The Orphan Nuclear Receptor Small Heterodimer Partner as a Novel Coregulator of Nuclear Factor-κB in Oxidized Low Density Lipoprotein-treated Macrophage Cell Line RAW 264.7*

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Small heterodimer partner (SHP), specifically expressed in liver and a limited number of other tissues, is an unusual orphan nuclear receptor that lacks the conventional DNA binding domain. In this work, we found that SHP expression is abundant in murine macrophage cell line RAW 264.7 but was suppressed by oxidized low density lipoprotein (oxLDL) and its constituent 13-hydroxyoctadecadienoic acid, a ligand for peroxisome proliferator-activated receptor γ. Furthermore, SHP acted as a transcription coactivator of nuclear factor-κB (NFκB) and was essential for the previously described NFκB transactivation by palmitoyl lysophosphatidylcholine, one of the oxLDL constituents. Accordingly, NFκB, which was transcriptionally active in the beginning, became progressively inert in oxLDL-treated RAW 264.7 cells as oxLDL decreased the SHP expression. Thus, SHP appears to be an important modulatory component to regulate the transcriptional activities of NFκB in oxLDL-treated, resting macrophage cells.

The nuclear receptor superfamily includes receptors for a variety of small hydrophobic ligands such as steroids, T3, and retinoids as well as a large number of related proteins that do not have known ligands, referred to as orphan nuclear receptors (for review, see Ref. 1). The receptor proteins are direct regulators of transcription that function by binding to specific DNA sequences named hormone response elements in promoters of target genes. Nearly all the superfamily members bind as dimers to DNA elements. Although some apparently bind only as homodimers, thyroid hormone receptors, vitamin D receptor, retinoic acid receptors (RARs),1 the peroxisome proliferator-activated receptors (PPARs), and several orphan nuclear receptors bind their specific response elements with high affinity as heterodimers with the retinoid X receptors. Receptors have dimerization interfaces in both their DNA binding domain and ligand binding domain. The DNA binding domain interfaces are quite different for each receptor, whereas the ligand binding domain interface is primarily based on a conserved motif referred to as the ninth heptad or the I-box (2). This motif is required for both heterodimerization and homodimerization of many nuclear receptors, including retinoid X receptor, thyroid hormone receptor, RAR, hepatocyte nuclear factor 4, and chicken ovalbumin upstream promoter transcription factor (2–4).

Small heterodimer partner (SHP) is an orphan nuclear receptor specifically expressed in liver and a limited number of other tissues, and its activities are in some ways opposite to those of retinoid X receptor (5). SHP, like the orphan nuclear receptor DAX-1 (for review, see Ref. 6), lacks the conventional DNA binding domain. Both direct biochemical and the yeast two-hybrid results demonstrated that SHP interacts with many members of the receptor superfamily (5). As expected from its lack of a DNA binding domain, addition of SHP inhibited in vitro DNA binding by nuclear receptors with which it interacted, and in mammalian cell cotransfections, SHP repressed their transactivation. In addition, the SHP sequences required for interaction with other superfamily members have recently been localized to the central portion of SHP (7), distinct from the I-box (2–4).

Macrophages are thought to play critical pathogenic roles in several chronic inflammatory diseases, including atherosclerosis (for review, see Ref. 8). The formation of an atherosclerotic lesion is a complex process involving intracellular lipid accumulation and various signal transduction pathways in vascular cells, such as endothelial cells, smooth muscle cells, and monocytes/macrophages. Each vascular cell is capable of oxidatively modifying low density lipoprotein (LDL) to generate oxidized LDL (oxLDL) (for review, see Ref. 9). Circulating monocytes adhere to activated endothelium, migrate into intima, and subsequently differentiate into macrophages that express various...
scavenger receptor genes. Accumulation of oxLDL through scavenger receptors mediates the formation of foam cells from macrophages and consequently fatty streaks, the earliest visible lesions of atherosclerosis. The oxidative modification results in a number of important biological activities of LDL, including the induction of a number of genes encoding cytokines and growth factors (10). Among the growth factors and cytokines that are secreted from vascular cells, a regulatory network is thought to exist, and a lesion may result from their collective action.

Cross-talk between transcription factors of distinct families is an important phenomenon in regulating gene transcription and has recently become the subject of intensive investigation. In particular, the transcription factor nuclear factor-κB (NFκB) has been shown to functionally interact with numerous other transcription factors, including members of the nuclear receptor superfamily (for review, see Ref. 11), resulting in mutually repressed biological activity of these transcription factors. These include glucocorticoid receptor, estrogen receptor, progesterone receptor, and androgen receptor (11). More recently, PPARγ and RAR/retinoid X receptor were also added to the list of nuclear receptors functionally interacting with NFκB (12–15). NFκB, composed of homo- and heterodimeric complexes of members of the Rel (NFκB) family of polypeptides, is important for the inducible expression of a wide variety of cellular and viral genes (for review, see Ref. 16). In vertebrates, this family comprises p50, p65 (RelA), c-Rel, p52, and RelB. These proteins share a 300-amino acid region, known as the Rel homology domain, that binds to DNA and mediates homo- and heterodimerization. This domain also is the target of variousIkB inhibitors. In the majority of cells, NFκB exists in an inactive form in the cytoplasm, bound to the inhibitory IkB proteins. Treatment of cells with various inducers results in the degradation of IkB proteins. The bound NFκB is released and translocates to the nucleus where it activates appropriate target genes.

In this study, we identified SHP as a novel transcription coactivator of NFκB. We have further presented the experimental results indicating that the modulation of SHP expression appears to function as a distinct regulatory component of the transcriptional activities of NFκB in oxLDL-treated, resting macrophage cells. These results could have an important implication for the differentiation mechanism of resting macrophage cells into foam cells and resulting atherogenesis.

**EXPERIMENTAL PROCEDURES**

**Preparation of Oxidized LDL, Plasmids, and Chemicals—**Human LDL (d = 1.019–1.063 g/ml) and oxLDL were prepared from the plasma of healthy volunteers as described previously (12, 17). The mammalian expression vectors for p65, SHP, HA-SHP, and steroid receptor coactivator-1 (SRC-1; SRC-1); the LexA, B42, T7, or GST vectors to express SHP, p65, p56N, p50, SRC-C, SRC-D, SRC-E, and RAR; the transfection indicator construct pRSV-β-gal; and the reporter constructs eB-LUC and PPRE-LUC were as previously described (5, 18–20). Troglitazone and 13-hydroxyoctadecadienoic acid (13-HODE) were purchased from Cayman Chemical (Ann Arbor, MI). Palmitoyl lysophosphatidylcholine (lysoPC) and actinomycin D were purchased from Sigma.

**Yeast Two-hybrid Test—**For the yeast two-hybrid tests, plasmids encoding LexA fusions and B42 fusions were cotransformed into Saccharomyces cerevisiae EGY48 strain containing the LacZ reporter plasmid SH18-34 (21). Liquid assays of β-galactosidase expression were carried out as described previously (21). Similar results were obtained in more than two similar experiments.

**Glutathione S-Transferase (GST) Pull-down Assays—**The GST fusion or GST alone was expressed in Escherichia coli, bound to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), and incubated with either cell lysates cotransfected with HA-SHP expression vector or labeled proteins expressed by in vitro translation by using the TNT-coupled Transcription-Translation System with conditions as described by the manufacturer (Promega, Madison, WI). Specifically

bound proteins were eluted from beads with 40 mM reduced glutathione in 50 mM Tris (pH 8.0) and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described previously (21). To introduce deletions into p65, the p65 expression vector was digested with the appropriate restriction enzymes before being subjected to the TNT-coupled Transcription-Translation System (Promega).

**Cell Culture and Transfections—**RAW 264.7, HeLa, and CV-1 cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2 (Life Technologies, Inc.). For transfections, cells were grown in six-well plates with medium supplemented with 10% fetal bovine serum for 24 h and transfected with the indicated plasmid(s) by the calcium phosphate coprecipitation technique (12). The total amounts of expression vectors were kept constant by adding the CMDS expression vector to transfections. After 24 h, cells were washed and incubated in serum-free Dulbecco’s modified Eagle’s medium in the absence or presence of either oxLDL or other reagent(s). Cells were harvested after the indicated time periods, luciferase activity was measured using the luciferase assay kit (Promega), and the results were normalized to the LacZ expression.

**Northern, Western, and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analyses—**Total RNA from cell monolayers was isolated using guanidinium isothiocyanate and phenol (22). RNA (20 μg) was separated on a 1% agarose-formaldehyde gel and transferred to nylon membranes (Hybond, Amersham Pharmacia Biotech). For RT-PCR analyses, total cDNA synthesized from 2 μg of total RNA was amplified for 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The oligonucleotide primer sets for 1141 and 1101 (− ΔΔCt) as described previously (21). To introduce deletions into p65, the p65 expression vector was digested with the appropriate restriction enzymes before being subjected to the TNT-coupled Transcription-Translation System (Promega). The total amounts of expression vectors were kept constant by adding the CMDS expression vector to transfections. After 24 h, cells were washed and incubated in serum-free Dulbecco’s modified Eagle’s medium in the absence or presence of either oxLDL or other reagent(s). Cells were harvested after the indicated time periods, luciferase activity was measured using the luciferase assay kit (Promega), and the results were normalized to the LacZ expression.

**RESULTS AND DISCUSSION**

**SHP as a Novel Transcription Coactivator of NFκB—**Many nuclear receptors have been shown to functionally interact with NFκB (for review, see Ref. 11). In this work, we examined whether the NFκB components p50 and p65 also interact with the unusual orphan nuclear receptor SHP. GST fusion to SHP interacted with radiolabeled p65 but not with luciferase or p50 (Fig. 1A, left). In addition, SHP interacted with SRC-1, a transcription activator of nuclear receptors and other transcription factors (for review, see Ref. 23) including NFκB (18). According to GST fusion to SHP specifically interacted with the radiolabeled C-terminal subregions of SRC-1 (i.e. SRC-D and SRC-E containing the SRC-1 residues 759–1141 and 1101–1441, respectively) (Fig. 1A, left). It is noted that these regions are distinct from the region containing the previously known receptor binding sites (i.e. SRC-C consisting of the SRC-1 residues 568–779) that includes the nuclear receptor-interacting LXXLL motifs (24, 25) in which L and X denote leucine and any amino acid, respectively. Similar results were also obtained with the mammalian two-hybrid-based tests (results not shown). The SHP interaction interface was further mapped to the N-terminal 283 residues of p65 (Fig. 1A, right). In addition,
HeLa nuclear extracts cotransfected with HA-tagged SHP expression vector retained with GST fusion to p65 but not with GST alone contained SHP as demonstrated by Western analyses with HA-monoclonal antibody (Fig. 1B). Consistent with these results, B42 fusion to the N-terminal domain of p65 (i.e. p65N consisting of the p65 residues 1–283), but not B42 alone or B42 fusion to p50, stimulated the LexA-SHP-mediated transactivation of the LacZ reporter construct controlled by upstream LexA binding sites (Fig. 2A, bottom). SHP also interacted with SRC-D and SRC-E but not with SRC-C in yeast (Fig. 2A), whereas RAR interacted with SRC-C as well as the C-terminal region of SRC-1 (i.e. SRC-E) as previously shown (23).

Confirming the functionality of these interactions, cotransfected SHP enhanced transactivation by NFκB in a dose-dependent manner either alone or in synergy with SRC-C (Fig. 2B). In contrast, SHP efficiently suppressed transactivation by RAR as previously described (5) and had no significant effect on transactivation directed by Gal4 fusion to VP16 (results not shown). These results clearly demonstrate that SHP directly interacts with p65 and is a positive transcriptional coregulator of NFκB. This positive regulatory role is in sharp contrast to the previously reported inhibitory role of SHP on a variety of receptor-dependent signaling pathways (5).

**Down-regulation of SHP by oxLDL in RAW 264.7—**SHP was previously demonstrated to be specifically expressed in liver and a limited number of tissues (26). During our recent search for additional tissues or cell lines that may express SHP, we found that SHP mRNA is abundant in the mouse macrophage cell line RAW 264.7. Surprisingly this expression was significantly repressed by oxLDL, but not by native LDL (nLDL), in a time-dependent manner (Fig. 3A). However, the repression was not observed in the presence of the endocytosis blocker cytochalasin B (12) (Fig. 3B), suggesting that the inhibitory effect of oxLDL likely involves endocytosis of oxLDL. We have previously shown that 13-HODE, a constituent of oxLDL and a ligand for PPARγ, functions in an endocytosis-dependent man-
SHP-mediated Activation of NFκB in Macrophage

**Fig. 4.** SHP as an essential factor for oxLDL-mediated transactivation of NFκB. A, B, and D, CV-1 cells were transfected with LacZ expression vector, expression vectors for SHP and PPARγ, and a reporter gene, κB-LUC or PPRE-LUC, as indicated. Normalized luciferase expressions from triplicate samples were calculated relative to the LacZ expressions, and the results were expressed as fold activation (n-fold) over the value obtained with the reporter alone. The data are representative of three similar experiments, and the error bars are as indicated. The cells were incubated for 4 h with nLDL (100 μg/ml) or oxLDL (100 μg/ml) (A), troglitazone (15 μM) (B), and lysoPC (10 μM) and/or 13-HODE (20 μg/ml) (D) as indicated. C, RT-PCR (RT) and Western blot (W) analyses of PPARγ in RAW264.7 cells were executed as described previously (21). Total RNA or cell lysate was prepared from cells treated with oxLDL (100 μg/ml) for the time periods as indicated. The 250-base pair PCR fragment of PPARγ as well as the antibody-directed PPARγ band are as shown. Equivalent loading of RNA and protein was verified by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR product and actin protein, respectively.

**Fig. 5.** Time-dependent transactivation of NFκB and PPARγ. A, RAW264.7 cells were transfected with the indicated reporter constructs along with PPARγ expression vector (10 μg) and LacZ expression vector and treated for the indicated time periods with either vehicle only (ethanol), nLDL (100 μg/ml), oxLDL (100 μg/ml), lysoPC (10 μM), or 13-HODE (20 μg/ml). Normalized luciferase expressions from three independent experiments performed in triplicate samples are presented relative to the LacZ expressions, and the error bars are as indicated. B, our results suggest a model in which SHP serves as a transcriptional coactivator molecule of NFκB and PPARγ. In particular, SHP appears to be essential for the NFκB transactivation by oxLDL as indicated by a solid line. In addition, resting macrophage cells, like RAW 264.7 cells, may express a putative repressor molecule of PPARγ that is likely subjected to down-regulation by oxLDL and dominates over the stimulating activity of SHP on the PPARγ transactivation (see the text for further details).
coactivator of NFkB and have further presented the experimental results indicating that targeted expression of SHP appears to function as a distinct regulatory component of the transcriptional activities of NFkB in oxLDL-treated, resting macrophage cells.

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