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C/EBP-related protein 2 confers lipopolysaccharide-inducible expression of interleukin 6 and monocyte chemoattractant protein 1 to a lymphoblastic cell line

(transcription factor/cytokine/inflammation)

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ABSTRACT **C/EBP-related proteins 2 and 3 (CRP2 and CRP3) are differentially expressed by P388 lymphoblasts and their derivative P388D1(IL1) macrophages. We have ectopically expressed CRP2, the predominant CRP in macrophages, in P388 lymphoblasts. The expression of CRP2 is sufficient to confer the lipopolysaccharide (LPS)-inducible expression of interleukin 6 and monocyte chemoattractant protein 1 to lymphoblasts, which normally do not display LPS induction of inflammatory cytokines. Consistent with these findings, the expression of CRP2 antisense RNA blocks the LPS induction of IL-6 expression in P388D1(IL1) macrophages. This work clearly establishes the essential role of CRP2 in the induction of cytokine genes by LPS. Additionally, these data add MCP-1 to the list of cytokines showing an involvement of CRP2 in their expression.**

C/EBP related proteins (CRPs) are a family of basic region–leucine zipper transcription factors (1, 2). These proteins dimerize through a leucine zipper and bind to DNA through an adjacent basic region (3, 4). Several lines of evidence implicate CRPs in the regulation of inflammatory cytokines. The promoter regions for the interleukin (IL) 6, IL-1α, IL-1β, IL-8, tumor necrosis factor α, and granulocyte colony-stimulating factor genes contain known or predicted CRP binding sites (5–8). Both CRP2 (1) [also known as NF-IL6 (5), LAP (9), IL-6DBP (10), AGP/EBP (11), and C/EBPβ (2)] and CRP3 (1) [also known as NF-IL6β (12) and C/EBPδ (2)] can transactivate a reporter gene driven by the IL-6 promoter in transient expression assays (5, 12). Additionally, the lipopolysaccharide (LPS)-induced expression of IL-1β (7, 8) and granulocyte colony-stimulating factor (5, 13, 14) requires one or more elements that bind a CRP-like activity.

Among the hematopoietic lineages, mature macrophages and granulocytes are specific in their high level expression of CRP2 and/or CRP3 (15, 16). Macrophages are also notable for LPS-induced transcription of genes for the various inflammatory cytokines, the same genes whose promoters possess CRP binding sites. Indeed, CRP2 and CRP3 expression is LPS inducible (5, 12), and the CRP binding sites of the IL-1β and granulocyte colony-stimulating factor genes are required for the LPS response (7, 8, 13, 14). These data suggest that CRP2 and/or CRP3 may be necessary for the LPS-induced cytokine response in macrophages.

The evidence for the function of CRP2 and CRP3 in the regulation of IL-6 and other cytokines has rested on transient transactivation of promoter–reporter genes rather than activation of endogenous cytokine genes containing a full complement of regulatory sequences. In this paper, we have directly assessed the capacity of CRP2 for conferring LPS-induced cytokine expression to a lymphoblastic cell line normally lacking this activity. We have found that ectopic expression of CRP2 in the lymphoblastic P388 cell line (17) confers LPS-inducible expression of the genes encoding IL-6 and monocyte chemoattractant protein 1 (MCP-1). Moreover, the expression of CRP2 antisense RNA blocks the induction of IL-6 by LPS in P388D1(IL1) macrophages. These results establish the critical role of CRP2 in the LPS-induced activation of cytokine genes.

MATERIALS AND METHODS

Cells and Cell Culture. P388 murine lymphoblasts are $P388D_1$ cells (ATCC CCL 46). We have denoted these cells as P388 to avoid confusion with their macrophage derivative, which is usually referred to as P388D1 (17). P388 cells are lymphoblastic in morphology and lack appreciable expression of Mac-1, Mac-2, Mac-3 as determined by fluorescent-activated cell sorting. P388D1(IL1) murine macrophages are $P388D_1$ (IL-1) cells (ATCC TIB 63). These cells are macrophage-like in morphology and express Mac-1, Mac-2, and Mac-3 at high levels. Cells were cultured in RPMI 1640 medium with 5% (vol/vol) fetal calf serum and 50 μM 2-mercaptoethanol.

Transfections. Transfections of G418-resistant vectors were carried out with 10^6 cells, 5 μg of DNA, and 40 μg of Lipofectin (GIBCO/BRL) in 3 ml of Opti-MEM I medium (GIBCO/BRL). Cells were incubated in the transfection cocktail for 16 h followed by the addition of RPMI 1640 with 20% fetal calf serum. After 72 h the medium was replaced with the standard growth medium with G418 (GIBCO/BRL) at 0.67 mg/ml.

Expression Vectors. pSV(X)Neo is pZIP-NEO SV(X)1 (18). pSV(X)CRP2 and pSV(X)CRP2AS were constructed by insertion of the 1.55-kb *Nco* I/*Eco*RI genomic fragment encoding rat CRP2 (2) into the *Bam*HI site of pSV(X)Neo in both sense and antisense orientations.

Nucleic Acid Isolation and Analysis. Cytoplasmic RNA was isolated as described (19). RNAs were electrophoresed through 1% agarose/formaldehyde gels. Transfers to membranes were hybridized and washed to a stringency of 0.1 × SSPE at 65°C. Hybridization probes were prepared with a random priming kit (United States Biochemical) with the incorporation of 5′-[α-^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq; DuPont/NEN). The probe for CRP2 was the genomic fragment described for expression vectors. The glyceraldehyde phosphate dehydrogenase (GAPDH) probe was a 1.3-kb rat cDNA (20). The IL-6 probe was a 0.65-kb murine cDNA (from N. Jenkins and N. Copeland, National Cancer Institute–Frederick Cancer Research and Development Center).

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Abbreviations: CRP, C/EBP-related protein; IL, interleukin; LPS, lipopolysaccharide; MCP-1 monocyte chemoattractant protein 1; GAPDH, glyceraldehyde phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay.

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The MCP-1 probe was a 0.58-kb murine cDNA (21). The IL-1α probe was a 1.7-kb murine cDNA (22). The IL-1β probe was a 1.0-kb murine cDNA (23).

Western Analysis. Nuclear extracts were prepared as described below. The extracts (50 μg) were adjusted to 1× Laemmli sample buffer (24) and processed on 12% PAGE gels. The gels were transferred onto Immobilon-P membranes (Millipore), and antigen–antibody complexes were visualized with the enhanced chemiluminescence kit (Amersham).

Electrophoretic Mobility Shift Assays (EMSAs). Nuclear extracts were prepared as described by Lee et al. (25) except that the samples were not dialyzed into buffer D. Protein was incubated with a double-stranded oligonucleotide probe homologous to nucleotides −107 to −90 of the rat albumin promoter (DEI site; 5′-TCGACTATGATTTTGTAATGGGGC-3′ annealed with 5′-TCGAGCCCCATTACAAAATCATAG-3′) or to a probe containing an optimal C/EBP binding site (5′-GATCCTAGATATCCCTGATTGCGCAATAGGCTCAAAGCTG-3′ annealed with 5′-AATTCAGCTTTGAGCCTATTGCGCAATCAGGGATATCTAG-3′). All binding reactions were performed at 23°C in a 25-μl mixture containing 6 μl of nuclear extract (1 mg/ml in buffer C), 6% (vol/vol) glycerol, 4% (wt/vol) Ficoll, 10 mM Hepes (pH 7.9), 10 mM dithiothreitol, 0.25 μg of bovine serum albumin, 0.06% bromophenol blue, 1 μg of poly(dI-dC), and 1.25 ng of probe. Samples were electrophoresed through 6% polyacrylamide gels in 0.5 × TBE at 150 V. For "supershifts", nuclear extracts were preincubated with antisera for 30 min at 4°C prior to the binding reaction. Binding reactions with purified recombinant bacterial CRP2 and CRP3 contain 0.25 μl of normal rabbit serum.

Metabolic Labeling and Immunoprecipitation. Cells were washed twice in methionine-deficient DMEM (GIBCO/BRL) and then labeled in the same medium containing 200 μCi of EXPRE^{35}S^{35}S protein labeling mix (DuPont/NEN) and 5% dialyzed fetal calf serum. After 3 h, cells were collected and lysed, and immunoprecipitation was performed and analyzed as described (26).

Antisera. Rabbit anti-CRP2 antiserum was generated by immunization with a peptide corresponding to amino acids 1–12 of CRP2 (1). Rabbit anti-CRP3 antiserum was generated by immunization with a peptide corresponding to amino acids 255–266 of CRP3 (1).

RESULTS

Differential CRP2 and CRP3 Expression Between P388 Lymphoblasts and Their Macrophage Derivative P388D1(IL1). Upon screening a panel of hematopoietic cell lines for differential CRP2 and CRP3 expression, we found that the P388 B-lymphoblastic cell line lacked CRP2 and CRP3 transcripts, while its macrophage derivative P388D1(1) expressed these transcripts abundantly (Fig. 1*A*). C/EBP transcripts were not detected in either cell line (data not shown). These findings suggested that this pair of cell lines could provide a model system in which to test the capacity of CRP2 and CRP3 to confer macrophage characteristics to a lymphoid cell line not normally expressing CRP2 and CRP3. In particular, we sought to examine the LPS-induced transcription of genes encoding inflammatory cytokines.

Having observed differential expression at the RNA level, we sought to confirm that P388D(IL1) expressed active CRP2 and CRP3 proteins while P388 did not. Western blot analysis of nuclear extracts from the two cell lines revealed the presence of CRP2 protein in P388D1(IL1) cells but not in P388 cells (Fig. 1*B*). An EMSA showed that the nuclear extract of P388D1(IL1) cells formed several complexes (a, b, and c) that could be supershifted by incubation with an antiserum specific for the CRP2 amino terminus (Fig. 1*C*). Complex a comigrates with the species obtained using bacterial CRP2, whereas complexes b and c represent modified or heterodimeric CRP2 binding forms. Complex d was not supershifted and may contain the LIP protein, a truncated

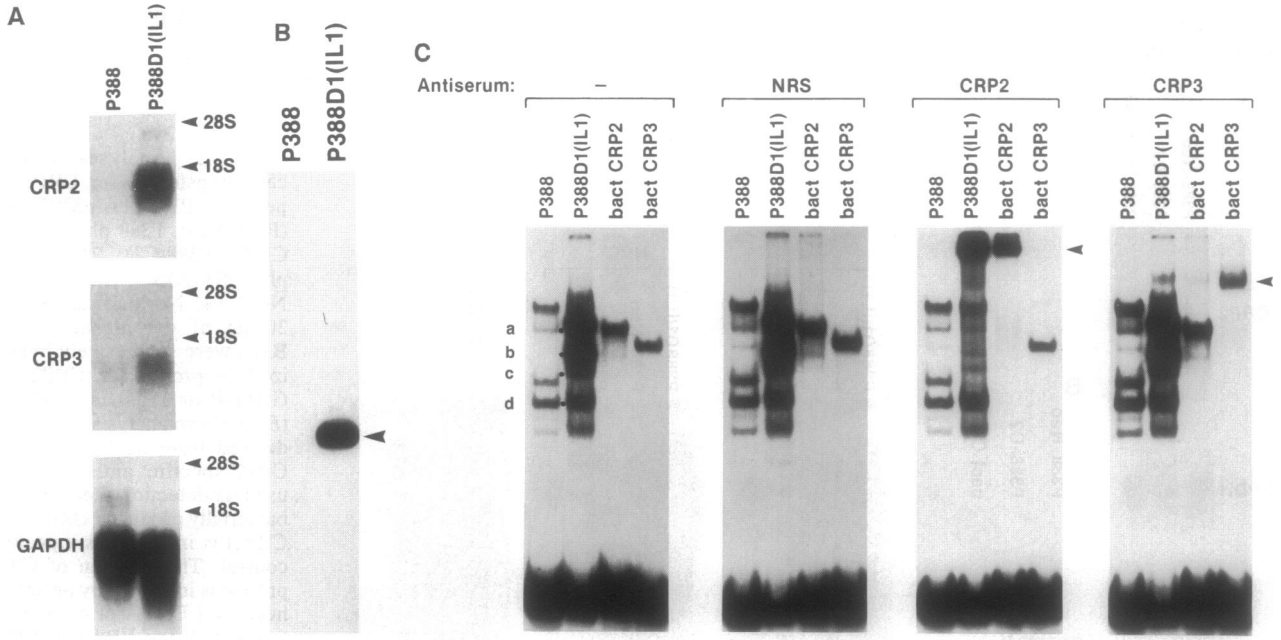

FIG. 1. Analyses of differential CRP2 and CRP3 expression between P388 and P388D1(IL1) cells. (*A*) Northern analysis of 20 μg of cytoplasmic RNA. The same blot was successively hybridized to probes for CRP2, CRP3, and GAPDH. Hybridization to GAPDH served as a loading control. Positions of ethidium bromide-stained 28S and 18S rRNAs are marked. (*B*) Western analysis of proteins derived from nuclear extracts. CRP2-specific antiserum was used for detection. (*C*) EMSA of nuclear extracts with ^{32}P-labeled optimal C/EBP binding site as probe. Samples were treated in the absence of antiserum (−) and in the presence of normal rabbit serum (NRS), CRP2 antiserum (CRP2), and CRP3 antiserum (CRP3). Recombinant bacterially produced CRP2 (bact CRP2) and recombinant bacterially produced CRP3 (bact CRP3; a truncated form of CRP3 beginning at Met-68) were analyzed as positive controls for DNA binding and supershift. Complex a represents CRP2 homodimer, while b and c probably contain heterodimeric forms of CRP2; all are supershifted with CRP2 antiserum. Species d is not supershifted and may represent LIP (27). The positions of species supershifted by antisera are indicated by arrowheads.

form of CRP2 (27) that would not react with our amino-terminal-specific antiserum. CRP3 protein was not observed in a Western blot analysis (data not shown), but a supershift species was detectable with CRP3-specific antiserum in an EMSA (Fig. 1*C*). It was not possible to determine which EMSA complex gave rise to this supershifted species. Nonetheless, it is clear that P388D1(IL1) macrophages display CRP binding activity absent in P388 B lymphoblasts. CRP2 homo- and heterodimers represent the major CRP binding species in the macrophage cell line, whereas CRP3 is only a minor component of the binding activity. Therefore, we focused subsequent studies on the role of CRP2 in determining the differential ability of P388 and P388D1(IL1) cells to produce inflammatory cytokines.

Ectopic Expression of CRP2 in P388 B Lymphoblasts. The murine retrovirus vector pSV(X)Neo was used to ectopically express the CRP2 gene in P388 cells. Populations of P388 cells were transfected with pSV(X)CRP2 or the parental vector lacking an expressed insert, pSV(X)Neo. Pools of stably transfected cells were obtained after selection with G418. Those cells transfected with pSV(X)CRP2 were designated P388-C2 and those cells transfected with pSV(X)Neo were designated P388-Neo.

To assess whether the transfected cells were composed of a single clonal outgrowth or a complex population of many transfectants, we performed Southern blot analyses (data not shown). These studies demonstrated that the P388-C2 population possesses at least a haploid abundance of the CRP2 expression vector. P388-C2 is composed of either two clones, each with a single ectopic CRP2 integration, or a single clone with two ectopic CRP2 integrations.

CRP2 expression in the transfected populations was initially characterized by Northern blot analysis (Fig. 2*A*). P388-C2 cells expressed an ≈5.6-kb CRP2 RNA corresponding to the expected genome-length retroviral transcript. A Western blot analysis of nuclear extracts from the transfected populations confirmed the ectopic expression of the transfected CRP2 gene at the protein level in P388-C2 (Fig. 2*B*). In addition, EMSAs detected a supershifted protein–DNA complex in a P388-C2 nuclear extract that had been incubated with CRP2-specific antiserum (Fig. 2*C*). Neither CRP3 mRNA, protein (data not shown), nor a supershift species was observable in P388-C2 cells, indicating that endogenous CRP3 expression was not induced by CRP2 and that P388-C2 differed from P388-Neo only in its acquisition of CRP2 expression.

LPS-Induced Cytokine Expression Occurs in the Transfectants That Ectopically Express CRP2. Cultures of cells ectopically expressing CRP2 were treated with LPS (20 μg/ml) over a time course, and RNA was isolated from several time points. Control populations of P388 lymphoblasts and P388D1(IL1) macrophages, each transfected with pSV(X)Neo, were also subjected to time courses of LPS treatment, and RNA was isolated. Northern analyses were performed to detect transcripts for IL-6, MCP-1, IL-1α, and IL-1β. Transcripts for GAPDH were also examined as a normalization control. LPS was found to induce transcripts for IL-6 and MCP-1 in P388-C2 cells (Fig. 3). These inductions were generally comparable to those seen in the P388D1(IL1)-Neo macrophage control population. Transcripts for IL-1α and IL-1β were not induced by LPS in P388-C2 cells, whereas a vigorous induction of these genes was observed in the P388D1(IL1)-Neo macrophages. All four cytokines showed either no induction or very weak induction (MCP-1) upon LPS treatment of P388-Neo lymphoblasts. Untransfected P388 cells as well as P388 cells ectopically expressing CRP2 antisense RNA failed to respond to LPS treatment (data not shown).

Since the P388-C2 population is a clonal or biclonal outgrowth, we examined an independently derived population of pSV(X)CRP2 transfectants, P388-C2-2, to assure ourselves that the acquisition of LPS inducibility was a consequence of ectopic CRP2 expression rather than clonal variation. Similarly to P388-C2 cells, P388-C2-2 cells are a clonal population (data not shown). P388-C2-2 cells were treated with LPS over a time course, and RNA was isolated from each time point. A Northern analysis confirmed that transcripts for IL-6 were induced by LPS treatment in these CRP2-expressing cells (data not shown).

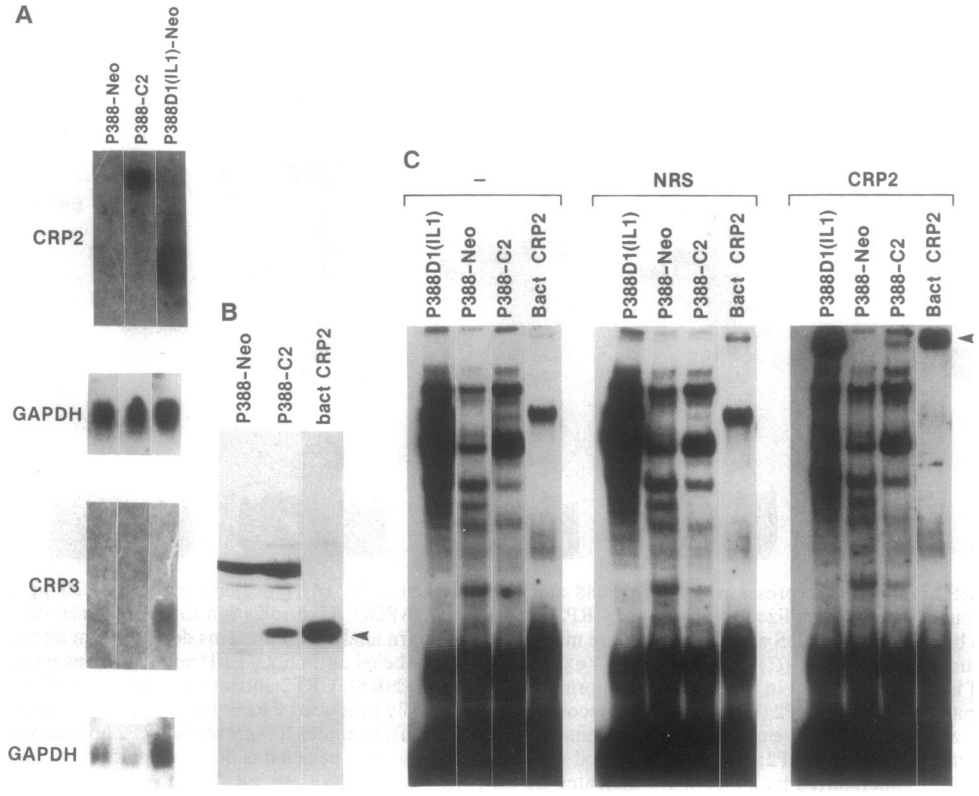

FIG. 2. Analyses of P388 cells transfected for CRP2 expression: P388 plus pSV(X)Neo (P388-Neo), P388 plus pSV(X)-CRP2 (P388-C2), P388D1(IL1) plus pSV(X)Neo [P388D1(IL1)-Neo]. (*A*) Northern analysis of 20 μg of cytoplasmic RNA. Blots were successively hybridized to probes for CRP2 and GAPDH or CRP3 and GAPDH. (*B*) Western analysis of proteins derived from nuclear extracts. CRP2-specific antiserum was used for detection. Recombinant bacterially produced CRP2 (bact CRP2) is included as a positive control. The position of CRP2 protein is indicated by an arrowhead. (*C*) EMSA of nuclear extracts with the ^{32}P-labeled DEI site in the rat albumin promoter as probe. Samples were treated in the absence of antiserum (–) and in the presence of normal rabbit serum (NRS) and CRP2 antiserum (CRP2). The position of the CRP2 EMSA species supershifted by CRP2 antiserum is indicated by the arrowhead.

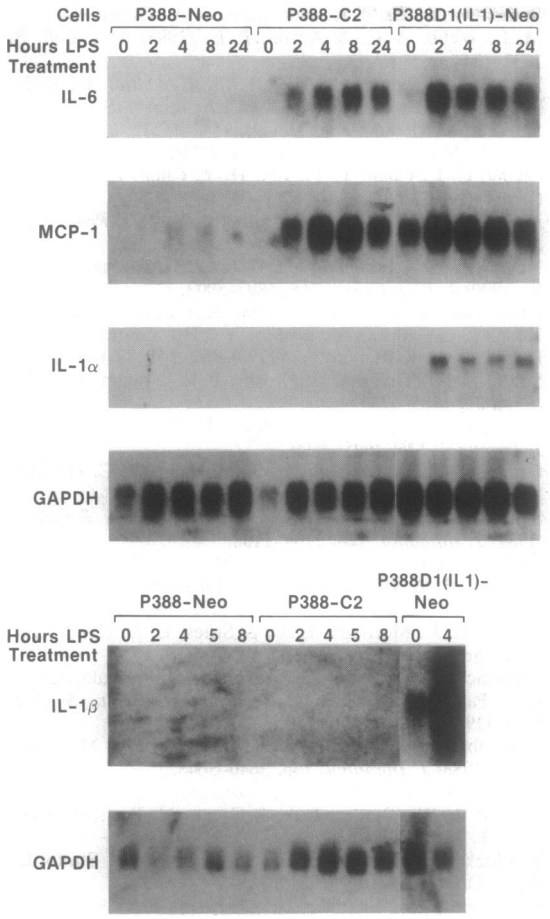

FIG. 3. Northern analyses for cytokine expression in P388 cells transfected for CRP2 expression. Cytoplasmic RNA was isolated over time courses of LPS treatment as indicated. Twenty micrograms of RNA was analyzed. Blots were successively hybridized to probes for IL-6, MCP-1, IL-1α, IL-1β, and GAPDH. The transfectants analyzed were P388 plus pSV(X)Neo (P388-Neo), P388 plus pSV(X)-CRP2 (P388-C2), and P388D1(IL1) plus pSV(X)Neo [P388D1(IL1)-Neo].

IL-1 and IL-6 have been reported to induce transcription of the MCP-1 (28) and IL-6 genes (29–32). Thus, LPS may act through the induction of IL-1 and IL-6. Experiments parallel to those described above were performed to examine the abilities of murine IL-1 and human IL-6 to induce transcription of the genes for inflammatory cytokines in the P388 transfectants. No induction of IL-6, MCP-1, IL-1α, or IL-1β was observed (data not shown). Thus, the inductions observed with LPS are most likely a direct effect. Alternatively, the lack of response to IL-1 and IL-6 in the P388 transfectants might be explained trivially by the absence of a receptor for these cytokines.

Inhibition of LPS-Induced Cytokine Expression by CRP2 Antisense RNA. The preceding experiments show that CRP2 expression is sufficient to confer LPS inducibility of IL-6 and MCP-1 transcription to P388 lymphoblasts. To address the question of whether CRP2 is essential for LPS-inducible expression of cytokines in macrophages, we sought to inhibit CRP2 function in P388D1(IL1) cells. This cell line expresses CRP2 and CRP3 (Fig. 1) and displays LPS-inducible expression of IL-6, MCP-1, IL-1α, and IL-1β (Fig. 3). To block CRP2 expression, a pSV(X) vector carrying the CRP2 gene in an antisense orientation [pSV(X)CRP2AS] was stably transfected into P388D1(IL1) cells. As a control, P388D1(IL1) cells were transfected with pSV(X)Neo. RNA was prepared from populations of these transfected cells in the presence or absence of a 4-h treatment with LPS (20 μg/ml). Northern analysis (Fig. 4 *Upper*) was performed to examine transcripts for IL-6 (highly induced in P388 cells ectopically expressing CRP2) and IL-1β (uninduced in P388 cells ectopically expressing CRP2). Both IL-6 and IL-1β transcripts failed to be induced in P388D1(IL1)-C2AS, a transfectant population carrying pSV(X)CRP2AS. In comparison, a vigorous induction of IL-6 and IL-1β transcripts was observed for P388D1(IL1)-Neo. It is noteworthy that IL-1β transcripts are unaffected by CRP2 expression in P388 cells, whereas they are dramatically inhibited by antisense CRP2 RNA in P388D1(IL1) cells. This most likely indicates that transcription of IL-1β requires activation of a transcription factor(s) not present in P388 cells that acts together with CRP2 to elicit an LPS response.

To confirm that the expression of antisense RNA actually reduced the level of CRP2, the transfected populations were metabolically labeled, and cellular lysates were subjected to immunoprecipitation with a CRP2-specific antiserum. The level of CRP2 protein was reduced in populations expressing antisense CRP2 RNA in comparison to the control population (Fig. 4 *Lower*). These results indicate that suppression of the LPS response by antisense CRP2 RNA is indeed due to inhibition of CRP2 protein synthesis.

DISCUSSION

The data presented in this paper demonstrate that CRP2 is sufficient to confer LPS-inducible expression of IL-6 and MCP-1 to P388 lymphoblasts. Furthermore, the expression of CRP2 antisense RNA in P388D1(IL1), a macrophage cell line derived from P388, blocks the ability of this cell line to induce IL-6 transcripts in response to LPS. These data establish the critical role of the CRP2 protein in the activation of endogenous IL-6 and MCP-1 genes by LPS and demonstrate that P388 lymphoblasts lack only one activity necessary for transcription of these genes. It is possible that CRP2 induces a set of regulatory genes whose products also contribute to activation of cytokine gene expression. However, in light of previous studies showing the ability of CRP2 to transactivate the proximal promoter region of IL-6 (5, 9), the simplest interpretation of our results is that CRP2 acts directly on these target cytokine genes.

To our knowledge, our results are the first indication that CRP2 may be involved in the regulation of MCP-1. An inspection of the MCP-1 promoter sequence reveals several potential CRP binding sites (33), consistent with the possi-

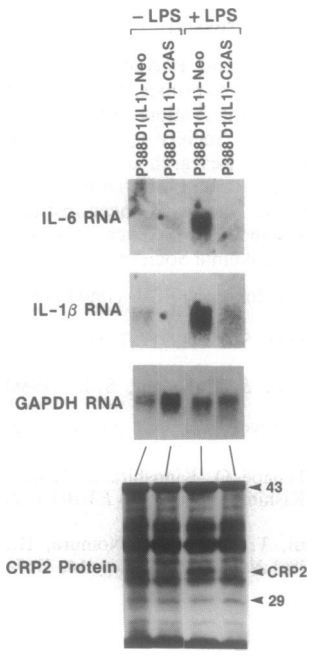

FIG. 4. (*Upper*) Inhibition of IL-6 and IL-1β expression by antisense CRP2 RNA in P388D1(IL1) cells: P388D1(IL1) plus pSV(X)Neo [P388D1(IL1)-Neo] and P388D1(IL1) plus pSV(X)-CRP2AS [P388D1(IL1)-C2AS]. Cells were either untreated (– LPS) or exposed to LPS for 4 h (+ LPS). Northern analysis of 20 μg of cytoplasmic RNA was successively hybridized to probes for IL-6, IL-1β, and GAPDH. (*Lower*) Metabolically labeled protein from identically treated cells was immunoprecipitated with CRP2-specific antiserum and analyzed by PAGE. The positions of CRP2 and marker proteins (in kDa) are indicated.

bility that MCP-1 transcription is controlled by CRP2. In contrast, although the promoters for the IL-1α and IL-1β genes also possess CRP binding elements, ectopic expression of CRP2 was not sufficient to confer responsiveness of these genes to LPS in P388 lymphoblasts. On the other hand, antisense RNA for CRP2 blocked LPS induction of IL-1β mRNA in P388D1(IL1) macrophages. While CRP2 may be essential for IL-1β expression, other factors are likely to be necessary for LPS induction of IL-1β. Indeed, other investigators have found that even though both MCP-1 and IL-1 are induced by LPS, there are critical differences in their regulation (34, 35). For example, agents that elevate intracellular levels of cAMP suppress the LPS induction of MCP-1 but do not affect induction of IL-1α and actually enhance the induction of IL-1β.

It is clear that LPS induction of IL-6 and MCP-1 in our system operates through either posttranscriptional alteration of CRP2 or induction of a necessary cooperating transcription factor. While we did not observe any alteration in the CRP2 DNA-binding species observed before and after LPS treatment (data not shown), it has recently been shown that 12-*O*-tetradecanoylphorbol 13-acetate increases the site-specific phosphorylation of CRP2 and enhances its activity (36). Many of the activities of LPS are mimicked by phorbol esters (37). In addition, LPS can induce NF-κB activity (38). NF-κB can functionally associate with CRPs (39), and the IL-6 promoter contains both an NF-κB and a CRP binding site (40, 41). Indeed, CRP2 and NF-κB have recently been shown to synergistically activate the transcription of IL-6 (42). Glucocorticoid receptor (43), AP-1 (44), and c-myb (45, 46) have been similarly implicated as transcription factors that can cooperate with CRPs, and their roles must also be considered in understanding the regulation of cytokine genes by CRP2.

The ability of CRP2 to confer LPS-inducible expression of the inflammatory cytokines, IL-6 and MCP-1, to a B-lymphoblast cell line has some parallels to the ability of NF-M (avian CRP2) and c-*myb* to activate the myeloid-specific *mim-1* and lysozyme genes in heterologous cell types such as erythroid and fibroblastic cells (45, 46). CRPs may have a role in determining the differentiated functions, and in promoting differentiation itself, of myelomonocytic cells. The examination of myeloid-specific gene expression in the CRP2-transfected B lymphoblasts of this study will further illuminate this issue. The relative abilities of other CRP family members, such as C/EBP and CRP3, to participate in cytokine induction as well as other differentiated functions of myelomonocytic cells must also be assessed.

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