Expression of Mixed Lineage Kinase-1 in Pancreatic β-Cell Lines at Different Stages of Maturation and during Embryonic Pancreas Development*

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Events controlling differentiation to insulin-secreting β-cells in the pancreas are not well understood, although β-cells are thought to arise from pluripotent ductal precursor cells. To search for signaling proteins that might be involved in β-cell maturation, we analyzed protein kinase expression in two developmentally and functionally distinct pancreatic β-cell lines, RIN-5AH and RIN-A12, by reverse transcriptase polymerase chain reaction. A number of tyrosine and serine/threonine kinases were identified in both lines. One protein kinase, mixed lineage kinase-1 (MLK-1), was expressed at both the RNA and protein levels in RIN-5AH cells, which display an immature β-cell phenotype, but was not detected in the more mature RIN-A12 cells. Furthermore, levels of MLK-1 mRNA and protein were increased after brief stimulation of RIN-5AH cells with either the differentiation inducer, sodium butyrate, or with serum after serum starvation. These increases in expression were independent of phenotypic markers such as insulin secretion or surface expression of major histocompatibility class I- and A2B5-reactive ganglioside. In addition, increases in MLK-1 expression in the stimulated RIN-5AH cells were accompanied by phosphorylation of MLK-1 on serine but not tyrosine. Antisense oligonucleotides to two distinct regions of MLK-1 caused RIN-5AH cells, but not RIN-A12 cells, to adopt a highly differentiated morphology, with a reduction in DNA synthesis and MLK-1 protein levels and elevated glucagon mRNA levels, but with no effect on insulin mRNA. In an immunohistochemical survey of embryonic mouse tissues, we found that temporal expression of MLK-1 was regulated in a tissue-specific manner. In the embryonic pancreas, MLK-1 expression was evident in ductal cells from day 13 to 16 but was not detected in late stage gestation or neonatal pancreases. These data suggest that MLK-1 is regulated in immature pancreatic β-cells and their ductal precursors at the level of functional maturity and may therefore play a role in β-cell development.

The pancreas responds to changes in blood glucose concentra-

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1 The abbreviations used are: PYK, protein-tyrosine kinase; MLK, mixed lineage kinase; RIN, rat insulinoma; FBS, fetal bovine serum; MHC, major histocompatibility complex; ODN, oligonucleotide; AS-ODN, antisense oligonucleotide; S-ODN, sense oligonucleotide; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.
entially expressed in the two β-cell lines. Because MLK-1 was expressed only in the more immature β-cell type, the expression of MLK-1 in these cells was examined under both resting and stimulated conditions and during pancreas development in the embryonic mouse. Furthermore, MLK-1 antisense oligonucleotides were used to link MLK-1 expression to the phenotypic status of the immature β-cell.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antiserum was raised in rabbits against a recombinant protein containing the kinase catalytic domain (residues 101–359) of human MLK-1 (18) fused to glutathione S-transferase. Antiserum was absorbed twice against glutathione S-transferase bound to glutathione-Sepharose beads. Antibodies were further purified by precipitation with 33% saturated ammonium sulfate followed by dialysis against phosphate-buffered saline (PBS). The antibodies were tested for recognition of MLK-1 on Western blots of lysates of COS cells transfected with a cDNA encoding the kinase catalytic domain of human MLK-1 without glutathione S-transferase. Control antibodies were derived from preimmune rabbits. Monoclonal anti-rat MHC class 1 (RTI.A) antibody, CL007A, was a gift from Cedars Laboratory (Ontario, Canada), and monoclonal anti-A2B5 was obtained from the American Type Culture Collection (Rockville, MD). Anti-phosphotyrosine antibodies (PY-20) were from Upstate (Schenectady, NY), and anti-phosphoserine antibody PSR-45 was from Sigma. Swine anti-rabbit horseradish peroxidase was from Dako (Carpinteria, CA), and sheep anti-rabbit and anti-mouse horseradish peroxidase was from Silenus Laboratories (Sydney, Australia).

Cell Culture and Functional Status—Rat insulinoma cells were continuous clonal cell lines established from a nude mouse heterotransplant of a transplantable islet cell tumor (19) maintained in culture at 37 °C in an atmosphere of 10% CO₂ in air, in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Insulin released by cultured cells was measured by radiomuonassay (Phadebus Insulin RIA, Pharmacia, Uppsala, Sweden) of culture supernatants 5 days after treatment. Cell surface expression of both MHC class 1 and II and the kinase catalytic domain of MLK-1 were examined in these two cell lines using commercially prepared phenol/guanidine isothiocyanate reagent (20). Total or poly(A)-selected RNA transcripts (purified using PolyATtract mRNA isolation system III, Promega) were reverse transcribed and amplified using a 320A sequenator (Applied Biosystems, Foster City, CA). The primer MLK-1 probe was a 1.07-kilobase pair cDNA fragment encoding the region corresponding to the kinase catalytic and leucine zipper domains as reported for human MLK-1 (18). The mouse MLK-2 probe was a 250-base pair cDNA fragment coding for the C-terminal end of the kinase catalytic domain and the beginning of the leucine zipper domain. Both MLK cDNAs were isolated from a mouse brain cDNA library in λ gt11 (CLONTECH, Palo Alto, CA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 1.4-kilobase pair fragment of rat GAPDH cDNA and was a gift of Dr. R. Ramsay (Peter MacCallum Cancer Institute, Melbourne, Australia).

Northern Analyses—Total RNA samples were run at high stringency (purified using PolyATtract mRNA isolation system III, Promega) were examined by Northern analyses performed by standard methodology (22). Briefly, RNA was electrophoresed on a 1% formaldehyde agarose gel and transferred to GeneScreen (Du Pont NEN, Boston, MA) using a 320A sequenator (Applied Biosystems, Foster City, CA). The mouse MLK-1 probe was a 1.07-kilobase pair cDNA fragment encoding the region corresponding to the kinase catalytic and leucine zipper domains as reported for human MLK-1 (18). The mouse MLK-2 probe was a 250-base pair cDNA fragment coding for the C-terminal end of the kinase catalytic domain and the beginning of the leucine zipper domain. Both MLK cDNAs were isolated from a mouse brain cDNA library in λ gt11 (CLONTECH, Palo Alto, CA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 1.4-kilobase pair fragment of rat GAPDH cDNA and was a gift of Dr. R. Ramsay (Peter MacCallum Cancer Institute, Melbourne, Australia).

Metabolic Labeling and Immunoprecipitation—Cells (2 × 10⁵) were plated in six-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) and allowed to adhere for 48 h prior to treatment with MLK-1 sense or antisense ODNs as described. [H]Thymidine (50 μCi/well) was added for 12 h in the presence of fresh ODNs. Treated cells were washed with ice-cold PBS prior to lysis with 0.5 M EDTA and precipitation with 10% trichloroacetic acid. Precipitated DNA was recovered by filtration over premoistened Whatman GF/C filters and washed three times with ice-cold PBS under vacuum. Air-dried filters were placed in scintillant for β-galactosidase assays.

For immunoprecipitations, an equivalent number of dpm of preimmune rabbit serum was added to RIN cells (2 × 10⁵) plated in six-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) and allowed to adhere for 48 h prior to treatment with MLK-1 sense or antisense ODNs as described. [H]thymidine (50 μCi/well) was added for 12 h in the absence of fresh ODNs. Treated cells were washed with ice-cold PBS prior to lysis in 0.5 M EDTA and precipitation with 10% trichloroacetic acid. Precipitated DNA was recovered by filtration over premoistened Whatman GF/C filters and washed three times with ice-cold PBS under vacuum. Air-dried filters were placed in scintillant for β-galactosidase assays.

For immunoprecipitations, an equivalent number of dpm of preimmune serum from RIN cells was incubated with 10 μl of a 1:1 slurry of protein A-Sepharose (Pharmacia) in PBS to which 5 μl of preimmune rabbit serum had been added. Supernatants were retained for subsequent experiments.

In phosphorylation experiments, cells at a density of 2 × 10⁵/well were washed with phosphate-free RPMI 1640 and biosynthetically labeled by culture for 6 h in 2 ml of methionine-free RPMI with 2% dialyzed FBS and 200 μCi of [³⁵S]methionine (Tran³⁵S-label, ICN). Labeled cells were washed three times in ice-cold PBS prior to lysis in 100 μl of lysis buffer A (10 mM CHAPS, 50 mM HEPES, 130 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, pH 7.4) for 5 min at 0 °C, then clarified by ultracentrifugation (10,000 × g), followed by incubation for 2 h at 4 °C with 10 μl of a 1:1 slurry of protein A-Sepharose (Pharmacia) in PBS to which 5 μl of preimmune rabbit serum had been added. Supernatants were retained for subsequent experiments.

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using commercially prepared reagent (Bio-Rad). Proteins from cell lysates (20 \mu g/lane) or immunoprecipitates (as above) were resolved by SDS-PAGE, electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Germany), and blocked overnight in 5% skim milk in Tris-buffered saline. Membranes were incubated in primary antibody diluted 1:500 (anti-MLK-1), 1:2000 (4G10), or 1:1000 (PSR-45) for 2 h at 25 °C, followed by four washes in Tris-buffered saline and incubation with anti-rabbit (for anti-MLK-1 primary) or anti-mouse (for 4G10 or PSR-45 primary) horseradish peroxidase-labeled secondary antibody diluted 1:1000 for 1 h at 25 °C. Following five washes in Tris-buffered saline, blots were developed with ECL reagent (Amersham International, Amersham, UK) and bands detected by autoradiography.

Immunocytochemistry—In experiments using cultured RIN cell lines, cells were grown on Teflon-coated microscope slides at 10^4 cells/slide chamber in 10% CO₂, 90% air until the cells were ~75% confluent (usually 2–3 days). Adherent cells were washed three times with PBS
Table I

| Reference | Protein kinase | Cell line |
|-----------|---------------|-----------|
| 53        | Lyn           | RIN-5AH   |
| 54        | Lck           | RIN-12    |
| 55        | Fyn           | RIN-5AH   |
| 56        | Yes           | RIN-5AH   |
| 57        | Abl           | RIN-5AH   |
| 58        | Raf           | RIN-5AH   |
| 18        | MLK-1         | RIN-5AH   |
| 18        | MLK-2         | RIN-5AH   |

* Number of clones representing each kinase sequence.

prior to fixation in methanol for 10 min followed by air drying. When tissue sections were employed, 4-mm cryostat sections were placed on aminosilane-coated slides, and tissues were identified using 1% toluidine blue staining of every sixth serial section. Sections were fixed in cold acetone (−20 °C) for 10 min and then air-dried and stored at −20 °C. Prior to indirect immunoperoxidase staining, slides of tissue sections were briefly dipped into −20 °C acetone. Previously fixed, frozen slides of cells were allowed to come to room temperature.

Slides were blocked for at least 10 min in PBS with 10% FBS followed by the addition of primary antibody (preimmune or anti-MLK-1) at a 1:500 dilution and incubation for 1 h in a humidified box. Slides were then washed three times (5 min/wash) with PBS and dipped in PBS with 10% FBS before adding horseradish peroxidase-conjugated swine anti-rabbit IgG diluted 1:800 (13 mg/ml stock) for 45 min at 25 °C, followed by three washes in PBS. Complexes were detected with diamobenzidine (Sigma) (100 mg/ml) in 0.006% hydrogen peroxide for 6 min and then washed and counterstained with hematoxylin.

RESULTS

Characterization of RIN Cell Lines—RIN-5AH and RIN-A12 cell lines display characteristic features of immature and mature β-cells, respectively. Fig. 1 shows that RIN-A12 cells possess a differentiated morphology, with extensive process formation. These cells synthesize high levels of insulin mRNA and secrete insulin. In addition, RIN-A12 cells express relatively low levels of MHC class 1 protein on the cell surface. In comparison, RIN-5AH cells are characterized by a flat, clustered, less differentiated morphology, low levels of insulin mRNA and secreted insulin, and relatively high surface expression of MHC class 1, with lower levels of A2B5-reactive gangliosides compared with those detected in RIN-A12 cells.

Detection of Protein Kinases from RIN-5AH and A12 Cell Lines—Polymerase chain reaction-amplified cDNAs from RIN-5AH and RIN-A12 cell lines contained sequences encoding the catalytic domains of eight different protein kinases from a total of 28 RIN-5AH and 43 RIN-A12 protein kinase-positive clones (Table I). Members of the src (lyn, lck, fin, and yes) and abl PYK subfamilies and of the rapf/mos protein serine/threonine kinase subfamily were present in both cell types. In RIN-5AH cDNA, a nucleotide sequence coding for MLK-1 (18) was found twice. Interestingly, this sequence was not found in any of the RIN-A12 clones tested. However, a sequence encoding a second member of the MLK family, MLK-2, which shares 75% amino acid identity with MLK-1 (25), was detected in both cell types.

MLK-1 Protein and mRNA Are Differentially Expressed in RIN Cells—To analyze and confirm the expression of MLK-1 in RIN cells, biosynthetically labeled proteins from lysates of RIN-5AH and RIN-A12 cells were immunoprecipitated with anti-MLK-1 antibodies, and the subsequent immune complexes were resolved by SDS-PAGE and visualized by autoradiography. Fig. 2A shows that MLK-1 (apparent molecular weight, 105,000) was precipitated from RIN-5AH cell lysate (lane 1), but not from RIN-A12 lysate (lane 3). No precipitated radioactivity was detected in either cell line with rabbit preimmune antibodies (lanes 2 and 4).

The expression of the MLK-1 gene at the RNA level was examined by Northern analyses of RNAs from RIN-5AH and RIN-A12 cell lines. Employing a cDNA probe for mouse MLK-1, a MLK-1 transcript was detected in RNA derived from RIN-5AH but not from RIN-A12 (Fig. 2B). In contrast, using a mouse MLK-2-specific cDNA probe, transcripts were detected in RNA from both cell lines tested (Fig. 2C). The integrity and amount of RNA from both cell lines was confirmed by reprobing the same blot with a GAPDH probe (Fig. 2D).

MLK-1 Expression in Stimulated Cells—The RIN-A12 cell line was originally cloned from parental RIN-5AH cells after culture in the presence of the differentiation inducer sodium butyrate (NaB) for 6 days (15). To determine the effect of transient cell stimulation on MLK-1 protein and mRNA levels, RIN-5AH cells were subjected to cell synchronization after serum deprivation followed by either serum replacement or a brief exposure (6 h) to NaB. Under these conditions, RIN-5AH cells displayed cellular morphology, cell growth rates, surface expression of both MHC class 1 and A2B5 reactive-ganglioside antigen, which were similar to control conditions (Fig. 3). However, Northern analyses revealed that mRNA transcripts for both MLK-1 and insulin were up-regulated in stimulated RIN-5AH cells (Fig. 4C). To assess the levels of MLK-1 protein after stimulation of RIN-5AH cells, cell lysates were analyzed by SDS-PAGE and probed with anti-MLK-1 or control antibodies by Western blotting. Fig. 4D shows that both serum stimulation (lane 5) and treatment with 1 mM NaB for 6 h (lane 6) resulted in increased levels of MLK-1 protein expression. No MLK-1 protein could be detected in RIN-A12 cells after stimulation with serum or NaB under identical conditions (Fig. 4D, lanes 2 and 3).

To further confirm the effect of NaB treatment on MLK-1 expression, anti-MLK-1 immunocytochemistry was performed on resting or stimulated RIN cells. Fig. 5 shows that, while no MLK-1 staining could be observed in the RIN-A12 cells under any of the conditions tested (panels D–F), RIN-5AH cells stained positively for MLK-1 in the cytoplasm (panel B), and the intensity of staining was increased following cell culture with 1 mM NaB (panel C).

Phosphorylation Studies—To examine the phosphorylation state of MLK-1 following stimulation, RIN-5AH cells were first

![Figure 2. MLK expression in RIN cells.](image)
biosynthetically labeled with \([^{32}P]\)orthophosphate prior to stimulation with serum or NaB. The labeled cells were then lysed, and the MLK-1 was immunoprecipitated and examined by SDS-PAGE and autoradiography. Fig. 6A shows that very little labeled MLK-1 was detected in cells cultured under basal conditions. Stimulation of RIN-5AH cells with either serum or NaB, however, resulted in increased phosphorylation of MLK-1.

To determine whether the phosphorylation of MLK-1 was on tyrosine or serine, we employed 4G10 anti-phosphotyrosine antibodies or PSR-45 anti-phosphoserine antibodies to immunoblot MLK-1 immunoprecipitates from RIN-5AH cells after biosynthetic labeling with \([^{32}P]\)orthophosphate treated as above. Fig. 6B and C. shows that phosphoserine, but no phosphotyrosine, immunoreactivity could be detected in MLK-1 immunoprecipitates after treatment with serum for 15 min or 1 mM NaB for 1 h. No phosphorylated MLK-1 was detected after precipitation with preimmune antibodies (results not shown).

**Antisense Studies**—To examine the relationship between MLK-1 expression and the differentiation state of RIN cells, we cultured the cells in the presence of antisense or sense MLK-1 phosphorothioate ODNs. Log growth phase RIN-5AH cells exposed to 5 \(\mu\)M AS-ODN-1 or AS-ODN-2 for 24 h displayed a more highly clumped, rounded, undifferentiated morphology compared with S-ODNs treated or untreated cells (Fig. 7, A–C). RIN-A12 cells displayed no morphological change after treatment with MLK-1 ODNs (Fig. 7, D–F). In addition, the ability of AS-ODN-1- or AS-ODN-2-treated cells to incorporate \([^{3}H]\)thymidine was reduced by 58 and 77%, respectively, in RIN-5AH cells but was unaffected in RIN-A12 cells (Fig. 7G). No effect was observed after treatment with the appropriate sense ODN. To confirm the effects of the AS-ODNs on cellular expression of MLK-1, AS-ODN-1- or AS-ODN-2-treated RIN-5AH cells were stimulated with 1 mM NaB for 6 h in the continued presence of AS-ODNs, and lysates of these cells were examined by Western blot. AS-ODN-treated cell lysates revealed little or no stimulation of MLK-1 protein expression after NaB treatment compared with NaB-stimulated, S-ODN-treated cells or cells incubated in medium control (Fig. 7H).

Because the levels of insulin gene expression are used to define the maturational status of \(\beta\)-cell lines, we examined the effects of AS-ODNs on RIN insulin gene transcription by Northern analyses. We observed no significant change in the levels of insulin mRNA after treatment of RIN-5AH cells (Fig. 7I) with either AS-ODNs or S-ODNs compared with basal levels. How-
ever, Fig. 8 shows that the very low levels of glucagon mRNA that can be detected in RIN-5AH cells after prolonged exposure of filters were increased by 60 and 80% after treatment with AS-ODN-1 and AS-ODN-2, respectively, compared with basal or S-ODN levels.

**MLK-1 Expression in the Developing Mouse Pancreas—**Because restricted expression of MLK-1 to immature β-cells was observed in vitro using transformed cells, we investigated the pattern of MLK-1 expression in the developing mouse pancreas by immunohistochemistry (Table II). Using fresh, frozen, acetone-fixed tissues from embryonic days 12–19 and young adult pancreatic tissue, we detected immunoreactive MLK-1 protein in embryonic pancreas from embryonic days 13–16. The staining pattern suggested that MLK-1 was restricted to the cytoplasm of cells organized into duct-like structures with the most intense staining occurring at embryonic days 14–16 (Fig. 9). At later gestational time points, MLK-1 could not be detected above background levels and was also absent in the neonatal pancreas. Each of the other organs surveyed displayed distinct developmentally regulated expression of MLK-1 (Table II).

**DISCUSSION**

The high proliferative capacity and potential for lineage-specific differentiation of RIN cells has allowed their use in a number of studies of β-cell growth and differentiation (12–14). Indeed, RIN cells have been demonstrated to be pluripotent, with multiple hormone-producing cell lines generated from single cells (16, 17). RIN-5AH cells represent a relatively undifferentiated cell type that secretes low levels of islet hor-

FIG. 4. Effects of cell cycle synchronization and NaB treatment on insulin and MLK expression in RIN-5AH cells. A, levels of insulin released into culture medium, measured 5 days after treatment as described under “Experimental Procedures.” Results are the mean ± S.D. of two experiments each performed in triplicate. B, Northern analysis of mRNA from RIN-5AH cells under basal conditions and after cell cycle synchronization (48 h of serum starvation followed by treatment with 10% FBS) or 6 h of culture with 1 mM NaB. Northern blots were probed with cDNAs for insulin and α-tubulin (control). C, Northern analysis of mRNA from unstimulated (basal) and NaB (6-h) stimulated RIN-5AH cells probed with MLK-1 and GAPDH cDNAs. D, immunoblot of RIN cell lysates (20 μg of protein/lane) with anti-MLK-1 antibodies after cells were treated with medium control (lanes 1 and 4), 10% FBS following serum starvation (lanes 2 and 5) or 1 mM NaB for 6 h (lanes 3 and 6). The positions of molecular weight markers are shown at the right, and the MLK-1 position is indicated by an arrow.

FIG. 5. Immunocytochemistry of RIN cells. RIN cells, cultured under either basal or stimulated conditions, were fixed and stained with preimmune (A and D) or anti-MLK-1 antibodies (B and E, basal (no treatment)); C and F, cells cultured in 1 mM NaB for 6 h) as described under “Experimental Procedures.”

FIG. 6. Analysis of phosphoproteins in RIN-5AH lysates after anti-MLK-1 immunoprecipitation. A, autoradiograph of SDS-PAGE separated proteins from anti-MLK-1 immunoprecipitates of lysed [32P]orthophosphate biosynthetically labeled RIN-5AH cells. B and C, ECL autoradiograph detection of phosphorylated amino acids by Western blotting. Anti-MLK-1 immunoprecipitates from [32P]-labeled RIN-5AH cell lysates were resolved by SDS-PAGE, blotted onto nitrocellulose membranes, and probed with PSR-45 anti-phosphoserine antibody (B) or 4G10 anti-phosphotyrosine antibody (C). In all three panels, cells were treated as described in the legend to Fig. 4. Positions of molecular weight markers are shown at the right, and the MLK-1 position is shown by an arrow.

mones and is unresponsive to the insulin secretagogue, glucose (19). Exposure of RIN-5AH cells to prolonged culture (6 days) with the differentiation inducer NaB led to the generation of several sublines of which RIN-A12 displayed a phenotype more characteristic of normal adult islet cells (15). In the present studies, the “immature” RIN-5AH (undifferentiated morphology, low insulin, high MHC class 1) and “mature” RIN-A12 (differentiated, high insulin, low MHC class. In the present
studies, the “immature” RIN-5AH (undifferentiated morphology, low insulin, high MHC class 1) and the “mature” RIN-A12 (differentiated morphology, high insulin, low MHC class 1) cell lines were employed as a model of β-cell lineage maturation to address the possible role of protein kinases in β-cell growth and differentiation. Messenger RNA from the two lines was subjected to reverse transcriptase-polymerase chain reaction with primers based on nucleotide sequences encoding conserved motifs within subdomains VI and IX of the catalytic domains of PYKs (20). Our findings show that both RIN-5AH and RIN-A12 cells express mRNAs for the cytoplasmic PYKs lyn, lck, fyn, and yes, which are members of the src subfamily of PYKs. The c-src family has nine known members (blk, c-fgr, fyn, hck, lck, lyn, c-src, c-yes, and yrk) that are believed to play critical roles in signal transduction via their three src homology domains. The expression patterns of c-src family PYKs (reviewed in Ref. 26) suggest that they function in a broad range of biological situations. For example, c-src PYKs can associate with activated receptor PYKs, including the receptors for platelet-derived growth factor (27) and epidermal growth factor (28), both of which have been shown to influence β-cell growth and differentiation (29). Expression of c-abl was similarly detected in both RIN cell lines. The mammalian c-Abl protein, although not linked to a receptor, has been shown to bind to specific DNA
sequences in the cell nucleus as well as to actin filaments (30), suggesting a possible role in signal transduction and cell cycle regulation. The cytoplasmic serine/threonine kinase, Raf-1, which is thought to play a critical role in the control of proliferation and differentiation (reviewed in Refs. 31 and 32), was also detected in both RIN-5AH and RIN-A12 cells. Raf-1 is thought to act as a key regulatory molecule that integrates upstream signals from protein kinase C and Ras-like proteins (33) and transmits them into the mitogen-activated protein kinase and nuclear factor \( \kappa B \) (NF-\( \kappa B \)) pathways.

MLK-1 expression was detected only in the RIN-5AH cells, although it is possible that very low levels of MLK-1 mRNA may exist in RIN-A12 cells. However, because polymerase chain reaction primers used to amplify protein kinase sequences may not necessarily recognize all possible templates in each cell with equal efficiency, the differential expression of MLK-1 in RIN-5AH and A12 cells required more direct confirmation. Using both Northern analysis and anti-MLK-1 immunoprecipitation from lysates of biosynthetically labeled cells, we were unable to demonstrate the presence of MLK-1 mRNA or protein in RIN-A12 cells. Both were detected in the RIN-5AH cells, however, in support of the findings by reverse transcriptase-polymerase chain reaction. In addition, immunocytochemistry with MLK-1-specific antibodies revealed a positive signal for MLK-1 in the cytoplasm of RIN-5AH cells, but not in RIN-A12 cells.

The MLKs represent a new family of protein kinases that share several distinctive structural features. All members described thus far have catalytic domains in which the primary sequence contains motifs conserved in both serine/threonine and tyrosine-specific protein kinases. In addition, immediately \( COOH \)-terminal to the kinase catalytic domain are two predicted leucine/isoleucine zipper domains, separated by a short spacer region (18). Leucine zipper sequences are usually associated with the dimerization of transcription factors that is critical for activation of transcription (34, 35). It is possible that the two leucine zippers within the MLK-1 protein may form helices that interact with one another as has been postulated for helix-loop-helix domain structures (36). Helix-loop-helix domains have been shown to be critical for the myogenic function of the cell type-determining protein, Myo-D, as well as the oncogenic activity of \( c-myc \) (37). In addition to the dual leucine zipper domain, MLK proteins all contain an extremely basic sequence C-terminal to the zipper region (18). Although this basic domain generally occurs N-terminal to the helix-loop-helix motif in most transcription factors (38), the ability of MLK-1 to bind DNA elements has not been excluded. MLKs 1–3 also contain a \( src \) homology 3 domain and a large C-terminal domain rich in serine, threonine, and proline residues that includes consensus sequences for phosphorylation by a number of other protein kinases. The presence of these domains raises the possibility that the MLKs may play roles in as yet undetermined signal transduction pathways.

Our observations suggest that the expression of MLK-1 may be regulated by the functional state of the cell. Because RIN-A12 cells were generated from RIN-5AH cells after prolonged (6-day) culture in 1 mM NaB, we examined the effects on RIN-5AH cells of short term (6-h) stimulation with NaB as well as cell cycle synchronization following serum starvation. Expression of MLK-1 mRNA and protein were both up-regulated after a short stimulation with NaB and to a lesser extent after cell synchronization at \( G_1/G_0 \) followed by serum replacement. These findings were confirmed by anti-MLK-1 immunocytochemistry on resting and stimulated cells. It is possible, therefore, that stimulated RIN-5AH cells, which also display in-
creased insulin mRNA synthesis, represent activated cells that may be at the threshold of the differentiation process. Unlike prolonged exposure to NaB, however, which drives RIN-5AH differentiation into a biochemically and morphologically more mature phenotype, a short stimulation with NaB did not affect the morphology or levels of insulin secretion of these cells. The fact that MLK-1 expression is up-regulated concomitant with phosphorylation on serine but not tyrosine at this early activated stage, raises the possibility of its involvement in early signaling cascades that lead to β-cell differentiation.

To further investigate a potential role for MLK-1 in the early inductive phase of β-cell activation we designed and synthesized two sense and antisense ODNs corresponding to regions within the kinase catalytic and N-terminal domains of MLK-1. The sequences of both these regions are unique to MLK-1 and in particular are not conserved in other members of the MLK family and were not found to match any sequences other than MLK-1 in data base searches. Antisense ODNs to both MLK-1 regions were capable of altering cell morphology and DNA synthesis of RIN-5AH cells but not RIN-A12 cells. In addition, treatment of RIN-5AH cells with AS-ODNs ablated the ability of 1 mM NaB to stimulate an increase in the levels of MLK-1 protein expression, resulting in an elevation of glucagon mRNA levels without affecting the levels of insulin mRNA expression. Sense ODN-treated cells consistently failed to display altered phenotypes. Taken together, these results suggest that the inability of RIN-5AH cells to regulate their levels of MLK-1 following AS-ODN treatment resulted in a quiescent, undifferentiated phenotype that displayed low levels of both glucagon and insulin gene expression, reminiscent of the parental RINm cell line (16, 17). In addition, these data suggest that although insulin gene regulation is a marker of cellular activation and ultimately differentiation, MLK-1 is not directly linked to pathways that regulate insulin mRNA expression. The effects of MLK-1 AS-ODNs on cellular morphology, however, are suggestive of a possible role for MLK-1 in activation pathways that modulate components of the cellular cytoskeleton.

Recently, it has been reported that all of the members of the MLK family contain a Cdc42/Rac-interactive binding motif and that proteins containing this motif may engage Cdc42 and/or Rac in a GTP-dependent manner and participate in downstream signaling events (39). Indeed, the Cdc42/Rac-interactive binding domain of MLK-3 has been directly demonstrated to bind to the GTP form of Cdc42, with weaker binding to Rac GTP (39). These observations are of interest because Rho GTPase subfamily members (Rac, Cdc42) are known to stimulate the formation of focal adhesions and the reorganization of cytoskeletal components such as stress fibers (40), membrane ruffling (41), and filopodia (42). These events are thought to be critical during the activation stage of cellular responses (43). In addition, in Drosophila melanogaster both Rac and Cdc42 are involved in extension of neuronal growth cones (44). Rac and Cdc42 activate the c-Jun N-terminal kinase pathway via a series of phosphorylation intermediates resulting in transcriptional activation of c-Jun (45, 46). These intermediates, such as the kinases that phosphorylate mitogen-activated protein/extracellular signal-regulated kinase and SAP/ERK kinase-1 (50–52). Despite these observations, a precise biological role for the MLK family is not yet known. Interestingly, a more distantly related MLK family member, the dual leucine zipper-bearing kinase, has been recently implicated as a potentially important mediator of synaptic vesicle trafficking in nerve terminals, possibly as a result of signal transduction events mediated by calcineurin. The function(s) of MLKs in other cellular systems is not yet understood. The above observations led us to ask whether MLK-1 was expressed during pancreatic development. In light of the restricted expression of MLK-1 to the less mature β-cell line, RIN-5AH, we reasoned that MLK-1 may be more highly expressed at early stages of pancreatic development, when precommitted ductal cells are thought to be pluripotent. We observed that in the embryonic pancreas, MLK-1 was primarily expressed in duct-like structures between embryonic days 13 and 16 of mouse embryogenesis. It is these ductal cells that are considered to be the precursors of mature endocrine cells, including β-cells. Interestingly, MLK-1 expression in the pancreas was not detectable in late gestation and young adulthood, mirroring the absence of MLK-1 from the more mature β-cell line, RIN-A12. These observations, taken together with the results of cell stimulation and antisense treatments, are consistent with a role for MLK-1 in the early inductive phases of β-cell development. Determination of interactions between MLK-1 and other cellular effector proteins during β-cell proliferation and differentiation should provide new insights into the developmental events leading to β-cell maturation.

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