

Regulation of macrophage inflammatory protein-2 gene expression in response to 2,4-dinitrofluorobenzene in RAW 264.7 cells

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Several skin sensitizers, like 2,4-dinitrofluorobenzene (DNFB), are known to provoke contact hypersensitivity responses after topical application. Here, we show that DNFB can upregulate macrophage inflammatory protein-2 (MIP-2) expression in RAW 264.7 cells via a mechanism that is largely dependent on mitogen-activated protein kinase (MAPK) signaling pathways. ELISA-based transcription factor activation assays and chromatin immunoprecipitation assays revealed that functional interaction between AP-1 and MIP-2 promoter element is necessary for MIP-2 gene expression by DNFB. Interestingly, topical application of DNFB to NC/Nga mice increased MIP-2 expression in dermis, suggesting that MIP-2 contributes to the leukocyte infiltration associated with atopic dermatitis. These results provide additional insight of the mechanism of contact hypersensitivity induced by contact sensitizers. [BMB reports 2008; 41(4): 316-321]

INTRODUCTION

Skin sensitizers, such as, 2,4-dinitrofluorobenzene (DNFB), 2,4-dinitrochlorobenzene (DNCB), 2,4,6-trinitrobenzene sulfonic acid (TNBS), and metal allergen nickel sulfate, provoke delayed hypersensitivity responses (1). For example, the repeated topical application of DNFB evokes a contact hypersensitivity reaction and induces a shift in the cutaneous cytokine milieu from a Th1 to a Th2 profile (2, 3), which could be induced by alterations in local cytokine levels, augmented eosinophil recruitment, or allergic inflammation.

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Langerhans cells (LCs), which are members of the dendritic cell (DC) family, are the major antigen-presenting cells in skin, and are important components of cutaneous immune responses to topically applied contact sensitizers (4, 5). Moreover, expressions of major histocompatibility (MHC) class II molecules and costimulatory molecules, such as, CD40, CD54, CD83, and CD86 are upregulated in DCs exposed to contact sensitizers (5-7). Furthermore, contact sensitizers reportedly activate p38 MAP kinase and ERK1/2 in the pathway leading to CD40 upregulation, and these activations are dependent on ROS (reactive oxygen species) (8, 9).

Macrophages also constitute a major subpopulation of cells in the mouse dermis (10), but the role they play in contact sensitizer-induced hypersensitivity reactions has not been investigated. Here, we show that MIP-2 is upregulated by DNFB in RAW 264.7 cells and suggest a molecular mechanism that explains this phenomenon.

RESULTS

MIP-2 expression in DNFB-stimulated RAW 264.7 cells

The expressions of chemokine genes were determined after stimulating cells with contact sensitizers. Time-course analysis was conducted to monitor the mRNA levels of MIP-2, CCL17, and CCL20. As shown in Fig. 1A, DNFB treatment failed to induce the expressions of CCL17 and CCL20, but greatly enhanced MIP-2 mRNA expression, which peaked at 2 h post-treatment (Fig. 1B). In contrast, the basal level of the MIP-2 mRNA expression was detected in cells that were stimulated with DNCB or DNP. However, DNP failed to induce MIP-2 mRNA at high concentration (20 µg/ml).

We then examined whether MIP-2 protein is released by RAW 264.7 cells in response to DNFB. ELISA results revealed the presence of MIP-2 protein in culture supernatants when cells were stimulated with DNFB (Fig. 1C), but not after stimulation with DNCB or DNP.

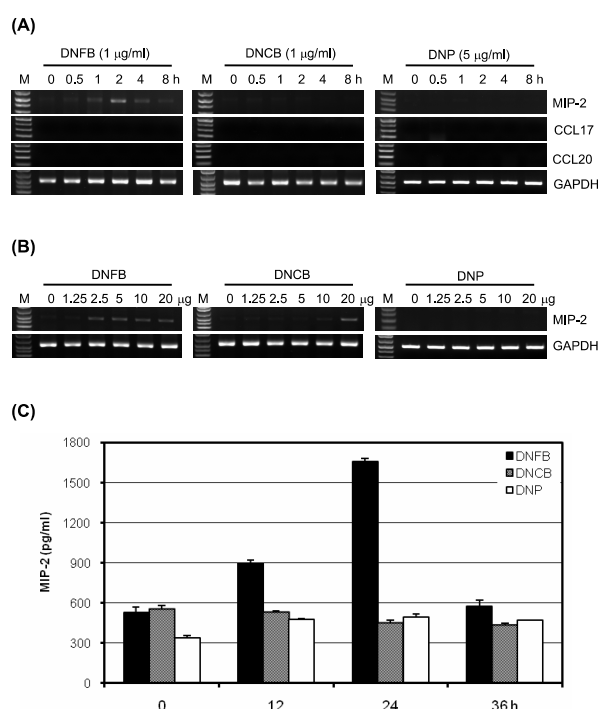


Fig. 1. DNFB-induced MIP-2 expression in RAW 264.7 cells. RAW 264.7 cells were treated with DNFB, DNCB, or DNP for the indicated times (A and C) and indicated concentrations for 2 h (B). (A) and (B) After extracting total RNA, the expressions of MIP-2, CCL17, and CCL20 were analyzed by RT-PCR. M, DNA standard marker. (C) Culture supernatants were harvested and MIP-2 levels were measured by ELISA.

ERK1/2 and p38 kinase activations were required for DNFB-induced MIP-2 gene expression

To identify the signal transduction pathways activated by DNFB, we investigated whether DNFB modulates the activations of NF- κ B and MAPK superfamily members, such as, SAPK/JNK, ERK1/2, and p38 kinase. In RAW 264.7 cells treated with 5 µg/ml of DNFB, p38 kinase and ERK1/2 phosphorylations and activations were observed at 10 min and 30 min post-treatment, respectively (Fig. 2A). However, no significant SAPK/JNK activation or I κ B α phosphorylation was observed. Furthermore, no significant activation of the NF- κ B or MAPK superfamilies was detected in the cells stimulated with DNCB or DNP (Fig. 2A).

To further explore whether MIP-2 gene expression is modulated by the activations of the NF- κ B and MAPK superfamily signal pathways, we examined MIP-2 mRNA levels after cells had been stimulated with DNFB in the presence of pathway-specific inhibitors, i.e., SP 600125 (20 µM; a SAPK/JNK inhibitor), PD 98059 (25 µM; an MEK1/2 inhibitor), PD 169316 (10 µM; a p38 MAP kinase inhibitor), or BMS345541 (10 µM; an IKK-2 inhibitor). MIP-2 gene expression was found to be reduced in the presence of PD 98059 and PD 169316, whereas

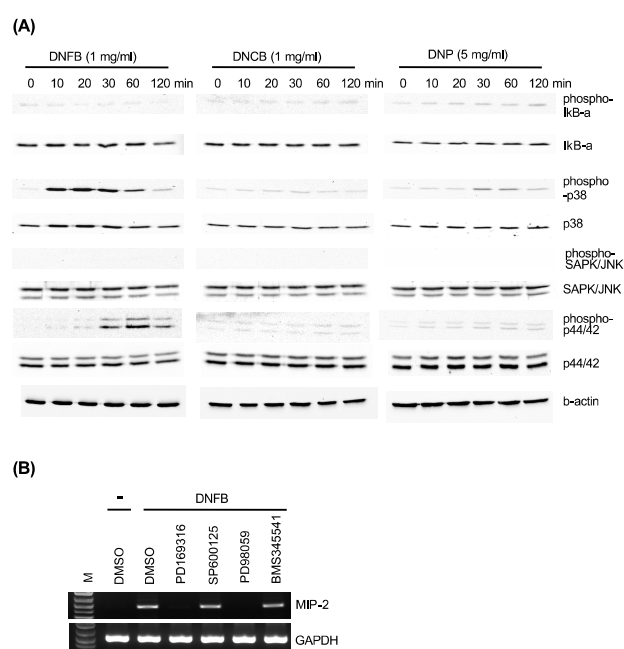


Fig. 2. DNFB-induced MIP-2 expression was found to be linked to the activations of p38 kinase and ERK1/2. (A) DNFB-induced activations of p38 kinase and ERK1/2. RAW 264.7 cells were treated with DNFB (1 µg/ml), DNCB (1 µg/ml), or DNP (5 µg/ml) for the indicated times. Cell lysates were analyzed by Western blotting using phosphospecific antibodies to I κ B α (Ser32/Ser36), SAPK/JNK (Thr183/Tyr185), ERK1/2 (Thr202/Tyr204), or p38 (Thr180/Tyr182). Amounts of I κ B α , SAPK/JNK, ERK1/2, or p38 are shown in the bottom panel of each pair. (B) Inhibition of DNFB-induced MIP-2 expression. RAW 264.7 cells were pretreated with 25 µM SP 600125 (a SAPK/JNK inhibitor), 10 µM PD 169316 (a p38 inhibitor), 25 µM PD 98059 (a MEK inhibitor), or 50 µM BMS345541 (an IKK-2 inhibitor). Cells were then stimulated for 2 h with DNFB or DMSO. MIP-2 mRNA (466 bp) expressions were analyzed by RT-PCR. GAPDH was used as an internal control (501 bp). PCR reactions were performed over 25 cycles. M: DNA standard markers.

the other two inhibitors had no effect (Fig. 2B).

These results indicate that MIP-2 gene expression is regulated by the ERK1/2 and p38 kinase pathways, rather than by MAPK and NF- κ B pathways in DNFB-stimulated RAW 264.7 cells.

DNFB induced binding between c-Jun and MIP-2 promoter

MIP-2 promoter contains binding sites for NF- κ B, AP-1, and Sp-1, and has been reported to contain the majority of sequences responsible for MIP-2 activation in cells treated with LPS or CpG-DNA (11, 12). To confirm that AP-1 binds to MIP-2 promoter in DNFB-stimulated RAW 264.7 cells, ELISA-based transcription factor activation assays were performed using TransAMTM AP-1 family kits. In addition, the DNA binding activities of c-Jun (a component of AP-1 transcription factor complex) and c-Fos were determined in the nuclear extracts of DNFB-treated cells (Figs. 3A&B). It was found that the DNA binding activity of c-Jun increased after DNFB treatment. To de-

termine the specificity of the protein-DNA complex, we conducted competition experiments using the MIP-2 promoter sequences -454 to -435 (AP-1up) or -161 to -142 (AP-1down), which contained either AP-1 binding elements or point mutation sequences of AP-1 binding elements. The results obtained showed that the protein/DNA complex was specifically dis-

rupted by adding AP-1 binding elements to the MIP promoter sequence. However, the pre-incubation of with point mutation sequences had no effect (Fig. 3A&B). These findings demonstrate that AP-1 binds directly to the binding sites of MIP-2 promoter when cells are stimulated with DNFB.

To further clarify the nuclear interaction between AP-1 and MIP-2 promoter *in vivo*, we performed ChIP assays after stimulating RAW 264.7 cells with DNFB. Accordingly, formaldehyde cross-linked chromatin fragments were isolated from cells, immunoprecipitated with an antibody to phospho-c-Jun, and analyzed by PCR. As shown in Fig 3C, specific DNA sequences encoding the AP-1 binding site (either AP-1 up or AP-1 down) of mouse MIP-2 promoter were clearly detected in a time dependent manner. Taken together, our *in vitro* and *in vivo* results suggest that AP-1 transcription factor complex binds directly to the AP-1 binding sites of MIP-2 promoter when RAW 264.7 cells are activated by DNFB.

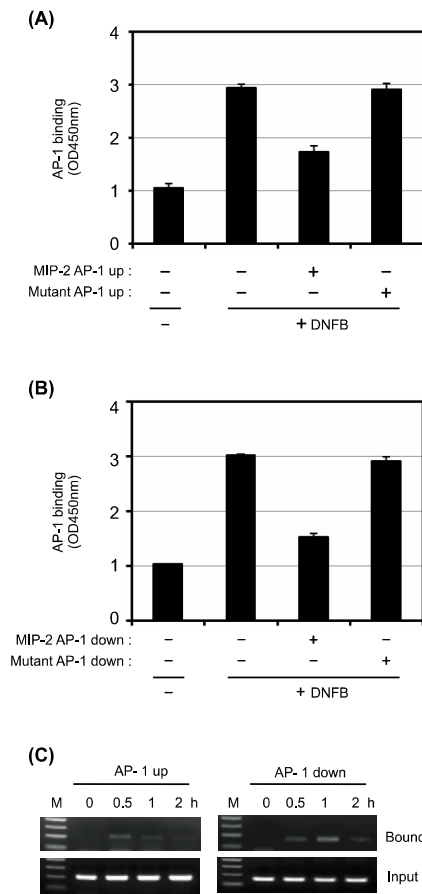


Fig. 3. Direct interaction between AP-1 transcription factor complex and MIP-2 promoter. (A) and (B) ELISA-based transcription factor activation assays were performed in a 96-well plate coated with an oligonucleotide containing an AP-1 consensus binding site. Microwells containing the DNA probe were incubated with nuclear extracts (10 μ g) from RAW 264.7 cells treated for 1 h with DNFB (5 μ g/ml; phosphorylated c-Jun in nuclear extracts specifically binds to DNFB). DNA-protein complexes were analyzed by performing competition assays using oligonucleotides for MIP-2 AP-1 or its mutant for transcription factor binding sites; protein-DNA complexes were detected using anti-phospho-c-Jun antibody. (C) Recruitment of AP-1 transcription factor complex by MIP-2 promoter *in vivo*. RAW 264.7 cells were incubated with DNFB for the indicated times. The cross-linked chromatin isolated from DNFB-treated RAW 264.7 cells was immunoprecipitated with phospho-c-Jun antibody. Immunoprecipitates were analyzed by PCR using the primer pairs described in "Materials and Methods" (Bound) to detect the presence of the MIP-2 gene promoter sequence. DNA-purified from sonicated chromatin preparations was directly analyzed by PCR as an input control (Input).

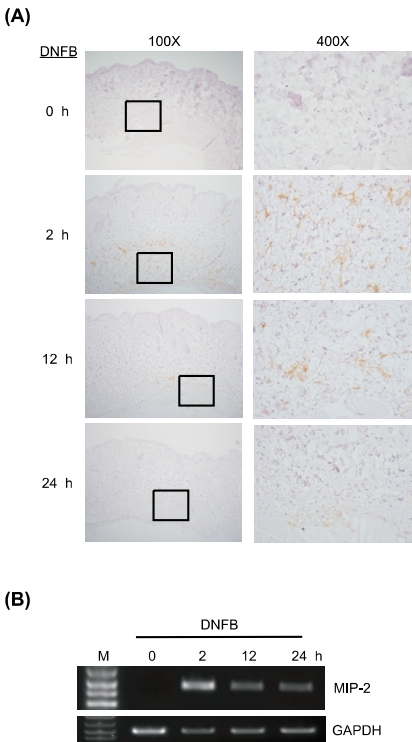


Fig. 4. DNFB-induced MIP-2 expression in NC/Nga mouse skin. DNFB (0.15%) in acetone/olive oil (3:1) was applied to dorsal skin for the indicated periods. (A) Immunohistochemical analysis of MIP-2. The paraffin sections of skin biopsies were stained for MIP-2 expression. The photomicrographs on the left were taken at an original magnification of X100, and those on the right panel at X400. (B) RT-PCR analysis of MIP-2 expression. After extracting total RNA from dorsal skin, MIP-2 mRNA expressions were analyzed by RT-PCR using the primer sets detailed in "Materials and Methods". GAPDH was used as an internal control. M, DNA standard marker.

Expression of MIP-2 in dermis after DNFB has been topically applied

Thymus-regulated and activation-regulated chemokine (TARC, CCL17) expressions have been reported to be significantly increased in NC/Nga mice that exhibit atopic dermatitis-like lesions (13). Here, we used NC/Nga mice as a contact hypersensitivity model to confirm MIP-2 expression after DNFB application, by performing immunohistochemical analyses of dorsal skin taken at different times after topically applying DNFB. Our results revealed that MIP-2 protein was rapidly and transiently expressed in the dermis after treatment (Fig. 4A). In addition, we also examined MIP-2 mRNA expression at sites of application, and as shown in Fig. 4B, RT-PCR revealed that MIP-2 mRNA was dramatically upregulated at only 2 h post application.

DISCUSSION

MIP-2 (a C-X-C ~6 kDa heparin binding chemokine) was first isolated from RAW 264.7 media after treating cells with Gram-negative bacterial LPS (14). The production of MIP-2 in response to LPS and MIP-2 upregulation, which is associated with inflammatory disease, is of particular clinical importance because MIP-2 promotes neutrophil recruitment during inflammation (15). However, MIP-2 expressional changes due to contact hypersensitivity response to skin sensitizers have not been previously described.

Macrophages and LCs in skin are crucial host defense elements, and hence, they are present in skin in huge numbers (10). However, because the majority of studies on contact sensitizers have been focused on LCs, the roles of macrophages during contact sensitizer-induced hypersensitivity reactions are comparatively unknown. Nevertheless, prior art indicates that DNFB activates MAPK family members and upregulates CD40 protein levels in skin-derived DCs (9). The present study shows, for the first time, that MIP-2 expression is induced by DNFB in RAW 264.7 cells. Furthermore, it provides clues about the molecular mechanism responsible.

Recently, we studied the mechanisms of MIP-2 gene expression. Initially, we identified NF- κ B, AP-1, and Sp-1 binding sites in MIP-2 promoter. Moreover, the functions these binding sites with regard to the expressional regulations of MIP-2 by LPS, CpG-DNA, or pyrrolidine dithiocarbamate have been previously characterized (11, 16, 17, 22). In the present study, we found that MAPK signaling pathways and AP-1 transcription factor complex contribute to DNFB-induced MIP-2 transcription. Moreover, of the three different MAPK signaling pathways, ERK1/2 and p38, but not JNK, were found to be activated by DNFB. Reportedly, the activation of the ERK1/2 pathway increases c-Fos expression (18) and induces c-Fos phosphorylation (phosphorylated c-Fos is also a component of AP-1 complex). Moreover, it has been reported that the p38 pathway activates a number of transcription factors, including Elk-1, which bind to

serum responsive element (SRE) (19), which in turn, is a component of the promoters of immediate early genes, including that of c-fos. Thus, activation of the p38 pathway may cause AP-1 activation via SRE. Moreover, in the present study, ELISA-based transcription factor activation assays and ChIP assays revealed that a functional interaction between AP-1 and the promoter element is required for the DNFB-induced expression of MIP-2 in RAW 264.7 cells.

Importantly, when DNFB was applied topically to the dorsal skin of NC/Nga mice, MIP-2 expression was clearly enhanced (Fig. 4), and although the source of this MIP-2 is unclear, our results suggests that MIP-2 may contribute to leukocyte infiltration and the atopic dermatitis-like lesions induced by DNFB. In view of the fact that macrophages constitute a major cellular subpopulation in the dermis and that MIP-2 is induced by DNFB in RAW264.7 cells, we speculate that MIP-2 production via AP-1 activation in skin macrophage cells contributes to DNFB-induced leukocyte infiltration.

In summary, our observations demonstrate that DNFB upregulates MIP-2 expression in the NC/Nga mouse dermis and in RAW 264.7 cells. The expression of MIP-2 gene was found to be dependent on MAPK signaling pathways, and in the present study AP-1 was found to contribute to DNFB-induced MIP-2 expression in RAW 264.7 cells. These results provide more evidence concerning the mechanisms underlying DNFB-induced contact hypersensitivity.

MATERIALS AND METHODS

Reagents

DNFB, DNCB and dinitrophenyl (DNP) were purchased from Sigma (St. Louis, MO). BMS-345541 (an IKK-2 inhibitor) and SP 600125 (a stress-activated protein kinase (SAPK)/Jun N-terminal kinase (a JNK inhibitor) were purchased from Calbiochem (San Diego, CA). PD 98059 (a MAPK/ERK kinase (MEK) inhibitor) and PD 169316 (a p38 inhibitor) were purchased from A.G. Scientific, Inc (San Diego, CA, USA). For experiments involving inhibitors, cells were preincubated with SP 600125 for 10 min plus BMS-345541, PD 98059, or PD 169316 for 1 h before being stimulated with DNFB.

Cell culture

RAW 264.7 cells (a mouse macrophage cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cell viabilities were typically greater than 95% by the trypan blue exclusion test.

RT-PCR analysis

RT-PCR was performed using total RNA isolated from cells or skins using RNeasy RNA isolation kits (Qiagen, Valencia, CA). One microliter of the cDNA mixture was subjected to standard

PCR over 25 cycles using the following primer sets: MIP-2, 5'-TGGGTGGGATGTAGCTAGTTCC-3' (sense), 5'-AGTTTGCTTGACCCTGAAGCC-3' (anti-sense); CCL17, 5'-CAGGAAGTTGTGAGCTGGT-3' (sense), 5'-TGTGTTCCCTGTAGTGCATA-3' (anti-sense); CCL20, 5'-GCTTTGGCATGGGTACTGCT-3' (sense), 5'-CTTTGGATCAGCGCACACAG-3' (anti-sense); mouse GAPDH, 5'-ATGGTGAAGGTCGGTGTGAACG-3' (sense), 5'-GTTGTCTGGATGATCTTGGCC-3' (anti-sense). PCR products were visualized with UV light after being resolved on 1% agarose gels.

Western blotting

SDS-PAGE and Western blotting were performed as previously described (11). Immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology Inc, Beverly, MA) and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

ELISA

Cell culture media were collected after cells had been incubated with DNFB for the indicated times. MIP-2 levels in culture supernatants were then determined using a commercially available mouse MIP-2 ELISA kit (R&D Systems, Minneapolis, MN).

ELISA-based transcription factor activation assays

TransAM™ AP-1 family transcription factor assay kits (Active Motif, Carlsbad, CA) were used to determine AP-1 binding activity in nuclear extracts, according to the manufacturer's instructions, but with several modifications (20). Briefly, nuclear extracts (10 µg) from RAW 264.7 cells treated with DNFB (5 µg/ml) were incubated for 1 h in a 96-well plate coated with an oligonucleotide containing the TPA-responsive element (TRE, 5'-TGAGTCA-3') consensus binding site, to which active phosphorylated c-Jun specifically binds in nuclear extracts. After washing, anti-phospho-c-Jun antibody was added to the wells, and then antibody binding was detected with a secondary HRP-conjugated antibody. Color development was quantified by spectrophotometry within 5 min at 450 nm using a reference wavelength of 655 nm. The provided WI-38 (TPA + calcium ionophore) nuclear extract was used as a positive control for c-Jun activation. For the competition assay, MIP-2 promoter fragments encompassing -454 to -435 and -161 to -142 [MIP-2 AP-1up (-454/-435) and MIP-2 AP-1down (-161/-142) probes, respectively] were synthesized by GenoTech (Daejeon, Korea). We then used oligonucleotides to encode the following sequences in sense and anti-sense orientations: MIP-2 AP-1up (-454/-435) wild type (MIP-2 AP-1up), 5'-GAGATGACTAAATTCAACA-3'; AP-1up (-454/-435) mutant (MIP-2 mAP-1up), 5'-GAGATGATGtgATTTCACA-3'; AP-1down (-161/-142) wild type (MIP-2 AP-1down), 5'-GTAGAATGAGGCAGGCAGCT-3'; AP-1down (-161/-142) mutant (MIP-2 mAP-1down), 5'-GTAGAATGAGGtgGGCAGCT-3'. Mutated sites are indicated by lower case letters. For the oligonucleotide competition experiment, we preincubated nuclear extracts with the oligonucleotides before adding cell extract to wells.

ChIP assays

Chromatins from RAW 264.7 cells treated with DNFB were prepared as previously described (21) with several modifications (22). ChIP assays were performed using 5 µg of anti-phospho c-Jun antibody. Immune complexes were eluted with 50 mM NaHCO₃ and 1% SDS, and DNA was analyzed by PCR using promoter-specific primer pairs for the AP-1 binding sites of MIP-2 promoter. The following primer sets were used: AP-1 up, 5'-CTCGTGCTCAGTACACCGCA-3' and 5'-GGGAACCTTAGT CATTAGGACTGA-3' (product size 197 bp); AP-1 down, 5'-GGTCACTTCAGCGCAGACATC-3' and 5'-CCTCATCAGGAAGCA CAGAGC-3' (product size 165 bp). The DNA purified from sonicated nuclear lysates was directly analyzed by PCR using the same primer set, and the PCR product was used as an input control.

NC/Nga mice and the topical application of DNFB

Seven-week-old male specific-pathogen-free (SPF) mice were obtained from Central Laboratory Animal Inc. (Seoul). Animal studies were approved by the Institutional Animal Care and Use Committee of Hallym University. Briefly, dorsal hair was removed using an electronic clippers three day before each experiment. DNFB (25 µl of 0.15%) in acetone/olive oil (3:1) was then applied to dorsal skin for the indicated times.

Histology and immunohistochemistry

Dorsal skin specimens from each animal were fixed in 4% buffered formalin solution, embedded in paraffin, and cut into 4 µm thick sections, which were then stained with hematoxylin-eosin or anti-MIP-2 antibodies using standard procedures.

Acknowledgments

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