Analysis of NOD2-mediated Proteome Response to Muramyl Dipeptide in HEK293 Cells

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NOD2, a cytosolic receptor for the bacterial proteoglycan fragment muramyl dipeptide (MDP), plays an important role in the recognition of intracellular pathogens. Variants in the bacterial sensor domain of NOD2 are genetically associated with an increased risk for the development of Crohn disease, a human chronic inflammatory bowel disease. In the present study, global protein expression changes after MDP stimulation were analyzed by two-dimensional PAGE of total protein extracts of human cultured cells stably transfected with expression constructs encoding for wild type NOD2 (NOD2WT) or the disease-associated NOD2 L1007fsinsC (NOD2SNP13) variant. Differentially regulated proteins were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) peptide mass fingerprinting and MALDI MS/MS. The limited overlap in the responses of the NOD2-overexpressing cell lines to MDP included a down-regulation of heat shock 70-kDa protein 4. A complex pro-inflammatory program regulated by NOD2WT that encompasses a regulation of key genes involved in protein folding, DNA repair, cellular redox homeostasis, and metabolism was observed both under normal growth conditions and after stimulation with MDP. By using the comparison of NOD2WT and disease-associated NOD2SNP13 variant, we have identified a proteomic signature pattern that may further our understanding of the influence of genetic variations in the NOD2 gene in the pathophysiology of chronic inflammatory bowel disease.

NOD2 belongs to a growing family of regulatory nucleotide-binding oligomerization domain proteins with a central nucleotide-binding oligomerization domain and N-terminal caspase recruitment domains that are involved in programmed cell death and immune responses. The domain structure consists of two adjacent N-terminal caspase recruitment domains, a central nucleotide binding domain, and 10 C-terminal leucine-rich repeats (1). The leucine-rich repeats of NOD2 are homologous to those seen in R proteins and Toll-like receptors, which recognize pathogen-associated molecular patterns, thus enabling an innate immune response. The identified proteins were categorized into functional groups, and their implications for pathophysiology of Crohn disease are discussed.

Materials and Methods

Cell Culture and Generation of Stable Transfectants—Human HEK293 and myelomonocytic THP-1 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were cultured in RPMI + 10% fetal calf serum. One day before transfection, the cells were seeded at a density of 10^6 cells per well.
5 × 10^5 cells/2 ml on 6-well plates. Transfections were performed with FuGENE 6™ (Roche Applied Science) according to the manufacturer’s manual, using 1 μg of the indicated plasmid/well. 48 h after the transfection, cells were selected over 4 weeks with 800 μg/ml G418 (Invitrogen) to select for stable transfectants. For each plasmid, 24 colonies were picked, after they had reached a diameter of 1 cm, and were assessed for stable expression of the encoded protein by Western blot. To avoid experimental bias from stable plasmid integration, six independent stable clones were pooled per plasmid to generate polyclonal cell lines. For confirmatory experiments, NOD2<sup>WT</sup>- and NOD2<sup>SNP13</sup>-expressing cells were generated with the FLP-IN system (Invitrogen). HEK293 cells containing the FRT recombination site were purchased from Invitrogen. Wild type and mutated (SNP13) NOD2 were inserted to the genomic recombination site by FLP recombinase enzyme. A control cell line was generated by transfecting an empty mock vector. All stable cell lines were sequence-verified using an ABI3700 sequencer (Applied Biosystems, Foster City, CA) prior to use. All primers were purchased from Eurogentec (Lie`ge, Belgium).

Protein Extraction—From five parallel cell cultures for each cell line, the cells were harvested by centrifugation, and the pellets were weighed. Proteins were extracted from the pellets using a modification of the protocol of Klose and Kobalz (13). In short, the cells were ground in a ceramics mortar cooled in a liquid nitrogen bath, adding the 1.25-fold weight of a buffer containing 50 mM Tris (pH 7.1), 20% (v/v) glycerol and 50 μg/ml hygromycin B (Invitrogen). Single colonies were picked and expanded in selection medium. Vector inserts were obtained from Bachem (Bubendorf, Switzerland) and the genomic DNA context in the selected clones were sequenced to verify the NOD2 sequence and proper integration in the genomic locus.

Expression of NOD2 (wild type and SNP13) was monitored by immunoblotting using anti-NOD2 (Cayman Chemicals, Ann Arbor, MI) antibody. MDP-LD and MurNAc-D-Ala-D-iso-Gln (MDP-DD) were generated by transfecting an empty mock vector. All stable cell lines were selected for hygromycin B resistance and consequently cultured in Dulbecco’s modified Eagle’s medium containing 1% penicillin/streptomycin and 50 mM Tris (pH 7.1), 20% (v/v) glycerol (Sigma), and a protease inhibitor mixture (Complete™, Roche Applied Science). After 15 min of grinding under continued cooling, the homogenates were transferred to fresh vials where they were mixed with urea and thiourea in the ratio 3:2:1 (w/w) under continuous stirring at room temperature for 5 min. After the urea had dissolved, diethiothreitol (700 mM) was added to a final concentration of 70 mM, and the samples were stirred for another 25 min at room temperature. After removal of cell debris by centrifugation at 15,000 x g for 15 min at room temperature, the supernatants were stored at −80 °C until analysis by two-dimensional GE.

**Two-dimensional Gel Electrophoresis**—Isoelectric focusing was performed with an Ettan IEPgphor<sup>TM</sup> apparatus (Amersham Biosciences) in 24-cm-long IEP strips with nonlinear gradients in the pH range of 3–10 (Amersham Biosciences). Prior to loading, the samples were centrifuged sharply for 30 min (22,000 × g; 25 °C) to remove insoluble aggregates and large organelles. Protein concentrations in the supernatant samples were determined with the two-dimensional Quant kit (Amersham Bio-
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FIGURE 3. Functional analysis (“clustering”) of the 198 proteins identified in this study. Numbers of proteins belonging to the functional classes are shown in the individual doughnut segments. For details of functional clustering, see “Results.”
spots were excised from the two-dimensional gel at an automatic gel excision work station (Proteineer SP, Bruker Daltonics, Bremen, Germany) by using an excision tool with a diameter of 1.5 mm. The gel samples containing the protein spots were delivered into the wells of the modified MTPs. In situ tryptic digestion was performed according to Shevchenko et al. (16) with some modifications. First, the excised gel plugs were washed three times for 20 min in 100 l each of washing buffer (50 mM NH₄CO₃ (pH 8), 50% (v/v) ethanol) and then dehydrated for 5 min with 100 l of ethanol. Removal of buffers and ethanol between incubation steps was performed by centrifugation of the gel-containing MTPs for 1 min at 1000 x g. The gel pieces were then placed for 15 min in a vacuum centrifuge (Savant, SVC100H) in order to completely remove any remaining liquid. For digestion of proteins, a trypsin solution (Roche Applied Science) containing 10 ng/µl trypsin in 50 mM NH₄CO₃ (pH 8) was freshly prepared and kept on ice. 4 µl (40 ng of trypsin) of the trypsin solution was added to each gel piece, and these were incubated for 30 min at 4 °C. Subsequently, 8 µl volumes of 50 mM NH₄CO₃ (pH 8) buffer were added to each sample, and the digestion was performed at 37 °C for 4 h. The digestion was stopped by addition of 10 µl of extraction buffer containing 0.5% trifluoroacetic acid and 2 mM n-octyl glycopyranoside. The extracted peptides were recovered by centrifugation into fresh MTPs placed under the modified plates.

Mass Spectrometry—MALDI sample preparation was performed on MALDI 600–384 AnchorChip sample plates (Bruker Daltonics, Bremen, Germany) as described previously (17, 18). A TeMO automatic liquid handler (Tecan, Switzerland) was used to deposit 1-µl aliquots of the tryptic digests onto pre-formed microcrystalline layers of the MALDI matrix α-cyano-4-hydroxycinnamic acid. After the solvent had evaporated, the samples were briefly washed with 0.1% trifluoroacetic acid. Mass spectra of positive ions in the m/z range 640–4,000 were recorded on an Ultraflex LIFT and a Reflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in the reflector mode using delayed ion extraction. Fragment ion mass spectra of selected peptides, recorded on the MALDI-TOF/TOF instrument, were used for verification of uncertain identification results. Selection of the first monoisotopic signals in the spectra was performed using the signal detection algorithm SNAP implemented in the FlexAnalysis software (Bruker Daltonics, Bremen, Germany). The spectra were calibrated using a recently described procedure (19) based on a combination of external calibration (using polyethanolglycol) followed by internal calibration using signals from tryptic autodigestion products (monoisotopic masses (MH⁺) are as follows: 842.51, 1045.562, and 2211.1045 Da) and two peptide standards (human angiotensin I and human ACTH-(18–39); 1296.6853 and 2465.1989 Da, respectively).
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The calibrated mass lists were then filtered, removing calibration masses and common contaminants. Protein identification was performed with the Mascot database search engine (version 1.8) querying primarily the SwissProt database (SwissProt www.expasy.org/spprot/) and secondarily the NCBI database (www.ncbi.nlm.nih.gov/). The minimum MOWSE scores used for identification were 52 for SwissProt searches (setting for organism Homo sapiens) and 66 for NCBI (setting for organism mammalia), corresponding to a statistical significance level of \( p < 0.005 \) in both searches. The identification of a protein was considered reliable if it was identified with this level of significance from at least two gels (without conflicting identifications in any parallel gels).

SDS-PAGE and Immunoblotting—Western blotting was performed as described (9). 15 \( \mu g \) of protein extract was separated by denaturing SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking, the membranes were probed with specific primary antibodies, washed, and incubated with horseradish peroxidase-conjugated IgG as secondary antibody. The PDX2 and PDX4 antibodies were purchased from ACRIS (Littleton, CO), TCPH-\( \eta \) from Santa Cruz Biotechnology (Santa Cruz, CA), and YB1 from Abcam (Cambridge, MA). The detected proteins were visualized by chemiluminescence (ECL, Amersham Biosciences).

siRNA Transfection and Luciferase Assay—Stable FLP-IN HEK293 cells were transfected with siRNAs against PDX4 (Invitrogen Stealth select HSS116212 and HSS116213) or a scrambled control without any homology to known transcripts together with 400 \( ng \) of pRL-TK Renilla reference plasmid and 100 \( ng \) of pRL-TK Renilla reference plasmid by using a standard protocol. 8 h later, transfection medium was replaced with regular cell culture medium. After 24 h MDP was added at the indicated concentration. Luciferase activity was determined after 8 h with a dual luciferase reporter gene kit from Promega according to the manufacturer’s manual. The cells lysates were analyzed with a MicroLumatPlus LB96V microplate luminometer (EG & G Berthold, Wellesley, MA) after automatic injection of the necessary substrate solutions. All samples were at least measured in quadruplicates in three independent experiments. The results for firefly luciferase activity were normalized to Renilla luciferase activity.

RNA Isolation and RT-PCR—Total RNA was isolated using the RNeasy kit from Qiagen. 500 \( ng \) of total RNA were reverse-transcribed as described elsewhere (20). Primers used were as follows: NOD2_\( F \), GTG TCT TCT GTA GCC TCT CC, and NOD2_\( R \), TTC ATT GGC CTA AGT CC; PDX2_\( F \), CTT CTC TGC TCA TCG ATG GAG AA, and PDX2_\( R \), ATT CTC ATT GGC CCA AGT CC; PDX4_\( F \), GTG TTC TCC GCC AGA TCA CT, and PDX4_\( R \), TGG GCT TAA TCG TGT CAT TG; TCPH_\( F \), CCG AGC AGT TTA TGG AGG AG, and TCPH_\( R \), CAA AGC CAG CAT TGT CAT AC; YB1_\( F \), TGG GCG TCG ACC ACA GTA TT, and YB1_\( R \), GCT GCT GAC CCT GGG TCT CA. For PCR, denaturation was for 5 min at 95 °C, 28 cycles of 30 s at 95 °C, 20 s at 58 °C, 60 s at 72 °C; final extension was for 5 min at 72 °C. To confirm the use of equal amounts of RNA in each experiment, all samples were checked in parallel for \( \beta \)-actin mRNA expression. All amplified DNA fragments were analyzed on 1% agarose gels and subsequently documented by a BioDoc Analyzer (Biometra, Göttingen, Germany).

RESULTS AND DISCUSSION

This study presents for the first time an analysis of the changes in the cellular proteome upon activation of NOD2 by using its ligand, the bacterial cell wall component MDP-LD, as a stimulus. The experimental design is schematically depicted in Fig. 1. The following three stable transfectants of HEK293 cells were used in the study: cells stably overexpressing NOD2\( ^{WT} \), cells overexpressing the mutant NOD2\( ^{SNP13} \), and mock transfected control cells (mock transfectant) (Fig. 2A). Samples were harvested from five parallel cell cultures under normal growth conditions and 4 and 24 h after the stimulation of the cells by MDP. Protein extracts were analyzed by two-dimensional GE. Transfected HEK293 cells represent an accepted model system for exploration of NOD2 signaling pathways, as the cells normally do not express NOD2 at detectable levels (1) (Fig. 2). The use of polyclonal cell lines excludes the risk of experimental bias from the stochastic chromosomal integration of the respective plasmids. The function of the stably expressed NOD2 protein was assessed using IL-8 enzyme-linked immunosorbent assay, which demonstrated a significant up-regulation of IL-8 secretion upon MDP stimulation (1 \( \mu g/ml \)) only in the NOD2\( ^{WT} \) transfected cells (Fig. 2B).

Following spot detection and matching, each protein spot was assigned an identification number (SuperSpotID) by which it can be tracked on all gel images where it was detected. Normalization of protein staining intensities, as described above, allowed quantitative comparisons of protein abundance between any samples in the study. To confirm that changes in abundance by NOD2\( ^{WT} \) or NOD2\( ^{SNP13} \) overexpression and/or MDP stimulation. These proteins are listed in supplemental Table 1, including their averaged, normalized spot densities in the different analyses. The averaged gel images for each sample are shown in supplemental Fig. 1, annotated with SuperSpotIDs.

Classification of Regulated Proteins—The identified proteins were sorted into functional groups derived from information obtained from SwissProt/TrEMBL and, for proteins with no assigned function in those...
data bases, through InterPro searches (GO classification, www.ebi.ac.uk/interpro/). In order for each protein to appear only in one category, the following hierarchy of protein functions was defined: regulator/DNA repair and replication/nucleic acid binding/protein folding and assembly/heat shock protein/protein modification and degradation/protein biosynthesis/nucleotide biosynthesis/carbohydrate metabolism/oxidoreductase/other metabolism/transport/structural protein/unknown function.

The result of this functional analysis is shown in Fig. 3. Of the 198 protein spots identified, 33 have been described in the literature as regulator proteins or putative regulator proteins; 22 were nucleic acid-binding proteins, DNA repair or replication proteins (without proven involvement in any regulation); 32 proteins were involved in the biosynthesis, modification, or degradation of proteins; and 25 were heat shock proteins or involved in protein folding or assembly. Of the 16 protein spots containing proteins of unknown function, 10 were novel proteins without assigned functions so far (supplemental Table 1). The observed protein distributions resembles those reported in previous work on the general influences of muramyl dipeptide on DNA and protein synthesis (21–24).

FIGURE 6. Silencing of PDX4 enhances MDP-induced NF-κB activation in NOD2WT-expressing cells. A, Western blot (WB) analysis of stable FLP-in cells HEK293 cells with recombinase-based integration NOD2WT or the empty vector cassette. Cells were transfected with two different siRNAs against PDX4 or a control siRNA in the presence of the reporter plasmids pNF-κB-Luc and pRL-TK as described (29). After 24 h, silencing of PDX4 was assessed using Western blot. Note that the FLP-IN cells carry a single copy of the NOD2 expression cassette. B, in parallel, identically treated cells were stimulated with the indicated doses of MDP for 8 h. Luciferase activity was determined by dual luciferase assay as described. Normalization regarding transfection efficiency was performed by concomitant measurement of Renilla luciferase activity. All samples were at least measured in quadruplicate in three independent experiments. Results are expressed in mean relative light units ± S.E.; *, p < 0.05; **, p < 0.01.

As expected for proteomic studies, the functional distribution observed in this study is slightly skewed if compared with the molecular function data available for all human genes from EBI (www.ebi.ac.uk; 19,779 entries as of 14.01.2005). For example, according to EBI data, about 19.9% regulators (adding up the classes GO:0030528, GO:0030234 and GO:0004871) and 15.5% of genes with nucleic acid binding function would be expected, although in our study the corresponding numbers are 16.6 and 9%, respectively. This discrepancy can be explained with the existence of genes coding for regulatory and nucleic acid-binding RNAs and with the occurrence of tissue-specific and low level regulatory proteins, which are not normally detected on two-dimensional gels of whole-cell lysates. Proteins involved in metabolic processes are, on the other hand, over-represented, as would be expected because of their predominantly high cellular concentrations. The functional classes of transport and structural proteins are covered in this study with the frequency expected from genomic data (about 5% each).

Altered Protein Abundances in Unstimulated NOD2WT and NOD2SNP13 Transfectants—Overall, 32 proteins were detected that differed in abundance between the unstimulated mock transfectants and the NOD2WT (Fig. 1, comp1). The significantly regulated proteins are depicted in a heat map (Fig. 3A). That this number is relatively large may...
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**TABLE 1**

| SuperSpot ID | Protein name | Gene name      | Protein description                                                                 | Regulation factor |
|--------------|--------------|----------------|--------------------------------------------------------------------------------------|-------------------|
| 34291        | MAT3         | MATR3          | Matrin 3                                                                              | 2.365             |
| 33385        | TPIS         | TPI1           | Triose-phosphate isomerase (TIM)                                                     | 2.213             |
| 33569        | PP11         | PTPN           | Phosphatidylinositol transfer protein α isoform (PI-TP-α)                            | 1.804             |
| 34061        | ALFA         | ALDOA          | Fructose-bisphosphate aldolase A (lung cancer antigen NY-LU-1)                       | 1.722             |
| 33588        | Q96GK4       | MESP0          | Hypothetical protein MGC272                                                         | 1.667             |
| 33589        | Q96H1        | 11148          | FSC1_HUMAN, Fascin 1, p55, 55-kDa actin-binding protein                               | 1.638             |
| 33538        | PRSX         | PSMC6          | 26 S protease regulatory subunit S10B (proteasome subunit p42)                       | 1.629             |
| 34167        | ROC          | HNRPc          | Heterogenous nuclear ribonucleoproteins C1/C2                                       | 1.523             |
| 33379        | SERA         | PHGDH          | D-3-phosphoglycerate dehydrogenase (3-PGDH)                                          | 0.651             |
| 33373        | SREC         | PSAT1          | Phosphoserine aminotransferase (PSAT)                                                | 0.613             |
| 33683        | PPCM         | 8725           | Mitochondrial phospho-enolpyruvate carboxykinase (PEPCK-M)                          | 0.579             |
| 34475        | Q9NPB2       | NTSC           | 5′(3′)-Deoxyribonucleotidase, cytosolic type                                          | 0.570             |
| 33697        | PD61         | PDCD61P        | Programmed cell death 6-interacting protein, HP95                                   | 0.512             |
| 33981        | TERA         | VCP            | Transitional endoplasmic reticulum ATPase                                           | 0.391             |
| 34080        | Q9Y6K4       | PPP1R7         | Protein phosphatase-1 regulatory subunit 7α2                                          | 0.336             |

*TPIS indicates triose-phosphate isomerase.

The regulation factors given are ratios of protein spots levels in unstimulated cells carrying NOD2<sup>SNP31</sup> divided by their levels in unstimulated cells carrying NOD2<sup>WT</sup>. These results emphasize the importance of the concept of the "basal activation" of NOD proteins, i.e. auto-activation by high expression levels (27). In the experimental system used for our initial screen, NOD2<sup>WT</sup> and NOD2<sup>SNP31</sup> are expressed at high levels. Proteins, which were identified to be differentially regulated by NOD2 expression in the absence of the NOD2-ligand, may be regulated in an MDP-dependent manner in other cell types expressing low endogenous levels of NOD2 (e.g. monocyes) as demonstrated for PDX4 (Fig. 5, A and B). However, NOD2 can be expressed in high levels (e.g. in intestinal epithelial cells under inflammatory conditions). Thus, it will be interesting to assay further the contribution of auto-activation for NOD2-induced signaling.

**Time Course of MDP-induced Changes in Protein Abundance**—The kinetics of proteome responses to MDP was assessed following 4 and 24 h of stimulation. The response of the three cell lines to MDP stimulation differed significantly. Fig. 7 shows the regulated proteins clustered according to their co-regulation among the three cell lines, after 4 and 24 h of MDP stimulation. As evident from Fig. 7 and Supplemental Fig. 3, a number of proteins exhibited transient regulation by MDP, as observed after 4 h of stimulation, although other proteins showed sustained regulation by MDP even after 24 h. For example, in the mock transfectant, transient induction was detected for the putative protein phosphatase-1 regulatory subunit 7α2 (Q9Y6K4, encoded by the PPP1R7 gene). This protein might be active in regulating phosphorylation events relevant to cell division (30). In unstimulated cells, the levels of Q9Y6K4 are also elevated in NOD2<sup>WT</sup>, but not in NOD2<sup>SNP31</sup>, if compared with levels in the control cell line.

Among the proteins regulated in a NOD2<sup>WT</sup>-specific manner are the Crk-like protein and phosphoglycerate kinase 1 (PGK1). Crk-like protein is an oncogene and an adaptor protein involved in several signaling processes. It has been shown that it associates with WASP (31), ASAP1 (32), BCR-ABL (33, 34), DOCK2 (35), and STAT5 (36), among others. Furthermore, this protein provides a link between Jak kinases and downstream cascades that act in interferon-dependent transcriptional regulation (37). The PGK1 protein is an enzyme of the glycolytic pathway, but as a primer recognition protein it also modulates DNA replication and repair in mammalian cells (38, 39). PGK1 may also be secreted and participates in angiogenic processes as a disulfide reduc-

be due to the fact that expression of NOD2 above physiological levels may activate downstream signaling pathways to a certain extent (1). The corresponding number for the comparison between the unstimulated mock transfectants and NOD2<sup>SNP31</sup> (Fig. 1, comp) is 20 proteins. Among these two sets, 9 proteins share a similar regulation, including nucleic acid-binding proteins ROG and H31, protein biosynthesis proteins SYR and DSP4, DNA repair protein MSH2, and pereoxiredoxin 4 (PDX4). PDX4 has been implicated in the modulation of the NF-κB pathway (25), and its regulation in the two-dimensional GE (Fig. 2B) was verified by Western blot analysis (Fig. 4C). Parallel regulation in cell lines overexpressing NOD2<sup>WT</sup> and NOD2<sup>SNP31</sup> might reflect the conserved ability of NOD2<sup>SNP31</sup> to induce basal signaling upon overexpression despite a clear defect in MDP-induced NF-κB transactivation (26, 27).

Most interestingly, we could confirm the NOD2-mediated PDX4 up-regulation in THP-1 cells, which express endogenous levels of NOD2 (28). Upon stimulation with the NOD2-ligand MDP-LD, a prolonged PDX4 transcript and protein up-regulation were detectable by Western blot and RT-PCR (Fig. 5, A and B). To exclude an unspecific effect, the MDP-DD stereoisomer was employed, which is not recognized by NOD2 (2, 3). Stimulation with this compound did not result in a significant up-regulation of PDX4 (Fig. 5A).

We further investigated the putative influence of PDX4 on NOD2-induced signaling pathways. NF-κB activation after MDP stimulation was assessed in the isogenic stable HEK cell populations (FLP-IN). PDX4 gene expression was silenced using two different siRNAs, which independently down-regulated PDX4 protein levels (Fig. 6A). A robust NF-κB activation after MDP stimulation (1 μg/ml) was seen exclusively in the NOD2<sup>WT</sup>-transfected cells. The NF-κB transcriptional significance was significantly enhanced by PDX4 knock down (Fig. 6B). Thus, the NOD2-mediated induction of PDX4 could be part of a negative feedback loop modulating NF-κB activation downstream of NOD2.

Comparison between unstimulated NOD2<sup>WT</sup> and NOD2<sup>SNP31</sup> cell lines (comp2) revealed several differentially expressed proteins (Table 1), including a regulatory subunit of protein phosphatase 1 (Q9Y6K4, see below), two proteasome complex proteins (PSA5 and PSE2), and cyclophilin A (PPIA) which is involved in protein folding.
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The observed up-regulation of PDX2, YB1, and TCPH in the two-dimensional GE experiments was again confirmed in THP-1 cells differentially stimulated with MDP-LD and MDP-DD by Western blot analysis (Fig. 5A). Only endogenous NOD2 activation by MDP-LD led to a marked increase of PDX2, YB1, and TCPH protein levels, whereas the inactive stereoisomer MDP-DD had no effect. To establish whether the observed changes are caused by transcriptional activation or reflect post-transcriptional regulations, cDNA from stimulated THP-1 cells was amplified using specific primers for PDX2, PDX4, YB1, and TCPH. Here a clear increase of mRNA levels of PDX2 and PDX4 paralleling the protein up-regulation was observed, whereas only a moderate increase of TCPH and YB1 mRNA could be detected (Fig. 5B). Similar results were obtained using isogenic stable HEK293 cells (FLP-IN) carrying a single copy of NOD2WT, NOD2SNP13, or the empty vector cassette (supplemental Fig. S4).

The NOD2-dependent changes in protein abundance after MDP stimulation were further analyzed by comparing protein levels in NOD2WT with those in the mock transfectant after MDP stimulation (Fig. 1, comp10 and comp11). Significant up-regulation of proteins by MDP in NOD2WT (relative to unstimulated cells, Fig. 1, comp4 and comp7) concomitant with elevated levels of those proteins in the NOD2WT cell line (relative to the control) was detected for 8 protein spots. Down-regulation by MDP and by NOD2WT overexpression was observed for 4 protein spots (see supplemental Table 1). For interpretation of the data, it is important to examine whether the protein spots are isoforms that might be differentially regulated. In Table 2, the data are listed for those significantly regulated proteins that display only one isoform in our study and that are consequently amenable to interpretation. Only a single protein with regulation by MDP in the mock transfectant and with concomitant changes in NOD2WT was detected (namely, spot 33545, which is an isoform of β-actin).

The PSE1 protein (product of the gene PSEM1) was observed at elevated levels throughout the experiments in NOD2SNP13, relative to levels in the mock transfectant, although in NOD2WT it was elevated significantly only after 24 h of MDP stimulation. This protein, also known as REG-α or PA28a, is involved in immunoproteasome assembly and required for efficient antigen processing, and it has been reported to be up-regulated by interferon γ (51). Most interestingly, PSE2, another subunit of the PA28 activator complex, has also been identified in this study, but this protein shows different patterns of regulation.

From the presented data, it is clear that distinct effects of MDP exist in all three cell lines. Shared effects between groups, e.g. a down-regulation of the heat shock 70-kDa protein 4 (HSP74) at 4 and 24 h in the NOD2WT and NOD2SNP13 cell lines, point to common pathways that are responsible for the overlap. One possible explanation could be a background expression of endogenous wild type NOD2. Although this possibility cannot be completely ruled out, several lines of evidence argue against this explanation as follows. (i) NOD2 mRNA and protein are not detectable in HEK293 cells (1). (ii) MDP activates canonical NF-κB signaling via NOD2, which is only observed in HEK293 cells stably or transiently transfected with NOD2 (1). (iii) There is a clear set of proteins that is specifically up-regulated in the NOD2WT-transfected cells and cannot be found in any other group (Fig. 7).

MDP has also been described as the ligand for the inflammasome protein NALP3/CIAS1, which has been implicated in the generation of mature IL-1β (52). We detected neither NALP3 mRNA nor protein in our cell lines at any time point (data not shown), making it tempting to

FIGURE 7. Venn diagrams identify clusters of specifically regulated proteins upon MDP stimulation in each cell line. Significantly regulated proteins in the three cell lines 4 and 24 h after MDP stimulation (relative to unstimulated cells) are illustrated in Venn diagrams to show common as well as group-specific responses. Up-regulated proteins are shown in red; down-regulated proteins are shown in green, and in the overlapping areas proteins regulated in opposing directions are shown in blue.
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TABLE 2
Selected examples of NOD2-dependent changes in protein abundance after MDP stimulation

Regulation by MDP was assessed in the NOD2WT-overexpressing cell line, with regulation factors expressed relative to protein levels in unstimulated NOD2WT cells (1st column, comp4 and comp7, respectively), whereas NOD2-dependent changes are expressed as ratios of NOD2WT levels relative to levels in corresponding samples from the mock transfectant (2nd column, comp10 and comp11, respectively).

| SuperSpot ID | Protein name | Gene name | Protein description | After MDP | MDP regulation (1) | WT to mock (2) |
|--------------|--------------|-----------|---------------------|-----------|-------------------|---------------|
| 34116        | Q9NX63       | CHCHD3    | Hypothetical protein FLJ20420 | 4 h       | 1.93              | 1.96          |
| 34204        | H2BF         | HIST1H2BN | Histone H2B.F (H2B/F/H2B.1) | 4 h       | 1.91              | 2.17          |
| 33858        | PGK1         | PGK1      | Phosphoglycerate kinase 1 (PRG2) | 24 h      | 1.57              | 1.59          |
| 33525        | Q8WFX1       | PSPE1     | Parasepelle protein 1 | 24 h      | 0.52              | 0.43          |
| 34081        | PA1G         | PAFAH1B3  | Platelet-activating factor acylhydrolase IB γ subunit | 24 h      | 0.59              | 0.53          |

speculate that MDP exerts its common effects via NOD2/NALP3-independent mechanisms. A recent report has demonstrated that surface calreticulin can act as an alternate receptor for muramyl dipeptide and induces apoptotic signaling in RK13 cells (53, 54). In accordance to previous reports (1, 5), we did not detect significant cell death induced by MDP in HEK293 cells; however, the possibility of nonapoptotic signaling via calreticulin is under current investigation.

The significant regulation of proteins triggered by MDP stimulation in the NOD2SNP13 cells points to a signaling capacity of the truncated protein, which may be different from the NOD2WT protein. In fact, we detected a cluster of proteins up-regulated by MDP stimulation in NOD2SNP13 cells, which were specific to this genetic variant. For example, MTTx2 (metaxin 2), a mitochondrial membrane protein up-regulated in lipopolysaccharide-induced liver injury (55), was significantly up-regulated after 4 and 24 h. Similarly, transient down-regulation of cathepsin D was seen only in the NOD2SNP13 cells after 4 h. Cathepsin D is a major lysosomal protease mediating programmed cell death induced by interferon-γ, Fas/APO-1, and tumor necrosis factor-α (56). The observation of NOD2SNP13-dependent regulation is in contrast to the established view that NOD2SNP13 is a loss of function mutation. In fact, a recent study also could demonstrate that macrophages from mice with a mutation corresponding to human NOD2SNP13 show increased mitogen-activated protein kinase activation and cytokine release upon MDP stimulation (57). Further studies will be required to molecularly dissect signaling downstream of activated NOD2SNP13.

The only proteins described so far as being expressed NOD2-dependently are IL-8 (9) and IL-1β (58), and α-defensins (11, 59) were not detected in this analysis. The low abundance of these proteins might preclude monitoring the amounts of these proteins by two-dimensional GE because of the detection limit of the system, which is ~0.015 µg of protein per spot (corresponding to 0.0075% of total protein). Therefore, the presented proteome responses depict the changes only in medium to highly abundant protein species, although low expressed proteins may not be detected. Still, two-dimensional GE provides a powerful technology that has been used successfully to study altered protein expression patterns on a global level, for example in the identification of mitogen-activated protein kinase pathway signaling targets (60) or in deciphering the activation response of lipopolysaccharide-primed monocytes (61).

It should be mentioned that none of the regulated proteins detected in this study have been described as NF-κB target genes. Thus, the investigation of other signaling pathways, e.g., the activation of heat shock responsive elements, may lead to a novel understanding of NOD2-mediated signaling in intestinal disease.

Some of the regulated proteins emerging from our study have already been implicated in the pathophysiology of inflammatory bowel disease. For example, the down-regulation of tumor suppressor protein p16 (CDN2; INK4; gene product of CDKN2A) and mismatch repair gene MSH2 have been implicated in inflammation-associated carcinogenesis (62–65).

The metabolic enzyme triose-phosphate isomerase (TPIS, Table 1) and the regulator proteins GDIR and PA1G have been described in the same type of regulation after stimulation of monocytes with lipopolysaccharide (61) as observed in our study after MDP stimulation. PA1G is the platelet-activating factor acylhydrolase γ subunit (PAFAH 29-kDa subunit, also called PA1B3; gene product of PAFAH1B3) and is down-regulated in NOD2WT cells upon 24 h of MDP treatment (Table 2). PAFAHs catalyze the removal of the acetyl group of the glycerol backbone of PAF, producing biologically inactive lyso-PAF. Most interestingly, PAF has been described as a potent and endogenous mediator in inflammatory intestinal processes, including inflammatory bowel disease. PAF activity is decreased in the ileal mucosa of patients with Crohn disease, and PAF activity in plasma of Crohn disease patients is inversely related to disease activity (66, 67). In addition, elevated levels of the “natural killer cell enhancing factor” peroxiredoxin 2 (NKEF8; PDX2) in cells carrying NOD2WT is in accordance with the DNA microarray data reported for samples from patients with Crohn disease (68) and may define a pathway of clinical relevance.

The description of changes in proteomic patterns in the three different HEK293 cell lines gives several clues regarding the physiological cellular responses to the bacterial cell wall component MDP. (i) It defines a part of the complex pro-inflammatory program regulated by NOD2 that encompasses a regulation of key genes involved in protein folding, DNA repair, cellular redox homeostasis, and metabolism. On the other hand, NOD2-mediated up-regulation of peroxiredoxin 4 could serve as part of a negative feedback loop attenuating the NF-κB activation upon ligand recognition. (ii) It unveils a common response pattern to MDP, which includes the regulation of a protein phosphatase-1 subunit, indicating the existence of a general MDP sensor that is independent from NOD2 and NALP3. Although the observed changes describe alterations of abundant proteins in the cell, the results also describe the complexity and dynamics of the cellular proteome, which may not reflect simple pathways (e.g. NF-κB activation). (iii) The comparison of the programs initiated by wild type NOD2 and the mutated NOD2 form, which renders individuals susceptible for Crohn disease, may provide a signature pattern that could lead to a deeper understanding of the influence of defective NOD2 in the complex barrier dysfunction observed in chronic inflammatory bowel disease.

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