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Protein Kinase C Activation Increases Binding of Transcription Factor PU.1 in Murine Tissue Macrophages

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PU.1 is a transcription factor found in macrophages, B cells, neutrophils, and hemopoietic stem cells. In macrophages PU.1 regulates a number of genes, including *c-fms*, CD11b, CD18, and FcγR1b. Previously, in primary macrophages PU.1 binding to the sequence GAGGAA was found to be induced by treatment with bacterial lipopolysaccharide (LPS) and interferon-γ (IFN-γ). Here we investigated the role of protein kinase C (pKC) in the induction of PU.1 binding in macrophages. We report that pharmacological activation of pKC increases PU.1 binding, while inactivation of pKC inhibits the increases in PU.1 binding by agents which activate pKC in macrophages (LPS and tumor necrosis factor-α), but not by an agent which does not activate pKC (IFN-γ). pKC activation may therefore be one pathway by which PU.1 binding may be increased in primary macrophages.

PU.1 is a transcription factor found in macrophages, mast cells, B cells, neutrophils, and hemopoietic stem cells (1–3). In macrophages PU.1 has been shown to regulate several genes, including *c-fms*, CD11b, FcγR1b, and CD18 (4–7). In primary macrophages PU.1 binding to a core consensus sequence of GAGGAA, is increased by treatment with bacterial lipopolysaccharide (LPS) and interferon-γ (IFN-γ), as measured by electrophoretic mobility shift assay (EMSA). (8). Since LPS both induces PU.1 binding (8) and activates pKC activity in macrophages (for review, 9), we hypothesized that pKC activation may play a role in increasing PU.1 binding in macrophages. Here we show that pharmacological activation of pKC by two different agents induces PU.1 binding, while inhibition of pKC activity lessens PU.1 binding induction by LPS and tumor necrosis factor-α (TNF-α), but not by IFN-γ treatment.

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Abbreviations used: pKC, protein kinase C; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; LPS, bacterial lipopolysaccharide; EMSA, electrophoretic mobility shift assay; CIP, calf intestinal phosphatase; TPA, tetradecanoyl-phorbol-13-acetate; EMSA, electrophoretic mobility shift assay.

EXPERIMENTAL PROCEDURES

Materials. Tissue culture media were purchased from MediaTech (Washington, DC) and fetal bovine serum from HyClone Laboratories (Logan, UT). All tissue culture reagents contained less than 0.125 ng/ml endotoxin (LPS), as quantified by the *Limulus* amoebocyte assay supplied by Associates of Cape Cod, Inc. (Woods Hole, MA). Dupont NEN Research Products (Boston, MA) was the source of all radiolabeled chemicals. rIFN-γ was a gift of Schering-Plough (Kenilworth, NJ). LPS from *Escherichia coli* 026:B6 was purchased from Difco (Detroit, MI). TNF-α was purchased from Boehringer Mannheim (Mannheim, Germany). Tetradecanoyl-phorbol-13-acetate (TPA), *sn*-1-2-octanoyl-gylcerol, staurosporine, and dIdC were obtained from Sigma Chemical Co. (St. Louis, MO). Calf intestinal phosphatase (CIP), Klenow fragment, and T4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA).

Cell culture. Specific pathogen-free inbred C57B1/6J mice (6 to 8 weeks old) were purchased from Charles River Breeding Laboratories (Raleigh, NC). Thioglycolate-elicited macrophages were obtained and cultured as previously reported (10). All macrophage cultures were routinely found to contain greater than 98% macrophages, as determined by Giemsa stain or by histochemical assay for nonspecific esterase. After 24 h, the macrophages were treated as indicated.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared, protein was quantified, and oligonucleotides prepared as previously described (11). Each experiment used 2.5×10^7 macrophages/treatment. DNA binding proteins present in the nuclear extracts were analyzed using 3 μgs of protein to bind the synthetic oligonucleotide 5′-TCCAATGCTGATTGGTTCCTCACTTGGGACCAACC-3′ (*I-Ab* Y-box) (8). For experiments involving CIP, oligonucleotides 5′-TCCAATGCTGATTGGTTCCTCACTTGGGACC-3′ and 5′- GGTCCCAAGTGAGGAACCAATCAGCATTGGA-3′ where annealed and Klenow labeled with α^{32}P-CTP according to manufacturer's instructions (New England Biolabs). Macrophage nuclear extracts were CIP treated by adding 1 μl CIP to 5 μl nuclear extracts (3 μgs protein) in modified Dignam solution C (8,11), and incubating on ice for 15 min. Oligonucleotide, dIdC, and H_2O were added to a final volume of 20 μl as previously described (11). Unless otherwise mentioned, all treatment times were 6 h. Data represent the results of at least 3 experiments.

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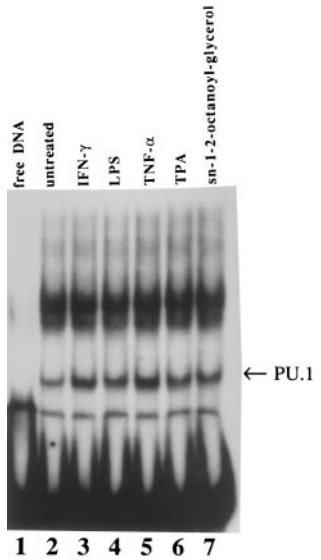

FIG. 1. EMSA of nuclear proteins from macrophages examining the induction of PU.1 binding to the *I-Ab* Y-box oligonucleotide probe by various stimuli. Lane 1, free DNA; lane 2, untreated; lane 3, IFN-γ; lane 4, LPS; lane 5, TNF-α; lane 6, TPA; lane 7, *sn*-1-2-octanoyl-gylcerol. [IFN-γ], 20 units/ml; [LPS], 10 ng/ml; [TNF-α], 3,000 units/ml; [TPA], 1 μg/ml; [*sn*-1-2-octanoyl-gylcerol], 10 μM.

RESULTS

To examine the role of pKC on PU.1 binding we treated macrophages with IFN-γ, LPS, TNF-α, TPA, and *sn*-1-2-octanoyl-gylcerol. As previously found, IFN-γ and LPS induced PU.1 binding to an oligonucleotide containing the sequence GAGGAA (*I-Ab* Y-box) (8) (Fig. 1, lanes 3 and 4). TNF-α, a cytokine known to induce pKC activity in macrophages (12), also induced PU.1 binding (Fig. 1, lane 5). Similarly, two pharmacological activators of pKC, TPA and *sn*-1-2-octanoyl-gylcerol (13, 14), induced PU.1 binding (Fig. 1, lanes 6 and 7). The identification of the protein as PU.1 was previously determined through cold oligonucleotide competition, footprint analysis, and by use of an anti-PU.1 serum (8). TPA treatment over time demonstrated that PU.1 binding was increased by TPA treatment as early as 0.25 h after treatment, with maximal induction occurring at roughly 3 h (Fig. 2).

To investigate the role of pKC activation in increasing PU.1 binding after LPS, TNF-α, and IFN-γ treatment, pKC activity was inhibited by two methods: (1) by chronic TPA treatment for 24 h prior to treatment, followed by treatment in the continued presence of the same concentration of TPA and (2) a 10 to 12 h preexposure to the relatively specific pKC inhibitor staurosporine prior to treatment, followed by treatment in the continued presence of the same concentration of staurosporine. As shown in Figures 3A and 3B, both TPA and staurosporine pretreatment partially inhibited the ability of LPS and TNF-α to increase PU.1 binding. TPA was usually a more effective inhibitor than staurosporine. Similarly, pKC inhibition more effectively inhibited PU.1 binding induction induced by LPS than that induced by TNF-α (compare Fig. 3A and 3B). PU.1 binding induction by IFN-γ was not significantly effected by pKC inhibition (Fig. 3C).

Since this data indicates that pKC activity and possibly PU.1 phosphorylation, plays a role in inducing PU.1 binding, we hypothesized that phosphatase treatment of the macrophage nuclear extracts may suppress PU.1 binding. To test this hypothesis we prepared an internally labeled *I-Ab* Y-box oligonucleotide resistant to dephosphorylation by CIP (see Methods and Materials). As shown in Figure 4, CIP lessened the LPS-mediated increases in PU.1 binding to an internally labeled *I-Ab* Y-box oligonucleotide (Fig. 4, compare lanes 6 and 7). Similar results were found for binding induced by TNF-α and IFN-γ (data not shown). As expected T4 polynucleotide kinase end-labeled oligonucleotide was dephosphorylated by CIP, while Klenow internally labeled oligonucleotide was not (Fig. 4, compare lanes 1 and 2, to 3 and 4).

Lastly, we investigated the effect of different combinations LPS, INF-γ, and TNF-α upon increased PU.1 binding. As shown in Figure 5, although all 3 signals induced PU.1 binding, no combination of the 3 resulted in significantly increased binding over that of any stimulus alone.

DISCUSSION

PU.1 is a transcription factor known to regulate a number of genes in macrophages (4–7). Treatment of macrophages with IFN-γ and LPS has been found to increase PU.1 binding to the sequence GAGGAA in the EMSA (8). Previously, treatment of the U937 macrophage-like cell line with TPA for 72 h resulted in

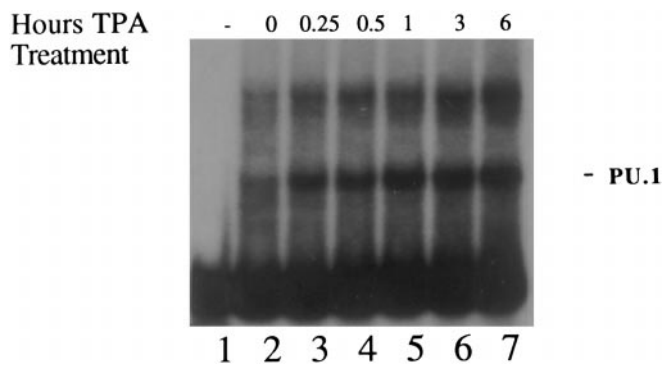

FIG. 2. EMSA of nuclear proteins from macrophages examining the induction of PU.1 binding to the *I-Ab* Y-box oligonucleotide over time. Lane 1, free DNA; lane 2, untreated; lane 3-7 have TPA at 0.25, 0.5, 1, 3, and 6 h respectively. [TPA], 1 μg/ml.

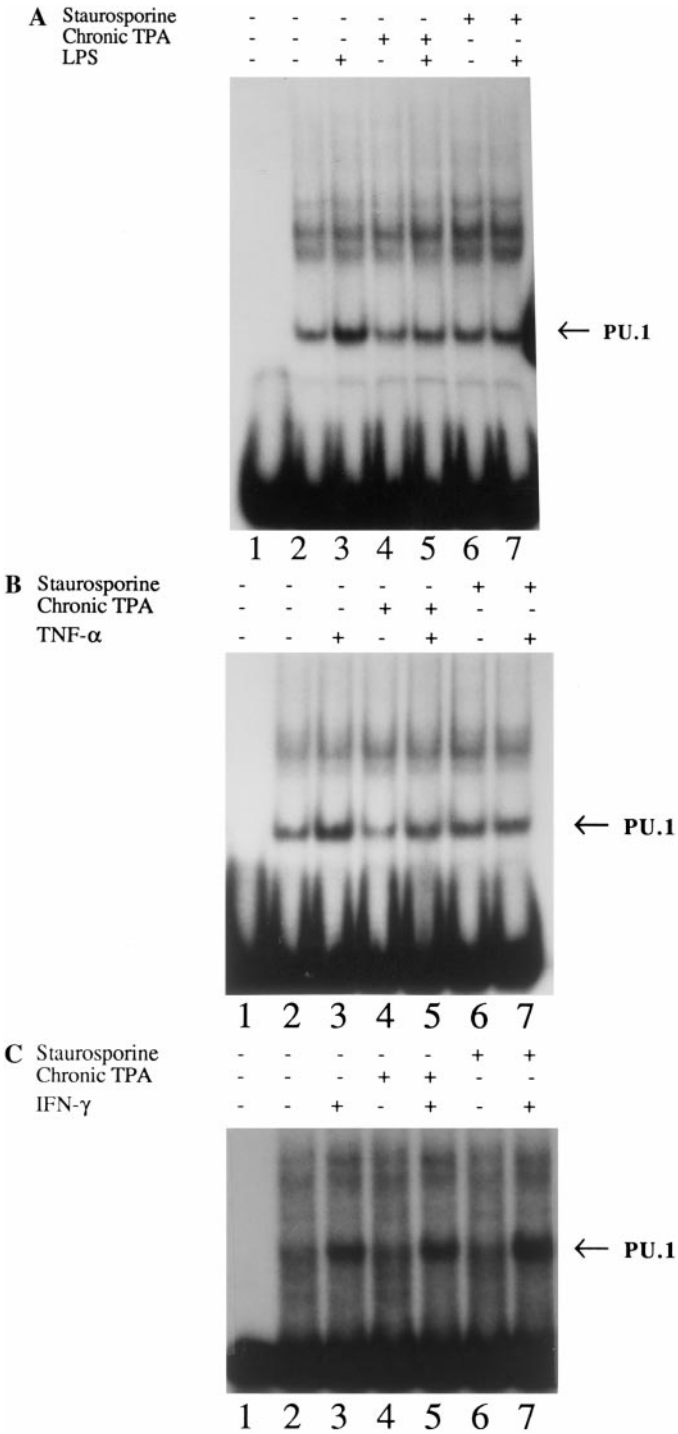

FIG. 3. (A) EMSA demonstrating the effect of pKC on LPS induced PU.1 binding to the *I-Ab* Y-box oligonucleotide. Lane 1, free DNA; lane 2, untreated; lane 3, LPS; lane 4, TPA; lane 5, LPS + TPA; lane 6, staurosporine; lane 7, LPS + staurosporine. [LPS], 10 ng/ml. [TPA], 1 μg/ml; [staurosporine], 20 nM. (B) EMSA demonstrating the effect of pKC inhibition on TNF-α induced PU.1 binding to the *I-Ab* Y-box oligonucleotide. Lane 1, free DNA; lane 2, untreated; lane 3, TNF-α; lane 4, TPA; lane 5, TNF-α + TPA; lane 6, staurosporine; lane 7, TNF-α + staurosporine. [TNF-α], 3,000 units/ml. [TPA], 1 μg/ml; [staurosporine], 20 nM. (C) EMSA demonstrating the effect of pKC on IFN-γ induced PU.1 binding to the *I-Ab* Y-box oligonucleotide. Lane 1, free DNA; lane 2, untreated; lane 3, IFN-γ; lane 4, TPA; lane 5, IFN-γ + TPA; lane 6, staurosporine; lane 7, IFN-γ + staurosporine. [IFN-γ], 20 units/ml. [TPA], 1 μg/ml; [staurosporine], 20 nM.

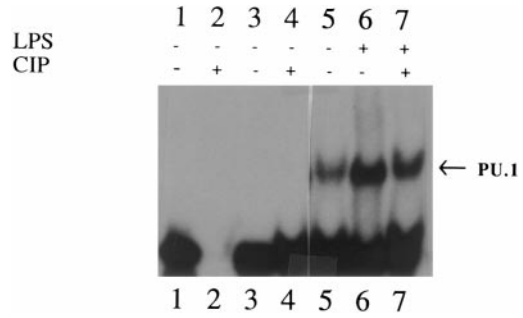

FIG. 4. EMSA demonstrating the effect of CIP treatment of LPS treated macrophage nuclear extracts on PU.1 binding to the *I-Ab* Y-box oligonucleotide. Lane 1, T4 labeled *I-Ab* Y-box oligonucleotide; lane 2, CIP + T4 labeled *I-Ab* Y-box oligonucleotide; (lanes 3-7 used Klenow labeled *I-Ab* Y-box oligonucleotide) lane 3, oligonucleotide only; lane 4, CIP + oligonucleotide; lane 5, untreated; lane 6, LPS; lane 7, LPS + CIP. [LPS], 10 ng/ml.

increased in PU.1 mRNA and protein binding to a PU.1 binding site (15). This later observation was attributed to events linked to TPA-induced cellular changes and not due to a direct effect of pKC activity upon PU.1 transcription or binding to DNA. Here we have demonstrated that (1), treatment with two pharmacological activators of pKC (TPA and *sn*-1-2-octanoyl-gylcerol) (13, 14) are capable in increasing PU.1 binding in primary macrophages by themselves, and (2), pharmacological inhibition of pKC activity inhibits increases in PU.1 binding initiated by stimuli which activate pKC (LPS and TNF-α), but does not effect increases in PU.1 binding by an agent which does not (IFN-γ) (11, 15). Lastly, we show that phosphatase treatment of nuclear extracts from LPS, TNF-α, and IFNγ treated macrophages results in a partial suppression of induced PU.1 binding. While CIP treatment does not identify the kinases/phosphatases involved in regulating PU.1 phosphorylation, the data does indicate that the phosphorylation state of PU.1 effects its binding to DNA. This finding is not surprising as LPS treatment was previously found to induce both PU.1 binding to DNA in primary macrophages, and serine phosphorylation by casein kinase II, in the RAW264.7 murine macrophage-like cell line (16). When macrophages were treated with different combinations of LPS, INF-γ, and TNF-α, no combination of agents induced PU.1 more than each stimulus alone, indicating that at least for PU.1 binding to the GAGGAA sequence, no synergism of the agents was found.

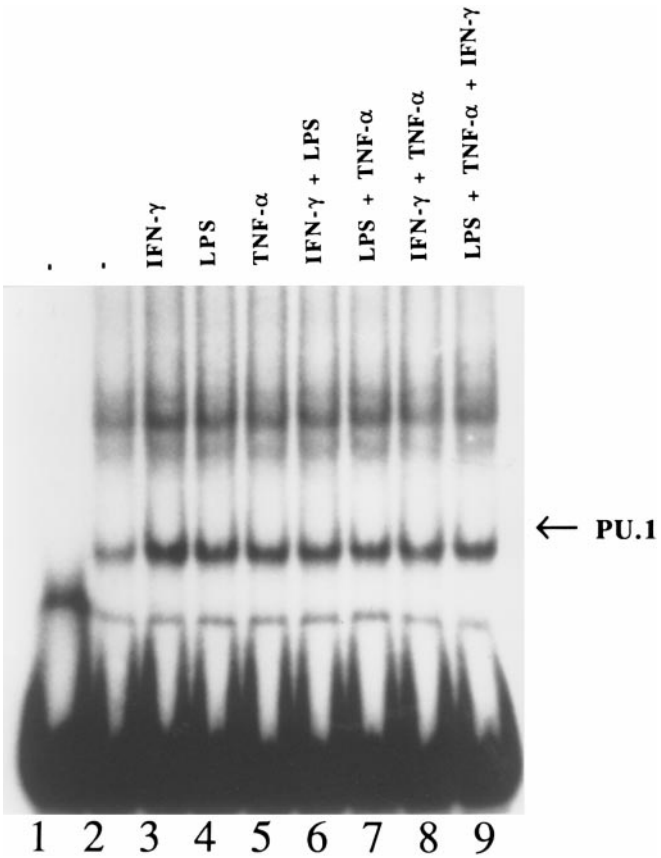

FIG. 5. EMSA of nuclear proteins from macrophages examining the induction of PU.1 binding to the *I-Ab* Y-box oligonucleotide by various stimuli. Lane 1, free DNA; lane 2, untreated; lane 3, IFN-γ; lane 4, LPS; lane 5, TNF-α; lane 6, IFN-γ + LPS; lane 7, LPS + TNF-α; lane 8, IFN-γ + TNF-α; lane 9, LPS + TNF-α + IFN-γ. [IFN-γ], 20 units/ml; [LPS], 10 ng/ml; [TNF-α], 3,000 units/ml.

Here we have shown pKC activation may play a role in increasing PU.1 binding in primary macrophages. Taken with previous data (16), PU.1 is the target of at least two different cellular kinases, pKC and casein kinase II, that act in response to external signals to alter PU.1 binding.

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