

MAPK Kinase Kinase-1 Is Essential for Cytokine-Induced c-Jun NH₂-Terminal Kinase and Nuclear Factor- κ B Activation in Human Pancreatic Islet Cells

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OBJECTIVE—The transcription factor nuclear factor- κ B (NF- κ B) and the mitogen-activated protein kinases (MAPKs) c-Jun NH₂-terminal kinase (JNK) 1/2 are known to play decisive roles in cytokine-induced damage of rodent β -cells. The upstream events by which these factors are activated in response to cytokines are, however, uncharacterized. The aim of the present investigation was to elucidate a putative role of the MAPK kinase kinase-1 (MEKK-1) in cytokine-induced signaling.

RESEARCH DESIGN AND METHODS—To establish a functional role of MEKK-1, the effects of transient MEKK-1 overexpression in β TC-6 cells, achieved by lipofection and cell sorting, and MEKK-1 downregulation in β TC-6 cells and human islet cells, achieved by dicer–small interfering RNA treatment, were studied.

RESULTS—We observed that overexpression of wild-type MEKK-1, but not of a kinase dead MEKK-1 mutant, resulted in potentiation of cytokine-induced JNK activation, inhibitor of κ B (I κ B) degradation, and cell death. Downregulation of MEKK-1 in human islet cells provoked opposite effects, i.e., attenuation of cytokine-induced JNK and MKK4 activation, I κ B stability, and a less pronounced NF- κ B translocation. β TC-6 cells with a downregulated MEKK-1 expression displayed also a weaker cytokine-induced iNOS expression and lower cell death rates. Also primary mouse islet cells with downregulated MEKK-1 expression were protected against cytokine-induced cell death.

CONCLUSIONS—MEKK-1 mediates cytokine-induced JNK- and NF- κ B activation, and this event is necessary for iNOS expression and cell death. *Diabetes* 57:1896–1904, 2008

Type 1 diabetes is an autoimmune disease that results in destruction of the insulin-producing β -cells (1). Cytokines, such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ), induce β -cell death in vitro, and the local release of the same cytokines, by islet-infiltrating lymphocytes and macrophages, has been proposed to mediate pancreatic β -cell destruction in vivo (2). Levels of pro-inflammatory cytokines have been correlated to insulinitis and β -cell destruction in NOD mice (3) and in human pancreatic biopsies from patients with recent-onset type 1

diabetes (4). After receptor activation, cytokine-induced signaling involves the activation of the mitogen-activated protein kinases (MAPKs) c-Jun NH₂-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 (5,6). Interestingly, inhibition of JNK or p38 results in protection against cytokine-induced β -cell death (7,8), which points to a prominent role of these MAP kinases in cytokine-induced β -cell death. In addition to the MAPKs, IL-1 β - and TNF- α -induced signaling results in activation of the transcription factor nuclear factor- κ B (NF- κ B) (9,10). In rodent islets, cytokine-induced cell death is caused by increased nitric oxide (NO) production, which results from activation of NF- κ B-mediated inducible NO synthase (iNOS) gene transcription (2).

The MAPKs JNK, ERK, and p38 transduce a large variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, proliferation, and apoptosis (11). MAPKs are activated by dual phosphorylation of threonine and tyrosine residues and are organized in signaling cascades consisting of a three-kinase set. Consequently, an MAPK kinase kinase (MAP3K, MKKK, or MEKK) phosphorylates an MAPK kinase (MAP2K, MKK, or MEK), which in turn activates an MAPK (11).

The 196-kDa serine/threonine protein kinase MEKK-1, which belongs to the MAP3K family, mediates death signals when persistently activated. Studies in various cell types have reported that MEKK-1 promotes apoptosis in response to genotoxic stimuli, such as UV irradiation, etoposide, and cisplatin (12), and nongenotoxic stimuli, including Fas stimulation, anoikis, and pro-inflammatory cytokines (13). In most cell types, MEKK-1 activates all three major MAPK pathways (12,14), with the strongest effect on the JNK pathway through phosphorylation of MKK4 (15). Besides its serine/threonine kinase activity, MEKK-1 also acts as a ubiquitin E3 ligase (16).

The transcription factor NF- κ B is activated by a wide range of stimuli, including stress signals and proinflammatory cytokines (17). The classical NF- κ B pathway involves the release of the p50/p65 subunits from the inhibitor of κ B (I κ B) complex in the cytosol, a step induced by phosphorylation of I κ B by I κ B kinases (IKKs). When released from I κ B, the p50/p65 dimer translocates to the cell nucleus and regulates gene expression (17). Activation of NF- κ B has been shown to be protective in most nonislet cells (17). However, in pancreatic islets, the role of NF- κ B is far from clear, and several recent reports propose a pro-apoptotic role for NF- κ B in pancreatic β -cells (18,19).

The chain of events that promote cytokine-induced activation of JNK and NF- κ B in β -cells is essentially unknown. Considering the prominent role of MEKK-1 as an activator of both JNK and NF- κ B (15,20), the aim of the present study was to investigate the effect of genetic gain

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or loss of MEKK-1 function on MAPK and NF- κ B activation in response to proinflammatory cytokines.

RESEARCH DESIGN AND METHODS

Cell culture. Murine β TC-6 cells (American Type Culture Collection, Manassas, VA) at passage numbers 20–30 were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% FCS (Sigma Chemicals, St. Louis, MO), 2 mmol/l L-glutamine, 0.1 mg/ml streptomycin, and 100 units/ml benzylpenicillin. Islets from NMRI mice (Naval Medical Research Institute-established; Mølle and Bomholt, Denmark) were isolated by collagenase digestion as described previously (8) and incubated free floating in working solution. Isolated human pancreatic islets were provided by Dr. Olle Korsgren (Department of Radiology, Oncology, and Clinical Immunology, Uppsala University Hospital, Uppsala, Sweden). Human islets were maintained in RPMI 1640 (Sigma) supplemented as above, and the glucose concentration of the medium was lowered to 5.6 mmol/l. All cells were grown at 37°C in a humidified air incubator with 5% CO₂.

Plasmids. pcDNA3 (Invitrogen, Carlsbad, CA) plasmids containing either the full-length mouse MEKK-1 or the kinase inactive MEKK-1 [MEKK-1 K(1253)→M] (mut MEKK-1) cDNA were provided by Pär Gerwins (Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden). The plasmids were control sequenced at insertion sites and at the mutation site to verify the integrity of construct before experimentation. For green fluorescent protein (GFP) expression, the pd2EGFP-N1 (Clontech Laboratories, Mountain View, CA) vector was used. To overexpress the MEKK-1 constructs in β TC-6 cells, Lipofectamine 2000 was used as previously described (21).

Diced small-interfering RNA-mediated downregulation of MEKK-1 in human islet cells. Human islets, in groups of 100, were trypsinized (0.5%) for 5 min at 37°C and then treated with 30 units/ml DNase I (Amersham Life Science, Piscataway, NJ) for 2 min. The resulting free islet cells were placed in nonattachment plates and transfected with 100 ng diced small-interfering RNA (d-siRNA) directed against mouse MEKK-1 or GL3 (a firefly luciferase gene). d-siRNA directed against the 3'-end of coding region of the mouse MEKK-1 gene and 5'-end of Photinus Pyralis GL3 luciferase gene was synthesized as described previously (22). The *in vitro* transcription templates were amplified from cloned cDNAs using PCR and the following primers: MEKK-1 forward, 5'-CGGTAATACGACTACTATAGGGCTG AAGTTCTA AGCAGCGCACG-3'; MEKK-1 reverse, 5'-GCGTAATACGACTACTA TAG GGAGACAGGATATGCAACCGGGAG-3'; GL3 forward, 5'-GCGTAATACGA CTCACTATAGGAACAATTGCTTTTACAGATGC-3'; and GL3 reverse, 5'-GCGTAATA CGACTACTATAGGAGCCAGACAGTAGATCC-3'. The T7 polymerase promoter sequence is shown in bold. d-siRNA was introduced into islet cells during a 2-h incubation using Lipofectamine 2000 in 200 μ l serum-free culture medium. The transfection medium was then replaced by full culture medium, and the cells were cultured for 24–48 h.

In vitro treatment of cells and evaluation of cell viability. Transfected cells were either left untreated or treated with a mixture of cytokines (50 units/ml IL-1 β , 1,000 units/ml IFN- γ , and 1,000 units/ml TNF- α) for 48 h. Viability of β TC-6 or mouse islet cells was determined by staining the cells with 20 μ g/ml propidium iodide (Sigma) and 5 μ g/ml bisbenzimidazole (Sigma) for 10 min at 37°C. After careful washing, cells were trypsinized and analyzed by fluorescence microscopy using Openlab 3.0.4 software. Total number of cells and number of propidium iodide-positive cells were counted using the NIH Image 1.63 software by investigators not aware of sample identity.

Immunoprecipitation of MEKK-1. Cells (10⁸) were washed twice in ice-cold PBS and resuspended in lysis buffer (50 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mmol/l EDTA, 20 mmol/l β -glycerophosphate, 2 mmol/l sodium orthovanadate, 10 mmol/l sodium fluoride, 5 mmol/l sodium diphosphate decahydrate, and 0.2% protease inhibitor cocktail; Sigma) on ice for 20 min. The lysed cells were cleared by centrifugation, and remaining extracts were incubated with 5 μ g MEKK-1 rabbit polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) or 5 μ g purified rabbit IgG (control) (Sigma) for 2.5 h on ice. Immune complexes were purified by binding to 50 μ l protein A Sepharose (Amersham Biosciences) for 1 h at 4°C and thereafter washed three times with lysis buffer and once with H₂O. The Sepharose beads were resuspended in SDS sample buffer (2% SDS, 0.15 mol/l Tris, pH 8.8, 10% glycerol, 5% β -mercaptoethanol, bromphenol blue, and 2 mmol/l phenylmethylsulfonyl fluoride), and immunoprecipitates were resolved by electrophoresis.

Immunoblotting. Cells were washed with ice-cold PBS and directly lysed in SDS-sample buffer, boiled for 5 min, and separated on SDS-PAGE. Proteins were electrophoretically transferred to Immobilon filters (Amersham Biosciences). Filters were blocked in 5% BSA for 1 h, after which they were probed with anti-phospho-MEKK-1 T1383 (Abgent Envision Proteomics), anti-MEKK-1 (c22), NOS-2, p65, I κ B (all Santa Cruz), P-JNK, P-ERK, P-p38, p-MKK4, total JNK, and total ERK (all Cell Signaling, Beverly, MA) antibodies.

Bound antibodies were removed from Immobilon filters by incubating for 40 min at 55°C in 2% (wt/vol) SDS and 0.1 mmol/l β -mercaptoethanol. Horseradish peroxidase-linked goat anti-rabbit or anti-mouse were used as secondary antibody. The immunodetection was performed as described for the ECL immunoblotting detection system (Amersham Biosciences) and using the Kodak ImagerStation 4000MM. The intensities of the bands were quantified by densitometric scanning using Kodak Digital Science ID software (Eastman Kodak, Rochester, NY).

Extraction of nuclear proteins and electromobility shift assay. Cells were treated with IL-1 β (50 units/ml) for 30 min, after which proteins were extracted for electromobility shift assay (EMSA), which was performed as previously described (10). p65 antibody (0.2 μ g) (Santa Cruz) was used for supershift.

RESULTS

Transient overexpression of MEKK-1 in β TC-6 cells.

To evaluate the effect of MEKK-1 overexpression in insulin-producing cells, murine β TC-6 cells were transiently transfected using Lipofectamine 2000 together with an enhanced GFP (EGFP) expression vector alone or the EGFP vector together with either wild-type or a kinase inactive mut MEKK-1 vector. To increase the percentage of transfected cells, GFP-positive cells were sorted out by fluorescence-activated cell sorting 24 h after transfection. By using this approach, it is possible to enrich the number of GFP-positive β TC-6 cells to up to 80% (23). MEKK-1 overexpression was assessed by Western blot analysis, and 2 days after the transfection, we observed a strong increase in MEKK-1 immunoreactivity in cells transfected with wild-type and mut MEKK-1 when compared with cells transfected with GFP alone (Fig. 1A). MEKK-1 immunoreactivity was often observed as a double band, indicating that the MEKK-1 protein is posttranslationally modified in insulin-producing cells.

Cytokine-induced JNK phosphorylation is potentiated by MEKK-1 overexpression in β TC-6 cells.

To investigate the effect of MEKK-1 overexpression on cytokine-induced activation of the MAPKs JNK, ERK, and p38, we transiently transfected β TC-6 cells with GFP alone or together with either wild-type or mut MEKK-1 and treated the cells with a mixture of cytokines (50 units/ml IL-1 β , 1,000 units/ml IFN- γ , and 1,000 units/ml TNF- α). We did not detect any effect of MEKK-1 on the basal phosphorylation of JNK, ERK, and p38 in untreated cells (Fig. 1B). However, in cells treated with cytokines, we observed an increased JNK phosphorylation in wild-type MEKK-1-overexpressing cells when compared with cells overexpressing GFP or mut MEKK-1 (Fig. 1B). The MEKK-1 mutant did not significantly decrease JNK phosphorylation compared with cytokine-stimulated GFP cells. This finding indicates that the kinase dead MEKK-1 mutant acts as null mutation rather than a dominant-negative mutation. We also did not observe any effect of MEKK-1 on cytokine-induced ERK or p38 phosphorylation. These results suggest that JNK activation in response to cytokines is augmented by MEKK-1.

MEKK-1 is involved in NF- κ B signaling in β TC-6 cells.

Next, we investigated whether overexpression of wild-type or mut MEKK-1 had any effect on cytokine-induced NF- κ B activation. Thus, β TC-6 cells were transiently transfected with GFP alone or together with either wild-type or mut MEKK-1 and then treated with a cytokine mixture for 30 min. Expression of MEKK-1 did not affect I κ B levels in cells not exposed to the cytokines (Fig. 1C). However, cytokine-exposed β TC-6 cells overexpressing wild-type MEKK-1 displayed enhanced I κ B degradation when compared with cells overexpressing GFP or mut MEKK-1 (Fig.

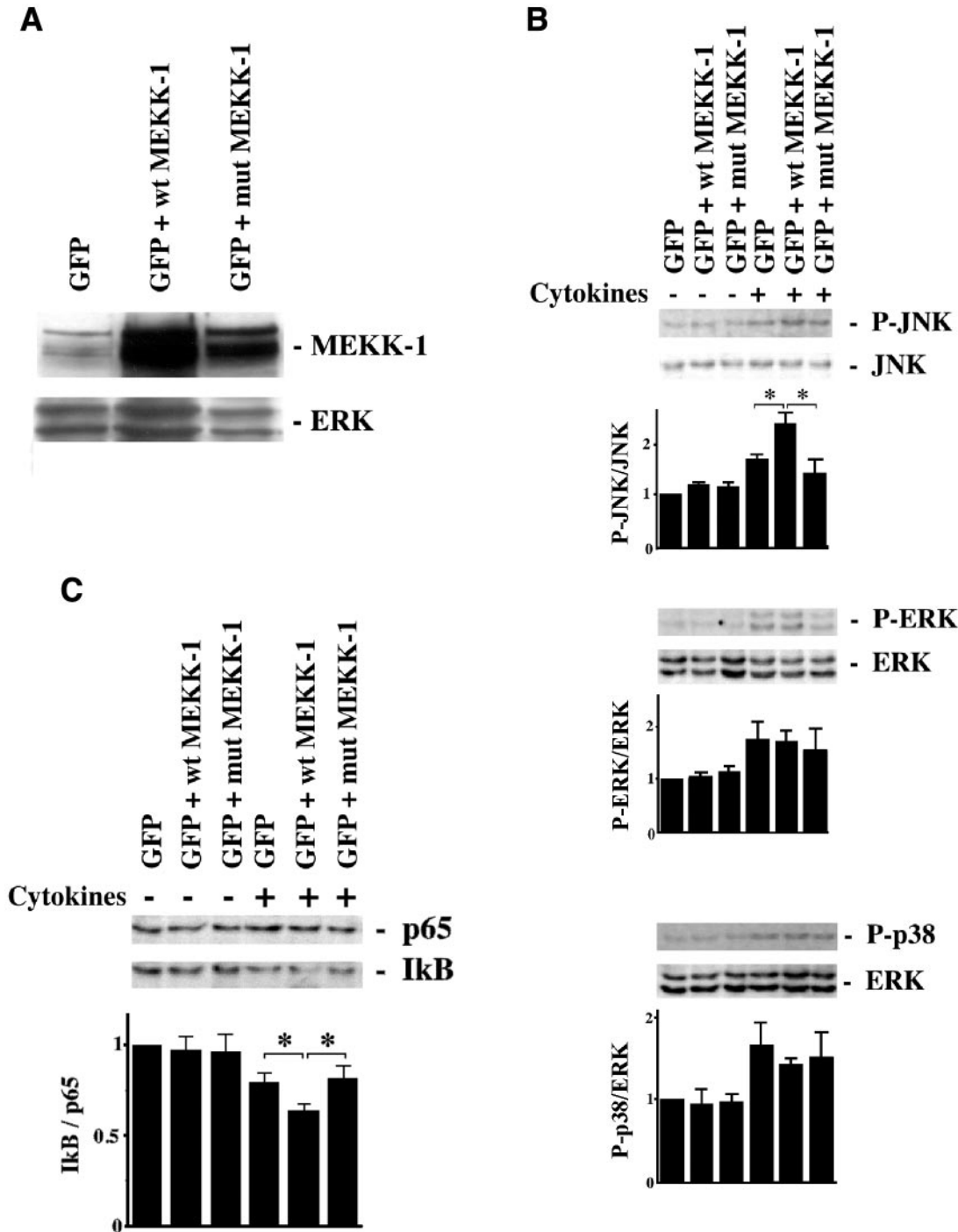


FIG. 1. Cytokine-induced JNK phosphorylation and NF- κ B activation is potentiated by MEKK-1 overexpression in β TC-6 cells. **A:** β TC-6 cells were transfected with GFP, GFP + wild-type MEKK-1, or GFP + kinase inactive mutant (mut) MEKK-1. The GFP-expressing cells were enriched by fluorescence-activated cell sorting. Two days after the transfection and cell sorting procedure, the cells were lysed, and proteins were separated by SDS gel electrophoresis and analyzed by immunoblotting with MEKK-1 and ERK antibodies. A representative blot from one out of three experiments is shown. **B:** β TC-6 cells transiently overexpressing GFP, GFP + wild-type MEKK-1, or GFP + mut MEKK-1 were either left untreated or treated with a mixture of cytokines (50 units/ml IL-1 β , 1,000 units/ml IFN- γ , and 1,000 units/ml TNF- α) 2 days after the transfection and cell sorting procedure. After 30 min of cytokine exposure, the cells were lysed and proteins were separated by SDS gel electrophoresis and analyzed by immunoblotting with phospho-specific antibodies against JNK, ERK, or p38. Results from immunoblots were quantified by densitometry. Values of phospho-protein bands were related to those of non-phospho-specific protein bands. Data are presented as means \pm SE for three individual experiments. * P < 0.05 using one-way ANOVA followed by Student's t test. **C:** β TC-6 cells transiently overexpressing GFP, GFP + wild-type MEKK-1, or GFP + mut MEKK-1 were either left untreated or treated with a cytokine mixture (50 units/ml IL-1 β , 1,000 units/ml IFN- γ , and 1,000 units/ml TNF- α). After 30 min of cytokine exposure, the cells were lysed, and proteins were separated by SDS gel electrophoresis and analyzed by immunoblotting with p65 and I κ B antibodies (*top panels*). Results from immunoblots as the one shown in the *top panel* were quantified by densitometry (*bottom panel*). Values of I κ B bands were related to those of p65 bands. Data are presented as means \pm SE for three individual experiments. * P < 0.05 using one-way ANOVA followed by Student's t test.

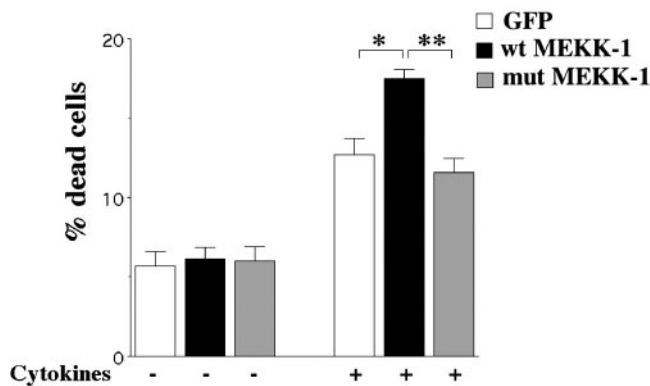


FIG. 2. Transient overexpression of MEKK-1 augments cytokine-induced cell death in β TC-6 cells. β TC-6 cells transiently overexpressing GFP, GFP + wild-type MEKK-1, or GFP + mut MEKK-1 were either left untreated or treated with a mixture of cytokines (50 units/ml IL-1 β , 1,000 units/ml IFN- γ , and 1,000 units/ml TNF- α) for 48 h corresponding to days 3–4 after the transfection and cell sorting procedure. The cells were then analyzed for cell death by fluorescence microscopy. Results from three separate observations are presented as means \pm SE. * P < 0.05 and ** P < 0.01, respectively, using one-way ANOVA and Student's t test.

1C). The data obtained from these experiments indicate that MEKK-1 may take part in the signaling pathway leading to NF- κ B activation in insulin-producing cells.

Transient overexpression of MEKK-1 augments cytokine-induced cell death in β TC-6 cells. β TC-6 cells transiently transfected with GFP alone or together with either wild-type or mut MEKK-1 were treated with a mixture of cytokines. No effect of MEKK-1 was observed in untreated cells (Fig. 2). However, β TC-6 cells overexpressing wild-type MEKK-1 showed increased rates of cytokine-induced cell death when compared with cells overexpressing GFP or mut MEKK-1 (Fig. 2). Taken together, these results indicate that MEKK-1 participates in cytokine-induced signaling, leading to the death of insulin-producing cells.

Cytokine treatment induces MEKK-1 1383T phosphorylation in β TC-6 cells. We next studied whether MEKK-1 becomes activated in response to cytokine treatment. MEKK-1 activation can be assessed by Western blot analysis of MEKK-1 T1383 phosphorylation using phospho-specific antibodies. The T1383 site is located in the MEKK-1 activation loop and becomes autophosphorylated on activation (24). β TC-6 cells were therefore left untreated or treated with a mixture of cytokines for 0.5, 1, or 3 h followed by immunoprecipitation of the MEKK-1 protein. Cytokine treatment for 1 h induced a significantly increased phosphorylation of MEKK-1 compared with untreated cells (Fig. 3).

MEKK-1 knockdown by d-siRNA in human islet cells. MEKK-1 overexpression in primary islet cells is problematic because the liposome-mediated transfection of primary islet cells is less efficient than that of β -cell lines (25). However, in a previous study, we achieved successful knockdown of target genes in dispersed primary islet cells by using liposomal reagents and d-siRNA (26). The d-siRNA technique uses the *in vitro* activity of recombinant dicer to yield a pool of d-siRNA, which seems to be more efficient than synthetic siRNA molecules (22). Therefore, we proceeded to investigate effects of genetic MEKK-1 loss of function in primary human islet cells. Dispersed human islet cells were transfected either with GL3 Luciferase (GL3 d-siRNA) or MEKK-1 d-siRNA. We observed a pro-

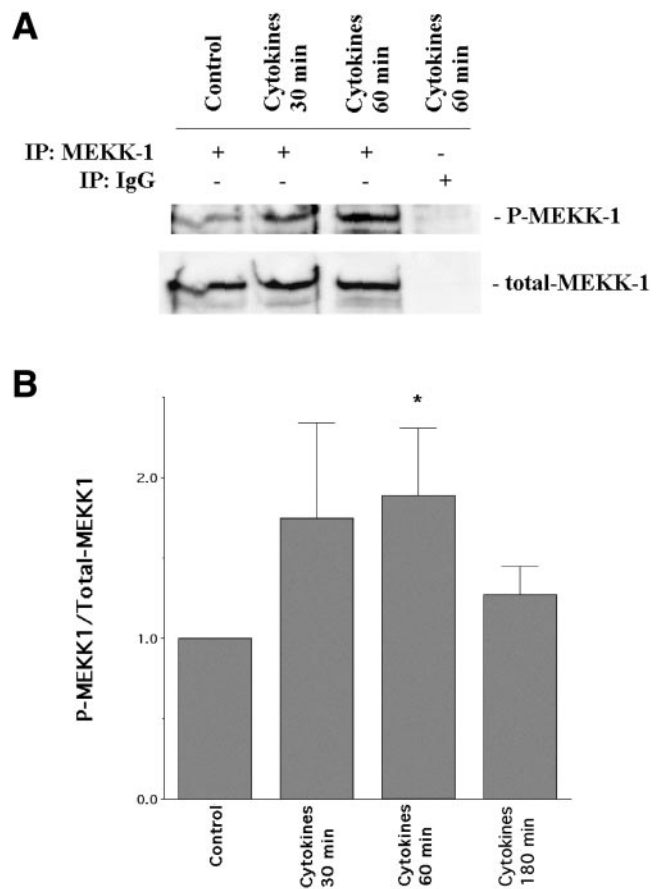


FIG. 3. Cytokine treatment induces MEKK-1 T1383 phosphorylation in β TC-6 cells. β TC-6 cells were either left untreated or treated with a mixture of cytokines (50 units/ml IL-1 β , 1,000 units/ml IFN- γ , and 1,000 units/ml TNF- α) for 0.5, 1, or 3 h. After cytokine treatment, the cells were lysed and immunoprecipitated (IP) using MEKK-1 antibody or rabbit IgG as control. The immunoprecipitated proteins were separated by SDS gel electrophoresis and analyzed by immunoblotting using T1383 phospho-MEKK-1 and MEKK-1 antibodies (A). Results from immunoblots, as the one shown in the *top panel*, were quantified by densitometry (B). MEKK-1 phosphorylation was determined by relating P-MEKK-1 bands to those of total MEKK-1. Data are presented as means \pm SE for four individual experiments. * P < 0.05 vs. control using Student's t test.

nounced downregulation of MEKK-1 protein levels in human islet cells treated with MEKK-1 d-siRNA when compared with GL3 d-siRNA (Fig. 4A).

Effects of MEKK-1 d-siRNA on JNK and MKK4 activation in human islet cells treated with cytokines. Dispersed human islet cells were treated with GL-3 or MEKK-1 d-siRNA, and 2 days after the transfection, the cells were exposed to a mixture of cytokines for 30 min. We observed a less pronounced cytokine-induced JNK phosphorylation in the MEKK-1 d-siRNA-exposed cells than in the control cells (Fig. 4B). Phosphorylation of p38 and ERK was, however, not affected by the MEKK-1 d-siRNA treatment. We also analyzed MKK4 phosphorylation in response to IL-1 β and observed a weaker activation at 0.5 and 1 h in cells treated with MEKK-1 d-siRNA compared with control cells (Fig. 4C). This indicates that IL-1 β -induced MKK4 and JNK activation requires MEKK-1 activity in human islet cells.

MEKK-1 is required for IL-1 β -induced NF- κ B signaling in human islet cells. Dispersed human islet cells were transfected with GL3 d-siRNA or MEKK-1 d-siRNA. Two days after the d-siRNA treatment, the cells were

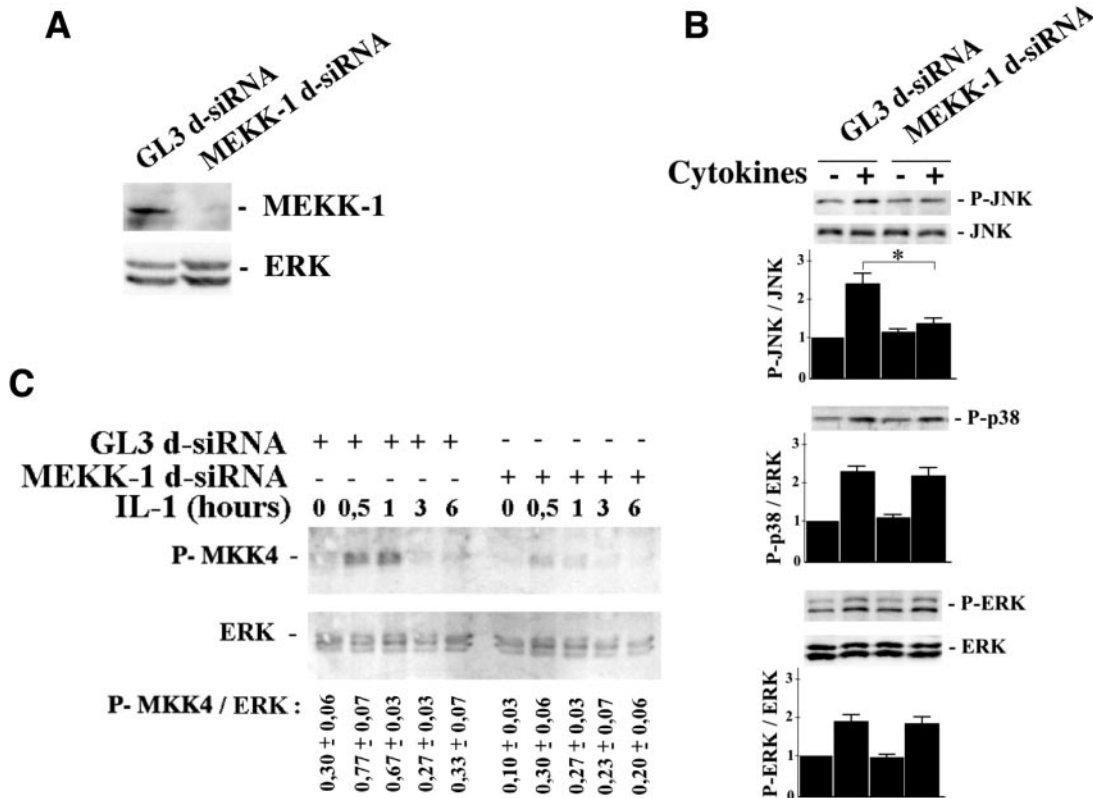


FIG. 4. Effects of MEKK-1 d-siRNA on MEKK-1 levels and JNK, p38, ERK, or MKK4 activation in dispersed human islet cells. **A:** Dispersed human islet cells were treated with GL-3 or MEKK-1 d-siRNA. Two days after the d-siRNA treatment, the cells were lysed, and proteins were separated by SDS gel electrophoresis and analyzed by immunoblotting with MEKK-1 antibodies. A representative blot from one out of two experiments is shown. **B:** Dispersed human islet cells were transfected with GL3 d-siRNA or MEKK-1 d-siRNA and either left untreated or treated with a mixture of cytokines (50 units/ml IL-1 β , 1,000 units/ml IFN- γ , and 1,000 units/ml TNF- α). After the 30-min cytokine exposure, the cells were lysed, and proteins were separated by SDS gel electrophoresis and analyzed by immunoblotting with phospho-specific JNK, ERK, and p38 antibodies. The results were quantified by densitometric scanning of three experiments and expressed as means \pm SE. **C:** Dispersed human islet cells were treated with GL-3 or MEKK-1 d-siRNA. Two days after the d-siRNA treatment, the cells were exposed to 50 units/ml IL-1 β . After 30 min of IL-1 β treatment, the cells were lysed, and proteins were separated by SDS gel electrophoresis and analyzed by immunoblotting. MKK4 activation was determined by calculating the ratios between P-MKK4 intensities and ERK intensities. Values are means \pm SE for three observations.

exposed to IL-1 β for 30 min. In dispersed islet cells treated with GL-3 d-siRNA, we observed increased degradation of I κ B after IL-1 β incubation (Fig. 5). However, in MEKK-1 d-siRNA-treated cells, no IL-1 β -induced I κ B degradation could be detected (Fig. 5), indicating that MEKK-1 is required in IL-1 β -induced NF- κ B signaling in primary human islet cells. To further support this finding, we studied the nuclear translocation of NF- κ B using an EMSA. β TC-6 cells and dispersed human islet cells were treated with GL-3 or MEKK-1 d-siRNA, and 2 days after d-siRNA incubation, the cells were either left untreated or treated with IL-1 β for 30 min. Nuclear extracts were prepared, and the presence of active NF- κ B was determined by EMSA. In both β TC-6 cells (Fig. 6A) and dispersed human islet cells (Fig. 6B), IL-1 β induced translocation of NF- κ B to the nuclei. Furthermore, MEKK-1 d-siRNA-treated β TC-6 cells and dispersed human islet cells showed a pronounced reduction in the IL-1 β -induced translocation of NF- κ B. No effect of MEKK-1 d-siRNA was observed in untreated cells (Fig. 6). These data give further support to the notion that MEKK-1 activates NF- κ B in primary human islet cells in response to stimulation with IL-1 β .

Effects of MEKK-1 knockdown on iNOS induction in β TC-6 cells. We also investigated whether MEKK-1 knockdown affects IL-1 β -induced iNOS induction. β TC-6 cells were treated with GL3 or MEKK-1 d-siRNA, and 2

days after the d-siRNA treatment, the cells were treated with IL-1 β overnight. IL-1 β treatment resulted in an induction of the iNOS protein in cells treated with GL-3 or MEKK-1 d-siRNA (Fig. 7). However, the iNOS induction was markedly inhibited in MEKK-1 d-siRNA-treated cells when compared with cells treated with GL-3 d-siRNA (Fig. 7).

MEKK-1 d-siRNA protects β TC-6 cells from cytokine-induced cell death. Finally, we determined the effect of MEKK-1 knockout on cytokine-induced cell death. Because the effect of the d-siRNA treatment is transient and because human islet cells require >7 days of cytokine exposure for increased apoptosis and necrosis to occur (27), only the rodent β -cell line and primary mouse islets, which both respond more rapidly to cytokine-induced cell death, were used for cell viability analysis. β TC-6 cells and dispersed mouse islet cells that had been transfected with GL3 or MEKK-1 d-siRNA were treated with a mixture of cytokines for 48 h and analyzed for cell death by vital staining with bisbenzimidazole and propidium iodide. Cytokine exposure resulted in increased cell death in both cell types treated with GL-3 d-siRNA, whereas cells treated with MEKK-1 d-siRNA were protected from cytokine-induced cell death, when compared with GL-3 treated cells (Fig. 8A and B). These results indicate that MEKK-1 downregulation protects β TC-6 cells and mouse islet cells

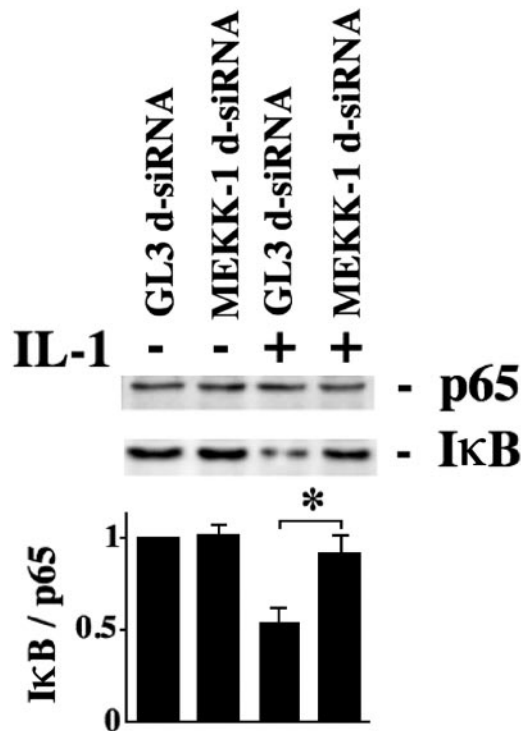


FIG. 5. MEKK-1 is required for IκB degradation in human islet cells. Dispersed human islet cells were transfected with GL3 d-siRNA or MEKK-1 d-siRNA. Two days after the d-siRNA treatment, the cells were incubated with 50 units/ml IL-1β for 30 min. The cells were then lysed, and proteins were separated by SDS gel electrophoresis and analyzed by immunoblotting with p65 and IκB antibodies (*top panel*). Results from immunoblots as the one shown in the *top panel* were quantified by densitometry. Intensities of IκB and p65 bands were used for calculation of IκB over p65 ratios. Data are presented as means ± SE for three individual experiments. **P* < 0.05 using one-way ANOVA followed by Student's *t* test.

from cytokine-induced cell death by inhibition of the JNK/NF-κB-iNOS-NO signaling cascade.

DISCUSSION

Both JNK and NF-κB, the latter presumably via IKKβ phosphorylation, are activated in insulin-producing cells when exposed to the cytokines IL-1β and TNF-α (5,8,10). The third cytokine presently used, IFN-γ, however, activates Signal transducer and activator of transcription (STAT)-1/3 and augments the actions of IL-1β, possibly by stimulating AP-1 expression and MAPK activation (28,29). Prolonged and pronounced activation of JNK, which occurs in response not only to pro-inflammatory cytokines but also to islet isolation and amyloid formation, leads to β-cell death (30,31). Activation of NF-κB is also a pro-apoptotic event in rodent β-cells because it participates in iNOS induction leading to the generation of toxic levels of NO. A number of protein kinases have been identified as activators of IKKβ and JNK in response to proinflammatory cytokines. For example, phosphorylation of JNK and/or IKK requires transforming growth factor-β-activated kinase-1 (TAK1) (32) or MEKK-2 (33) in synovocytes, mixed lineage kinase 3 (MLK3) in T-cells (34), MEKK-1 in embryonic stem cells (35), NF-κB-inducing kinase (NIK) in HeLa cells (36), and apoptosis signal regulating kinase-1 (ASK1) in human umbilical vein endothelial cells (HUVECs) (37). However, the main intracellular activator(s) of JNK/IKKβ in β-cells has, to our knowledge, hitherto not been identified. With this back-

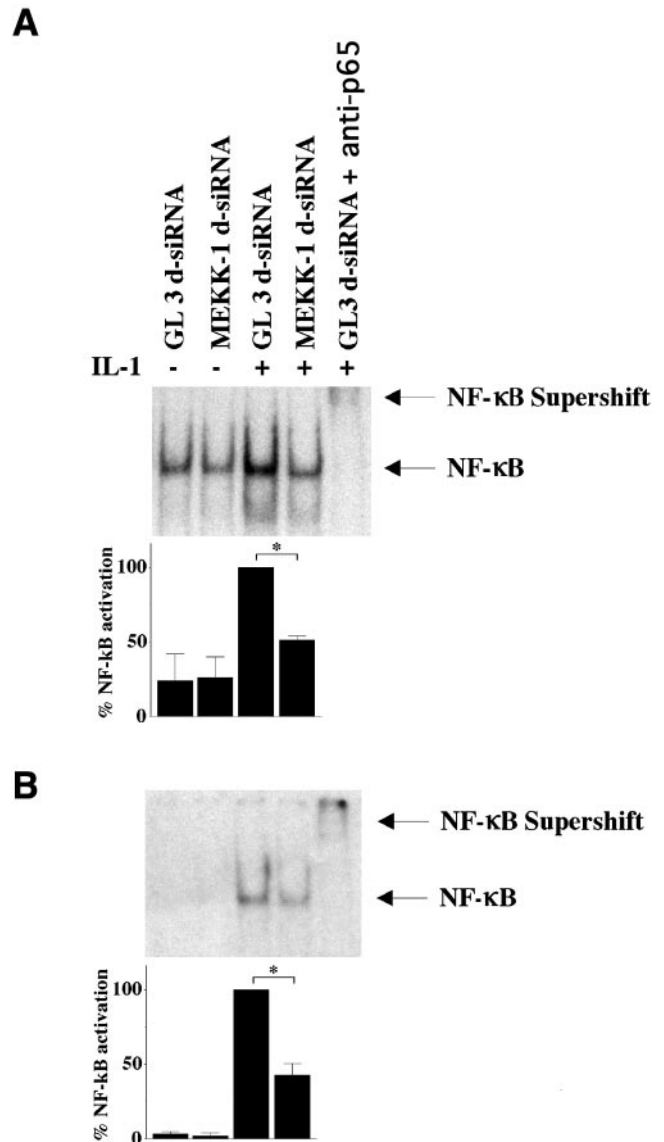


FIG. 6. MEKK-1 is required for IL-1β-induced nuclear translocation of NF-κB. BTC-6 cells (*A*) and human islet cells (*B*) were treated with GL3 or MEKK-1 d-siRNA. Two days after the d-siRNA treatment, the cells were exposed to 50 units/ml IL-1β for 30 min. Nuclear extracts were prepared and NF-κB activity was determined by EMSA. The last lane contains nuclear extracts from IL-1β-exposed cells and the p65 antibody to induce a supershift. The *bottom panels* show densitometric scanning results. Results are means ± SE for three separate experiments. **P* < 0.05 vs. GL3 + IL-1β using Student's *t* test.

ground, and using the genetic gain and loss of MEKK-1 function approach, we presently propose an essential role of MEKK-1 in cytokine-induced β-cell JNK/IKKβ activation. More specifically, we observed expression and cytokine-induced phosphorylation of MEKK-1 in β-cells, and that loss of MEKK-1 resulted in a weaker activation of MKK4, JNK, IκB degradation, NF-κB translocation, and iNOS induction in response to cytokines. In line with these results, MEKK-1 downregulation protected also against cytokine-induced cell death. It is well established that enhanced production of NO is the main cause of rodent β-cell death and that this event requires NF-κB activity (2).

An interesting issue is whether MEKK-1 knockdown would have protected against cytokine-induced death of human β-cells. Human β-cells do not benefit from iNOS inhibitors when exposed to cytokines (38,39). This raises

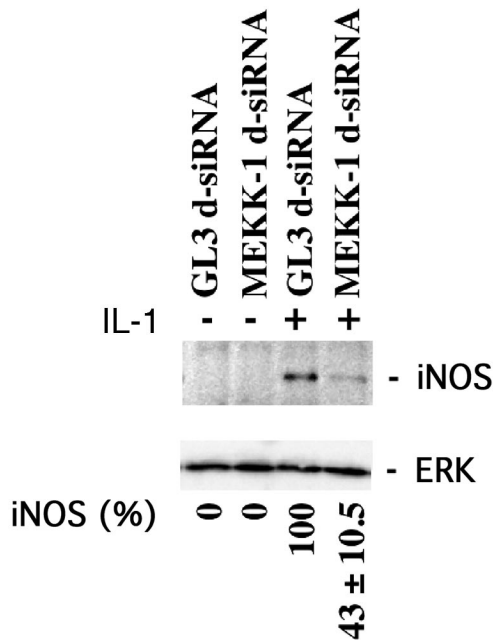


FIG. 7. MEKK-1 knockdown attenuates IL-1 β -induced iNOS induction in BTC-6 cells. BTC-6 cells were treated with GL3 or MEKK-1 d-siRNA. Forty-eight hours after d-siRNA treatment, the cells were either left untreated or treated with 50 units/ml IL-1 β overnight. The cells were then lysed, and proteins were separated by SDS gel electrophoresis and analyzed by immunoblotting with iNOS and ERK antibodies. The percentage of iNOS induction was calculated by relating iNOS bands to those of ERK-1. Data are presented as means \pm SE for three individual experiments. * P < 0.05 using Student's t test.

the possibility that NF- κ B could exert an anti-apoptotic effect in human islet cells, as it does in many other cell types (17), and that the pro-apoptotic effects of JNK activation are in part neutralized by NF- κ B, thereby explaining the weaker apoptotic response of human β -cells compared with rodent β -cells. Unfortunately, the effect of siRNA in islet cells is only transient (1–3 days) (26), and the toxic effect of cytokines requires >7 days of cytokine exposure (27). Thus, a different approach than that presently used would have been necessary to clarify this particular issue.

Previous investigations on the role of MEKK-1 in β -cells are scarce. However, it has been reported that expression of the constitutively active kinase domain of MEKK-1 promotes JNK activation (40). It has also been reported that expression of the MEKK-1 kinase domain resulted in a lowered insulin gene transcription (41), a finding that concurs well with the present study. In addition, in a very recent study, we report that MEKK-1 participates in NO-, hydrogen peroxide-, and streptozotocin-induced islet cell death (21), which indicates that multiple forms of stress converge at MEKK-1 in insulin-producing cells.

We presently report that MEKK-1 activates JNK, but not ERK or p38, in insulin-producing cells when stimulated with cytokines. This finding is in line with studies performed on non- β -cells showing that increased MEKK-1 activity leads to the preferential activation of JNK over p38 and ERK (15,42). In this context, it should also be noted that β -cells seem to use a TAB1-dependent, MKK3/6-independent p38 autophosphorylation mechanism to activate p38 in response to cytokines (43) or NO (44). Interestingly, inhibition of either JNK or p38 leads often to improved β -cell survival (7,8). This indicates that a pro-

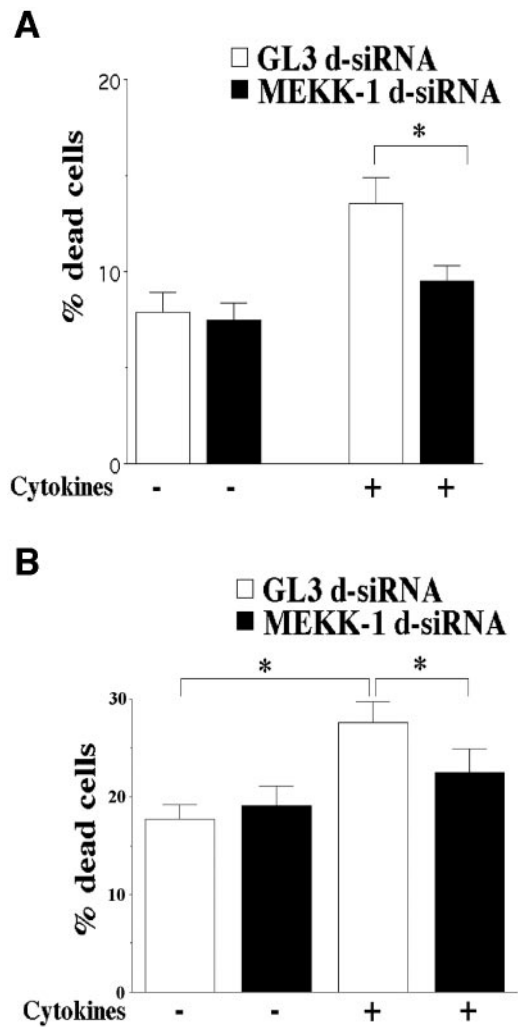


FIG. 8. MEKK-1 downregulation protects against cytokine-induced β TC-6 and mouse islet cell death. BTC-6 cells (A) and dispersed mouse islet cells (B) that had been transfected with GL3 or MEKK-1 d-siRNA were either left untreated or treated with a mixture of cytokines (50 units/ml IL-1 β , 1,000 units/ml IFN- γ , and 1,000 units/ml TNF- α) for 48 h and analyzed for cell death by vital staining with bisbenzimidazole and propidium iodide followed by fluorescence microscopy. Results from three separate observations are shown in A and are given as means \pm SE. * P < 0.05 using Student's paired t test. B: Results from eight separate observations are shown as means \pm SE. * P < 0.05 using repeated measurement two-way ANOVA followed by Student's t test.

nounced activation of one of the two MAPKs, is required for certain forms of β -cell death.

MEKK-1 was phosphorylated on the threonine residue 1383 in response to cytokines. This is in line with previous studies identifying the amino acid positions T1383 and T1395 as activation sites in MEKK-1 (24,45). The mechanisms by which MEKK-1 is phosphorylated and subsequently activated are not well understood, but it has been suggested that Rac1 (46), receptor-interacting protein (47), TRAF2 (48), and RhoA (49) by a direct interaction promote MEKK-1 autophosphorylation and activation.

In summary, our results support a pivotal role of MEKK-1 in cytokine-induced death of rodent β -cells and possibly also that of human islet cells. Because many other cell types rely on other MAP3Ks, such as ASK1, TAK, MEKK-2, DLK, and MLK3, for inflammatory signal transduction, it may be that developing and applying MEKK-1-specific inhibitors can selectively target the inflammation

of β -cells that occurs in diabetes. In this context, it is noteworthy that the 5q11-q13 region, which contains the MEKK-1 gene, has been reported to be associated with type 1 diabetes of Scandinavian families (50). Thus, there might exist polymorphisms of the MEKK-1 gene that differentially affect β -cell death in both type 1 and type 2 diabetes.

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