Interleukin-1β Stimulation of c-Jun NH_2-Terminal Kinase Activity in Insulin-Secreting Cells

Evidence for Cytoplasmic Restriction

Christopher D. Major and Bryan A. Wolf

Cytokines have been shown to have dramatic effects on pancreatic islets and insulin-secreting β-cell lines. It is well established that cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and γ-interferon (IFN-γ) inhibit β-cell function and are cytotoxic to human and rodent pancreatic islets in vitro. Despite the pleiotropic effects of cytokines on β-cells, the specific signal transduction pathways and molecular events involved in β-cell dysfunction remain largely unresolved. In this report, we have examined IL-1β stimulation of c-Jun NH_2-terminal kinase (JNK) activity in insulin-secreting clonal cell lines. We demonstrate that IL-1β transiently activates 46- and 54-kDa isoforms of JNK in cultured RINm5F β-cells. Furthermore, IL-1β stimulation of JNK activity is specific, because TNF-α and IFN-γ were without effect. Stable overexpression of JNK1 in RINm5F cells increased levels of activated JNK without affecting kinase activity. JNK-interacting protein (JIP) associates with endogenous as well as overexpressed JNK, suggesting that JIP may serve to regulate JNK activity. Finally, we demonstrate that activated JNK is fully retained in cytoplasmic and membrane compartments without any nuclear translocation. Together, these data indicate that IL-1β–stimulated JNK activity may be distinctly targeted to cytoplasmic and/or membrane compartments in clonal insulin-producing cells, and that JIP may serve to localize JNK activity to specific substrates. *Diabetes* 50:2721–2728, 2001

Mitogen-activated protein kinase (MAPK) signaling cascades consist of a core module of three protein kinases. MAPKs are activated by phosphorylation within a T-X-Y domain by one of several dual-specificity (serine/threonine and tyrosine) kinases (MAP2Ks) (1). Altogether, 13 mammalian MAPKs have been identified and classified on the basis of sequence homology and differential activation by agonists. The stress-activated subgroup of MAPKs (SA-MAPK) includes the c-Jun NH_2-terminal kinase (JNK) and p38 MAPK family members, which are activated by cellular stress, including UV irradiation, Fas, free radicals, heat shock, and proinflammatory cytokines (2–4).

The physiological role of JNK and p38 MAPK activity has been a matter of much study and debate. The SA-MAPKs have been implicated in a variety of cell responses, including cell proliferation, apoptosis, differentiation, and inflammation (5). Available evidence suggests that their roles are likely cell- and stimulus-specific. A role for SA-MAPKs in apoptosis was first reported in a serum-starved neuronal cell model, in which expression of kinase-inactive JNK or p38 MAPK activators prevented apoptosis, whereas constitutively active forms of the same activators promoted apoptosis (6). Analogous to the expression of mutant upstream activators of JNK or p38 MAPK, expression of nonphosphorylatable substrates also prevents apoptosis (7). Direct evidence supporting a role for JNK in apoptosis comes from transgenic mouse models with targeted deletions of *jnk1*, *jnk2*, or *jnk3* genes. $JNK3^{-/-}$ mice are resistant to kainate-induced neuronal apoptosis (8), whereas mice deficient in both JNK1 and JNK2 are embryonic-lethal, presenting with severe dysregulation of apoptosis in brain development (9). Additionally, kinase-inactive JNK1 and JNK2 prevent Fas-induced neuroblastoma apoptosis (10). These reports suggest a critical role for JNK in neuronal apoptosis; however, the role of JNK in the apoptotic process is not straightforward. In contrast to neuroblastoma cells, Fas-mediated apoptosis of Jurkat T-cells is JNK-independent (11,12). Further reports indicate that the role of JNK in apoptosis is largely dependent on the system examined. JNK antisense oligonucleotides prevent etopside- and camptothecin-induced apoptosis of human myeloid leukemic cells (13), whereas expression of kinase-inactive JNK1 mutants prevents UV-induced apoptosis in a small-cell lung cancer model (14). However, expression of mutant JNK activators fails to protect from

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BSA, bovine serum albumin; DMRIE, 1,2dimyristoyloxypropanyl-3-3dimethylhydroxy ethyl ammonium bromide; DTT, dithiothreitol; ERK, extracellular signal regulated kinase; IB-1, islet brain-1; IFN-γ, γ-interferon; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; IUF-1, insulin upstream factor-1; JIP, c-Jun NH_2-terminal kinase–interacting protein; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MAP2K, MAPK kinase; MEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SA-MAPK, stress-activated MAPK; TBS, Tris-buffered saline; TNF-α, tumor necrosis factor-α.

TNF-α– or extracellular matrix detachment–induced apoptosis in MCF7 breast cancer or epithelial cell lines (15,16). Furthermore, because expression of kinase-inactive JNK1 or JNK2 in an ovarian cancer model cell line only transiently inhibits paclitaxel-induced apoptosis, the role of JNK in the apoptotic process is controversial at present (17).

Equally confounding in the study of SA-MAPK signaling is the reported mechanism of JNK activity. As the name implies, JNK phosphorylates the transcription factor c-Jun. It is well established that the JNK protein kinases also phosphorylate the transcriptional activation domains of other factors, including ATFs, JunD, Elk-1, and Sap-1 (18). The phosphorylation of these factors is thought to increase transcriptional activity and protein synthesis (19). As such, phosphorylation of c-Jun by JNK is necessary for the apoptotic response in neurons (20,21). However, evidence is accumulating that the role of JNK may involve a mechanism independent of transcriptional activation. Recently, JNK1/2-deficient fibroblasts were shown to be resistant to UV-induced apoptosis resulting from a defect in the mitochondrial death pathway (22). Apoptosis in these cells does not require mRNA synthesis or new protein synthesis, in contrast to excitotoxic-induced apoptosis in neurons (8). The authors suggest that JNK may be involved in the release of cytochrome c from mitochondria by phosphorylating apoptotic regulatory proteins, such as bcl-2 and bid. As such, the association of bcl-2 and JNK and the phosphorylation of bcl-2 by JNK have been reported (23,24). Furthermore, JNK has recently been shown to mediate anisomycin-induced phosphorylation of insulin receptor substrate-1, a key mediator of insulin signaling, supporting a role for JNK signaling beyond transcriptional control (25).

There is accumulating evidence of JNK and p38 MAPK activity in insulin-secreting β-cells (26–29). IL-1β activates the three major MAPK pathways, extracellular signal regulated kinase (ERK), JNK, and p38 MAPK in islets (27,30). Based on pharmacological studies, p38 MAPK is suggested to mediate transcription of inducible nitric oxide synthase (iNOS) as well as the activity of insulin upstream factor-1 (IUF-1) in cultured islets or clonal insulin-producing cells, respectively (27,28). A putative role for p38 MAPK and possibly JNK has also been suggested in IL-1β–induced iNOS expression in astrocytes and rat glomerular mesangial cells (31,32). At present, pharmacological inhibitors of JNK are not commercially available, although a proprietary indocarbazole, CEP-1347, has been reported to inhibit the JNK signaling pathway and block motoneuron and auditory hair cell apoptosis (33,34). Whereas pharmacological studies implicate p38 MAPK activity in transcriptional regulation, the role of cytokine-mediated JNK activation and subsequent JNK-mediated signaling remains unknown in β-cells.

In this study, we sought to characterize cytokine-mediated SA-MAPK signaling pathways in β-cells using clonal insulin-producing cell lines. We report that among the proinflammatory cytokines, IL-1β specifically activates JNK and p38 MAPKs. We further report that JNK activity is mainly restricted to the cytosolic compartment with absolute nuclear exclusion and that JNK activity may be regulated by the JNK interacting protein (JIP).

RESEARCH DESIGN AND METHODS

Reagents and antibodies. Cell culture media, serum, media additives, and 1,2dimyristoyloxypropanyl-3-3dimethyl-hydroxy ethyl ammonium bromide (DMRIE)-C liposome reagent were purchased from Life Technologies (Grand Island, NY). Hybond C nitrocellulose, enhanced chemiluminescence detection system, and Hyperfilm were purchased from Amersham (Arlington Heights, IL). Recombinant mouse IL-1β, TNF-α, and IFN-γ were purchased from Genzyme (Cambridge, MA). Antibodies for JNK, p38 MAPK, phospho-JNK, phospho-p38 MAPK, and recombinant c-Jun and ATF-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-FLAG epitope (M2) antibody was purchased from Sigma Chemicals (St. Louis, MO). [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). The cDNA constructs pcDNA3.1-JNK1(wt) and pcDNA3.1-JNK1(APF) were the kind gift of Roger J. Davis (Howard Hughes Medical Institute, University of Massachusetts, Worcester, MA).

Cell culture. βTC3 insulinoma cells were obtained through the University of Pennsylvania Diabetes Endocrinology Research Center from Dr. D. Hanahan (University of California, San Francisco, CA). RINm5F insulinoma cells were purchased from the American *Type Culture* Collection (Rockville, MD). βTC3 and RINm5F cells were cultured in complete RPMI-1640 (11 mmol/l glucose) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mmol/l L-glutamine at 37°C under conditions of 95% air/5% CO_2. The medium was changed twice weekly and on the day before each experiment. Cells were trypsinized and passaged weekly. βTC3 cells were used exclusively between passages 38 and 52; RINm5F cells were used between passages 23 and 52. For cytokine studies, the indicated cytokines were added in a phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA) vehicle to complete RPMI-1640.

Stable transfection of insulin-secreting cells. RINm5F cells were transfected using DMRIE-C reagent, a 1:1 (mol/l) liposome formulation of the cationic lipid DMRIE, and cholesterol. Briefly, 32 μg DNA and 48 μl DMRIE-C reagent in a preformed lipid/DNA complex were added to 2×10^6 cells in a 10-cm tissue culture dish. Cells were incubated at 37°C for 5 h to allow uptake of plasmid DNA. The medium was subsequently changed and cultured for an additional 48 h. Cells were subsequently cultured in the presence of 200 μg/ml for 5 weeks to generate stable clones. Altogether, 20 stable clones of either empty vector (pcDNA3.1) controls, JNK1(wt), or JNK1(APF) were isolated and subcultured for an additional 5 weeks. From the original 20 clones that were isolated, 10 of each were chosen at random and screened for FLAG expression by Western blotting with anti-FLAG M2 monoclonal antibody. Clones expressing the FLAG epitope were either subcultured or aliquotted as frozen stocks.

Immunocomplex kinase assays. JNK and p38 activity were measured by in vitro immunocomplex kinase assays in the presence of [γ-^{32}P]ATP and exogenous substrate. Briefly, 1×10^6 cells in 6-well tissue culture dishes were stimulated with indicated amounts of IL-1β, IFN-γ, TNF-α, or H_2O_2 for times indicated. Cells were washed with ice-cold PBS and lysed in either JNK lysis buffer (PBS pH 7.40, 1% Triton X-100, 0.5% NP-40, 0.1% SDS, 25 mmol/l NaF, 1 mmol/l $NaVO_3$, 10 μg/ml of aprotonin and leupeptin, and 10 μg/ml phenylmethylsulfonyl fluoride [PMSF]) or p38 MAPK lysis buffer (20 mmol/l Tris-HCl pH 7.4, 1% Triton X-100, 10% glycerol, 137 mmol/l NaCl, 2 mmol/l EDTA, 25 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, and 2 mmol/l $Na_4P_2O_7$). Lysates were cleared (10,000*g* for 10 min at 4°C), and JNK or p38 MAPK were immunoprecipitated with 1 μg anti-JNK or p38 MAPK per 300 μg of total protein and 35 μl of a 50% protein-A Sepharose slurry. Immunocomplexes were washed twice with lysis buffer and twice with kinase buffer (for JNK: 20 mmol/l MOPS pH 7.6, 2 mmol/l EGTA, 10 mmol/l $MgCl_2$, 0.1% Triton X-100, 1 mmol/l dithiothreitol [DTT], and 1 mmol/l Na_3VO_4; for p38 MAPK: 25 mmol/l HEPES, 25 mmol/l β-glycerophosphate, 25 mmol/l $MgCl_2$, 2 mmol/l DTT, and 0.1 mmol/l Na_3VO_4). Kinase reactions were carried out by the addition of 50 μl of complete kinase buffer (kinase buffer containing 1 μg c-Jun, 10 μCi [γ-^{32}P]ATP, and 15 μmol/l ATP per reaction for JNK; kinase buffer containing 1 μg ATF-2, 10 μCi [γ-^{32}P]ATP, and 50 μmol/l ATP for p38 MAPK) to precipitated immunocomplexes and incubation at 30°C for 20 min. Reactions were terminated by the addition of 25 μl of 3× sample buffer (375 mmol/l Tris pH 6.8, 6% SDS, 15% glycerol, 0.009% bromophenol blue, and 45 mg/ml DTT). Samples were boiled for 5 min and centrifuged at 10,000*g* for 10 min to pelleted Sepharose. Reaction products (25–50 μl) were separated by SDS-PAGE. Gels were dried under vacuum and [^{32}P]-incorporated c-Jun or ATF-2 was visualized using a Molecular Dynamics Phosphorimager and ImageQuant Software.

Western blotting. RINm5F or βTC3 were lysed in radioimmunoprecipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 25 mmol/l NaF, 1 mmol/l $NaVO_3$, 10 μg/ml of aprotonin and leupeptin, and 10 μg/ml PMSF), and protein concentration was determined by a bicinchoninic acid protein assay. Lysates were denatured in sample buffer (125 mmol/l Tris pH 6.8, 2%

SDS, 5% glycerol, 0.003% bromophenol blue, and 15 mg/ml DTT final concentration) and boiled for 5 min before separation of protein on 10% SDS-PAGE mini-gels at 175 V for ~60 min. Proteins were subsequently transferred to Hybond C nitrocellulose membranes at 100 V for 1.5 h. Blots were blocked in Tris-buffered saline (TBS) with 3% BSA for 2 h at room temperature and probed with rabbit anti-JNK1 (C-17 or FL) or anti-p38 overnight at 4°C. Blots were rinsed with TBS/0.5% Tween-20 and washed three times with the same solution. Proteins were detected by incubation with horseradish peroxidase–conjugated secondary antibody and visualized using enhanced chemiluminescence with subsequent exposure to Hyperfilm film.

Coimmunoprecipitation analysis. βTC3, RINm5F, or RINm5F cells expressing JNK1(wt) or JNK1(APF) were lysed in Tris lysis buffer (50 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 1% NP-40, 5 mmol/l EGTA, 5 mmol/l EDTA, 20 mmol/l NaF, 0.1 mmol/l Na_3VO_4, 1 mmol/l PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotonin). Lysates were cleared by the addition of 25 μl of a 50% protein-G slurry and centrifuged at 10,000*g* for 10 min at 4°C. Lysates were immunoprecipitated with goat anti-JIP1 antibody (E-19) and 25 μl protein-G slurry overnight at 4°C. Immunoprecipitates were washed twice with lysis buffer, resuspended in 50 μl of 1× sample buffer (125 mmol/l Tris pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, and 15 mg/ml DTT), and boiled for 5 min. Immunoprecipitated proteins were separated on a 10% SDS-PAGE mini-gel and transferred to nitrocellulose. Membranes were blocked as described and probed with rabbit anti-JNK1 (C-17) antibody. JNK was identified by incubating with horseradish peroxidase–conjugated goat anti-rabbit secondary antibody and visualized using enhanced chemiluminescence.

Subcellular fractionation. RINm5F cells in 15-cm dishes were treated as indicated. Cells were washed twice with ice-cold PBS and once with fractionation buffer (0.25 mol/l sucrose, 50 mmol/l Tris-HCl pH 7.4, 25 mmol/l KCl, 5 mmol/l $MgCl_2$, 20 μmol/l leupeptin, 20 μmol/l aprotonin, 0.2 mmol/l PMSF, 50 mmol/l NaF, and 1 mmol/l Na_3VO_4). Cells were scraped in 0.3 ml fractionation buffer and homogenized in a tight-fitting (0.2-mm clearance) Dounce homogenizer tube with a motor-driven pestle for 20 strokes. As determined by cell counting, efficiency of cell shearing was 95%. Nuclear fractions were obtained by centrifuging homogenates at 600*g* for 10 min. The nuclear pellet was lysed in TNT buffer (1% Triton X-100, 150 mmol/l NaCl, 10 mmol/l Tris-HCl pH 7.4, 1 mmol/l EGTA, 1 mmol/l EDTA, 0.2 mmol/l Na_3VO_4, 20 μmol/l leupeptin, 20 μmol/l aprotonin, 0.2 mmol/l PMSF, and 50 mmol/l NaF), and centrifuged at 15,000*g* for 10 min to produce the nuclear lysate fraction. The initial supernatant (homogenate minus nuclear fraction) was subsequently centrifuged at 100,000*g* for 60 min to obtain a cytosolic extract (supernatant) and membrane fraction (pellet). Membrane pellets were dissolved in TNT buffer to obtain membrane-associated proteins.

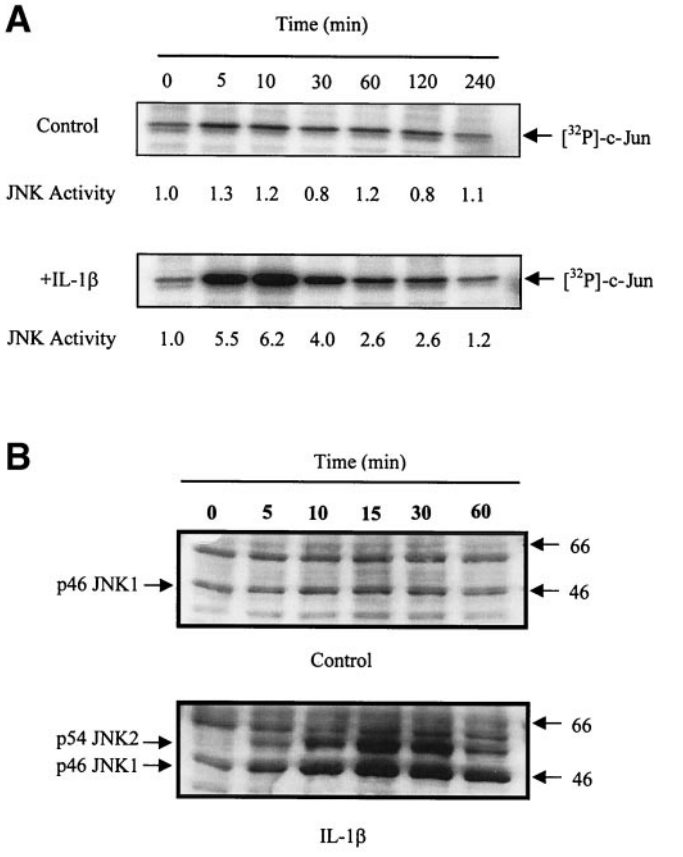

FIG. 1. IL-1β activates JNK in RINm5F cells. *A*: JNK activity in insulin-secreting β-cells. RINm5F cells were stimulated in the presence or absence of 100 units/ml IL-1β for the times indicated. After treatment, cells were lysed and JNK was immunoprecipitated with anti-JNK1 antibody. Anti-JNK1 immunoprecipitates were subject to an in vitro kinase assay in the presence of [^{32}P]ATP and recombinant c-Jun as substrate. [^{32}P]-incorporated c-Jun was separated by SDS-PAGE and visualized on a phosphorimager. c-Jun phosphorylation was quantitated using ImageQuant software, and activity is expressed as the fold activity over unstimulated cells. *B*: Activation of JNK isoforms in RINm5F cells. RINm5F cells were treated with vehicle controls (upper panel) or 100 units/ml IL-1β (lower panel) for the times indicated. Whole-cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. Phosphorylated JNK isoforms were detected by immunoblotting with antibodies specific for phosphorylated JNK. Proteins were visualized by enhanced chemiluminescence and subsequently exposed to film. Results are representative of three independent experiments.

RESULTS

IL-1β activates JNK in insulin-secreting cells. The proinflammatory cytokines IL-1β and TNF-α activate JNK and p38 MAPKs in a variety of cell types and models. To determine whether SA-MAPKs are activated by cytokines in insulin-producing cells, RINm5F cells were treated with IL-1β (100 units/ml), and JNK activity was determined by immunoprecipitation of JNK1 followed by in vitro kinase analysis of c-Jun phosphorylation in the presence of [γ-^{32}P]ATP. Results indicate that IL-1β transiently stimulates JNK activity 6.2-fold over untreated controls, peaking within 10 min of stimulation (Fig. 1*A*). Similar results were obtained with the βTC3 cell line (data not shown).

Because JNK can be expressed as 46- and 54-kDa isoforms by alternative splicing, we also probed for JNK activation by immunoblotting with phosphospecific antibodies recognizing the JNK phosphorylation motif T-P-Y. As shown in Fig. 1*B*, phosphorylation of 46- and 54-kDa JNK transcripts was observed, and JNK activation was correlated with the increased JNK activity seen in Fig. 1*A*.

Specificity of SA-MAPK activity in insulin-secreting cells. Because JNK can be activated by various cell stress agents, we sought to determine whether other stress agents could activate JNK in insulin-producing cells. Cells were treated with or without TNF-α (1,000 units/ml), IFN-γ (500 units/ml), low (0 mmol/l) or high (15 mmol/l) glucose, or 200 μmol/l H_2O_2. IL-1β potently activated JNK, with TNF-α and IFN-γ having no stimulatory effect on JNK activity (Fig. 2*A*). Although hyperglycemia (15 mmol/l glucose) and oxidative stress (H_2O_2) stimulated JNK activity 2- to 3-fold over control levels, neither treatment approached the JNK activation induced by IL-1β (22.4-fold), indicating that JNK activation is specific for IL-1β in insulin-secreting β-cells.

p38 MAPK is also activated by cell stress. To determine whether p38 MAPK, like JNK, is activated by cytokines or cell stress in β-cells, RINm5F cells were assayed for p38 MAPK activity using exogenous ATF-2 as a substrate in in vitro kinase assays. Results indicate that IL-1β potently stimulates p38 MAPK activity in both cell lines, whereas TNF-α and IFN-γ have no effect (Fig. 2*B*). Similar results were obtained for the βTC3 line (data not shown). Furthermore, compared with JNK, H_2O_2 induced potent p38 MAPK activity, indicating that p38 MAPK may also mediate the effects of oxidants on insulin-producing cells.

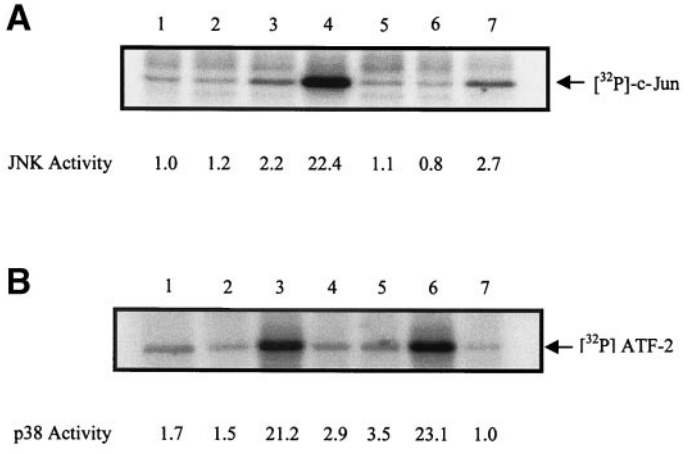

FIG. 2. IL-1β specifically activates JNK and p38 MAPKs in insulin-secreting β-cells. *A*: RINm5F cells were treated for 20 min with various cell stress agents (*lane 1*, untreated; *lane 2*, 0 mmol/l glucose; *lane 3*, 15 mmol/l glucose; *lane 4*, 100 units/ml Il-1β; *lane 5*, 1,000 units/ml TNF-α; *lane 6*, 100 units/ml IFN-γ; and *lane 7*, 200 μmol/l H_2O_2). After treatment, cells were lysed and JNK activity was determined as described. Results are expressed as the fold JNK activity of untreated controls. *B*: RINm5F cells were treated as above (*lane 1*, 0 mmol/l glucose; *lane 2*, 15 mmol/l glucose; *lane 3*, 100 units/ml IL-1β; *lane 4*, 1,000 units/ml TNF-α; *lane 5*, 100 units/ml IFN-γ; *lane 6*, 200 μmol/l H_2O_2; and *lane 7*, untreated). p38 MAPK was immunoprecipitated, and activity was determined in an in vitro kinase assay using recombinant ATF-2 as substrate. Results are representative of three independent experiments.

These data indicate that among inflammatory cytokines implicated in β-cell dysfunction, IL-1β specifically activates SA-MAPKs in insulin-secreting cells.

Overexpression of JNK1 and kinase-inactive JNK1 in insulin-secreting cells. To determine the role of JNK in β-cells, RINm5F cells were transfected with FLAG-epitope–tagged versions of JNK1 or a nonphosphorylatable mutant JNK1, whereas the canonical T-P-Y activation motif has been mutated to A-P-F [JNK1(APF)] (2). Based on reports of JNK1(APF) expression in a small-cell lung cancer model, kinase-inactive JNK is predicted to behave as a competitive inhibitor of endogenous JNK, thereby inhibiting JNK-mediated signal transduction (14). Stable cell lines of each transfectant were screened for FLAG or JNK expression (Fig. 3*A*). To determine whether expression of JNK1(APF) inhibits endogenous JNK in RINm5F cells or, conversely, whether JNK1 overexpression enhances endogenous JNK activity, immunoprecipitation kinase assays were performed on cells treated with or without IL-1β. Expression of JNK1 had no additive effect on JNK activity immunoprecipitated from IL-1β–treated cells, as determined by c-Jun phosphorylation (Fig. 3*B*). In the stable clones tested, wild-type JNK1 expression did not significantly increase JNK activity versus parental or vector controls, despite overexpression of the JNK1 protein. Conversely, JNK1(APF) had no quantitative inhibitory effect on JNK activity. Additionally, expression of the JNK1(APF) did not affect the activation of endogenous JNK, as determined by immunoblot analysis of IL-1β–treated JNK1(APF)-expressing clones (Fig. 3*C*), further indicating that JNK1(APF) does not function as a competitive inhibitor of endogenous JNK in RINm5F cells. Interestingly, expression of wild-type JNK1 did lead to enhanced phosphorylation of JNK (Fig. 3*C*), which did not correlate with an increase in JNK activity, raising the

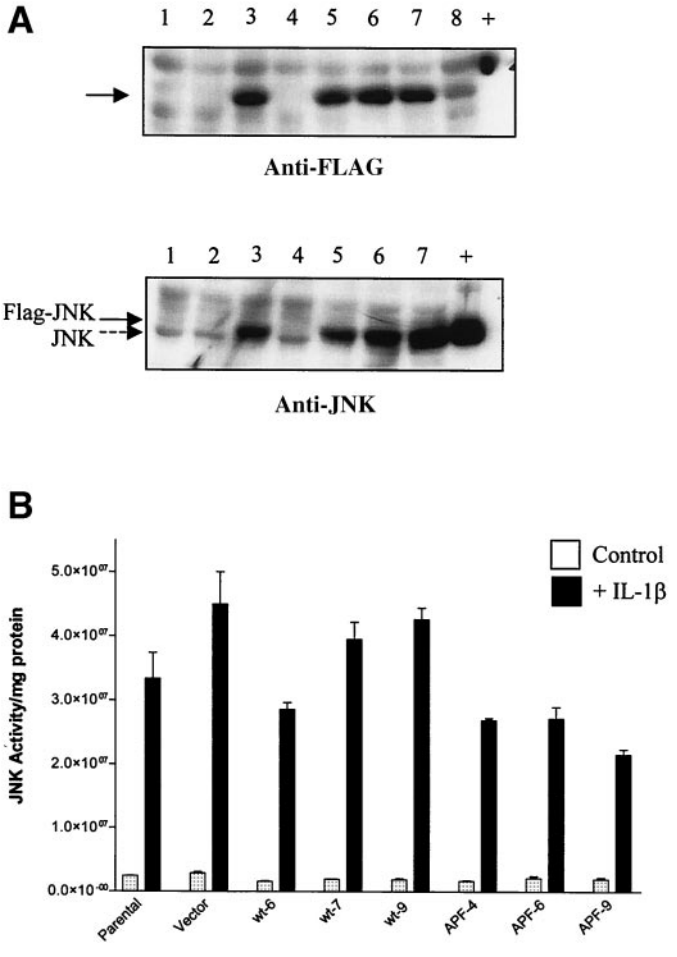

FIG. 3. Effect of ectopic JNK1 expression on RINm5F JNK activity. *A*: RINm5F cells (parental cells; *lane 1*) were stably transfected with empty vector (*lane 2*) or pcDNA3.1 vector encoding either FLAG-JNK1(wt) (*lanes 3*, *5*, and *7*) or FLAG-JNK1(APF) mutant constructs (*lanes 4*, *6*, and *8*). Cells were screened for expression of FLAG-tagged JNK proteins (upper panel) or total JNK (lower panel). Upper control lanes (+): FLAG-tagged BAP. Lower control lanes: GST-JNK1 recombinant protein. *B*: Parental, empty vector control or stable clones expressing JNK1 (wt-6, wt-7, wt-9) or JNK1(APF) mutants (APF-4, APF-6, APF-9) were stimulated in the presence or absence of IL-1β for 20 min. JNK activity was determined by immunoprecipitation-kinase assays as described and normalized to lysate protein. Results represent the means ± SE from duplicate observations from three independent experiments. *C*: Empty vector (EV) or stable clones overexpressing JNK1 (*lane 1*) or JNK1(APF) mutant (*lanes 2*, *3*, *4*) were treated with or without 100 units/ml IL-1β for 20 min. JNK activation was determined by immunoblotting with antibodies specific for activated JNK. Proteins were visualized by enhanced chemiluminescence and subsequently exposed to film. Results are representative of two independent experiments.

hypothesis that JNK activity may be tightly regulated in insulin-producing cells.

JNK interacts with JIP. Recently, JIP has been described as a cytosolic scaffolding protein capable of bind-

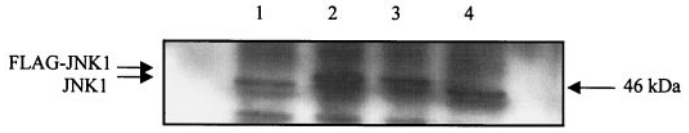

FIG. 4. JNK coimmunoprecipitates with JIP in β-cells. βTC3 (*lane 1*), RINm5F cells expressing FLAG-JNK1 (*lane 2*) or FLAG-JNK1(APF) (*lane 3*), and parental RINm5F cells (*lane 4*) were lysed and immunoprecipitated with anti-JIP antibody. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-JNK1 antibody. Proteins were visualized by enhanced chemiluminescence and subsequently exposed to film. Results are representative of three independent experiments.

ing JNK as well as upstream components of the JNK pathway (35,36). Therefore, if a JNK scaffolding protein is highly expressed in islet β-cells, JIP may be a regulatory component of JNK signaling in β-cells. To determine whether JNK is associated with JIP in insulin-producing cells, JIP-1 was immunoprecipitated from βTC3, RINm5F, or stable clones expressing FLAG-tagged JNK1 constructs. JIP-1 immunoprecipitates were then immunoblotted with anti-JNK to probe for association. Figure 4 indicates that JNK coprecipitates with JIP in βTC3 and RINm5F cells (*lanes 1* and *4*, respectively). Furthermore, JIP coprecipitates FLAG-tagged JNK1, because a doublet was observed in stable clones expressing either FLAG-JNK1 or FLAG-JNK1(APF) (*lanes 2* and *3*, respectively). Because JIP associates with endogenous as well as overexpressed JNK, this suggests that JIP may potentially regulate JNK in RINm5F and βTC3cells.

Subcellular localization of JNK in insulin-secreting cells. JIP-1 was originally described as an inhibitor of JNK, preventing nuclear translocation (35). A current view is that JIP may serve as a scaffolding protein for specific kinases in the JNK pathway (1). Furthermore, localization of JIP to the tip of extending neurites suggests that JNK may be distinctly targeted to subcellular locales for interaction with putative substrates (37). Conversely, JNK translocation to the nucleus has been reported in the ischemic heart (38), and an epitope-tagged JNK1 or a constitutively active JNKK2-JNK1 fusion protein localizes to the nucleus after UV treatment (39). These results suggest that JNK localization may be cell type–specific and, furthermore, that JNK may have cytosolic as well as nuclear substrates. To determine the subcellular localization of JNK, parental RINm5F or FLAG-JNK1–expressing cells were stimulated in the presence or absence of IL-1β for 20 min, and cytosolic, nuclear, and membrane preparations were obtained via differential centrifugation, as described. In parental RINm5F cells, JNK expression is enriched in the cytosolic fraction and is also present in crude membrane fractions (Fig. 5, upper panel). JNK expression was not detected in the nuclear fraction of quiescent RINm5F cells, although minimal JNK translocation to the nucleus was observed upon IL-1β treatment. In cells overexpressing JNK1, the kinase was identified in cytosolic, nuclear, and membrane fractions, with the majority being cytoplasmic (Fig. 5, lower panel). No significant translocation was observed upon IL-1β stimulation.

To determine the subcellular localization of active JNK, RINm5F and cells overexpressing JNK were stimulated in the presence or absence of IL-1β, and cell fractions were probed for phosphorylated JNK using phosphospecific

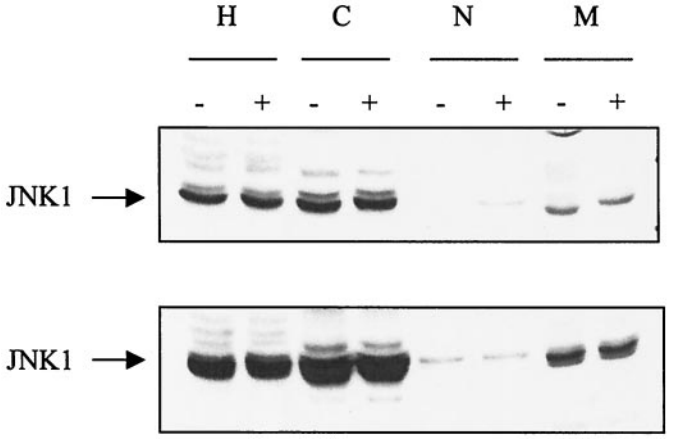

FIG. 5. Subcellular localization of JNK1 in insulinoma cells. Parental (upper panel) or FLAG-JNK1–expressing RINm5F (lower panel) cells were treated with or without 100 units/ml IL-1β for 20 min. Subcellular fractions were prepared by ultracentrifugation as described. (H, homogenate; C, cytosolic fraction; N, nuclear fraction; M, membrane fraction.) Equal protein amounts were separated by SDS-PAGE, and JNK expression was detected using anti-JNK1–specific antibody. Results are representative of three individual experiments.

antibodies. The rationale for this experiment is that if activated JNK translocates to the nucleus upon IL-1β stimulation, a detectable shift in JNK localization from the cytosolic compartment to the nucleus should be observed. In contrast, if JNK is targeted to cytosolic or membrane substrates, phosphorylated JNK should be detected in specific fractions. Our data indicate that in nontransfected cells, activated JNK1 remained predominantly cytoplasmic and was excluded from the nuclear compartment (Fig. 6, upper panel). Phosphorylated JNK was also detected in membrane fractions, suggesting potential membrane or cytoskeletal localization. In FLAG-JNK1–expressing cells, similar distribution of phosphorylated JNK was observed. JNK phosphorylation was observed mainly in cytosolic fractions, with minor localization to membranes (Fig. 6, lower panel). Furthermore, consistent with results in nontransfected cells, phosphorylated JNK was absent in nuclear fractions. Cytosolic and/or membrane localization of phos-

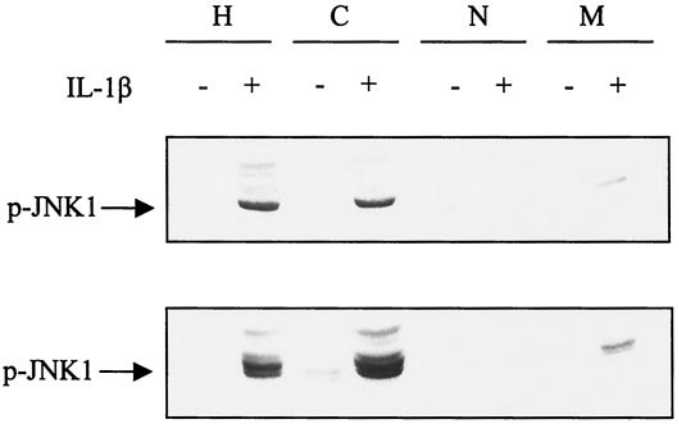

FIG. 6. Subcellular localization of activated JNK1 in insulinoma cells. Parental (upper panel) or FLAG-JNK1(wt)–expressing RINm5F (lower panel) cells were treated with or without 100 units/ml IL-1β for 20 min. Subcellular fractions were prepared as described (H, homogenate; C, cytosolic fraction; N, nuclear fraction; M, membrane fraction.). Equivalent proteins were separated by SDS-PAGE, and JNK expression was detected using anti–phospho-JNK1–specific antibody. Results are representative of three individual observations.

phorylated JNK, coupled with evidence that JNK associates with JIP, suggests that JNK activity may be targeted to the cytoplasmic- or membrane-associated substrates.

DISCUSSION

In this study, RINm5F and βTC3 insulin-producing cell lines were used to study SA-MAPK activity in β-cells. IL-1β transiently increased JNK activity in both RINm5F and βTC3 lines, supporting previous observations in rat islets (27). JNK activation was largely IL-1β–specific because TNF-α, which activates JNK in other systems (40), and IFN-γ had no effect on kinase activity (Fig. 2*A*). Additionally, IL-1β and H_2O_2 also stimulated p38 MAPK activity to comparable levels (Fig. 2*B*). Because IL-1β plays a key role in the pathogenesis of type 1 diabetes (41–44), SA-MAPK has become a potential mediator of the effects of IL-1β on β-cells. JNK and p38 MAPK activity has been reported in β-cells, but their role is thus far unclear. Pharmacological inhibition of p38 MAPK and ERK in rat islets prevents iNOS expression and partially inhibits nitric oxide (NO) production, whereas inhibition of p38 MAPK alone is implicated in transcriptional activity of IUF-1 (27,28). In an effort to specifically inhibit the JNK pathway, we chose to express kinase-inactive JNK1 in insulin-producing cell lines. Expression of JNK1(APF) and JNK2(APF) has been shown to inhibit endogenous JNK activity (10,14). Furthermore, ectopic expression of this kinase inactive mutant inhibits Fas-, paclitaxel-, and UV-induced apoptosis (10,14,17), indicating that a molecular approach to kinase inhibition may provide insights into IL-1β–mediated β-cell dysfunction.

Wild-type or kinase-deficient JNK1 was stably expressed in RINm5F cells, as determined by expression of the FLAG epitope (Fig. 3*A*). However, expression of wild-type JNK1 in RINm5F cells did not affect IL-1β–induced JNK activity, despite an increase in levels of phosphorylated or activated JNK (Fig. 3). Conversely, expression of kinase-inactive JNK did not inhibit either endogenous JNK activation or JNK activity. This unexpected result could not be explained by the expression of a nonfunctional kinase, because expression of epitope-tagged JNK1 encoded a functional kinase, as determined by anti-FLAG immunocomplex kinase assays (data not shown). In contrast, expression of kinase-inactive JNK1 did encode a nonfunctional kinase, as expected (data not shown). Therefore, expression of kinase-dead JNK1 did not interfere with endogenous JNK activity as predicted. One possible explanation could be the functional redundancy of JNK2 signaling in clonal insulin-producing cells. IL-1β stimulated the activation of both 46-kDa JNK1 and 54-kDa JNK2 in RINm5F cells (Fig. 1*B*). It may therefore be necessary to inhibit JNK2 as well as JNK1 in β-cells to completely inhibit JNK signaling. Correspondingly, expression of JNK1(APF) or JNK1(wt) did not alter the susceptibility of RINm5F cells to IL-1β–induced iNOS expression or NO accumulation (data not shown). Recent evidence from compound knockout mice demonstrates the redundancy of JNK activity (22). JNK1- or JNK2-deficient mouse embryonic fibroblast (MEF) cells maintain UV- and fetal bovine serum–stimulated JNK activity. In contrast, MEF cells derived from $JNK1^{-/-}$ or $JNK2^{-/-}$ mice possess no JNK activity.

An alternate explanation as to why expression of either wild-type or kinase-inactive JNK1 in RINm5F cells did not alter endogenous JNK activity is that JNK activity may be tightly regulated in β-cells. Interestingly, JIP, a putative molecular scaffold, was originally reported (and named) as a JNK inhibitory protein (35). It was reported that overexpression of JIP-1 localized to the cytoplasm and served to inhibit JNK signaling by sequestering JNK in the cytoplasm. Furthermore, the mouse homologue of JIP is named islet brain-1 (IB-1) because expression of the protein is most pronounced in pancreatic islets and brain (45). Paradoxically, JIP proteins have been implicated in inhibition as well as augmentation of JNK activity in the same laboratory (35,46). Paradox notwithstanding, JIP proteins may serve as regulators of JNK signaling and, as such, have been shown to bind MAP2Ks and MAP2K kinases (MAP3Ks) of the JNK pathway (46). We demonstrate here that JIP associates with JNK in both βTC3 and RINm5F cells and, furthermore, binds ectopically expressed JNK1, suggesting that JIP may regulate JNK activity in insulin-producing cells. Interestingly, a recent report suggests JIP is a key mediator of apoptosis in insulin-secreting β-cells because cells expressing an inducible IB-1 antisense RNA display reduced IB-1 expression and an increased apoptotic rate in response to IL-1β (47). These data further implicate JNK and JNK regulation in IL-1β–mediated β-cell dysfunction.

To determine the putative mechanism of JNK action in β-cells, we sought to determine the subcellular localization of JNK as well as IL-1β–activated JNK in insulin-producing cells. MAPKs can undergo a pronounced nuclear translocation upon activation. The process is best understood as pertaining to ERK signaling (48). Correspondingly, nuclear translocation of JNK has been reported during ischemia and reperfusion of the rat heart (49), and a JNKK2-JNK1 fusion protein that acts as a constitutively active JNK predominantly locates to the nucleus, stimulating c-Jun transcriptional activity (39). Additionally, both nuclear and cytosolic JNK activity, with no apparent translocation, has been reported in epidermal keratinocytes (50). In contrast, JNK has also been implicated in the regulation of cytoplasmic proteins. Activated JNK has been localized to microtubules as well as mitochondria (24,51). We show here that JNK activity in RINm5F cells is predominantly cytosolic, with minor expression in membrane fractions (Fig. 5). We further demonstrate that IL-1β–activated JNK is fully retained in the cytosol with no apparent nuclear translocation (Fig. 6). We propose that IL-1β–mediated JNK signaling may play a role in the phosphorylation of cytosolic or membrane targets and, furthermore, that it is regulated by interaction with JIP proteins.

In summary, we have shown that IL-β transiently activates JNK and p38 MAPKs in insulin-producing cells, JNK associates with JIP proteins, and activated JNK is targeted to the cytoplasm with absolute nuclear exclusion. We propose a role for JNK activity in the phosphorylation of cytoplasmic targets in IL-1β–mediated β-cell dysfunction. Further studies are necessary to define specific targets of JNK activity in β-cells, as well as determine the mechanisms of JNK downregulation and its role in β-cell responses to IL-1β.

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