycolytic enzyme [32]. However, it has been reported that insulin and cellular ATP levels regulate both the activity and synthesis of several glycolytic enzymes [32].

Protein synthesis, chaperones and protein folding. Proteins involved in protein synthesis were generally down-regulated after maturation of the beta-cell phenotype (the first six proteins in this group, Table 1). IL-1β has been shown to inhibit gene transcription and protein synthesis [13, 33] and to decrease preproinsulin biosynthesis in particular [34]. Therefore, a global decrease in protein synthesis could reduce the capacity of a beta cell to survive IL-1β exposure.

Citrullinaemia is an autosomal disorder of the urea metabolism characterized by high circulating levels of citrulline as a result of a deficiency in the activity of the urea cycle enzyme argininosuccinate synthetase (ASS) [35]. In addition, mutations in ASS cause high levels of citrulline. The expression level of ASS was significantly ($p<0.0001$) lower in the beta-cell phenotype compared to the pre-beta-cell phenotype. This could result in increased levels of citrulline in beta cells. Beta-cell sensitivity to IL-1β is in part mediated through iNOS expression. iNOS converts arginine and oxygen to citrulline and NO [36]. Arginine might be provided by uptake from the extracellular fluid, by intracellular protein degradation or by synthesis from

citrulline [37]. Thus, it is conceivable that the down-regulation of ASS expression results in increased levels of citrulline in beta cells that in turn could induce higher intra-cellular levels of arginine. The increased pool of arginine could then serve as substrate for iNOS and result in increased production of NO [37]. However, our previous analyses have shown that despite the different sensitivity to IL-1β, no significant difference in IL-1β induced NO production could be detected between the two phenotypes [12]. Nevertheless, it has been shown that IL-1β induces the citrulline-NO cycle in beta cells, and that extracellular arginine or citrulline is required for NO production [38]. Furthermore, the arginine level has been shown to be increased in IL-1β exposed beta cells [38]. Thus, it is conceivable that the beta-cell phenotype could contain increased concentrations of arginine due to low ASS expression as an acquired trait occurring during beta-cell maturation.

During beta-cell maturation seven protein spots involved in the formation and maintenance of the actin/myosin cytoskeleton changed expression level. One example is caldesmon also shown to be down-regulated in IL-1β exposed rat islets [14]. Whether this influences beta-cell stability and IL-1β induced toxicity is yet unknown.

The remaining 15 proteins of this group are all involved in chaperone functions and/or protein folding. The ζ and γ subunits of the T-complex protein-1 were also changed in WF rat islets after IL-1β exposure (¤; Table 1) [14]. This complex is involved in molecular chaperoning and in the production and folding of native actin and tubulin [39]. We identified two proteins found in more than one spot (probable protein disulfide isomerase ER-60 and protein disulfide isomerase) specifically involved in the re-arrangement of both intra-chain and inter-chain disulfide bonds in proteins to form native protein structures. Interestingly, spots containing these two proteins were also previously shown to change expression levels in rat islets exposed to IL-1β [14, 16].

Of the heat shock protein spots (HSPs) eight changed their level of expression after beta-cell maturation and several of these also in response to IL-1β (Table 1; ¤, #). HSPs function as molecular chaperones assisting in protein folding, transport, translocation and degradation [40], and could have protective functions against exposure to several damaging stimuli, such as heat [41], cytokines [42] and NO [43]. Furthermore, lower expression levels of HSP have been shown in streptozotocin induced diabetic rats [44].

Signal transduction, regulation, differentiation and apoptosis. A protein spot containing PKC-interacting cousin of thioredoxin (PICOT), a Jun N-terminal kinase (JNK) inhibitor [45], was down-regulated during beta-cell maturation. JNK is activated in response to different stress factors such as cytokines, heat and oxi-

dative compounds. Dependent upon the type of stimulation, signalling through JNK activates programmed cell death (apoptosis), differentiation/proliferation or tumour development [46], and has been shown to be critically involved in IL-1β mediated beta-cell destruction [47].

Apoptosis plays an important role during cell maturation [48]. Apoptosis is characterized by a set of cellular events including cell shrinkage, chromatin condensation and DNA fragmentation. Many factors have been shown to be involved in this process [49]. Toxic levels of cytokines have been shown to induce apoptosis [50, 51]. In line with this, a cleavage product of lamin A was up-regulated after IL-1β exposure of rat islets in vitro [14]. After maturation of the beta-cell phenotype protein spots containing lamin B1 and C2 were up-regulated. Proteolysis of lamins, the major structural proteins of the nuclear envelope, are observed in cells undergoing apoptosis [52]. The relevance of this in relation to beta-cell sensitivity to IL-1β is not clear. However, it has been shown that inhibitors of lamin cleavage prevent apoptosis [53]. Voltage-dependent anion channel (VDAC) 1 was down-regulated while VDAC 2 was up-regulated after beta-cell maturation. VDAC 1 was also changed after IL-1β exposure of rat islets [14]. VDAC proteins form pores in the outer mitochondrial membrane serving as the major permeability pathway for metabolite flux between the cytoplasm and the mitochondria [54]. Pro-apoptotic proteins Bax and Bak bind to VDAC channels and induce apoptosis by releasing cytochrome C, whereas the anti-apoptotic protein bcl-x_L closes the VDAC channels and inhibits apoptosis [55].

Cellular defence. Gluthatione-S-tranferase (GST) is involved in the glutathione pathway where gluthathione peroxidase and GST work together with glutathione in the decomposition of hydrogen peroxide (H_2O_2) or other organic hydroperoxides [56]. In mammalians, GSTs are divided into six classes (α, μ, κ, θ, π and σ). Protein spots containing GST (class π and μ) were both down-regulated during beta-cell maturation, suggesting that the beta-cell phenotype has impaired ability to reduce H_2O_2 compared to the pre-beta-cell phenotype. This reduction in cellular defence was also seen in the IL-1β exposed BB rat islet [16], since glutathione synthase was down-regulated. This is in line with previous analysis showing an impaired ability of islet beta cells to reduce H_2O_2 [57]. Glutathione has been shown to protect a human insulinoma cell-line against the toxic effects of tumour necrosis factor α (TNF-α) [58], and together with catalase, glutathione peroxidase protects RIN cells against H_2O_2, reactive oxygen species and cytokines [59].

Cellular transport. Nuclear or cellular transport is important for the survival and function of specialized cells and 14 proteins with such functions changed their levels of expression during beta-cell maturation. The Coatomer delta subunit was also up-regulated in IL-1β exposed rat islets [14]. Maturation into the beta-cell phenotype was also associated with an increased expression level of clathrin light chain, suggestive of increased capacity for exocytosis in the beta cells. This correlates well with maturation of the beta-cell phenotype, and it could be associated with increased insulin-production secreted through exocytosis in clathrin-coated vesicles [60] in the stimulatory culture condition (11.5 mmol/l glucose) used in these experiments.

We previously hypothesized that cytokines induce a race between protective and deleterious mechanisms in beta cells, and when the deleterious mechanisms prevail, beta-cell destruction and T1DM develop [4]. In addition we suggested that IL-1β sensitivity is an acquired trait during beta-cell maturation. This is supported by our previous studies of the two phenotypes [12]. Of the 97 proteins of changed expression patterns following beta-cell maturation some could be crucial for the required sensitivity to cytokines and resulting PiPP in response to IL-1β [14, 16]. Thus, the beta cell may "pay a price" influencing several common cellular pathways to become a highly specialized insulin-producing beta cell.

Based upon the Copenhagen Model of T1DM we attempted simple mathematical modelling of the earliest phase of the pathogenesis of T1DM [61]. Resulting from this, one could view the unperturbed beta cell as being in a parameter space characterized by dynamic stability separated by a virtual parameter surface from a parameter space characterized by dynamic instability. The closer to the parameter surface in its still dynamic instability space a beta cell is, the easier it is "pushed" into the space of dynamic instability or ongoing destruction. Maturation of a pre-beta cell (NHI-glu) into a beta-cell phenotype (NHI-ins) might position the beta cell closer to the parameter surface. When metabolically active the beta cell could, although still in the dynamic stability space, be even closer to penetrate the surface, since its sensitivity to cytokine toxicity is increased. Exposure to IL-1β or a mixture of cytokines might be what "pushes" beta cells through the parameter space surface and into dynamic instability and destruction. Dependent on the stimulus this could involve the interaction of several different proteins and pathways and the outcome might in part be genetically determined.

In conclusion, differentiation and maturation from a stem cell to a beta cell is a complex series of events. Future functional analyses are needed to further elucidate the role of the proteins identified in this study for the last maturation stage towards insulin production and accompanying acquired sensitivity to IL-1β and other cytokines. We anticipate that such knowledge could provide useful information for the design of novel and rational preventive and/or curative strate-

gies in T1DM, both in relation to beta cell growth and differentiation as well as in inhibition of cytokine-mediated toxicity.

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