Molecular Regulation of Monocyte Chemoattractant Protein-1 Expression in Pancreatic β-Cells
Burak Kutlu,1 Martine I. Darville,1 Alessandra K. Cardozo,1,2 and Décio L. Eizirik1

Pancreatic β-cells are selectively destroyed during the course of type 1 diabetes. In the early stages of the disease, inflammatory infiltrates of mononuclear cells, containing predominantly monocytes and T-cells, are present in the islets (insulitis). Chemokines, such as monocyte chemoattractant protein-1 (MCP-1), play a key role in the recruitment and activation of these immunocytes. We have previously described cytokine-induced MCP-1 gene expression in human and rat pancreatic islets. In the present study, the transcriptional regulation by cytokines of the rat MCP-1 gene in fluorescence-activated cell sorting–purified rat β-cells, insulin-producing INS-1E cells, and RINm5F cells was investigated. Transient transfections with luciferase-reporter constructs identified an interleukin (IL)-1β–responsive enhancer region between –2,180 bp and –2,478 bp. Mutation of either of the two nuclear factor (NF)-κB sites present in this region abrogated IL-1β–induced MCP-1 promoter activity. Binding of NF-κB to the two sites was shown in vitro by gel shift assays, while supershift assays revealed the presence of p65/p50 heterodimers and p65 homodimers. In vivo binding of NF-κB was confirmed by chromatin immunoprecipitation assay. Blocking of NF-κB activation in cytokine-exposed primary β-cells by an adenovirus overexpressing a nondegradable form of IκBα or by pyrrolidine dithiocarbamate decreased IL-1β–induced MCP-1 mRNA expression. We conclude that NF-κB plays an important role for MCP-1 expression in β-cells. This transcription factor may be an interesting target for ex vivo gene therapy before islet transplantation. Diabetes 52: 348–355, 2003

There is strong evidence that type 1 diabetes develops as a consequence of autoimmunity, leading to β-cell destruction (1). In the early stages of insulitis, activated macrophages and T-cells are attracted to the islets and produce cytokines and free radicals, which contribute to β-cell dysfunction and death (2,3). The mechanisms regulating the attraction of monocytes and T-cells to the islets remain to be clarified. We have recently described that human and rat islets, and islets isolated from prediabetic NOD mice, express the chemokine MCP-1 (4,5). Subsequent studies by other groups confirmed that human islets produce and secrete biologically active MCP-1 (6). Importantly, MCP-1 released by pancreatic islets plays a relevant role for the clinical outcome of islet allografts in type 1 diabetic patients, as suggested by the observation that high MCP-1 secretion is negatively correlated with successful engraftment and long-lasting insulin independence (6). These intriguing observations suggest that MCP-1 and other chemokines produced by the cytokine-exposed islet cells (7,8) are involved in the recruitment and activation of immune cells during both insulitis and islet graft destruction.

MCP-1 belongs to the C-C family of chemokines (9–11) and is produced by diverse cell types in response to cytokines, oxidized LDL, and bacterial lipopolysaccharide (12–14). The differential induction and binding of activated transcription factors to the promoter region of the MCP-1 gene provides a critical regulatory step, allowing expression of the chemokine in a cell- and stimulus-specific manner (15). MCP-1 attracts monocytes (16), T-cells (17), and natural killer cells (18) to the site of inflammation, and it also regulates the release of arachidonic acid and the respiratory burst by monocytes (19,20). Local release of MCP-1 probably contributes to the inflammatory response in rheumatoid arthritis, atherosclerosis, glomerular diseases, and pulmonary granulomatous vasculitis (21–27). Of note, transgenic mice expressing MCP-1 under control of the insulin promoter develop an intense insulinitis (28).

In vivo footprinting in murine fibroblasts showed that tumor necrosis factor (TNF)-α induces site occupancy in both proximal and distal regions of the MCP-1 promoter (29). The distal region is located more than 2.2 kb upstream of the transcription start site and is required for induction of MCP-1 expression by TNF-α and IL-1β in mouse fibroblasts (30) and in human mesangial cells (31). This enhancer region contains two binding sites for NF-κB that are conserved in the human, mouse, and rat MCP-1 genes (30,32). Moreover, in the human gene a proximal NF-κB site cooperates with an adjacent AP-1 site for induction by TNF-α or IL-1β (33). In human osteoblastic cells interferon (IFN)-γ stimulates MCP-1 transcription through a complex element in the promoter region, namely an IFN response-inhibitory sequence (IRIS) adjacent to an IFN-γ activation site (γ-activated sequence [GAS]) (34). This element is conserved in the human, mouse, and rat genes (35). It is noteworthy that IFN-γ does not stimulate MCP-1 expression in rat β-cells or human.
pancreatic islets (5), suggesting that regulation of this gene in β-cells differs from other cell types and is mostly dependent on IL-1β. Unfortunately, there are no available data on the promoter regulation of MCP-1 gene expression in insulin-producing cells.

In the present study, we investigated the transcriptional regulation by cytokotines of the rat MCP-1 gene in β-cells. To achieve this goal, we carried out transfection experiments in an insulinoma cell line, INS-1E, and in fluorescence-activated cell sorting (FACS)-purified rat primary β-cells. We demonstrated by site-directed mutagenesis and gel-shift assays that NF-κB is involved in the induced activity of the MCP-1 promoter in vitro. In vivo binding of NF-κB to the enhancer region in the rat MCP-1 promoter was confirmed by chromatin immunoprecipitation (ChIP) assay, followed by real-time PCR quantification. Finally, by using an adenovirus expressing a nondegradable form of IκB, or the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC), we showed that NF-κB translocation to the nucleus is necessary for MCP-1 mRNA induction in response to cytokotines.

**RESEARCH DESIGN AND METHODS**

**Plasmid constructs and mutagenesis.** The MCP-1 promoter and enhancer region were isolated by PCR using the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany) on 0.5 μg rat genomic DNA prepared from liver as described (36). The primers used were based on the Genbank sequence (accession number AF070313). For the promoter region, the forward primer (position −515 to −499) was 5′-CGAATTCTAGCTTCGTTGG with an added EcoRI site, and the reverse primer (position +31 to +53) was 5′-GGCCATCGCTAGTGAGAAG with an added BamHI site. For the enhancer region, the forward primer (position −2479 to −2450) was 5′-GGCTCTAGAAGTCGAGGCTTACA and the reverse primer with an added XbaI site, and the reverse primer (position −2464 to −2180) was 5′-GGCCATCTTCTTCTGTTCAAC with an added EcoRI site. The amplified products were cloned into pBluescript KS (Stratagene, La Jolla, CA). Clones were sequenced and shown to be 98% identical to the published sequence. The promoter region (−514 to +53) is isolated from pBluescript by digestion with EcoRI (5′ end) and BamHI (3′ end). The promoter was in vitro transcribed in the absence of adenylate cyclase activators (41). INS-1E and βH9262 cells were selected based on response studies (2 and B.K. and D.L.E., unpublished data). Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega). Values obtained for the test plasmids in unstimulated cell extracts were at least 90-fold higher than values for the promoterless vector pGL3-Basic (0.1 light unit). Test values were corrected for the luciferase activity value of the transfection with pRL-CMV (Promega) as an internal control, by lipofection with lipofectAMINE.

Promoter and enhancer fragments were ligated into the pMCP-1-514 luc construct. To build the pMCP-1-514(enh) luc construct, the MCP-1 promoter and enhancer fragments containing BglII sites of pGL3-Basic. Mutations for the two NF-κB sites were as follows (upper strand shown): MCP-1 enhancer NF-κB site A, 5′-agAGGTTGGTAAGAAGAATGAG; NF-κB site B, 5′-agAGAATGGAGATTCCACTGCTCTT. The lucerase reporter constructs added nucelotides to allow end labeling with the Klenow fragment.

**ChIP assays.** Cross-linked chromatin from 5×10⁶ RINm5F cells, left untreated or exposed to IL-1β for 20 min, 2 h, or 4 h, was prepared as described (44), with the following modifications: β-glycerophosphate and sodium butyrate were omitted and 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin were added as protease inhibitors. Samples were sonicated with a Branson Sonifier 250 for 40 to 50 s. SDS was added at a final concentration of 1%, and samples were rotated for 1 h at room temperature and centrifuged at 12,000 rpm for 15 min. Supernatant was further sonicated for two 20-s pulses to an average size of 500–1,500 bp. For immunoprecipitation, 60 μl chromatin corresponding to 5×10⁶ cells was diluted 10-fold in 0.01% SDS, 1.1% Triton X-100, 155 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 8) with protease inhibitors. The chromatin solution was precleared with 20 μl 50% polyethylene glycol 8000 and 100 μg/ml sonicated herring sperm DNA for 45 min at 4°C. Precleared chromatin (20 μl saved as total input chromatin) was rotated without or with 3 μg rabbit polyclonal antibody overnight at 4°C. The antibody was directed against p65. As a negative control, cross-linked chromatin was incubated overnight in the absence of antibody. Protein A-Sepharose (50 μl) containing BSA and herring sperm DNA was added, and the incubation was continued for 3 h at 4°C. The immune complexes were washed twice with each of the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, and 20 mM Tris-HCl, pH 8), high-salt buffer (0.1% SDS, 1% Triton X-100, 500 mM NaCl, 2 mM EDTA, and 20 mM Tris-HCl, pH 8), LiCl buffer (0.25 mol/l LiCl, 1% Igepal, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8). TE buffer (1 mM EDTA and 10 mM Tris-HCl, pH 8). Immune complexes were eluted with two 200 μl 0.1 mol/l NaOH. 1% SDS for 15 min. Cross-links were reversed by addition of NaCl at a final concentration of 200 mM and incubation overnight at 65°C. Samples were treated with 40 μg/ml proteinase K for 1 h at 42°C, extracted with phenol-chloroform and chloroform, and ethanol-precipitated. Pellets were resuspended in 25–30 μl H₂O.

For quantification of the precipitated sequences, real-time PCR of MCP-1 enhancer sequence was performed with a single-step PCR on a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) as described previously (45,46). The rat MCP-1 enhancer region was specifically detected using the same primers as those used for the plasmid construction (see above). PCR amplification using a LightCycler instrument was carried out in 20 μl reaction volume containing 0.25 mol/l MgCl₂, 200 μmol/l forward and reverse primers, 2 μl FastStart SYBR Green 1 mix (Roche), and 2 μl template genomic DNA/plasmid in a glass capillary tube. The amplification of the MCP-1 enhancer was started with an initial activation step at 95°C (10 min) followed by 45 rounds of amplification at 95°C (10 s) for denaturation, 58°C (10 s) for annealing, and 72°C (14 s) for extension.
RESULTS

cystokine regulation of MCP-1 expression in INS-1E cells. To determine whether cytokines induce MCP-1 mRNA expression in INS-1E cells, as previously described for primary β-cells and RINm5F cells (4,5), these cells were exposed for 6 or 24 h to IL-1β, IFN-γ, or a combination of both cytokines, and MCP-1 mRNA content was measured by RT-PCR. There was no detectable MCP-1 expression in unstimulated cells or cells exposed to IFN-γ alone (Fig. 1), but MCP-1 expression was induced in cells exposed to IL-1β for 6 and 24 h, with higher value at 6 h. This increase was not potentiated by IFN-γ. These findings are similar to our previous observations indicating that IL-1β-induced MCP-1 expression in rat β-cells and human islets is not modified by IFN-γ (4,5). Thus, most of the subsequent experiments on MCP-1 promoter regulation were performed in the presence of IL-1β alone.

Identification of the cytokine-responsive element in the MCP-1 promoter. To determine the MCP-1 promoter regions responsive to IL-1β, the promoter fragment from nucleotides −514 to +53 relative to the transcription start site, with or without the enhancer fragment between −2,180 to −2,478 relative to the transcription start site (Fig. 2), were cloned into the luciferase reporter plasmid pGL3 and transfected into FACS-purified β-cells treated or not with IL-1β. In IL-1β-treated cells, the luciferase activity of the constructs pMCP-1-514(enh) luc was induced threefold, whereas pMCP-1-514 luc did not significantly respond to IL-1β (Fig. 3). This lack of response of the promoter region alone, even though there is a potential NF-κB binding site in this region, indicates a role for the potential NF-κB binding sites in the enhancer region (Fig. 2). Similar results were observed in INS-1E cells transfected with the human and rat pancreatic islets (50), a finding confirmed in the present series of experiments (data not shown).

RT-PCR analysis. RT-PCR was performed on poly(A)+ RNA as described (8). Primers for MCP-1 were 5′-ACCTGCTGCTACTTCAC-3′ and 5′-CTACAG AAGTGCTTGAGGTG-3′ (325 bp). The primers for glyceraldehyde-3-phosphate dehydrogenase were described previously (7). The ethidium bromide–stained agarose gels were photographed under ultraviolet transillumination using a Kodak Digital Science DC 120 camera (Eastman Kodak, Brussels, Belgium). Abundance of the PCR products were assessed by Biomax one- dimensional image analysis software (Kodak), and MCP-1 mRNA contents were expressed as optical densities corrected for GAPDH. Cytokines do not modify GAPDH expression in primary β-cells (8,51 and present data).

Statistical analysis. Results are given as means ± SE. Comparisons versus the respective control groups were performed using the Student’s paired t test.

human and rat pancreatic islets (50), a finding confirmed in the present series of experiments (data not shown).
same constructs. Thus, IL-1β induced a significant increase in the activity of pMCP-1-514[enh] but not of pMCP-1-514 luc (Fig. 4). Due to the large number of cells required, the subsequent experiments to elucidate the regulatory elements in the MCP-1 enhancer were performed in INS-1E cells.

The enhancer region contains two potential NF-κB binding sites (Fig. 2). Therefore, we mutated each NF-κB site separately and denoted the mutated constructs as pMCP-1-514[enh]mutA luc and pMCP-1-514[enh]mutB luc. The fold induction of MCP-1 promoter activity in response to IL-1β was reduced by 75 and 80% for mutA and mutB, respectively (Fig. 4). These results indicate that both binding sites are important for IL-1β–induced MCP-1 promoter activity.

NF-κB binding to the MCP-1 promoter in vitro. To characterize the factors binding to the IL-1β–responsive elements in the enhancer region, we performed EMSAs using probes spanning the NF-κB sites from nucleotides −2,272 to −2,297 containing the first NF-κB motif of the enhancer (site A) and from −2,242 to −2,266 containing the second NF-κB motif of the enhancer (site B). Based on our previous observation that the maximal IL-1β–induced NF-κB binding activity in vitro in insulin-producing cells takes place after 30 min (42,52), this time point was selected for in vitro determination of nuclear factor binding. Two complexes, denoted by “a” and “b,” were induced in cells stimulated with IL-1β, with a higher affinity for the probe of site B (Fig. 5A and B). The nature of complexes a and b was further identified by competition and supershift experiments. The two complexes were specifically competed by an excess of unlabelled probe (Fig. 5A, lanes 3–4 and 10–11) or with specific antibodies against p60 or p65 (lanes 5–6 and 12–13): a, p65 homodimers; b, p65/p50 complexes. The figure is representative of two to three similar experiments.
NF-κB binding to the MCP-1 promoter in vivo. To confirm in vivo our in vitro observations indicating NF-κB binding to the enhancer region, we performed ChIP assays with an antibody directed against the NF-κB p65 protein. ChIP assay allows for detailed analyses of protein-DNA interactions in a native chromatin environment (53), and the detection of association of rare transcription factor complexes can be achieved by real-time PCR quantification (45). We currently observed IL-1β-induced NF-κB binding in vivo by quantification of the rat MCP-1 enhancer region with real-time PCR (see RESEARCH DESIGN AND METHODS). NF-κB binding was detected as early as 30 min following IL-1β exposure, with continuously increasing values at 2 and 4 h (Fig. 6). The rat MCP-1 enhancer was undetectable in unstimulated control cells.

Effect of an IκBα super-repressor protein on cytokine-mediated MCP-1 gene expression. Overexpression of the IκB super-repressor by an adenovirus, AdIkB(SA)2, with serine 32 and 36 substituted by alanine residues, specifically blocks NF-κB activation without interfering with other transcription factors (47,48). We currently observed that AdIkB(SA)2 partially blocked cytokine-induced MCP-1 mRNA expression (Fig. 7A). Thus, the optical density (OD) values of AdIkB(SA)2 plus cytokines were 55 ± 9% of the values observed in noninfected cells exposed to the same cytokines (n = 3; P < 0.05), while infection with the control virus AdGFP did not modify the effects of cytokines (Fig. 7A; OD 86 ± 33% of the values observed in noninfected cells exposed to cytokines; n = 3). To further evaluate the role of NF-κB for cytokine-induced MCP-1 expression, rat islets were exposed to cytokines in the presence of the NF-κB blocker PDTC (50; Fig. 7B). There was no detectable MCP-1 expression in unstimulated cells or cells exposed to PDTC alone (Fig. 7B). MCP-1 mRNA was induced by cytokines after 6 and 24 h, and this induction was, to a major extent, prevented by PDTC (Fig. 7B). The results, expressed in OD after correction for GAPDH mRNA, for experiments 1 and 2, respectively, were as follows: cytokines-6 h, 1.96 and 1.61; PDTC plus cytokines-6 h, 0.54 and 0.47 (inhibition 72 and 71%, respectively); cytokines-24 h, 1.09 and 1.17; and PDTC plus cytokines-24 h, below detection limit (Fig. 7B).

DISCUSSION

MCP-1 expression is induced by cytokines in pancreatic β-cells and probably plays a role in attracting monocytes and T-cells to the site of insulitis (5,7,54). Indeed, the temporal pattern of MCP-1 expression, along with other CC chemokines, in the islets of NOD mice is well correlated with the progression of insulitis and β-cell destruction (5,54), and transgenic MCP-1 expression in β-cells induces mononuclear cell infiltration (28). In this context, it is of relevance to understand the molecular regulation of MCP-1 expression in the β-cells.

In the present study, we identified the transcription factor NF-κB as a key regulator of MCP-1 promoter activity. Mutation of either of the two NF-κB binding sites, located in the enhancer region at −2,242 and at −2,272, respectively, abrogated IL-1β-induced MCP-1 promoter activity. Moreover, we detected the binding of NF-κB p50 and p65 factors to these two sites by EMSAs and supershift experiments. In vivo binding of NF-κB to the enhancer region was confirmed by ChIP assays. Finally, overexpression of an IκB super-repressor, AdIkB(SA)2, or preculture in the presence of the NF-κB blocker PDTC...
decreased cytokine-induced MCP-1 mRNA expression. The proximal promoter region by itself does not respond to cytokines, suggesting no role in insulin-producing cells for neither the putative NF-κB and API binding sites (33), nor for the IRIS and GAS elements (34) located in this region.

Sterospecific alignment between adjacent cis elements has been observed in several genes, including those containing κB motifs (55). The two NF-κB binding sites in the enhancer region of MCP-1 promoter, site A and site B, are not identical in sequence, suggesting that they may serve distinct functions. Although higher affinity of NF-κB binding was observed for site B than A (Fig. 5), the fold induction of MCP-1 promoter activity in response to IL-1β was reduced by 75 and 80% for mutated site A and B, respectively (present data). The presence of these sites was also described as necessary to maintain TNF-induced MCP-1 promoter activity in these cells (30). Taken together with our findings, these results suggest that both binding sites for NF-κB are important for the IL-1β–induced MCP-1 promoter activity in insulin-producing cells.

Mammalian cells contain five NF-κB family members: p50, p52, p65, c-Rel, and RelB. Interactions between these proteins have been characterized in detail (56). P65/p50 heterodimers, the most frequent dimers, and p65 homodimers are activators of gene transcription, whereas p50 homodimers, which lack a transactivation domain, are transcriptionally inactive (57). Our supershift experiments with antibodies directed against p65 and p50 proteins suggest a role for p65/p50 and p65/p65 dimers in the regulation of MCP-1 in insulin-producing cells. The differential induction and binding of activated transcription factors to the promoter region of the MCP-1 gene provides a critical regulatory step, allowing expression of the chemokine in a cell- and stimulus-specific manner (15). Of note, we have observed the presence of the same active dimers of NF-κB in the context of cytokine regulation of Fas expression in primary β-cells (42).

In vitro determination of transcription factor binding to a promoter does not necessarily reflect the complex environment and the three-dimensional conformation of chromatin in a living cell. Indeed, it has been shown that the compact nature of chromatin can modulate accessibility to some DNA binding sites (45,53). Therefore, to confirm in vivo our observations of NF-κB binding to the rat MCP-1 promoter, we have performed ChIP assay, a novel method that allows detection of transcription factor binding in situ (53). We detected NF-κB binding in vivo at 30 min after IL-1β stimulation, with subsequent increases at 2 and 4 h (present data). Interestingly, it has been previously reported the presence of two distinct waves of early and late NF-κB recruitment to target promoters in a murine macrophage cell line, depending on the acetylation state of chromatin (58). NF-κB binding to the MCP-1 enhancer was detected only after 90 min of LPS exposure to macrophage cells (58). Our data suggest an early and sustained IL-1β–induced binding of NF-κB to the MCP-1 promoter in β-cells (Fig. 6), helping to explain the long-lasting effect of IL-1β on MCP-1 mRNA expression (Fig. 1 and 4,5,7,8).

The results described above suggest that NF-κB translocation to the nucleus is necessary for MCP-1 mRNA induction in β-cells exposed to cytokines. Indeed, rat β-cells infected with an adenovirus overexpressing the nondegradable IκBα super-repressor protein (AdIkB(SA)2) have a 50% lower induction of MCP-1 mRNA expression in response to IL-1β. The partial blocking observed (Fig. 7A) may be due to the limited efficiency of infection by the adenoviral system used, which is ~70–80% (47). These data are in good agreement with our previous microarray results, showing a 50–60% inhibition of cytokine-induced MCP-1 mRNA expression in β-cells previously infected with the AdIkB(SA)2 (8) and with the present observations that PDTC, a potent inhibitor of NF-κB, blocked cytokine-induced MCP-1 mRNA expression >70% in cytokine-exposed islets. However, the fact that the observed inhibitory effect of NF-κB blockers on MCP-1 expression in vitro is not complete suggests that other transcription factors may also contribute to MCP-1 regulation in β-cells.

As a whole, these findings suggest an essential role for NF-κB in the regulation of MCP-1 in β-cells. Of note, in vivo overexpression of IκBα, a natural inhibitor of NF-κB, significantly decreases local MCP-1 and ICAM-1 expression, as well as recruitment of macrophages following iliac artery balloon angioplasty in rabbits, thus reducing arterial restenosis (59). This, and the fact that high basal MCP-1 production by human islets correlates with a poor clinical outcome following islet transplantation in patients with type 1 diabetes (6), suggests that adenovirus- or lentivirus-mediated NF-κB inhibition might be a useful approach to decrease mononuclear cell infiltration and graft dysfunction/destruction following islet transplantation.

ACKNOWLEDGMENTS

This work was supported by grants from the Juvenile Diabetes Foundation International, the Funds voor Wetenschappelijk Onderzoek (FWO), and the Fonds National de la Recherche Scientifique (FNRS), Belgium.

We thank the personnel from the Diabetes Research Center, VUB, involved in β-cell purification; M.A. Neef, J. Schoonheydt, and M. Urbain, Laboratory of Experimental Medicine, ULB, for islet isolation; and E. Quartier for sequencing.

REFERENCES
7. Cardozo AK, Kruhoffer M, Leeman R, Orntoft T, Eizirik DL, Identification...