Proteomics Analysis of Cytokine-induced Dysfunction and Death in Insulin-producing INS-1E Cells

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Cytokines released by islet-infiltrating immune cells play a crucial role in β-cell dysfunction and apoptotic cell death in the pathogenesis of type 1 diabetes and after islet transplantation. RNA studies revealed complex pathways of genes being activated or suppressed during this β-cell attack. The aim of the present study was to analyze protein changes in insulin-producing INS-1E cells exposed to inflammatory cytokines in vitro using two-dimensional DIGE. Within two different pH ranges we observed 2214 ± 164 (pH 4–7) and 1641 ± 73 (pH 6–9) spots. Analysis at three different time points (1, 4, and 24 h of cytokine exposure) revealed that the major changes were taking place only after 24 h. At this time point 158 proteins were altered in expression (4.1%, n = 4, p ≤ 0.01) by a combination of interleukin-1β and interferon-γ, whereas only 42 and 23 proteins were altered by either of the cytokines alone, giving rise to 199 distinct differentially expressed spots. Identification of 141 of these by MALDI-TOF/TOF revealed proteins playing a role in insulin secretion, cytoskeleton organization, and protein and RNA metabolism as well as proteins associated with endoplasmic reticulum and oxidative stress/defense. We investigated the interactions of these proteins and discovered a significant interaction network (p < 1.27e–05) containing 42 of the identified proteins. This network analysis suggests that proteins of different pathways act coordinately in a β-cell dysfunction/apoptotic β-cell death interactome. In addition the data suggest a central role for chaperones and proteins playing a role in RNA metabolism. As many of these identified proteins are regulated at the protein level or undergo post-translational modifications, a proteomics approach, as performed in this study, is required to provide adequate insight into the mechanisms leading to β-cell dysfunction and apoptosis. The present findings may open new avenues for the understanding and prevention of β-cell loss in type 1 diabetes. Molecular & Cellular Proteomics 6:2180–2199, 2007.

The hallmark of type 1 diabetes is β-cell dysfunction and apoptotic β-cell death mediated by infiltrating immune cells, especially macrophages and T-cells, into the islets of Langerhans. Cytokines, such as interleukin-1β (IL-1β), interferon-γ (IFN-γ), and tumor necrosis factor α, secreted by these immune cells are one of the main causes of β-cell death (1, 2). β-Cells are highly specialized cells primarily oriented toward tightly regulated insulin synthesis and secretion. Because of the high demand of proinsulin/insulin processing, they possess a highly developed endoplasmic reticulum (ER) compartment. The correct folding of proinsulin in this ER compartment is mediated by a specific subset of proteins, the molecular chaperones. Altered expression of several of these genes may result in misfolded proinsulin molecules, which are subsequently recognized and degraded by the quality control machinery of the ER. In this way, β-cells may be forced to produce less insulin, resulting in a partial or complete loss of β-cells and hence in the onset of type 1 diabetes. Therefore, an understanding of the molecular chaperones and their ability to cope with stress is of major importance to the understanding and possibly treatment of type 1 diabetes. This paper is available online at http://www.mcponline.org

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1 The abbreviations used are: IL-1β, interleukin-1β; BiP/GRP78, immunoglobulin heavy chain-binding protein/glucose-regulated protein, 78 kDa; DDx1, DEAD box protein 1; DDx3X, DEAD box protein 3 X-chromosomal; ER, endoplasmic reticulum; hnRNP, heterogeneous nuclear ribonucleoprotein; HSPA8, heat shock 70-kDa protein 8; HSPA9, mortalin; IFN-γ, interferon-γ; NO, nitric oxide; ORP150, oxygen-regulated protein, 150 kDa; PTM, post-translational modification; PRDX, peroxiredoxin; ROS, reactive oxygen species; SOD2, manganese-superoxide dismutase; STAT1, signal transducer and activator of transcription 1; 2D, two-dimensional; 2DE, two-dimensional gel electrophoresis; TXNL1, thioredoxin-like protein 1; BLAST, Basic Local Alignment Search Tool; NCBI, National Center for Biotechnology Information; iHOP, Information Hyperlinked over Proteins.
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Chaperones in response to different stress situations has been associated with the pathogenesis of type 1 and even type 2 diabetes (3).

Not surprisingly, one of the major changes in β-cells upon exposure to inflammatory cytokines is an increased proinsulin/insulin ratio and loss of insulin secretion in response to glucose (4–6). Upon prolonged exposure of the β-cells to cytokines this functional impairment leads to apoptotic β-cell death as shown convincingly by in vitro exposure of human or rodent β-cells to cytokines (2, 7, 8). Many studies have been undertaken in an attempt to unravel the pathways involved in cytokine-induced β-cell destruction, revealing that stress response genes, either protective or deleterious for β-cell survival, are induced, whereas several genes related to differentiated β-cell functions are down-regulated (7, 9). Central factors in the regulation of these cytokine-induced genes are nuclear factor κB and signal transducer and activator of transcription 1 (STAT1) (7, 10, 11). The strength of the microarray technique used for the majority of these studies is that it allows analysis of a very large number of genes; however, the technique yields little information on the functional state of the cells as changes in transcript levels are not always reflected at the protein level (12). In addition, many forms of regulation take place at a post-transcriptional level, such as post-translational modifications (PTMs), proper protein folding, and selective proteolytic degradation (13, 14). It is thus crucial to complement cDNA array analysis with a global evaluation of protein expression. Previous proteomics studies have been performed on mechanisms of β-cell death mainly on whole rat islets. Most of these studies made use of classical two-dimensional gel electrophoresis (2DE) and focused on the effects of IL-1β alone on rat islets (15–17). Although these contributed greatly to our current understanding on the mechanisms involved in β-cell death, we believe that the use of other inflammatory stimuli, such as a combination of IL-1β and IFN-γ, will add valuable data improving our knowledge on the mechanisms involved in cytokine-induced β-cell death. Indeed although exposure of purified human or rodent β-cells to IL-1β alone will induce β-cell dysfunction, it is not sufficient to induce apoptosis (2). Combining IL-1β with IFN-γ will induce an extensive level of apoptosis, as reported in human and rodent islets as well as in INS-1E cells, even at relatively low cytokine concentrations. This suggests that IFN-γ signal transduction synergizes with IL-1β signaling to trigger β-cell apoptosis (3).

With the recent advances in differential dye labeling and the introduction of the 2D DIGE technique (18) as well as the development of more sophisticated MS equipment for protein identification, an attractive technology for the quantitative analysis of differences in protein profiles has become available (19, 20). In the present project we investigated by 2D DIGE analysis the complex processes involved in cytokine-induced β-cell death, making use of the rat insulin-producing INS-1E cell line. These are well differentiated cells and have been found to be a stable and reliable β-cell model (21) with a global pattern of cytokine-induced gene expression similar to that observed in primary β-cells (9, 22). The effects of single cytokine treatments as well as a combined treatment with IL-1β and IFN-γ were analyzed, each covering a broad pH range by using an overlap of two different IPG strips (pH 4–7 and 6–9). This allowed us to conclude that, at the concentrations presently used, IL-1β and IFN-γ act synergistically in the induction of apoptosis, concurrent with major alterations in protein levels, as compared with single cytokine treatment alone. Interestingly the minor changes observed in single cytokine treatments point to a protection or adaptation of the INS-1E cells toward the treatment. For the combined treatment, where we were able to identify 119 differentially expressed proteins, the changes are consistent with an induction of the apoptotic cell type. Importantly many of the regulations observed occur at a post-translational level, for instance for the ER chaperones immunoglobulin heavy chain-binding protein/glucose-regulated protein, 78 kDa (BiP/GRP78) and oxygen-regulated protein, 150 kDa (ORP150) and for the heterogeneous nuclear ribonucleoproteins (hnRNP). A protein interaction analysis of the differentially expressed proteins revealed a significant network (p < 1.27e−05) containing 42 of these proteins. These results suggest that many of the pathways involved act in a coordinate way with a central role for molecular chaperones and proteins involved in RNA metabolism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Conditions**—INS-1E cells, a kind gift from Prof. C. Wollheim (Centre Medical Universitaire, Geneva, Switzerland), were cultured in RPMI 1640 medium (Cambrex, Verviers, Belgium) supplemented with 10 mmol/liter HEPES, 10% (v/v) heat-inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mmol/liter sodium pyruvate, and 50 μmol/liter β-mercaptoethanol. Cells used for experiments ranged from passage 64 to 70 (21).

**Cell Death Analysis and Nitrite Determination**—The percentage of living, apoptotic, and necrotic cells was assessed by cell counting. INS-1E cells were cultured in 96-well plates (7500 cells/well). Typically after 24 h of control or cytokine-treatment (10 units/ml IL-1β, 500 units/ml IFN-γ, or the combination of the two cytokines with the same concentrations) cells were incubated for 15 min with propidium iodide (10 μg/ml) and Hoechst HO 342 (20 μg/ml) (23). A minimum of 500 cells was counted in each experimental condition by two researchers counting independently of each other on a Zeiss Axioskop 40 CFL fluorescence microscope (Zeiss, Jena, Germany).

For nitrite determination, 50 μl of culture supernatant was collected after 24 h from the cell cultures for the proteomics experiments (2.5 × 10⁶ cells/100-mm dish). Nitrite determination was performed using Griess reagent as described previously (24), and the concentration was determined spectrophotometrically at 546 nm.

**Sample Preparation for 2D DIGE Analysis**—INS-1E cells were incubated for 1, 4, and 24 h with 10 units/ml recombinant human IL-1β (a kind gift from Dr. C. W. Reinolds, National Cancer Institute, National Institutes of Health, Bethesda, MD) and 500 units/ml recombinant rat IFN-γ (R&D Systems, Minneapolis, MN). For the 24-h condition, cells were also incubated with individual cytokines using the same concentrations. For each condition, 12.5 × 10⁶ INS-1E cells (2.5 × 10⁶ cells/100-mm dish) were collected. For each time point or treatment
condition quadruplicate experiments were performed, originating from four independent experiments. The cells were washed twice in phosphate-buffered saline and resuspended in 100 μl of lysis buffer (7 M urea, 2 M thiourea, 4% (v/v) CHAPS, 40 mM Tris base, 1% (v/v) DTT, and a mixture of protease inhibitors (Complete protease inhibitor, Roche Diagnostics)). Samples were sonicated (UP50H, Hielser, Teltow, Germany) five times for 10 s after which they were centrifuged for 13 min at 20,000 × g (4 °C) to precipitate all nonsoluble proteins and membrane fractions. The supernatant was desalted by dialysis (PlusOne Mini Dialysis kit, GE Healthcare), and the pH was adjusted to 8.5. Protein concentration was determined using the Bradford assay (25). 50 μg of protein sample was labeled with 200 pmol of Cy3 or Cy5 (two “forward” and two “reverse” gels were made at each time point), whereas 50 μg of pooled internal standard was labeled with 200 pmol of Cy2 (18). The internal standard consisted of a pool of all control and treated samples of all four replicate experiments. The labeling reaction was carried out for 30 min on ice and quenched with 10 μl lysine (15 min on ice). Labeled protein extracts were pooled, and sample loading buffer was added (7 M urea, 2 M thiourea, 4% (v/v) CHAPS, 0.5% (v/v) IPG buffer, and depending on the pH range of the strips, 1% (w/v) DTT for IPG strips pH 4–7 or 1.2% (v/v) Destreak (GE Healthcare) for pH 6–9).

2D DIGE and Gel Imaging—IPG strips (24 cm) were rehydrated overnight in 450 μl of rehydration buffer (7 M urea, 2 M thiourea, 4% (v/v) CHAPS, 0.5% (v/v) IPG buffer, 0.05% (w/v) OrangeG, and either 1% (w/v) DTT (for IPG strips pH 4–7) or 1.2% (w/v) Destreak (for IPG strips pH 6–9)). The pooled samples containing sample loading buffer were loaded onto the rehydrated strips using anodic cup loading and separated according to their isoelectric point on an Ettan IPGphor II manifold. The complete process was tracked using the Ettan IPGphor control software (version 1.01.03) (GE Healthcare). The first dimension was ended when the current reached a stable phase (at ~60 kV-h).

Prior to the second dimension, the strips were equilibrated during two intervals of 15 min each in an equilibration buffer (6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 50 mM Tris-HCl, pH 8.8) containing 1% (w/v) DTT in the first step and 4% (w/v) iodoacetamide and 0.02% bromophenol blue in the second step. Equilibrated strips were placed on top of 12.5% SDS-polyacrylamide gel and separated on an Ettan DaltSix system (GE Healthcare).

Scanning of the gels was performed using a Typhoon 9400 (GE Healthcare) at 100-μm pixel size. Prior to analysis with the DeCyder™ Version 6.5 software, gel images were cropped using ImageQuant TL. Spot detection and matching was performed automatically using the “Batch Processor” module of the DeCyder Version 6.5 software followed by careful manual rematching of wrongly matched spots or unmatched spots. The 12 spot maps corresponding to the four gels from each time point and each pH range were used to calculate average abundance.

Spot Digestion and Protein Identification by MALDI-TOF/TOF Analysis—For spot picking, two preparative gels for each pH range were run (350 μg of protein lysate each). First and second dimension runs were performed as described above except that CyDye labeling was omitted. Glass plates were pretreated with BindSilane, and two reference markers were applied to enable automatic picking. The gels were poststained using Deep Purple (GE Healthcare). Matching with the analytical gels was performed using the biological variation analysis module of the DeCyder Version 6.5 software. A pick list was generated and exported into the Spot Picker Version 1.20 software that controls the Ettan Spot Picker (GE Healthcare).

Spots were picked in MilliQ water, transferred to 100 μl of fixation solution (50% (v/v) methanol, 5% (v/v) acetic acid, and 45% (v/v) MilliQ) and rinsed three times with MilliQ water and three times with ACN (LC-MS quality, CHROMASOLV®, Sigma-Aldrich). The gels were hydrated in a 100 mM NH₄HCO₃ solution for 10 min followed by a dehydration step in 100% ACN for 10 min with vigorous vortexing. This step was repeated twice prior to dehydrating the gel pieces in a SpeedVac.

Gel pieces were rehydrated in digestion buffer (50 mM NH₄HCO₃ and 5 mM CaCl₂) containing 5 ng/μl modified trypsin (Promega, Madison, WI) and incubated overnight at 37 °C. The resulting peptides were extracted out of the gel plugs in four steps: once with 50 mM NH₄HCO₃, twice with 50% (v/v) ACN and 5% (v/v) formic acid, and once with 95% ACN and 5% formic acid (30 min each). Supernatants were dried in a SpeedVac.

Upon concentrating and desalting the tryptic fragments using Milipore C₁₈ ZipTips, the samples were mixed in a 1:1 (v/v) ratio with α-cyano-4-hydroxy cinnamic acid matrix (saturated solution in 50% ACN and 2.5% TFA in HPLC water), spotted onto the MALDI target plate, and allowed to air dry. MS/MS analyses were performed on a 4800 MALDI-TOF/TOF instrument (Applied Biosystems, Foster City, CA). The instrument was calibrated with Applied Biosystems Calibration Mixture 1. Measurements were taken in the positive ion mode between 900 and 3000 m/z. Sequences were automatically acquired by scanning first in MS mode and selecting the 15 most intense ions for MS/MS using an exclusion list of peaks arising from tryptic auto-digestion. Air was used as the collision gas, whereas the collision energy was adapted automatically. Data interpretation was carried out using the GPS Explorer software (Version 3.5), and database searching was carried out using the Mascot program (Version 2.0.00).

Because all experiments were performed on rat INS-1E cells, MS/MS searches were conducted with the following settings: NCBI (taxonomy set on Rattus, 33,734 protein entries) and MSDB (Mass Spectrometry Protein Sequence Database) (taxonomy set on Rodentia, 88,439 protein entries) as database, MS/MS tolerance for precursor and fragment ions between 0.2 and 1 Da depending on the sample, methionine oxidation as variable modification, and carbamidomethylation of cysteine as fixed modification. As enzyme, trypsin was selected, and a maximum of one missed cleavage was allowed. Using these parameters the probability-based MOWSE (Molecular Weight Search) scores greater than the given cutoff value for MS/MS fragmentation data were taken as significant (p < 0.05).

For protein identifications where no hit was found in the rat databases, protein identity was based on comparison with the orthologous mouse sequence because the mouse genome is better annotated compared with the rat genome. In these cases the number of identical amino acids is indicated for each peptide (Supplemental Table 1). For all identified proteins, each sequenced peptide was individually aligned using BLAST (26). For protein identification where all the individual peptides completely matched to more than one Swiss-Prot database accession number/protein name, both protein sequences were aligned using BLAST (26). If this alignment resulted in 100% sequence identity, thus pointing to a single protein present in the database under different names/accession numbers, the Swiss-Prot accession number of the entry with the best description is consistently given in the first place to eliminate redundancy. For completeness also the second accession number is reported (Supplemental Table 1). For peptides matching to different isoforms or to multiple members of a protein family, the following criteria were used for selecting which one to report. 1) If at least one of the identified peptides matched exclusively to a specific isoform or protein member, this protein isoform/member could be identified unambiguously. 2) The experimental molecular weight and pl obtained from the 2DE gel was compared with the theoretical molecular weight and pl of the different isoforms/protein members. In case no single protein could be ruled out based on these criteria or possibly more than one member of the protein group could be present in a single spot, the different names/accession numbers are reported, including the percent identity between them (Supplemental Table 1).
Constructing a Human Protein Interaction Network—We constructed a human protein interaction network as reported recently (27, 28). Data were downloaded from MINT 48, BIND 49, IntAct 50, KEGG (Kyoto Encyclopedia of Genes and Genomes), and Reactome. To increase the coverage of interactions, interolog data 53 (the transfer of protein interactions between orthologous protein pairs in different organisms) were included. Interactions were transferred from 17 eukaryotic organisms and added to the network. Orthology was assigned using the InParanoid database 55 using stringent thresholds. The statistical significance of the networks was estimated using a randomization scheme in which networks were generated from random input sets of the same size as in the present experiment. We performed 1000 randomizations from which we derived a probability distribution that was used to calculate the significance of the networks in this study (28).

Statistical Analysis—For all experiments the paired Student’s t test was used. To analyze differences in protein levels, the DeCyder Version 6.5 software was used, and a p value of less than or equal to 0.01 was considered statistically significant.

RESULTS

Effects of Cytokines on Cell Viability and Nitric Oxide Production—INS-1E cells were exposed for 24 h with IL-1β (10 units/ml), IFN-γ (500 units/ml), or the combination of both cytokines. In parallel to the cell culture experiments performed for proteomics analysis, we investigated the effects of the cytokines on cell viability and nitric oxide (NO) production. As reported previously (9), only a minor increase in the proportion of apoptotic cells was observed with IL-1β (from 1.88 ± 0.83 to 5.40 ± 2.09%, p < 0.005, n = 4) or IFN-γ alone (from 1.88 ± 0.83 to 7.10 ± 2.09%, p < 0.05, n = 4), whereas a synergistic effect on apoptosis was seen in the combination-treated cells (from 1.88 ± 0.83 to 21.42 ± 4.93%, p < 0.0005, n = 4). No significant alterations in the proportion of necrotic cells were observed. In parallel with this increase in apoptosis, NO production increased from 29.31 ± 20.7 pmol of nitrite/10⁴ cells in control cells to 624.38 pmol of nitrate/10⁴ cells in IL-1β-exposed cells or to 809.85 ± 170.7 pmol of nitrate/10⁴ cells in IL-1β + IFN-γ-exposed cells (p < 0.001, n = 4). For IFN-γ alone, no significant rise in NO production was detected (35.27 ± 12.9 pmol of nitrate/10⁴ cells). These findings are in accordance with previously performed studies investigating alterations in mRNA levels in cytokine-induced INS-1E cells under similar conditions (9).

Differential Proteomic Profile of Cytokine-exposed INS-1E Cells—Proteomic profiles of cytokine-exposed INS-1E cells were determined at two early time points (1 and 4 h) and at one late time point after cytokine induction (24 h) in an attempt to distinguish between early and late effects induced by IL-1β and IFN-γ. In addition, after 24 h of incubation, also the effects of IL-1β and IFN-γ alone were investigated. For each of these conditions quadruplicate experiments were performed at two different pH ranges, comparing cytokine-exposed with non-exposed control INS-1E cells. In the pH 4–7 range, 2328 ± 151 spots could be matched for IL-1β and IFN-γ alone, and 2214 ± 164 spots could be matched for the combination of IL-1β and IFN-γ. Similarly for the pH 6–9 range a total of 1821 ± 184 spots were matched for IL-1β alone and IFN-γ alone, and 1641 ± 73 spots were matched for the combination. In the pH 4–7 range 31, 23, and 116 spots were observed as differentially expressed after 24-h incubation with IL-1β alone, IFN-γ alone, or IL-1β + IFN-γ, respectively (p ≤ 0.01) (Fig. 1A and Supplemental Fig. 1). In addition, only 1 and 19 spots were differentially expressed after IL-1β + IFN-γ treatment at the early time points of 1 and 4 h of incubation, respectively. Because some of these spots were differential in different treatment conditions or at different time points, a total of 159 spots was observed to be differential. For the pH range 6–9, 11, 0, and 30 spots were observed as differentially expressed after treatment with IL-1β alone, IFN-γ alone, and IL-1β + IFN-γ, respectively (p ≤ 0.01, 24 h). For the early time points no spots were differentially expressed after 1 h of incubation, and only two spots were differentially expressed after 4 h of incubation with the combination treatment (p ≤ 0.01). Taken together, a total of 40 different differential spots were observed when analyzing this pH range (Fig. 1B and Supplemental Fig. 2).

Identification and Classification of Differentially Expressed Proteins—We obtained the following results from our 2D DIGE experiments for the combination of IL-1β + IFN-γ. Of a total of 199 differentially expressed spots, we were able to identify 141 spots (70.9%) by MALDI-TOF/TOF, corresponding to 92 different proteins. Of note, 12 of these spots were found to contain two proteins. Therefore, it is at present not possible to conclude which protein in the spot actually changed expression upon cytokine treatment. Further analysis using narrow
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pH range zoom strips could tackle this problem. The identified proteins were grouped in different functional classes: 1) metabolism; 2) protein synthesis, modification, and secretion; 3) ionic channels, ion transporters, and related proteins; 4) processing of peptides and neurotransmitters; 5) cell adhesion, cytoskeleton, and related genes; 6) RNA synthesis/turover; 7) cell cycle and related genes; 8) defense/repair; 9) apoptosis/endoplasmic reticulum stress-related; and 10) miscellaneous (Tables I–IV). The same functional classes were used in our previous microarray analysis (9). The complete list of identified proteins is shown in Tables I–IV and includes information on the -fold regulation, Swiss-Prot accession number, number of identified peptide sequences, number of unique peptides, and percent sequence coverage. Additional information is shown in Supplemental Table 1. Although the majority of protein identifications are based on multiple peptide sequences, three identifications are single peptide-based protein identifications. For these latter proteins the peptide sequence, precursor m/z and charge ratio, and the score/Expect value are shown in Table V, and the annotated MS/MS spectra are shown in Supplemental Fig. 3. The identified proteins play a role in many different pathways, such as insulin biosynthesis (summarized in Fig. 2), energy metabolism (summarized in Fig. 3), cytoskeleton reorganization (summarized in Fig. 4) (29–33), RNA metabolism (such as hnRNP H, hnRNP K, DEAD box protein 1 (DDX1), DEAD box protein 3 X-chromosomal (DDX3)), oxidative stress (such as manganese-superoxide dismutase (SOD2), peroxiredoxin-1 (PRDX1), and peroxiredoxin-3 (PRDX3)), and molecular chaperones (BiP/GRP78, ORP150, heat shock 70-kDa protein 8 (HSPA8), and mortalin (HSPA9)).

Interestingly 34.8% of the identified proteins are represented in more than one spot, suggesting that they are undergoing PTM (indicated in Tables I–IV). The two ER-residing chaperones, BiP/GRP78 and ORP150, for example, are present in a train of spots suggestive for phosphorylation. For both chaperones the unmodified form is down-regulated by cytokine treatment (p < 0.01 for BiP/GRP78 and p < 0.05 for ORP150), whereas the modified forms, although not all of them at a significant level, are induced by cytokine treatment (Fig. 5). Also HSPA8, a cytosolic chaperone, was represented by three different isoforms, which were all down-regulated upon cytokine treatment. The position of the isoforms in the gel suggests that one of them is phosphorylated (spot 931 compared with spot 922 (native form)), whereas the other is undergoing another PTM or possibly alternative splicing (spot 971). The fourth differentially expressed chaperone, the mitochondrial HSPA9, was observed in two spots. The isoform in spot 1013 (pH 4–7) was induced by cytokine treatment, whereas the second isoform (spot 994 (pH 4–7)) co-localized with another protein, i.e. the neuroendocrine convertase 1 (PCSK1). Therefore it is difficult at present to make a conclusion on the possible up-regulation of this second isoform. A better separation of these spots, e.g. by using narrow range IPG strips, is necessary to allow conclusions on the differential expression of either of these proteins.

Another protein undergoing PTM is the cytoplasmic PRDX1 (spot 2014, pH 6–9), which was strongly induced (7.6-fold, p ≤ 0.01), whereas an isoform of PRDX1 (spot 1975, pH 6–9) was down-regulated (2.7-fold, p ≤ 0.01). Another group of proteins prone to PTM are the hnRNPs. Two different members have been identified (hnRNP H1 and hnRNP K), represented in a total of eight different spots; all were down-regulated (p ≤ 0.01). Especially hnRNP K seems to be prone to PTMs because six different spots contained this protein. These can be divided into two distinct groups of phosphorylated isoforms, with molecular mass of ~70 and 51 kDa, respectively. Also of note, for PCSK1 and PCSK2, two and six isoforms were identified, respectively, all with the same molecular weight and all significantly down-regulated (p ≤ 0.01).

Upon exposure to individual cytokines, we were able to identify 38 (30 and 17 identifications for IL-1β and IFN-γ, respectively) proteins of a total of 57 (40 and 23 spots for IL-1β and IFN-γ respectively) differentially expressed proteins (75% of the spots identified for IL-1β and 74% of the spots identified for IFN-γ). These are listed in Tables III and IV, classified using the same functional groups as above. An important subset of proteins identified in IL-1β-exposed cells were proteins involved in metabolism, whereas the main group of proteins altered by IFN-γ plays a role in defense and repair pathways. Of interest, more than half of the proteins identified in the single cytokine condition were not altered significantly in combination-exposed cells. In addition, more than 80 additional proteins were identified in combination-exposed cells compared with single cytokine-exposed cells, confirming the synergistic nature of the combined cytokine exposure.

Network Analysis—Because the majority of changes in protein profile were observed by treatment with the combination of IL-1β and IFN-γ, and this after 24 h of stimulation, concurrent with a major increase in apoptosis, this condition was chosen for further network analysis. Considering that most proteins do not act as single entities but work together in networks, we analyzed the interactions between the proteins identified in this work. This allowed us to examine whether the differentially expressed proteins form one or more functional pathways and to place them in a framework of cellular systems. For this purpose the identified proteins were placed in a protein interaction network recently developed by us (27, 28). This analysis revealed that 42 of the differentially expressed proteins identified constitute a significant interaction network (p < 1.27e−05). This suggests extensive cross-talk between the different proteins and the pathways in which they are involved (Fig. 6 and Supplemental Material 3) with some proteins such as the chaperones BiP/GRP78, HSPA8, and HSPA9 and the RNA synthesis/turover proteins placed at the center of different networks.

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| pH | Spot no. | Full protein name | Swiss-Prot accession no. | -Fold regulation | No. peptides sequenced | No. of unique peptides | Sequence coverage % |
|---|---|---|---|---|---|---|---|
| 4–7 | 1443 | Aldehyde dehydrogenase family 7 member A1 | ALDH7A1 Q9DBF1 | 1.21 | 2 | 2 | 6 |
| 4–7 | 2231 | N-Ethylmaleimide-sensitive factor attachment protein, α | NAPA P54921 | 1.48 | 6 | 6 | 29 |
| 4–7 | 2683 | Catechol O-methyltransferase | COMT P22734 | 1.32 | 3 | 3 | 16 |
| 4–7 | 803 | Cytoplasmic dynein 1 intermediate chain 2 | DYNC1I2 Q62871 | 1.81 | 3 | 3 | 6.3 |
| 4–7 | 1074 | WD repeat protein 1 | WDR1 Q5RK0 | 1.35 | 12 | 12 | 31 |
| 4–7 | 1254 | Chaperonin subunit | CCT8 XP_213673 | 1.24 | 10 | 10 | 16 |
| 4–7 | 1634 | Ezrin-radixin-moesin-binding phosphoprotein 50 | SLC9A3R1 Q9JJ19 | 3.06 | 3 | 3 | 13 |
| 4–7 | 2013 | Nucleophosmin | NPM1 P13084 | 1.21 | 4 | 4 | 16 |
| 4–7 | 2778 | Splicing factor, arginine/serine-rich 3 | SFRS3 P84104 | 1.47 | 2 | 1 | 14 |
| 4–7 | 1667 | NSFL1 cofactor p47, XY40 protein | NSFL1C O35987 | 1.17 | 11 | 11 | 42 |
| 6–9 | 1037 | Annexin 11 | ANXA11 Q5X177 | 1.47 | 3 | 3 | 11 |
| 4–7 | 1233 | Inositol 1,4,5-trisphosphate receptor type 2 | ITPR2 P29995 | 1.88 | 2 | 1 | 1 |

*Abbreviated protein name according to iHOP and Swiss-Prot.

*The Swiss-Prot accession number (if no Swiss-Prot accession number was available, the NCBI accession number was used). If two different accession numbers were found with 100% identity, both are reported, the first one consistently the one with the best description. If an isoform or individual protein of a protein member could not be ruled out, different accession numbers are given, including the percent homology with the first one.

*Fold regulation as calculated with the DeCyder 6.5 software ($p \leq 0.01$).

*Number of peptides sequenced by MS/MS.

*Number of unique sequences (in terms of amino acid sequence) when individually aligned using BLAST.

*Mouse accession number. For full information, please see Supplemental Table 1.
| pH  | Spot no.        | Full protein name                                      | Protein name | Swiss-Prot accession no. | -Fold regulation | No. peptides sequenced | No. of unique peptides | Sequence coverage |
|-----|-----------------|--------------------------------------------------------|--------------|---------------------------|-----------------|------------------------|------------------------|--------------------|
|     |                 |                                                        |              |                           |                 |                        |                        |                    |
|     |                 |                                                        |              |                           |                 |                        |                        |                    |
| 1.  | Metabolism      |                                                        |              |                           |                 |                        |                        |                    |
|     | Up-regulated    |                                                        |              |                           |                 |                        |                        |                    |
| 6–9 | 498, 529, 530, 533 | Aconitate hydratase, aconitase                         | ACO2         | Q9ER34                    | 1.59, ↑1.31     | 9, 3, 4, 11            | 9, 3, 4               | 16, 5, 8, 19       |
| 4–7 | 882             | Glycerol-3-phosphate dehydrogenase                     | GPD2         | P35571                    | 1.38            | 4                      | 4                      | 8.4                |
| 6–9 | 985, 1009       | Glutamate dehydrogenase 1                              | GLUD1        | P10860                    | 1.51, ↑1.27     | 2.3                     | 2.3                    | 6, 9                |
| 6–9 | 995             | Leucine aminopeptidase 3                               | LAP3         | Q68FS4                    | 1.25            | 7                      | 7                      | 15                 |
| 6–9 | 1009            | Leucine aminopeptidase 3 isofrm 2                      | GLS          | Q68FS4-2 (100%)           |                 |                        |                        |                    |
| 6–9 | 1019            | Glutaminase                                            | GLS          | P13264                    | 1.27            | 3                      | 3                      | 7                  |
| 6–9 | 1037            | Nicotinamide phosphoribosyltransferase 1               | NAMPT        | Q80Z29                    | 1.47            | 6                      | 6                      | 16                 |
| 6–9 | 1190, 1195      | Similar to NADP⁺-specific isocitrate dehydrogenase     | IDH2         | P56574                    | 1.29, ↑1.21     | 10, 8                  | 8, 6                   | 25, 18              |
|     |                 |                                                        |              |                           |                 |                        |                        |                    |
| 6–9 | 1286            | Citrate synthase                                       | CS           | Q8VHF5                    | 1.51            | 7                      | 1                      | 16                 |
| 6–9 | 1286            | Phosphoglycerate kinase 1                              | PGK1         | P16617                    | 1.51            | 2                      | 1                      | 10                 |
| 6–9 | 1286            | Creatine kinase                                        | CKMT1        | Q5BJ79                    | 1.60            | 2                      | 1                      | 9                  |
| 6–9 | 1463            | Glyceraldehyde-3-phosphate dehydrogenase               | GAPDH        | P04797                    | 2.04            | 4                      | 0                      | 15                 |
| 6–9 | 1484            | Transaldolase                                          | TALDO1       | Q9EQ02                    | 1.56            | 1                      | 1                      | 4                  |
| 6–9 | 1627            | Fumarylacetocetate hydrolase domain-containing 2A       | FAHD2A       | XP_215851                 | 1.34            | 5                      | 5                      | 18                 |
|     |                 |                                                        |              |                           |                 |                        |                        |                    |
| Down-regulated | 6–9       | Aconitate hydratase, aconitase                         | ACO2         | Q9ER34                    | 2.31            | 9                      | 9                      | 15                 |
| 4–7 | 814, 815, 816   | NADH dehydrogenase (ubiquinone) Fe-S protein 1         | NDUF1        | Q66HF1                    | 1.44, ↓1.87     | 4, 2, 3                 | 4, 2, 3               | 6, 3, 7             |
| 6–9 | 953             | Pyruvate kinase isozymes M2                            | PKM2         | P11980-2                  | 1.24            | 9                      | 0                      | 24                 |
| 6–9 | 1030            | Inosine 5’-monophosphate dehydrogenase 2               | IMPDH2       | Q69U9                     | 1.25            | 4                      | 4                      | 11                 |
| 6–9 | 1207            | Pyruvate dehydrogenase E1 α 1                         | PDHA1        | Q4FZZ4                    | 1.25            | 10                     | 1                      | 24                 |
| 4–7 | 1262            | Inosine 5’-monophosphate dehydrogenase 1               | IMPDH1       | XP_342651                 | 1.58            | 7                      | 7                      | 14                 |
| 6–9 | 1389            | Arginase-1                                             | ARG1         | P07824                    | 1.15            | 12                     | 12                     | 40                 |
| 4–7 | 1414            | α -Enolase                                             | ENO1         | P04764                    | 1.99            | 7                      | 6                      | 16                 |
| 6–9 | 1415            | α -Enolase                                             | Q5EB49 (99.5%)| Q5BJ93 (100%)           | 1.77            | 6                      | 6                      | 26                 |
| 6–9 | 1731            | Acetyl-coenzyme A acetyltransferase 2 Ab2-076          | ACAT2        | Q5Xi22                    | 1.77            | 6                      | 6                      | 26                 |
| 6–9 | 1750            | Fumarylacetocetate hydrolase domain-containing 2A       | FAHD2A       | XP_215851                 | 1.32            | 7                      | 7                      | 24                 |
| 6–9 | 1899            | Triose-phosphate isomerase 1                           | TP11         | P48500                    | 1.28            | 5                      | 1                      | 30                 |
| pH    | Spot no. | Full protein name                                      | Protein name\(^a\) | Swiss-Prot accession no.\(^a\) | -Fold regulation\(^c\) | No. peptides sequenced\(^d\) | No. of unique peptides\(^e\) | Sequence coverage % |
|-------|----------|--------------------------------------------------------|---------------------|-------------------------------|-----------------------|-------------------------------|------------------------|-------------------|
| 4–7   | 1962     | Ester hydrolase C11orf54 homolog                       | C11orf54            | Q5U2Q3                        | ↓1.37                 | 2                             | 2                      | 8                 |
| 6–9   | 2155     | Nucleoside-diphosphate kinase B                        | NM2                 | P19804                        | ↓1.21                 | 9                             | 5                      | 54                |
| 4–7   | 2187     | Glyoxalase domain-containing protein 4                 | GLOD4               | Q5I0D1                        | ↓1.37                 | 6                             | 6                      | 23.2              |

2. Protein synthesis, modification, and secretion

Up-regulated

| pH    | Spot no. | Full protein name                                      | Protein name\(^a\) | Swiss-Prot accession no.\(^a\) | -Fold regulation\(^c\) | No. peptides sequenced\(^d\) | No. of unique peptides\(^e\) | Sequence coverage % |
|-------|----------|--------------------------------------------------------|---------------------|-------------------------------|-----------------------|-------------------------------|------------------------|-------------------|
| 4–7   | 242      | 150-kDa oxygen-regulated protein                       | HYOU1               | Q63617                        | ↑1.51                 | 7                             | 7                      | 9                 |
| 6–9   | 958, 985 | Elongation factor 2                                    | EEF2               | P05197                        | ↑1.75, ↑1.51          | 8                             | 3                      | 12, 7             |
| 4–7   | 1013     | GRP 75 kDa                                            | HSPA9               | P48721                        | ↑1.30                 | 4                             | 3                      | 8                 |
| 4–7   | 1504     | Dolichyl-diphosphooligosaccharide-protein glycosyltransferase | DDOST               | Q641Y0                        | ↑1.64                 | 2                             | 2                      | 5.8               |
| 4–7   | 2332     | Proteasome activator complex subunit 2                | Q63798              | PSME2                         | ↑1.44                 | 7                             | 6                      | 25.3              |
| 4–7   | 2962, 2963 | GRP78; BIP                                      | HSPA5               | P06761                        | ↑2.69, ↑3.52          | 9                             | 14                     | 19, 24            |

Down-regulated

| pH    | Spot no. | Full protein name                                      | Protein name\(^a\) | Swiss-Prot accession no.\(^a\) | -Fold regulation\(^c\) | No. peptides sequenced\(^d\) | No. of unique peptides\(^e\) | Sequence coverage % |
|-------|----------|--------------------------------------------------------|---------------------|-------------------------------|-----------------------|-------------------------------|------------------------|-------------------|
| 4–7   | 902      | GRP78; BiP                                             | HSPA5               | P06761                        | ↓2.68                 | 11                            | 11                     | 27                |
| 4–7   | 922, 931, 971 | Heat shock 70-kDa protein 8                          | HSPA8               | P63018                        | ↓1.46, ↓1.47          | 7, 11                        | 3, 2, 5               | 16, 21, 7         |
| 4–7   | 994      | GRP 75 kDa                                            | HSPA9               | P48721                        | ↓1.91                 | 5                             | 4                      | 10                |
| 4–7   | 1062     | Serine/threonine-protein kinase PAK 2                 | PK2                 | Q64303                        | ↓1.20                 | 4                             | 2                      | 11                |
| 4–7   | 1463     | 26 S protease regulatory subunit 6B                    | PRS6B               | Q63570                        | ↓1.33                 | 6                             | 6                      | 18                |
| 4–7   | 1760     | Ubiquitin-conjugating enzyme E22                      | UBE2Z               | Q3B7D1                        | ↓1.70                 | 9                             | 9                      | 29                |
| 4–7   | 2140     | N-Ethylmaleimide-sensitive factor attachment protein, α | NAPA               | P54921                        | ↓1.48                 | 6                             | 6                      | 29                |

3. Ionic channels, ion transporters, and related

Up-regulated

| pH    | Spot no. | Full protein name                                      | Protein name\(^a\) | Swiss-Prot accession no.\(^a\) | -Fold regulation\(^c\) | No. peptides sequenced\(^d\) | No. of unique peptides\(^e\) | Sequence coverage % |
|-------|----------|--------------------------------------------------------|---------------------|-------------------------------|-----------------------|-------------------------------|------------------------|-------------------|
| 6–9   | 1095     | H\(^+\)-transporting two-sector ATPase                 | ATP5A1              | P15999                        | ↑1.47                 | 12                            | 12                     | 31                |
| 6–9   | 1618     | Voltage-dependent anion-selective channel protein 1    | VDAC1               | Q9Z2L0                        | ↑1.30                 | 9                             | 9                      | 52                |
| 4–7   | 2386     | Chloride intracellular channel protein 4               | CLIC4               | Q9Z2W7                        | ↑1.52                 | 5                             | 4                      | 32.5              |
| 4–7   | 809      | Sodium channel protein type 9 α subunit                | SCN9A               | O08562                        | ↓1.95                 | 3                             | 3                      | 1.6               |

Down-regulated

| pH    | Spot no. | Full protein name                                      | Protein name\(^a\) | Swiss-Prot accession no.\(^a\) | -Fold regulation\(^c\) | No. peptides sequenced\(^d\) | No. of unique peptides\(^e\) | Sequence coverage % |
|-------|----------|--------------------------------------------------------|---------------------|-------------------------------|-----------------------|-------------------------------|------------------------|-------------------|
| 4–7   | 809      | Sodium channel protein type 9 α subunit                | SCN9A               | O08562                        | ↓1.95                 | 3                             | 3                      | 1.6               |
| 6–9   | 1951     | Voltage-dependent anion-selective channel protein 1    | VDAC1               | Q9Z2L0                        | ↓2.15                 | 6                             | 6                      | 36                |

4. Processing of peptides and neurotransmitters
| pH | Spot no. | Full protein name | Swiss-Prot accession no. | -Fold regulation | No. peptides sequenced<sup>a</sup> | No. of unique peptides<sup>a</sup> | Sequence coverage<sup>%</sup> |
|----|---------|------------------|-------------------------|-----------------|-------------------------------|-----------------------------|------------------|
| Down-regulated |        |                  |                         |                 |                               |                             |                  |
| 4–7 | 979, 994 | Neuroendocrine convertase 1 | PCSK1 P28840 | ↓ 1.71, ↓ 1.91 | 2, 4 | 2, 4 | 6, 7 |
| 4–7 | 1003, 1007, 1024, 1060, 1084, 1090 | Neuroendocrine convertase 2 | PCSK2 P28841 | ↓ 3.81, ↓ 5.70, ↓ 2.81, ↓ 1.48, ↓ 1.69, ↓ 2.24 | 2, 2, 3, 9, 2, 2, 3, 9 | 2, 2 | 4, 4, 6, 15, 4, 4 |
| 5. Cell adhesion, cytoskeleton, and related genes | | | | | |
| Up-regulated | | | | | |
| 4–7 | 1505 | Ezrin-radixin-moesin-binding phosphoprotein 50 | SLC9A3R1 Q9JJ19 | ↑ 3.55 | 3 | 3 | 13 |
| 4–7 | 1659 | Actin β | ACTB P60711 | ↑ 1.50 | 6 | 0 | 16 |
| 4–7 | 1665 | Actin γ | ACTG1 P63259 (98.9%) | ↑ 1.63 | 4 | 1 | 13.1 |
| 4–7 | 1668 | Actin β | ACTB P60711 | ↑ 1.70 | 4 | 0 | 15 |
| 4–7 | 2723 | Cofilin-1 | CFL1 P45592 | ↑ 2.25 | 6 | 6 | 44 |
| Down-regulated | | | | | |
| 6–9 | 670 | Septin 9 | SEPT9 Q9ZQR6 | ↓ 1.50 | 2 | 1 | 5 |
| 4–7 | 741 | Mitofilin | IMMT Q3KR86 | ↓ 1.33 | 7 | 7 | 16 |
| 4–7 | 753 | Gial fibrillary acidic protein | GFAP P47819 | ↓ 1.49 | 2 | 1 | 6 |
| 4–7 | 814, 816 | Lamin-B2 | LMNB2 P21619<sup>f</sup> | ↓ 1.44, ↓ 2.23 | 4, 5 | 2, 5 | 7, 8 |
| 4–7 | 816 | Chaperonin subunit 6a, 7 | CCT6A Q3MH59 | ↓ 1.74 | 5 | 2 | 11 |
| 4–7 | 1049 | α -Internexin | INA P23565 | ↓ 1.46 | 7 | 7 | 19 |
| 4–7 | 1117 | Chaperonin subunit 10 | CCT8 XP_213673 | ↓ 1.46 | 10 | 10 | 16 |
| 4–7 | 1317 | Group-specific component | GC Q68FY4 | ↓ 2.07 | 2 | 2 | 6 |
| 4–7 | 2214 | Group-specific component | GC Q68FY4 | ↓ 1.94 | 5 | 1 | 17 |

6. RNA synthesis/turnover
| pH | Spot no. | Full protein name | Swiss-Prot accession no. | -Fold regulation | No. peptides sequenced | No. of unique peptides | Sequence coverage % |
|----|----------|-------------------|--------------------------|------------------|-----------------------|-----------------------|---------------------|
| Up-regulated | 6–9 | 529, 530 | ATP-dependent RNA helicase DDX1 | DDX1 Q641Y8 | 1.31, 1.44 | 6, 11 | 5, 10 | 9, 15 |
| | 6–9 | 590 | ATP-dependent RNA helicase DDX3X | DDX3X XP_228701 | 4.95 | 13 | 11 | 21 |
| | 4–7 | 1122 | Tyrosyl-tRNA synthetase | YARS Q4KM49 | 1.44 | 3 | 3 | 7 |
| Down-regulated | 4–7 | 706 | Eukaryotic translation initiation factor 4B | EIF4B Q5RKG9 | 1.62 | 3 | 3 | 7 |
| | 4–7 | 1049, 1056, 1075, 1174, 1211, 1216 | Heterogeneous nuclear ribonucleoprotein K | HNRPK P61980 | 1.46, 1.92, 3, 9, 2, 7, 6, 6, 6, 6, 7, 7, 25, 5, 23, 20, 16 |
| | 4–7 | 1262 | Heterogeneous nuclear ribonucleoprotein H1 | HNRPH1 Q8VHV7 | 1.58 | 4 | 1 | 19 |
| | 4–7 | 1279 | Heterogeneous nuclear ribonucleoprotein H1 | HNRPH1 Q8VHV7 | 1.72 | 4 | 0 | 17.8 |
| 7. Cell cycle and related genes | Down-regulated | 4–7 | N-Myc downstream regulated 1 | NDRG1 Q6JE36 | 1.48 | 6 | 6 | 12 |
| | 4–7 | 1571 | NSFL1 cofactor p47, XY40 protein | NSFL1C O35987 | 1.28 | 11 | 11 | 42 |
| | 4–7 | 2074 | Proliferating cell nuclear antigen, Cyclin | PCNA P04961 | 2.07 | 6 | 6 | 28 |
| 8. Defense/repair | Up-regulated | 6–9 | Mn-superoxide dismutase, mitochondrial | SOD2 P07895 | 2.50 | 5 | 5 | 40 |
| | 6–9 | 2014 | Peroxiredoxin-1, thioredoxin peroxidase 2 | PRDX1 Q63716 | 7.57 | 2 | 2 | 11 |
| | Down-regulated | 4–7 | Protein-disulfide isomerase A6/P5; thioredoxin d-c prot7 | PDIA6 Q63081 | 1.33 | 8 | 8 | 31 |
| | 4–7 | 1996 | Thioredoxin-like protein 1 | TXNL1 Q920J4 | 1.46 | 6 | 6 | 36 |
| | 6–9 | 1975 | Peroxiredoxin-1, thioredoxin peroxidase 2 | PRDX1 Q63716 | 2.67 | 8 | 8 | 47 |
| | 4–7 | 2613 | Peroxiredoxin III | PRDX3 Q9Z0V6 | 1.29 | 1 | 1 | 5 |
| 9. Apoptosis/endothelial cell stress-related | Up-regulated | 6–9 | Annexin 11 | ANXA11 Q5X177 | 1.47 | 4 | 3 | 11 |
| | 4–7 | 1097 | Inositol 1,4,5-trisphosphate receptor type 2 | ITPR2 P29995 | 2.19 | 2 | 1 | 1 |
DISCUSSION

In the present study we have identified 129 protein spots with an altered abundance or PTM by IL-1β, IFN-γ, or IFN-γ + IL-1β treatment of INS-1E cells, a rat insulin-producing cell line used as a model for β-cell dysfunction and death in type 1 diabetes. By applying the 2D DIGE technique to investigate the differential protein expression profiles we identified relevant proteins with a high confidence rate not possible by classical 2DE. The importance of the differentially expressed proteins was further supported by the close linkage they appear to have in the predicted interaction network. Indeed this suggests that the proteins identified, although belonging to a broad range of different classes and functional pathways, interact with each other in close relationships, creating a functional network that may regulate β-cell dysfunction and eventually β-cell death.

The concentrations of cytokines used in the present study clearly act synergistically in inducing apoptotic cell death in INS-1E cells. Indeed only minor alterations in the proportion of apoptotic cells were observed upon exposure to either IL-1β or IFN-γ alone, whereas the combined cytokines resulted in a dramatic increase in apoptosis. This is reflected in the observed changes in protein profiles where again a synergistic effect between IL-1β and IFN-γ was observed with major changes in protein levels only occurring in the combined treatment and this after a long incubation of 24 h.

Because the primary goal of β-cells is to process and secrete high amounts of insulin, it is not surprising that different proteins playing a crucial role in insulin biosynthesis were altered in expression following cytokine treatment (Fig. 2). In accordance with the reported increase in the proinsulin/insulin ratio and a decreased insulin secretion upon cytokine treatment (4, 6, 34), a clear down-regulation of the different isoforms of PCSK1 and PCSK2, the enzymes responsible for conversion of proinsulin to insulin, was observed. Another interesting protein important in the processing of insulin is thioredoxin-like protein 1 (TXNL1). TXNL1 reduces the disulfide bridges of insulin, thereby initiating the degradation of insulin (35). The observed down-regulation of this protein is yet another detrimental effect of IL-1β and IFN-γ on the insulin processing capacities in INS-1E cells.

In recent years, it has become clear that ER stress is implicated in β-cell death (3). One of the well known effects of cytokines on INS-1E cells is the formation of NO (2). Although inducible nitric-oxide synthase itself could not be detected in the 2D gels because of the high molecular weight of this protein, we observed two abundantly expressed ER chaperones, BiP/GRP78 and ORP150, that were clearly shifted toward more acidic forms suggestive for phosphorylation or ribosylation of these proteins. Although there are many studies implicating an important role of ER chaperones in β-cell death, very little information is available on the functional impact of their phosphorylation or ribosylation status. Over-

| pH Spot no. | Full protein name | Protein name | Sequence coverage % | pFold regulation | No. of unique peptides | No. of peptides sequenced | Swiss-Prot accession no. |
|---|---|---|---|---|---|---|---|
| 10. Miscellaneous | Serum albumin | ALB | 2.4 | 1.67 | 1 | 1.22 | P02770 |
| | Neogenin | NEO1 | 1.14 | 1.54 | 11 | 1.16 | P97603 |
| | Major vault protein | MVP | 19.3 | 1.54 | 11 | 1.16 | Q62667 |
| | Similar to stromal cell-derived factor 2 precursor | SDF2 | 31.6 | 1.16 | 3 | 3 | XP_213377 |

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2 Abbreviated protein name according to iHOP and Swiss-Prot.
3 The Swiss-Prot accession number (if no Swiss-Prot accession number was available, the NCBI accession number was used). If two different accession numbers were found with 100% identity, both are reported, the first one consistently the one with the best description. If an isoform or individual protein of a protein member could not be ruled out, different accession numbers are given, including the percent homology with the first one.
4 Fold regulation as calculated with the DeCyder 6.5 software (p < 0.01).
5 Number of unique peptides sequenced by MS/MS.
6 Number of unique sequences (in terms of amino acid sequence) when individually aligned using BLAST.
| pH | Spot no. | Full protein name | Protein name | Swiss-Prot accession no. | -Fold regulation | No. peptides sequenced | No. of unique peptides | Sequence coverage % |
|----|----------|-------------------|--------------|--------------------------|-----------------|----------------------|----------------------|----------------------|
| 6–9 | 304 | Aconitate hydratase, aconitase | ACO2 | Q9ER34 | ↑ 1.44 | 9 | 9 | 13.4 |
| 6–9 | 846 | Serine hydroxymethyltransferase | SHMT2 | Q5U3Z7 | ↑ 1.26 | 7 | 7 | 17.1 |
| 6–9 | 1308 | Creatine kinase | CKMT1 | P25809 | ↑ 3.08 | 9 | 6 | 21.1 |
| 6–9 | 1471 | Electron transfer flavoprotein subunit α | ETFA | P13803 | ↑ 1.36 | 3 | 3 | 16.9 |
| 4–7 | 1538 | Succinate-coenzyme A ligase | SUC1G2 | Q68FT4 | ↑ 1.15 | 7 | 7 | 23.6 |
| 4–7 | 305 | Aconitate hydratase, aconitase | ACO2 | Q9ER34 | ↓ 1.51 | 12 | 12 | 21.1 |
| 4–7 | 666 | Glycerol-3-phosphate dehydrogenase | GPD2 | P35571 | ↓ 1.61 | 6 | 6 | 10.1 |
| 4–7 | 815 | NADH-ubiquinone oxidoreductase 75-kDa subunit | NDUFS1 | Q66HF1 | ↓ 1.38 | 12 | 12 | 23.4 |
| 4–7 | 898 | NADH dehydrogenase (Ubiquinone) flavoprotein 1 | NDUFV1 | Q5XIH3 | ↓ 1.49 | 9 | 9 | 25.4 |
| 4–7 | 1414 | α-Enolase | ENO1 | P04764 | ↓ 1.87 | 5 | 4 | 15.7 |
| 4–7 | 735 | 75-kDa glucose-regulated protein, GRP75 | HSPA9 | P48721 | ↑ 1.20 | 11 | 10 | 18.5 |
| 4–7 | 962 | Syntaxin-binding protein 1-like 2 | STXBP1L2 | Q62991 | ↑ 1.19 | 5 | 5 | 14.4 |
| 4–7 | 1150 | Vacuolar ATP synthase subunit B, brain isoform | VATB2 | P62815 | ↑ 1.11 | 7 | 7 | 19.8 |
| 4–7 | 2386 | Chloride intracellular channel protein 4 | CLIC4 | Q9Z0W7 | ↑ 1.39 | 5 | 4 | 32.5 |
| 4–7 | 1024, 1084, 1090 | Neuroendocrine convertase 2 | PCSK2 | P28841 | ↓ 1.55, ↓ 1.44, ↓ 1.63 | 5, 6, 5 | 5, 6, 5 | 13, 13, 10 |

1. **Metabolism**
   - **Up-regulated**
   - **Down-regulated**

2. **Protein synthesis, modification, and secretion**
   - **Up-regulated**
   - **Down-regulated**

3. **Ionic channels, ion transporters, and related**
   - **Up-regulated**
   - **Down-regulated**

4. **Processing of peptides and neurotransmitters**
   - **Up-regulated**
   - **Down-regulated**

5. **Cell adhesion, cytoskeleton, and related genes**

### Notes
- The table includes the pH, spot number, full protein name, protein name, Swiss-Prot accession no., -Fold regulation, no. peptides sequenced, no. of unique peptides, and sequence coverage.
- The regulation values are indicated with arrows: ↑ for up-regulation and ↓ for down-regulation.
- The sequence coverage values are calculated as a percentage.
| pH     | Spot no. | Full protein name                   | Protein name<sup>a</sup> | Swiss-Prot accession no.<sup>b</sup> | -Fold regulation<sup>c</sup> | No. peptides sequenced<sup>d</sup> | No. of unique peptides<sup>e</sup> | Sequence coverage<sup>%</sup> |
|--------|----------|-------------------------------------|---------------------------|--------------------------------------|-------------------------------|------------------------------------|----------------------------------|-----------------------------|
| Up-regulated                          |          |                                     |                           |                                      |                               |                                    |                                 |                             |
| 4–7   | 1510     | Actin β                             | ACTB                      | P60711                               | †1.28                         | 5                                  | 0                                | 17.6                        |
|       |          | Actin γ                             | ACTG1                     | P63259 (98.9%)                      |                               |                                    |                                  |                             |
| Down-regulated                        |          |                                     |                           |                                      |                               |                                    |                                 |                             |
| 4–7   | 803      | Cytoplasmic dynein 1 intermediate chain 2 | DYNC1I2                  | Q62871                               | †1.12                         | 3                                  | 3                                | 6.3                         |
|       |          | Cytoplasmic dynein 1 intermediate chain 2 isof orm 2B | DYNC1I2                  | Q62871–2 (99.1%)                    |                               |                                    |                                  |                             |
|       |          | Cytoplasmic dynein 1 intermediate chain 2 isof orm 2C | DYNC1I2                  | Q62871–3 (95.9%)                    |                               |                                    |                                  |                             |
|       |          | DNCIC2 protein                       | DNCIC2                    | Q6AZ35 (98.6% identity)             |                               |                                    |                                  |                             |
| 4–7   | 2214     | Vitamin D-binding protein            | GC                        | P04276                               | †1.35                         | 4                                  | 4                                | 14.1                        |
|       |          | Group-specific component             |                           | Q68FY4 (99.8%)                      |                               |                                    |                                  |                             |
| 6. RNA synthesis/turndover             |          |                                     |                           |                                      |                               |                                    |                                 |                             |
| Up-regulated                          |          |                                     |                           |                                      |                               |                                    |                                 |                             |
| 4–7   | 545      | Eukaryotic translation initiation factor 4B | EIF4B                    | Q5RKG9                               | †1.35                         | 4                                  | 4                                | 8                           |
| Down-regulated                        |          |                                     |                           |                                      |                               |                                    |                                 |                             |
| 4–7   | 1075     | Heterogeneous nuclear ribonucleoprotein K | HNRPK                    | P61980                               | †1.81                         | 5                                  | 5                                | 13.6                        |
|       |          | Hnrpk protein                        |                           | Q5D059 (99.8%)                      |                               |                                    |                                  |                             |
| 8. Defense/repair                      |          |                                     |                           |                                      |                               |                                    |                                 |                             |
| Up-regulated                          |          |                                     |                           |                                      |                               |                                    |                                 |                             |
| 4–7   | 780      | Similar to guanylate nucleotide-binding protein 4 | GBP4                    | XP_227762                            | †1.67                         | 6                                  | 6                                | 9.2                        |
| 4–7   | 1305     | Protein-disulfide isomerase A6       | PDIA6                     | Q63081                               | †1.20                         | 4                                  | 4                                | 14                         |
| 6–9   | 1379     | Interferon-inducible GTPase          | IFI47                     | Q6NYB8                               | †2.08                         | 7                                  | 4                                | 22.2                       |
| 4–7   | 1994     | Mn-superoxide dismutase, mitochondrial | SOD2                     | P07895                               | †3.01                         | 4                                  | 4                                | 25.3                       |
| 4–7   | 2131     | Proteasome activator complex subunit 1 | PSME1                    | Q63797                               | †1.40                         | 6                                  | 1                                | 27.7                       |
| 4–7   | 2332     | Proteasome activator complex subunit 2 | PSME2                    | Q63798                               | †1.54                         | 7                                  | 6                                | 25.3                       |
| 10. Miscellaneous                     |          |                                     |                           |                                      |                               |                                    |                                 |                             |
| Up-regulated                          |          |                                     |                           |                                      |                               |                                    |                                 |                             |
| 4–7   | 321, 533 | Major vault protein                 | MVP                       | Q62667                               | †1.51, †2.01                  | 11                                 | 11                               | 14.7, 19.3                 |

<sup>a</sup> Abbreviated protein name according to iHOP and Swiss-Prot.

<sup>b</sup> The Swiss-Prot accession number (if no Swiss-Prot accession number was available, the NCBI accession number was used). If two different accession numbers were found with 100% identity, both are reported, the first one consistently the one with the best description. If an isoform or individual protein of a protein member could not be ruled out, different accession numbers are given, including the percent homology with the first one.

<sup>c</sup> -Fold regulation as calculated with the DeCyder 6.5 software ($p \leq 0.01$).

<sup>d</sup> Number of peptides sequenced by MS/MS.

<sup>e</sup> Number of unique sequences (in terms of amino acid sequence) when individually aligned using BLAST. For full information, please see Supplemental Table 1.
| pH | Spot no. | Full protein name | Protein name<sup>a</sup> | Swiss-Prot accession no.<sup>b</sup> | -Fold regulation<sup>c</sup> | No. peptides sequenced<sup>d</sup> | No. of unique peptides<sup>e</sup> | Sequence coverage<sup>%</sup> |
|----|---------|------------------|--------------------------|---------------------------------|-------------------|-----------------------------|-----------------------------|-----------------------------|
| 1. Metabolism | Up-regulated | 4–7 | Glucose-6-phosphate 1-dehydrogenase | G6PD P05370 | ↑ 1.43 | 10 | 10 | 23.9 |
| | Down-regulated | 4–7 | Pyruvate kinase isozyme R/L | PKLR P12928 | ↓ 1.34 | 6 | 6 | 12.4 |
| | | | Pyruvate kinase isozyme L | PKL P12928–2 (100%) | | | | 13.1 |
| 2. Protein synthesis, modification, and secretion | Up-regulated | 4–7 | Tryptophanyl-tRNA synthetase, cytoplasmic | WARS Q6P7B0 | ↑ 1.96 | 6 | 6 | 17.5 |
| | | 4–7 | Dolichyl-diphosphooligosaccharide-protein glycotransferase | DDOST Q641Y0 | ↑ 1.89 | 2 | 2 | 5.8 |
| 5. Cell adhesion, cytoskeleton, and related genes | Up-regulated | 4–7 | Actin β | ACTB P60711 | ↑ 1.53 | 5 | 0 | 17.6 |
| | | | Actin γ | ACTG1 P63259 (98.9%) | | | | 17.6 |
| | Down-regulated | 4–7 | Vitamin D-binding protein Group-specific component | GC P04276 | ↓ 1.27 | 4 | 4 | 14.1 |
| | | | | Q68FY4 (99.8%) | | | | 14.1 |
| 6. RNA synthesis/turnover | Down-regulated | 4–7 | Heterogeneous nuclear ribonucleoprotein K | HNRPK P61980 | ↓ 1.30 | 5 | 5 | 13.6 |
| 8. Defense/repair | Up-regulated | 4–7 | Interferon-induced GTP-binding protein Mx2 | MX2 P18589 | ↑ 1.93, ↑ 3.12 | 6 | 9 | 14.4, 18.4 |
| | | | Interferon-induced GTP-binding protein Mx3 | MX3 P18590 (98.8%) | | | | |
| | | | Myxovirus (influenza virus) resistance 2 | MX2 Q49903 (99.2%) | | | | |
| | | 4–7 | Similar to guanylate nucleotide-binding protein 4 | GBP4 XP_227762 | ↑ 3.70 | 6 | 6 | 9.2 |
| | | 4–7 | Interferon-inducible GTPase | IR47 Q6NYB8 | ↑ 9.32 | 7 | 4 | 22.2 |
| | | 4–7 | Interferon-γ -induced GTPase, Ac2–233 | ITP1 Q7TPJ1 | ↑ 9.82 | 7 | 4 | 6.1 |
| | | 4–7 | Proteasome activator complex subunit 1 | PSME1 Q63797 | ↑ 2.10 | 6 | 1 | 27.7 |
| | | 4–7 | Proteasome activator complex subunit 2 | PSME2 Q63798 | ↑ 2.01 | 7 | 6 | 25.3 |
| 9. Apoptosis/endoplasmic reticulum stress-related | Up-regulated | 4–7 | Inositol 1,4,5-trisphosphate receptor type 2 | ITPR2 P29995 | ↑ 2.92 | 2 | 1 | 0.9 |
| 10. Miscellaneous | Up-regulated | 4–7 | Major vault protein | MVP Q62667 | ↑ 1.57, ↑ 1.67 | 11 | 11 | 14.7, 19.3 |

<sup>a</sup> Abbreviated protein name according to iHOP and Swiss-Prot.
<sup>b</sup> The Swiss-Prot accession number (if no Swiss-Prot accession number was available, the NCBI accession number was used). If two different accession numbers were found with 100% identity, both are reported, the first one consistently the one with the best description. If an isoform or individual protein of a protein member could not be ruled out, different accession numbers are given, including the percent homology with the first one.
<sup>c</sup> -Fold regulation as calculated with the DeCyder 6.5 software (p < 0.01).
<sup>d</sup> Number of peptides sequenced by MS/MS.
<sup>e</sup> Number of unique sequences (in terms of amino acid sequence) when individually aligned using BLAST. For full information, please see Supplemental Table 1.
expression of BiP/GRP78 has a protective effect on β-cell death (36), and the present observations on decreased BiP expression is in agreement with our previous findings on an IFN-γ-induced decrease in BiP mRNA expression (37, 38). Furthermore phosphorylation of BiP results in a lower binding capacity for proteins (39, 40) that, together with the above described decrease in expression of non-phosphorylated BiP, may aggravate ER stress by interfering with the clearance of unfolded or misfolded proteins (41).

ORP150, another ER chaperone, also shifted toward expression of more acidic isoforms although less markedly than for BiP/GRP78. Increased ORP150 expression improves insulin resistance in animal models of type 2 diabetes and may have a protective role against β-cell death (42). In this context, it will be of interest to study the functional impact of the observed ORP150 PTM.

Another chaperone modified by cytokine treatment is HSPA8, which mainly resides in the cytosol. HSPA8 is an ATP-dependent chaperone (43) and has antiapoptotic effects (44). The observed down-regulation of this chaperone in all three different isoforms identified may hamper β-cell defenses. Opposite results have been described in neonatal rat islets where an increase in HSPA8 protein expression was observed upon treatment with IL-1β alone (45). Because in our experiments INS-1E cells were exposed to IL-1β + IFN-γ, this points toward an aggravating effect of IFN-γ on β-cell death via inhibition of molecular chaperones as shown previously in both INS-1E and primary β-cells (38). The fourth cytokine-modified member of the hsp70 family is HSPA9, a mitochondrial chaperone involved in protein folding and intracellular trafficking (46). HSPA9 has been implicated in the regulation of apoptosis through transcriptional inactivation of the tumor suppressor protein p53 (47).

Apart from NO formation, another detrimental effect of cytokine exposure to β-cells is the formation of reactive oxygen species (ROS). The observed induction of SOD2 points toward a protective response of the INS-1E cells against cytokine-induced ROS formation in agreement with published findings from microarray (9) or Western blot (10) analyses. However, a longer exposure of β-cells to cytokines will finally result in down-regulation of SOD2, further aggravating β-cell death (10). SOD2-mediated conversion of superoxide molecules will result in a high local production of H₂O₂. This hydrogen peroxide will be eliminated by reduction to H₂O by another group of proteins, the peroxiredoxins (48), and we have presently observed an up-regulation of the cytoplasmic PRDX1. It is puzzling why the other isoform of PRDX1 was down-regulated, which was also the case for the mitochondrial PRDX3, which exerts a function similar to that of PRDX1 (49). PRDX1 and PRDX3 use thioredoxins as electron donors to reduce hydrogen peroxide, and two proteins containing thioredoxin domains were presently identified: the ER protein-disulfide isomerase A6/P5 (PDIA6) and the cytoplasmic TXNL1. Cytokines down-regulated both these proteins, which may impair the β-cell ROS scavenging machinery. As a whole, these observations suggest a complex regulation of the SOD2-peroxiredoxin-thioredoxin system by cytokines that may initially contribute to free radical scavenging but, in the case of protracted exposure to cytokines, may contribute to cell death (10).

Different members of two large groups of proteins playing crucial roles in RNA metabolism were identified in the present study, namely the hnRNPs and the DEAD box family proteins.

### Table V

| Spot no. | Full protein name          | Protein name | Swiss-Prot accession no. | Ion score | No. peptides sequenced | Sequence coverage | Precursor m/z | Charge | Score | Expect | Sequences of MS/MS |
|----------|-----------------------------|--------------|--------------------------|-----------|------------------------|-------------------|---------------|--------|-------|--------|---------------------|
| 1484     | Transaldolase               | TALDO1       | Q9EOS0                   | 47        | 1                      | 1                 | 1392.5619    | +1     | 47    | 0.0011 | ALAGCDFLTISPK       |
| 2613     | Peroxiredoxin III           | PRDX3        | Q9Z0V6                   | 101       | 1                      | 1                 | 1476.8174    | +1     | 101   | 1.00e-08| DYGVLLESAGIALR      |
| 905      | Serum albumin               | ALB          | P02770                   | 75        | 1                      | 1                 | 1749.5309    | +1     | 75    | 2.10e-07| ECCHGDLLECADDR      |
The main functions of hnRNPs, a class of abundantly expressed proteins, are in transcription, processing of pre-mRNA, alternative splicing, and nucleocytoplasmic shuttling of proteins. hnRNPs themselves are prone to alternative splicing and PTM such as cleavage by caspases (50), altering their activation state. In turn, by having important functions in mRNA splicing (e.g., splicing of Bcl family members) and nucleocytoplasmic shuttling they play an important role in regulation of apoptosis (50). Especially hnRNP K (six spots, pH 4–7) seems to be prone to PTMs. The six different spots that have been identified can be divided into two distinct groups of modified isoforms. In addition, the location of the spots suggests a post-translational cleavage. Although this needs further investigation, cleavage by caspases, accompanied by subcellular translocation, may be involved in this process (50, 51). Two different members of the DEAD box protein family were also identified as altered by cytokine treatment, namely DDX1 and DDX3X. DEAD box proteins are implicated in diverse cellular processes such as RNA splicing, ribosome assembly, and translation initiation. Although the function of DDX1 and DDX3X is poorly understood, the increased expression of DDX3X correlates with its reported proapoptotic role in NIH-3T3 fibroblasts (52).

Compared with previous microarray data (7, 53, 54), few proteins, e.g., SOD2, PCSK1, PCSK2, and BIP/GRP78, were found to correlate with the present proteomics data. Microarray data of cytokine-treated rat or human \( \beta \)-cells (7, 9, 53, 55, 56) revealed many genes that were classified as playing a role in mononuclear cell infiltration/activation (e.g., chemokines), signal transduction (e.g., STAT1, IRF1, and c-jun), and dysfunction (e.g., insulin, pancreas/duodenum homeobox 1 (pdx-1) and insulin gene enhancer protein (isl-1)). Several of these genes were confirmed at the protein level by ELISA, Western blotting, or other methods (5, 57, 58). The majority of these proteins, however, were not detected in the present study. A weak point of 2DE in general is the limitation of protein separation for extreme molecular weight and pI, low abundance, or membrane proteins. This is probably the reason why we could not detect for instance inducible nitric-oxide synthase, chemokines, or transcription factors as modified by exposure to cytokines. It may be possible to overcome these problems, to a certain extent, by making use of narrower pH ranges or prefractionation techniques, but this...
Fig. 5. A, regulation of BiP/GRP78 24 h after IL-1β/IFN-γ treatment. Each panel consists of two representative three-dimensional images, one of control (Co) and one of cytokine-treated (Cyt) samples, and the graph view of the DeCyder analysis ($n = 4$) for each particular spot with the standardized log abundance in the y axis. a, spot 902, the unmodified isoform of BiP/GRP78; b and c, spot 2963/2962, BiP/GRP78 undergoing PTM. B, regulation of ORP150 24 h after IL-1β/IFN-γ treatment. a, representative three-dimensional view of the “spot train.” b–e, graph views of the DeCyder analysis ($n = 4$) for each particular spot with the standardized log abundance in the y axis. Spot 264 (b), the unmodified isoform of ORP150; spots 259 (c), 247 (d), and 242 (e), different forms of ORP150 undergoing PTM.

Fig. 6. Protein interaction network revealing that 42 of the differentially expressed proteins identified constitute a significant interaction ($p < 1 \times 10^{-6}$). Colored circles represent proteins that were identified in this study, and gray circles represent interconnecting proteins revealed by the network software program. A maximum of two interconnecting proteins is allowed. The identified proteins were colored according to their function (as classified in Tables I–IV): proteins involved in metabolism, blue; cell adhesion and cytoskeleton, green; RNA synthesis/turnover, yellow; protein synthesis/modification, pink; ionic channels and related proteins, purple; cell cycle, brown; and defense/repair mechanisms, orange. Central in the network are the molecular chaperones HSPA5 (BiP/GRP78), HSPA8, and HSPA9 (pink) and the RNA synthesis/turnover proteins (yellow). For full names of abbreviations, see Tables I–IV.
demanding approach was beyond the scope of the present study. On the other hand we identified many new proteins, not previously identified by microarray analysis, that play a role in for instance metabolism, insulin biosynthesis, cytoskeleton organization, ER stress and defense/repair, and RNA metabolism. Many of these proteins are abundantly expressed and are undergoing PTMs, which could of course not be detected by microarray analysis. It is therefore beyond any doubt that the present data are to a great extent complementary to the previously performed microarray results and add new interesting insights into the mechanisms involved in cytokine-induced β-cell dysfunction and apoptotic cell death.

In addition, treatment with a combination of IL-1β and IFN-γ clearly has much more pronounced effects on the global protein profile, as discussed above, as compared with the single cytokine treatments. Of note, although only 17 proteins were identified as changed by IFN-γ alone, some of these changes are very pronounced and not observed in the combination-treated cells, such as interferon-inducible GTPase (9.32-fold induced), interferon-γ-induced GTPase (9.82-fold induced), or guanylate nucleotide-binding protein 4 (3.70-fold induced). Interestingly these proteins are involved in cellular defense mechanisms. This suggests that treatment with IFN-γ alone at the concentration used may activate different protective mechanisms resulting in an adaptation of the cells against the cytotoxic effect of IFN-γ consistent with the finding that no induction of apoptosis when using the single cytokine is observed. Next to this, also an interesting picture is observed for IL-1β alone. Here most proteins that are affected are involved in β-cell function and not β-cell death. This is in concordance with previous observations that IL-1β plays a crucial role in inhibiting β-cell function (4, 5), supported by the intriguing recent observations that blocking IL-1β in type 2 diabetic patients partially restores β-cell function (59). The role of IL-1β in in vivo β-cell survival and death, however, is still controversial. Conflicting reports are present in the literature on whether IL-1β actually induces apoptosis in levels that are of relevant impact to affect β-cell mass in vivo or whether, under some conditions, it may promote β-cell survival (60, 61). Issues in the different reports are timing of exposure, dose of IL-1β used, and the presence of other cytokines that may amplify or inhibit IL-1β signaling (22, 60, 62).

Transferred to a more clinical situation where β-cells are under inflammatory attack by a combination of different cytokines the picture will be even more complex. Considering that we are dealing with a dynamic network of protein-to-protein interactions, it is most probable that perturbations of this network, induced by the local inflammatory environment, will result in β-cell dysfunction and eventually apoptotic cell death, leading to a diabetic phenotype. Molecular chaperones are suggested to play a crucial role in maintaining this dynamic network, and the present data suggest that the balance between the post-translationally modified and unmodified forms of the chaperones may be of relevance for the cellular outcome. Other central players are the proteins involved in RNA metabolism, such as the hnRNPs and DEAD box proteins. Use of the novel tools for network analysis (28) provides evidence for protein-to-protein interactions between these central players on the one hand and many of the proteins involved in energy metabolism and cytoskeleton organization on the other hand (Fig. 6) that will have a major impact on β-cell function. These findings lead us to hypothesize that, although upon an inflammatory attack the β-cell will eventually die from apoptosis, many important alterations are taking place that will affect the homeostasis of the cell state, resulting in a global β-cell dysfunction. This is also evidenced by the major detrimental effects on insulin processing and secretion, which are after all the primary tasks of this highly specialized cell. In conclusion, the present findings provide new insights into the protein pathways triggered in β-cells undergoing an inflammatory attack, pointing to a crucial role for chaperones and proteins involved in RNA processing. Together with previously published studies (9), a more comprehensive knowledge of the complexity of the networks involved will make it possible to develop new strategies for the treatment of type 1 diabetes.

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