Trans-repressor BEF-1 Phosphorylation

A POTENTIAL CONTROL MECHANISM FOR HUMAN ApoE GENE REGULATION*

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Human apolipoprotein E is a plasma lipoprotein that appears to play an important protective role in the development of atherosclerosis. While little is known about the regulation of apoE, recent studies have shown that cytokines repress apoE synthesis both in vivo and in vitro. Furthermore, we have recently shown that the endogenous apoE gene is negatively regulated by the nuclear trans-repressor BEF-1 in the human HepG2 cell line. In this study we demonstrate that treatment of HepG2 cells with the cytokine interleukin-1 and interleukin-6 resulted in the induction of an isoform of BEF-1, designated B1. The induction of the B1 isoform could be blocked by the protein kinase inhibitor staurosporine, suggesting that B1 is a phosphorylated form of BEF-1. As further support, the B1 isoform could also be induced by phorbol ester, and subsequently inhibited by staurosporine, implicating a role for protein kinase C-mediated phosphorylation. Quantitation of the levels of the BEF-1 isoforms, and studies in the presence of cycloheximide, provided evidence for the phosphorylation of an existing intracellular pool of BEF-1, with no change in the total intracellular level. Under conditions that generated increased levels of the B1 isoform, there was a concomitant and proportional decrease in the level of apoE mRNA. The effect did not appear to be the result of improved binding to the apoE regulatory region as the DNA binding affinity of B1 was identical to native BEF-1. Our data suggest that the regulation of apoE by BEF-1 is modulated by differential phosphorylation, possibly through the protein kinase C pathway.

Apolipoprotein E (apoE), a primary constituent of several classes of mammalian lipoproteins, functions in transport and redistribution of cholesterol and other lipids among various cells in the body (1–3). There is mounting evidence that apoE plays a major protective role in the development of arteriosclerosis (4–9). In addition, apoE has proposed roles in immunoregulation and in the genesis of Alzheimer’s disease (reviewed in Refs. 10 and 11).

The factors that regulate apoE expression from hepatic tissue, the primary source of this circulating lipoprotein, are not well understood. Using the human hepatoma cell line HepG2 as a model system, several groups have identified regulatory elements required for efficient expression of apoE (12, 13), and we previously determined that the nuclear repressor factor BEF-1 negatively regulated apoE gene expression in these cells (14). BEF-1 is a member of the NF-1 family of nuclear factors (15, 16), and studies have demonstrated that both its nuclear level and DNA binding activity are regulated via intracellular signaling, as demonstrated by effects mediated through the viral oncoprotein E1a and through a tyrosine phosphorylation that is required for its DNA binding activity (15, 17). In different cells, BEF-1 exists in two isoforms designated B1 and B2. While the functional role of each of these isoforms is unknown, phosphorylation studies have provided evidence suggesting that they represent a difference in serine/threonine phosphorylation (17). In confluent cultures of HepG2 cells, where apoE is under BEF-1-mediated repression (14), only the B2 isoform is expressed.

In a recent study, hamster hepatic apoE mRNA expression has been demonstrated to be repressed by the cytokines IL-1 and tumor necrosis factor (18). This result is consistent with previous studies demonstrating a similar repressive effect of IL-1 on macrophage apoE mRNA synthesis in culture (19). Because cytokines, including IL-1, are known to induce phosphorylation of many proteins (including transcription factors) (reviewed in Refs. 20 and 21), we have sought to investigate phosphorylation of BEF-1 as a potential mechanism for cytokine action in the repression of the apoE gene in HepG2 cells.

In this paper, we show that treatment of HepG2 cells with IL-1β, as well as IL-6, induces an isoform of BEF-1 (B1) that binds to the apoE regulatory region with equal affinity of the uninduced B2 isoform. This induction appeared to be due to increased phosphorylation, as B1 could also be induced by phorbol ester, and the induction could be blocked by the protein kinase inhibitor staurosporine. Furthermore we show that both IL-1 and IL-6 suppress the synthesis of apoE, and with increasing phosphorylation of BEF-1 we observed a proportional repression of apoE mRNA. Our data suggest that differential phosphorylation of trans-repressor BEF-1, possibly through the protein kinase C (PKC) pathway, plays a role in cytokine induced apoE gene repression.

EXPERIMENTAL PROCEDURES

Materials—Duolbeco’s modified Eagle’s medium/F-12 (31) and fetal bovine serum were purchased from Life Technologies, Inc. IL-1α and IL-6 were from R & D Systems. [α-32P]CTP and [γ-32P]ATP were purchased from Dupont NEN. Staurosporine was purchased from LC Laboratories. The MiniPlus SepaGels were from Integrated Separation Systems. The Biotec Ultraspec RNA isolation system was used for RNA isolation. The Ambion RPA II kit was used for RNA analysis. The Bio-Rad Prep-a-gene DNA purification kit was used for DNA isolations. The 3′ antisense apoE riboprobe was transcribed with SP6 RNA polymerase and [α-32P]CTP using the Promega Riboprobe Gemini II kit. All other reagents and chemicals were of the highest quality available.

Analysis of Total RNA—HepG2 cells were maintained as described previously (14), and total RNA was isolated as described by Chomczynski and Sacchi (22) using the Biotex Ultraspec RNA isolation system.
Apoe mRNA levels were analyzed by RPA (14, 23).

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from HepG2 cells as described by Dignam et al. (25). EMSAs were as described previously (14, 16, 26).

RESULTS

Induction of B1 Isoform of BEF-1—Initial experiments were performed to determine if the cytokine IL-1 had an effect on BEF-1. Nuclear extracts were prepared from confluent cultures of HepG2 cells treated with 10 ng/ml IL-1, and EMSAs were performed using the 5′ end-labeled ABEF-1 probe (14). As shown in the example in Fig. 1A, EMSAs performed using nuclear extracts from untreated HepG2 cells (lane 1) resulted in a single major DNA/protein complex (B2). In contrast, EMSAs performed using nuclear extracts prepared from IL-1-treated HepG2 cells gave two major DNA/protein complexes, B1 and B2. The minor complex migrating below B2 in each lane was not identified, but previous studies have demonstrated that it represents minor interaction of the BEF-1 site with NF-1 (15). Previously we have shown that HepG2 cell BEF-1 (forming predominately B2 EMSA complex and thus designated the B2 isoform of BEF-1, 17) and HeLa Cell BEF-1 (forming predominately B1 complex and designated the B1 isoform) are the same nuclear binding factor (14). We confirmed by competition studies that the IL-1-induced B1 displayed the same binding properties as the B2 isoform. As shown in Fig. 1B, cold ABEF-1-binding site competed both the B1 and B2 protein-DNA complexes with equivalent activity. Furthermore, similar competition experiments were repeated with identical results using cold prototype BEF-1 site as a competitor (14) and with labeled prototype binding site BEF-1 and cold ABEF-1 as a competitor (data not shown). These data indicate that B2 and the IL-1-induced B1 nuclear proteins are both BEF-1.

IL-1 Induction of B1 Appears to Be Due to Phosphorylation of B2—While previous studies with phosphatase-treated HeLa cell BEF-1 have suggested that complexes migrating as the B1 and B2 isoforms are due to differences in Ser/Thr phosphorylation (17), we performed experiments to determine if in fact the IL-1 induction of B1 in HepG2 cells was due to phosphorylation. An EMSA was performed using nuclear extracts derived from HepG2 cells treated either with IL-1 or with IL-1 plus the protein kinase inhibitor staurosporine (STS) (Fig. 2A). Compared with the untreated controls (lanes 2 and 3), IL-1 induced a significant level of B1. The IL-1-induced HepG2 B1 isoform comigrated with the HeLa cell B1 shown in lane 1. However, as shown in lanes 6 and 7, STS completely blocked the induction of B1 by IL-1. We saw no significant change in BEF-1 migration or level when cells were treated with STS alone.

In additional experiments, we quantitated the induction of B1 by IL-1 as well as by the PKC activator, phorbol 12-myristate 13-acetate (PMA). As shown in Fig. 2B, we consistently observed an induction of the B1 isoform by IL-1 that could be completely inhibited by STS. The treatment of HepG2 cells with PMA also resulted in an induction of the B1 isoform, although to a lesser extent than with IL-1 (Fig. 2B). Furthermore, the PMA-induced phosphorylation of BEF-1 was also
secreted HepG2 cell apoE, although IL-6 reduced apoE expression. Both IL-1 and IL-6 treatments reduced secreted HepG2 cell apoE, although IL-6 reduced apoE expression to a greater degree (60% with IL-6 versus 37% with IL-1).

Further analysis was performed to compare the degree of BEF-1 phosphorylation and mRNA levels in cells treated with or without IL-1 or IL-6. As shown in Fig. 4A, we observed the expected increase in IL-1-induced phosphorylation; however, we observed an even greater degree of BEF-1 phosphorylation with IL-6 in this and repeated experiments. As indicated for IL-1 above, there was no change in the total level of BEF-1 inhibited by STS. While we cannot rule out the possibility of some other post-translational modification leading to induction of B1, the above data suggest that the cytokine induction of B1 in HepG2 cells is due to increased phosphorylation of B2, possibly acting through the PKC pathway. Furthermore, the relative lack of effect of STS on the low basal level of B1, compared with its block of induced phosphorylation, suggests that pre-phosphorylated BEF-1 is relatively long-lived.

IL-1 Induction Does Not Increase the Total Amount of BEF-1. In repeated experiments, the degree of B1 induction by IL-1 was determined. As shown in Fig. 3A, IL-1 treatment of HepG2 cells did not increase the total amount of BEF-1. While approximately 15% of the total BEF-1 in resting HepG2 cells was in the B1 isoform, IL-1 consistently resulted in an induction of the B1 isoform to approximately 45–50% of the total (Fig. 3B). When examined in the presence and absence of CHX and as shown in Fig. 3C, we observed no difference in the degree of B1 induction following protein synthesis inhibition. These data together suggest that IL-1 treatment results in the phosphorylation of an existing intracellular pool of BEF-1.

Further, in experiments where cells were treated with and without CHX for up to 16 h we observed no significant decrease in the level of BEF-1, suggesting that this transcription factor is relatively long-lived.

Phosphorylation of BEF-1 Creates a More Potent Repressor—Having demonstrated that the BEF-1 nuclear protein becomes phosphorylated in response to IL-1, we were interested in determining if this change affects its apoE repressor activity. In initial experiments, HepG2 cells were treated with IL-1, and the level of apoE secreted was examined by immunoprecipitation analysis following procedures described in Ref. 19. In this study, we also analyzed the effect of another cytokine, IL-6, which preliminary studies had shown also increased phosphorylation of BEF-1. Both IL-1 and IL-6 treatments reduced secreted HepG2 cell apoE, although IL-6 reduced apoE expression to a greater degree (60% with IL-6 versus 37% with IL-1).

Further analysis was performed to compare the degree of BEF-1 phosphorylation and mRNA levels in cells treated with or without IL-1 or IL-6. As shown in Fig. 4A, we observed the expected increase in IL-1-induced phosphorylation; however, we observed an even greater degree of BEF-1 phosphorylation with IL-6 in this and repeated experiments. As indicated for IL-1 above, there was no change in the total level of BEF-1 phosphorylation following treatment of the cells with IL-6; only a shift in the degree of phosphorylated. As shown in Fig. 4B, we observed approximately a 50% decrease in apoE mRNA following IL-1 treatment but an 81% decrease following IL-6 treatment. The proportionally greater decrease in mRNA levels with increasing degree of phosphorylation, and no change in total BEF-1 binding activity, suggests that the B1 isoform is a more potent repressor than B2 at the BEF-1 repressor binding site in the apoE upstream regulatory region.

BEF-1 Phosphorylation Does Not Affect Its DNA Binding Affinity—We speculated that the apparent increase in BEF-1 repressor activity following phosphorylation might result from an increase in DNA binding affinity. Therefore, we determined the relative affinities of the B1 and B2 isoforms for the apoE DNA binding site. As shown in Fig. 5, both the B1 and B2 isoforms displayed saturable binding to the apoE-BEF-1 binding site. Using nonlinear regression analysis, the binding affinities for the two isoforms were determined to be identical, with an apparent $K_d$ of $17 \text{ nM}$. Thus, the modulation in repressive activity following phosphorylation of BEF-1 does not appear to result from an increase in affinity for the DNA binding site, suggesting that the mechanism may instead be the result of more efficient protein-protein interactions that result in inhibition of the transcriptional complex.

**DISCUSSION**

Changes in cellular gene transcription patterns induced by extracellular signals are an important part of many biological processes (reviewed in Ref. 27), and protein phosphorylation clearly has evolved as the most versatile post-translational modification for situations where rapid modulation of transcription factor activity is required in response to signals from receptors on the cell surface (reviewed in Refs. 28 and 29). Extracellular signaling molecules such as cytokines have been well described as affecting gene expression by modulating the phosphorylation of proteins directly involved in transcriptional control (reviewed in Ref. 20). These effects have been well described for the induction of expression mediated by NF-kB, c-jun, c-Fos, and NF-IL-6 (29, 30). In this study, we have provided evidence that the nuclear repressor factor BEF-1 becomes phosphorylated in response to cytokine activation of HepG2 cells, with a resulting increase in its functional repressor activity.

We were able to induce the phosphorylation of BEF-1 with IL-1, IL-6, and PMA. In preliminary studies, we also have
shown that transforming growth factor-β similarly induced the phosphorylation of BEF-1 generating the B1 isoform. Thus, extracellular signals from several agents appear to generate converging intracellular pathways that ultimately result in phosphorylation of nuclear repressor BEF-1. The PMA induction, and staurosporine inhibition of phosphorylation of B1, suggests PKC involvement in the differential phosphorylation of BEF-1. Researchers initially had attempted to implicate PKC in IL-1 signaling, since PMA, which directly activates PKC, mimics many of the actions of IL-1 (21). However, evidence has been presented questioning the importance of PKC in IL-1 action. Many studies have shown that inhibitors of protein kinase C such as staurosporine fail to block IL-1 responses in different cell types; and it has been suggested that staurosporine may even up-regulate IL-1 receptors and thereby increase the effect of IL-1 on some cells (reviewed in Ref. 21). In the case of BEF-1, however, we clearly show that STS blocked the induction by both cytokines and PMA. While our data would suggest that the mechanism for phosphorylation of B1 by these different agents is through a common pathway, it is possible that the induced phosphorylation by each agent is mediated through different kinases each inhibited by STS.

It is not clear how increased phosphorylation might facilitate the repressive activity of BEF-1 against apoE. Until we understand what factors bind to previously mapped elements in the apoE upstream regulatory region (12, 13, 31), and how they interrelate with one another and BEF-1, we can only speculate on the molecular mechanism of apoE repression. As reviewed recently by Hill and Treisman (32), a number of transcription factors contain signal-regulated transcription activation domains, and it is presumed that regulated phosphorylation facilitates their interaction with the basal transcriptional machinery or co-activator proteins. We have shown that the phosphorylation of BEF-1 does not alter its binding affinity for the DNA, suggesting that the effect on activity is likely a post-DNA binding mechanism. The increased repressor activity of phosphorylated BEF-1 on apoE gene expression possibly is the result of enhanced interactions with auxiliary transcription factors at the apoE promoter region. Overall, the balance between and interaction of various positively and negatively acting factors plays a critical role in controlling the expression of genes, and with regard to apoE, the differential phosphorylation of the nuclear repressor BEF-1 appears at least in part to play a role in determining its expression and response to cell signals. Understanding how factors such as BEF-1 control apoE gene regulation may aid in the development of agents to modulate its expression and ultimately in controlling implicated disease processes.

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