Down syndrome candidate region 1 increases the stability of the IκBα protein

IMPLICATIONS FOR ITS ANTI-INFLAMMATORY EFFECTS*

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Down syndrome candidate region 1 (DSCR1), an endogenous inhibitor of calcineurin, inhibits the expression of genes involved in the inflammatory response. To elucidate the molecular basis of these anti-inflammatory effects, we analyzed the role of DSCR1 in the regulation of NF-κB transactivation using glioblastoma cells stably transfected with DSCR1.4 or its truncation mutants (DSCR1.4 (1–133) and DSCR1.4 (134–197)). Overexpression of DSCR1.4 significantly attenuated the induction of cyclooxygenase-2 (COX-2) expression by phorbol 12-myristate 13-acetate (PMA) via a calcineurin-independent mechanism. Experiments using inhibitors of the signaling molecules for NF-κB activation showed that NF-κB is responsible for the induction of COX-2. Full-length and truncated DSCR1 decreased the steady-state activity of NF-κB as well as PMA-induced activation of NF-κB, which correlated with attenuation of COX-2 induction. DSCR1.4 did not affect the PMA-stimulated phosphorylation or degradation kinetics of IκBα; however, DSCR1.4 significantly decreased the basal turnover rate of IκBα and consequently up-regulated its steady-state level. In the same context, knockdown of endogenous DSCR1.4 increased the turnover rate of IκBα as well as COX-2 induction. These results suggest that DSCR1 attenuates NF-κB-mediated transcriptional activation by stabilizing its inhibitory protein, IκBα.

The human Down syndrome critical region 1 gene was isolated from chromosome 21q22, and it was thought to be located within a region that is involved in the expression of the Down syndrome phenotype (1). Refined maps of chromosome 21 revealed that the gene lies just outside this region (2), and the name of the gene was modified to Down syndrome candidate region 1 (DSCR1)² (3). DSCR1 encodes a protein that binds to the catalytic subunit of calcineurin, inhibiting its phosphatase activity (4). Consequently, it is also called modulatory calcineurin-interacting protein 1 or calcipressin 1 to reflect this function (5). The DSCRI gene consists of seven exons: exons 1−4 can be alternatively spliced, resulting in four different transcripts (denoted DSCR1.1 through DSCR1.4). DSCR1.1 and DSCR1.4 are widely expressed, particularly in the central nervous system, skeletal muscle, and heart. However, DSCR1.2 has been detected only in fetal liver and brain, and DSCR1.3 has not been detected in any tissue (5, 6).

Elucidation of the functional roles of DSCR1 in neuronal tissue is important because elevated levels of this protein have been implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer disease and Down syndrome (4, 7, 8). Recent studies in which the DSCRI gene was overexpressed in endothelial cells showed that DSCR1 down-regulates transcription of several pro-inflammatory factors and enzymes (9−12). In addition, the expression of DSCR1 is up-regulated by mitogens (9−11, 13), inflammatory cytokines (10, 12, 14), infection with Candida albicans (15), depolarization (10, 16), and oxidative stress (17), all of which are frequently implicated in the initiation and maintenance of the inflammatory process. Therefore, DSCR1 is likely to play a significant role as a negative regulatory element in the inflammatory reaction.

Nuclear factor κB (NF-κB) is a transcription factor involved in the regulation of many cellular target genes that play a central role in immune and inflammatory response (18). In resting cells, most NF-κB dimers are sequestered in the cytoplasm through association with inhibitory proteins known as inhibitors of κB (IκB), of which IκBα is the best characterized. NF-κB-activating signals generated by stimulation with mitogens or cytokines result in rapid degradation of IκBα, allowing NF-κB to localize to the nucleus, where it acts on its target genes. This stimulation-induced IκBα degradation is initiated by phosphorylation of IκBα at serines 32 and 36 by a large IκB kinase (IKK) complex (IKKα/IKKβ/IKKγ-NEMO), which labels IκBα for degradation by the ubiquitin/proteasome pathway (18−22). On the other hand, phosphorylation of the C-terminal
region of IκBα, which is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) residues (called the PEST domain), has been implicated as the proteolytic signal that determines the basal turnover rate of IκBα in resting cells (23, 24). Phosphorylation of the PEST domain is mediated by the constitutively active protein kinase casein kinase 2 (CK2) (25, 26). Down-regulation of CK2 expression by transforming growth factor β1 increases the half-life of IκBα, which is followed by a decrease in constitutive NF-κB activity in murine hepatocyte cells (27). In contrast, ultraviolet radiation increases CK2-mediated degradation of IκBα, resulting in activation of NF-κB in HeLa cells (28). These studies show that regulation of the basal turnover of IκBα is another potential means of controlling NF-κB activity.

Here, we investigated the molecular basis of the mechanism by which DSCR1 inhibits the expression of genes associated with inflammation. This study shows for the first time that DSCR1.4 decreases the basal turnover rate of IκBα by which DSCR1 inhibits the expression of genes associated with inflammation. This study shows for the first time that DSCR1 inhibits signal-independent degradation of IκBα in human U87MG glioblastoma cells. In addition, we demonstrate that this inhibition of the NF-κB pathway by DSCR1.4 is functionally significant by showing that overexpression of DSCR1.4 reduced induction of the NF-κB target genes cyclooxygenase-2 (COX-2) and interleukin-1β (IL-1β), which were induced by phorbol 12-myristate 13-acetate (PMA) or pro-inflammatory cytokines.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—Cyclosporin A, FK506, IL-1β, PD 98059, SB 203580, Ro-31-8220, calmodulin kinase II inhibitor, hypoestoxide, and tumor necrosis factor-α (TNF-α) were purchased from Calbiochem. PMA, A23187, and cycloheximide were purchased from Sigma. An antibody specific for COX-2 was purchased from Cayman Chemical (Ann Arbor, MI). Antibodies for NF-κB (p65), protein kinase C (PKC), IκBα (clone C-20), and IL-1β were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for IκBα, phospho-IκBα, phospho-IκBα-Thr-291, and phospho-ERK1/2 Thr-202/Tyr-204 were purchased from Cell Signaling Biotechnology (Beverly, MA).

**Cell Culture and Treatments**—Human U87MG glioblastoma cells were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in minimal essential medium supplemented with 0.1 mm nonessential amino acids, 1.0 mm sodium pyruvate, and 10% fetal bovine serum under an atmosphere of 5% CO₂ at 37 °C. For experiments, cells (5 × 10⁵/well) were plated in a gelatin-coated 6-well culture dish and incubated for 20 h in complete medium and then for 4 h under serum-reduced conditions (minimal essential medium with 0.1% fetal bovine serum) prior to treatment. For COX-2 induction experiments, cultures were stimulated with PMA (25 ng/ml) plus A23187 (1 μM), PMA (25 ng/ml) alone, TNF-α (10 ng/ml), or IL-1β (5 ng/ml) for the times indicated in the figure legends. In some experiments, cells were pretreated with inhibitors of signaling molecules for 15 min before PMA stimulation.

For IκBα turnover studies, quiescent cells were incubated for the indicated times in the presence of cycloheximide (20 μg/ml). At the ends of the experiments, whole cell extracts were prepared and subjected to immunoblot analysis as described below.

**Plasmid Construction**—To construct plasmids encoding recombinant DSCR1.4 protein containing an N-terminal (ntDSCR1) or C-terminal (ctDSCR1) green fluorescent protein (GFP) tag, human wild-type DSCR1.4 cDNA was amplified by PCR and cloned in-frame into the pcDNA3.1/NT-GFP-TOPO or pcDNA3.1/CT-GFP-TOPO vector (Invitrogen), respectively. Primer sets for PCR were as follows: for ntDSCR1, 5′-GGATCC-AATGATCTTTTAGAAAATCTTAAC-3′ (forward) and 5′-CTCGAGTCAGTGGGTGAGCGCCGTGTA-3′ (reverse); and for ctDSCR1, 5′-GAAGAAGAGATGCTTTAGAAGC-3′ (forward) and 5′-CAGTCCAGCTGAGGTGAGCGCCGTGTA-3′ (reverse).

To construct plasmids for expression of proteins without GFP fusion tags, full-length or truncated DSCR1.4 cDNA fragments were subcloned into the BamHI and EcoRI sites of the pIREs-hrGFP-2 vector (Stratagene, La Jolla, CA). Primer sets for PCR were as follows: for full-length DSCR1.4, 5′-GGATCC-CCAGCAAGATGCTTTAGAAGC-3′ (forward) and 5′-GAATTCTCGAGTGAGGTGAGCGCCGTGTA-3′ (reverse); for the DSCR1.4 mutant with a C-terminal deletion up to amino acid 133 (DSCR1.4C), 5′-GGATCCACGAAATGCTTTAGAAGC-3′ (forward) and 5′-GAATTCTCAATATAAGAGATCATA-3′ (reverse); and for the DSCR1.4 mutant with an N-terminal deletion up to amino acid 134 (DSCR1.4N), 5′-GGATCCAGATGCTTTAGAAGC-3′ (forward) and 5′-GAATTCTCAAGCTTTAGAAGC-3′ (reverse).

The derivatives of IκBα containing alanine replacements at Ser-32 and Ser-36 (IκBα-mutN) or at Ser-283 and Thr-291 (IκBα-mutC) were generated by overlap extension PCR (29). The PCR fragments were ligated in-frame into the pcDNA3.1D/V5-HisTOPO vector (Invitrogen) to generate vectors expressing V5-His-tagged mutant IκBα proteins. The overlapping primers used for the generation of IκBα-mutN and IκBα-mutC were 5′-CATGGCGTCCAGGCCGGCGCCGTTC-3′ and 5′-GGCGTCATAGCTCTCCTCATCTCCGGCCG-3′, respectively (with the mutation sites underlined). The N-terminal forward and C-terminal reverse primers for ligation into the expression vector were 5′-CACCACCGCGCATATGCTCC-3′ and 5′-TCAGATACAAGCTGAGCGCCG-3′, respectively (with the mutation sites underlined). The N-terminal forward and C-terminal reverse primers for ligation into the expression vector were 5′-CACCACCGCGCATATGCTCC-3′ and 5′-TCAGATACAAGCTGAGCGCCG-3′, respectively (with the mutation sites underlined). The N-terminal forward and C-terminal reverse primers for ligation into the expression vector were 5′-CACCACCGCGCATATGCTCC-3′ and 5′-TCAGATACAAGCTGAGCGCCG-3′, respectively (with the mutation sites underlined).

To generate an expression construct for the glutathione S-transferase (GST)-tagged PEST domain of IκBα (GST-PEST), a truncated IκBα cDNA was PCR-amplified using primers 5′-GGATCCACGCTGACACTAGAAAC-3′ (forward) and 5′-CTCGAGGCCATACGTCAGACCTG-3′ (reverse). The fragments were digested and then ligated into the BamHI and Xhol sites of pGEX4T-1 (Amersham Biosciences). The insert sequences of all constructs were confirmed by sequencing.

**Creation of Stable Cell Lines**—Parallel cultures of U87MG cells were transfected with vectors containing each of the DSCR1.4 constructs or with an empty vector using Lipo- vectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After selection against 400 μg/ml G418 (Roche Applied Science, Mannheim, Germany) for 3 weeks, GFP-positive populations were purified using a FACSCalibur machine.
Immunoblot Analysis—Immediately after the treatments were completed, cultures were lysed in an appropriate volume of lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM sodium fluoride, 1 mM sodium orthovanadate, and protease inhibitor mixture). After insoluble material had been removed by centrifugation, the supernatants were mixed with 3 × Laemmli sample buffer and denatured for 5 min at 90 °C. The proteins were separated by SDS-PAGE (7 or 12%) and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in 1% (w/v) Hammersten-grade casein in phosphate-buffered saline containing 0.05% Tween 20. Immunoblotting was done with appropriate antibodies in 0.5% casein in phosphate-buffered saline. The blots were incubated with horseradish peroxidase-conjugated anti-IgG secondary antibody (Sigma), washed three times with phosphate-buffered saline containing 0.05% Tween 20, and then visualized using the SuperSignal West Dura chemiluminescence substrate (Pierce). The band intensity was analyzed using an LAS-3000 image analyzer (Fujifilm, Tokyo, Japan).

RESULTS

**DSCR1.4 Inhibits PMA-induced COX-2 Expression via a Calcineurin-independent Mechanism**—To examine the effects of elevated DSCR1 levels on COX-2 induction, we generated derivatives of the U87MG cell line by transfection with expression vectors for recombinant DSCR1 proteins with GFP fusion tags at the N termini (ntDSCR1) or C termini (ctDSCR1) or with a vector expressing GFP alone as a control. The basal level of COX-2 expression in these cell lines was undetectable by Western blotting (data not shown). For induction of COX-2, cells were stimulated with PMA plus the calcium ionophore A23187 (PMA/A23187) for 8 h. In agreement with previous studies done in endothelial cell cultures (9, 11), the induction of COX-2 was markedly attenuated in cells transfected with ntDSCR1 or ctDSCR1 compared with control cells. This induction of COX-2 expression was also inhibited by the calcineurin inhibitors cyclosporin A and FK506 (Fig. 1A, upper panel), suggesting that calcineurin inhibition by DSCR1 plays a role in this process. In contrast, when COX-2 was induced by PMA alone (no A23187), the induction was reduced only by ntDSCR1 and not by ctDSCR1 or by pretreatment with calcineurin inhibitors (Fig. 1A, lower panel). These results indicate that DSCR1 reduces PMA-induced COX-2 expression via a calcineurin-independent mechanism.

To confirm these results, U87MG cells were stably transfected with an expression vector coding for DSCR1.4 without a fusion tag and then tested for stimulus-induced COX-2 expression. As shown in Fig. 1B, overexpression of DSCR1.4 attenuated both PMA/A23187- and PMA-induced COX-2 expression, whereas calcineurin inhibitors reduced only PMA/A23187-induced COX-2 expression.

**Knockdown of DSCR1.4 Promotes PMA-induced COX-2 Expression**—Next, we conducted knockdown experiments with endogenous DSCR1.4. Cells were transfected with synthetic siRNA oligonucleotides targeted against sequences in a region of exon 4 (DSCR1.4 siRNA). We found a 75–80% reduction in DSCR1.4 mRNA levels using real-time PCR analysis (data not shown). We applied this condition to investigate the effects of decreased endogenous DSCR1.4 levels on COX-2 induction by PMA. As shown Fig. 2, knockdown of DSCR1.4 enhanced COX-2 induction, resulting in a 2–4-fold increase in its expres-
DSCR1 Inhibits Signal-independent Degradation of IκBα

NF-κB Transactivation Is Attenuated by DSCR1.4—On the basis of the site of cleavage of DSCR1.4 by calpain (30), we generated plasmids encoding truncated forms of DSCR1.4 lacking a C-terminal region (amino acids 134–197) or an N-terminal region (amino acids 1–133), termed DSCR1ΔC and DSCR1ΔN, respectively. After stable transfection with DSCR1ΔC, DSCR1ΔN, or a vector coding for full-length DSCR1.4, U87MG cells were stimulated with PMA. As shown in Fig. 3A, full-length DSCR1.4, DSCR1ΔC, and DSCR1ΔN reduced COX-2 expression by 20, 77, and 80%, respectively.

Overexpression of DSCR1 inhibits the expression of genes associated with cell adhesion or inflammation such as COX-2, IL-8, monocyte chemotactc appetant protein 1 (MCP-1), tissue factor 1, E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) (9–11). It is worth noting that transcription of these genes is regulated by NF-κB (18). Therefore, we carried out a promoter assay to evaluate whether DSCR1.4 inhibits the transcriptional activity of NF-κB. Control and DSCR1-modified cell lines were transfected with an NF-κB-dependent luciferase reporter plasmid (Fig. 3B). Stimulation with PMA increased luciferase activity to a similar extent in the control and DSCR1-modified cell lines (~2.5-fold that of their respective non-stimulated controls). However, the luciferase activities of the DSCR1-modified cells were markedly compared with the control both at steady state and after stimulation with PMA.

Because DSCR1ΔN strongly down-regulated NF-κB activity and because overexpression of full-length DSCR1 or DSCR1ΔC caused aggregate formation,3 we used DSCR1ΔN in subsequent experiments. Moreover, DSCR1ΔN encodes a region that is common to all DSCR1 isoforms. Therefore, this fragment may be of use for elucidating the functions of other isoforms.

Inhibitors of Molecules Upstream of NF-κB Activation Decrease COX-2 Induction—Next, we determined whether reduction of NF-κB activity by DSCR1.4 is responsible for the observed attenuation of COX-2 induction. We first investigated signal transduction pathways that could lead to induction of COX-2 expression after stimulation with PMA. Among the inhibitors of signal molecules reportedly involved in PMA-mediated COX-2 expression (31–34), Ro-31–8220, PD 98059, and hypoestoxide inhibited the induction of COX-2 (Fig. 4A), indicating involvement of PKC, MEK1, and NF-κB signaling. We then determined whether PKC and MEK1 are upstream signal transduction molecules of NF-κB activation. The final obligatory step for activation of NF-κB is the degradation of IκB.

3 K.-O. Cho, Y. S. Kim, H. J. Lee, S.-K. Kim, S. Y. Kim, and Y.-J. Cho, unpublished data.
DSCR1 Inhibits Signal-independent Degradation of IκBα

Among the different IκB proteins, IκBα plays a major role in PMA-induced activation of NF-κB (35, 36). Therefore, we tested the effects of inhibitors of PKC and MEK1 on the degradation of IκBα after stimulation with PMA. Ro-31-8220 and PD 98059 significantly inhibited IκBα degradation (Fig. 4, B and C), indicating that PKC and ERK are upstream activators of NF-κB.

Next, we examined whether PKC and ERK activation is inhibited by DSCR1ΔN. PMA-stimulated PKC degradation (Fig. 4A, middle panel, third lane) or phosphorylation of ERK (Fig. 4D) was not decreased in DSCR1ΔN-transfected cells. In addition, PMA-stimulated phosphorylation at Ser-32 of IκBα was not inhibited by DSCR1ΔN, suggesting that DSCR1 affects a step downstream of IκBα phosphorylation in NF-κB-activating signaling.

DSCR1 Decreases the Basal Degradation and Increases the Steady-state Level of IκBα—Next, we investigated whether PMA-stimulated degradation of IκBα is inhibited by DSCR1ΔN. For this, the time course of IκBα degradation in DSCR1ΔN-transfected cells was compared with that in control cells following stimulation with PMA (Fig. 5, A and B). PMA stimulation resulted in a similar rapid reduction of IκBα in both control and DSCR1ΔN-transfected cells. However, the level of IκBα in DSCR1ΔN-transfected cells was higher than that in control cells throughout the time course. The steady-state level of IκBα was elevated by 30–40% in DSCR1ΔN-transfected cells compared with control cells.

To test whether the discrepancy in IκBα levels between the two cell lines is due to a difference in the stimulation-induced degradation rate of IκBα, we calculated the half-life of the initial “rapid degradation” phase of IκBα (0–30 min). As shown in Fig. 5 (C and D), the estimated half-lives of IκBα in DSCR1ΔN-transfected (19.8 min) and control (22.6 min) cells were not significantly different.

Next, we examined the basal turnover rate of IκBα in the same two cell lines. After incubation under serum-reduced conditions for 20 h, the cells were treated with cycloheximide. The protein level of IκBα was then observed every 2 h for 6 h. As shown in Fig. 6 (A and B), the IκBα half-life in control cells was 129.6 min, whereas that in DSCR1ΔN-transfected cells was 280.8 min, indicating a decrease in basal IκBα degradation in these cells.

Knockdown of DSCR1.4 Increases IκBα Degradation by an IKK- and CK2-independent Mechanism—We wished to determine whether endogenous DSCR1.4 has a role in the regulation of IκBα degradation. In addition, we also tested whether IKK-dependent phosphorylation of IκBα is involved in the mechanism by which DSCR1.4 affects IκBα degradation. For these experiments, cells were cotransfected with DSCR1.4 siRNA and an expression vector encoding IκBα-mutN, which has a double mutation at Ser-32 and Ser-36. The degradation of endogenous wild-type IκBα and IκBα-mutN was observed following treatments with cycloheximide. As expected, knockdown of DSCR1.4 increased the rate of wild-type IκBα degradation (Fig. 7A and B), suggesting that endogenous DSCR1.4 plays an important role in IκBα protein stability. Similarly, the half-life of IκBα-mutN was also shortened from 122.3 to 81.6 min (Fig. 7, A and C), suggesting that this function of DSCR1.4 does not involve phosphorylation of IκBα at Ser-32 and Ser-36.

Next, we tested whether CK2-dependent phosphorylation plays a role in the DSCR1.4-mediated stabilization of the IκBα protein. For this, we cotransfected cells with DSCR1.4 siRNA and IκBα-mutC, which has a double mutation at Ser-283 and Thr-291; these residues are the most critical phosphorylation sites for CK2 (23, 24). As expected, in the presence of cycloheximide, the double mutation increased the half-life of IκBα by 2.7-fold compared with that of endogenous wild-type IκBα. However, the half-life of IκBα-mutC was shortened from 327 to 234 min by cotransfection with DSCR1.4 siRNA (Fig. 8, A and B).

Taken together, these results show that knockdown of DSCR1.4 decreases the stability of the IκBα protein by a mechanism that is independent of IKK- and CK2-mediated phosphorylation of IκBα. Notably, cotransfection with DSCR1.4 siRNA decreased the steady-state expression levels of endogenous wild-type IκBα (Fig. 2, middle panel), IκBα-mutN (Fig. 7A), and IκBα-mutC (Fig. 8A) by 30, 22, and 37%, respectively. These results suggest that endogenous DSCR1.4 plays a significant role in maintaining the level of IκBα.
DSCR1 Inhibits Signal-independent Degradation of IκBα

**FIGURE 4.** DSCR1ΔN and inhibitors of PKC, MEK1, and IKK reduce PMA-stimulated degradation of IκBα and induction of COX-2 expression. A, control cells expressing an empty vector and cells expressing DSCR1ΔN were stimulated with PMA (25 ng/ml) for 6 h in the presence (+) or absence (−) of Ro-31-8220 (1 μM), PD 98059 (20 μM), SB 203580 (2 μM), calmodulin kinase II inhibitor (CamMInHtide; 1 μM) or hypoestoxide (50 μM) as indicated. The lysates prepared from these cells were then subjected to immunoblot analysis with antibodies specific for COX-2 and PKC. Equal loading of proteins was confirmed using anti-IκBα antibody. B, control cells expressing an empty vector and cells expressing DSCR1ΔN, RO31-8220, PD 98059, or hypoestoxide were stimulated with PMA (25 ng/ml) for 30 min in the presence (+) or absence (−) of Ro-31-8220 (1 μM), PD 98059 (20 μM), or hypoestoxide (50 μM) as indicated. The lysates prepared from these cells were then subjected to immunoblot analysis with anti-IκBα or anti-IκBα antibody to confirm equal loading of proteins. The relative densities of IκBα are the means from three experiments. *, p < 0.05; **, p < 0.001 (statistically significant differences between two groups as determined by unpaired Student’s t test). C, control empty vector-expressing and DSCR1ΔN-expressing cells were stimulated with PMA (25 ng/ml) for the indicated times. The lysates prepared from these cells were then subjected to immunoblot analysis for phosphorylated (p) ERK and IκBα levels or for NF-κB levels as a control.

**DISCUSSION**

DSCR1 has been shown to attenuate the expression of genes associated with inflammation. Although a previous study by Hesser et al. (9) showed that these anti-inflammatory effects are caused by inhibition of calcineurin signaling by DSCR1, the mechanism mediating this effect remains elusive. For example,

DSCR1A.4 Does Not Inhibit CK2-mediated Phosphorylation of IκBα Thr-291—Next, we tested whether DSCR1A.4 inhibits CK2-mediated phosphorylation of IκBα. For this, we conducted in vitro kination assays using GST-PEST as a substrate in the presence of whole cell extracts from cells stably transfected with full-length DSCR1A.4, DSCR1ΔC, DSCR1ΔN, and control vector. After incubation with cell extracts, the substrates were analyzed by immunoblotting with anti-phospho-IκBα Thr-291 antibody. As shown in Fig. 9 (A and B), overexpression of full-length DSCR1A.4 and its truncation mutants increased the phosphorylation of GST-PEST. The specificity of CK2-mediated phosphorylation was evidenced by complete inhibition of phosphorylation with heparin (Fig. 9A, fifth lane).

Next, we performed in vitro kination assays using a cell extract from DSCR1A.4 siRNA-transfected cells to test the effects of knockdown of DSCR1A.4 on CK2-mediated phosphorylation of IκBα. As shown Fig. 9B, transfection of DSCR1A.4 siRNA decreased GST-PEST phosphorylation.

DSCR1A.4 Attenuates TNF-α- and IL-1β-induced NF-κB Activation—The multiple signaling pathways that activate NF-κB converge at one critical step in which IκBα proteins are degraded by a proteasome system. Thus, the elevated steady-state level of IκBα in the DSCR1ΔN-transfected cell line would be predicted to attenuate NF-κB activation by diverse upstream signals and inhibit the expression of NF-κB target genes. To test this hypothesis, we assessed the effect of DSCR1A.4 and DSCR1ΔN on the expression of COX-2 and IL-1β after stimulation with IL-1β and TNF-α, respectively. As shown in Fig. 10A, DSCR1A.4 reduced IL-1β- and TNF-α-induced expression of their target genes. In addition, DSCR1A.4 attenuated the basal level of IL-1β expression (Fig. 10A, lower panel, second lane). As expected, its truncated version, DSCR1ΔN, also inhibited induction of COX-2 expression by IL-1β or TNF-α (Fig. 10B). Consistent with these results, DSCR1ΔN significantly attenuated NF-κB-driven luciferase activity that was stimulated with IL-1β or TNF-α (Fig. 10C).
DSCR1 affects the induction of very diverse types of genes and suppresses the expression of these genes more potently compared with pharmacological calcineurin inhibitors. These observations suggest the possibility that DSCR1 influences other signal transduction pathways that regulate inflammatory gene expression. We addressed this by overexpression and knockdown of DSCR1.4 because its expression is greatly elevated by inflammatory mediators such as calcium (37) and oxidative stress (17).

The results of this study confirm that DSCR1.4 attenuates PMA/A23187-induced COX-2 induction by inhibiting calcineurin activity. They additionally provide evidence for a novel mechanism of action for DSCR1.4 in that DSCR1.4 also reduces the activity of NF-κB and thereby reduces the induction of COX-2. We suggest that different regions of DSCR1.4 may be involved in these two mechanisms on the basis of two observations. First, tagging the C terminus of DSCR1.4 with GFP abolished the inhibitory effect of DSCR1.4 on PMA-mediated COX-2 induction, whereas inhibition of PMA/A23187-mediated COX-2 induction was preserved. Second, the region encoded by exon 7 of DSCR1 is necessary for binding to and inhibition of calcineurin (30, 38). However, the C-terminally truncated protein (DSCR1ΔC) lacks this region and yet more potently inhibited the activity of NF-κB and COX-2 induction compared with full-length DSCR1.4.

An important question is how DSCR1 decreases activation of NF-κB. Cytoplasmic retention of NF-κB by IκBα is the major

FIGURE 5. DSCR1ΔN has little effect on the kinetics of IκBα degradation induced by PMA. A and B, control cells expressing an empty vector and cells stably expressing DSCR1ΔN were stimulated with PMA (25 ng/ml) and then harvested at the indicated times for immunoblot analysis with antibodies specific for COX-2, IκBα, and NF-κB (as a control). IκBα protein levels are expressed as arbitrary densitometric units. C and D, control cells and cells stably expressing DSCR1ΔN were stimulated with PMA (25 ng/ml) for the indicated times. The lysates prepared from these cells were then subjected to immunoblot analysis for IκBα protein levels at each time point. Data represent the means ± S.D. from four independent experiments. The calculated half-lives (T1/2) of IκBα are indicated.

FIGURE 6. DSCR1ΔN extends the basal half-life of IκBα. A, control cells expressing an empty vector and cells stably expressing DSCR1ΔN were cultured under serum-reduced conditions for 20 h. Following the addition of cycloheximide (20 μg/ml), the cells were harvested at the indicated times. The lysates prepared from these cells were then subjected to immunoblot analysis for the IκBα protein. B, data represent the mean arbitrary densitometric units ± S.D. from four independent experiments. The calculated half-lives (T1/2) of IκBα are indicated.
mechanism that controls NF-κB activity. In this study, we found that both the half-life of IκBα in the presence of cycloheximide and the steady-state level of IκBα were increased in cells stably transfected with DSCR1ΔN. In the same context, knockdown of endogenous DSCR1.4 decreased both values. IκBα degradation is regulated mainly through phosphorylation. Several phosphorylation sites have been identified on IκBα, including the signal-induced IKK phosphorylation sites located at Ser-32 and Ser-36 (20) and the constitutive CK2 phosphorylation sites located in the PEST region (39). However, the IKK-dependent pathway does not appear to be inhibited in DSCR1ΔN-transfected cells because PMA-induced phosphorylation at Ser-32 and degradation of IκBα were not decreased. Moreover, the increase in IκBα degradation, induced by knockdown of DSCR1.4, was not blocked by the double mutation of Ser-32 and Ser-36.

Phosphorylation of the PEST region by CK2 is a well studied mechanism that modulates the basal turnover of IκBα (39). However, CK2 phosphorylation also did not appear to be affected by DSCR1.4: the double mutation of Ser-283 and Thr-291 did not block the decreased steady-state level of IκBα and the elevated turnover rate of IκBα that was induced by the knockdown of endogenous DSCR1.4. In addition, we have shown that overexpression of DSCR1.4 or its truncation mutants unexpectedly increased, rather than decreased, CK2-dependent phosphorylation of IκBα at Thr-291 using an in vitro kinase assay. Furthermore, a similar increase in CK2 activity due to DSCR1.4 overexpression was observed in a kinase assay using the synthetic peptide substrate RRRADDSDDDDD (data not shown). On the basis of these results, we suggest that DSCR1.4 modulates the degradation of IκBα by an IKK- and CK2-independent pathway.

Previous studies have demonstrated the calcineurin dependence of NF-κB activation. Calcineurin has been reported to syn-
ergize with other signaling molecules such as Raf (40) and PKC (41, 42) to activate the NF-κB pathway. Additionally, full activation of NF-κB requires simultaneous input through Ca²⁺-and PMA-activated signaling pathways (43). Finally, many genes are under the dual regulation of NFAT and NF-κB (12).

**FIGURE 9.** DSCR1.4 enhances phosphorylation of IκBα at Thr-291. A, whole cell extracts from cells stably transfected with DSCR1.4, DSCR1ΔC, DSCR1ΔN, or a control empty vector were subjected to in vitro kinase assay using the PEST domain of IκBα as a substrate. Phosphorylation was analyzed by immunoblotting with anti-phospho-IκBα Thr-291 antibody (p-IκBα(T291); upper panel). To confirm the equal amount of GST-PEST protein in each reaction mixture, the membrane was reprobed with anti-IκBα antibody (clone C-21; middle panel). Parallel gels containing cell lysates were blotted with anti-NF-κB antibody (lower panel). B, data represent the results of three independent immunoblot experiments. C, whole cell extracts from cells transiently transfected with DSCR1.4 siRNA (siDSCR1), CK2α siRNA (siCK2), or negative control siRNA (siControl) were subjected to in vitro kinase assay using the PEST domain of IκBα as a substrate.

**FIGURE 10.** DSCR1.4 attenuates TNF-α- and IL-1β-induced NF-κB activation. A, control cells expressing an empty vector and cells constitutively expressing DSCR1.4 were stimulated with TNF-α (10 ng/ml) or IL-1β (5 ng/ml) for 20 h in the presence or absence of cyclosporin A (CsA; 1 μM) or FK506 (50 nM) as indicated. The lysates prepared from these cells were then subjected to immunoblot analysis for COX-2 and IL-1β levels. Equal loading of proteins was confirmed using anti-NF-κB antibody. B, control cells and cells expressing DSCR1ΔN were stimulated with TNF-α (10 ng/ml) or IL-1β (5 ng/ml) for 20 h. The lysates prepared from these cells were then subjected to immunoblot analysis for COX-2 levels. C, control cells and cells expressing DSCR1ΔN were cotransfected with an NF-κB promoter-luciferase reporter plasmid (0.5 µg) and a herpes simplex virus thymidine kinase-Renilla luciferase reporter plasmid (0.2 µg). After 20 h, the cells were stimulated with TNF-α (10 ng/ml) or IL-1β (5 ng/ml) for 6 h, after which luciferase activities were measured. The results are presented as the means ± S.D.
cinerein would attenuate PMA/A23187 induction of some NF-κB target genes. Here, we have shown that DSCR1.4 inhibited NF-κB pathways as well as cinerein in separate mechanisms. This explains how expression of DSCR1.4 can inhibit gene expression by PMA/A23187 more potently compared with pharmacological cinerein inhibitors, as observed by Hesser et al. (9). In addition, DSCR1.4 is expected to be a broad-acting endogenous suppressor of NF-κB target gene expression because it elevates IκBα levels, affecting activation of NF-κB via the classical pathway. Consistent with this hypothesis, this study has shown that DSCR1.4 attenuated induction of NF-κB target genes by IL-1β and TNF-α, which utilize different adaptor molecules to activate IKK and subsequently degrade IκBα (44, 45).

Alterations in the expression of the DSCR1 gene have been implicated in several neurodegenerative conditions. For example, 2–3-fold increases in DSCR1 RNA were reported in postmortem brain samples from patients with Down syndrome and Alzheimer disease compared with age-matched controls (4, 7, 8). A >10-fold transient increase in DSCR1 RNA was observed in the penumbra area of mouse brain after induction of focal cerebral ischemia. However, the increase in DSCR1 expression in diseased brain does not prove that the protein plays either a beneficial or detrimental role in the disease pathology. Our finding that the expression of DSCR1.4 reduces activation of NF-κB and induction of its target genes, such as COX-2 and IL-1β, in a glial cell line suggests its significant role in preventing inflammatory reactions. This is supported by previous studies demonstrating that NF-κB expression is increased and can be activated by amyloid β-peptides in the brains of Alzheimer disease patients and that pharmacological prevention of this activation results in reduction of the inflammatory responses (46, 47). In Down syndrome cases, the density of activated microglia overexpressing IL-1 is as much as 30-fold higher than in age-matched control samples (48). During cerebral ischemia, NF-κB is involved in acute phase and inflammatory responses, which potentiate ischemic injury (49–51). In transgenic mice that express mutant IκBα with inhibited degradation, cerebral ischemia causes reduced NF-κB target gene expression and reduced infarct size (52). These finding suggest that DSCR1 could be a potential therapeutic target for treatment of these neurodegenerative diseases.

In conclusion, this study has shown that DSCR1.4 inhibits IκBα metabolism and may have physiologically relevant anti-inflammatory properties. The mechanism by which DSCR1.4 inhibits the basal turnover rate of IκBα and its pathophysiologically relevant role in neuroinflammatory diseases remain to be addressed in future studies.

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