Journal of Korean Association
of Cancer Prevention
2004; 9(4): 237-243

Role of Relish, a *Drosophila* NF-κB Protein, in Response to UV Irradiation

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NF-κB has emerged as a central component of the cellular signaling machinery that serves as an important regulatory role in inflammation, immunity, and oncogenesis. During UV exposure, a positive correlation between NF-κB/Rel activation and its translocation to the nucleus, and phosphorylation and degradation of I-κB protein have been known in mammal. While the mechanisms involved in NF-κB activation upon inflammatory response are well documented both in flies and mammals, little is known that *Drosophila* NF-κB plays any functional roles in UV irradiation. In this study, using a null mutant of Relish, *Drosophila* NF-κB, we investigated a role of *Drosophila* NF-κB in response to UV irradiation. We found UV-induced NF-κB activation through gel mobility shift assay with the κB binding sequences of *defensin* promoter region. *Relish* null mutant larvae were more sensitive to UV-induced pigmentation, immunosuppression and apoptosis of hemocytes than wild-type. These results suggest that *Drosophila* NF-κB protein is involved in immune response and serves as an anti-apoptosis factor against UV irradiation.

Key Words: NF-κB, Relish, UV, *Drosophila*

INTRODUCTION

Ultraviolet (UV) radiation is the primary environmental agent and has significant biological effects.[1)] Ultraviolet in sunlight may be divided into three components according to wavelength and biological activities: UVA (320～380 nm), UVB (290～320 nm) and UVC (180～290 nm).[2)] UVB and UVC wavelengths are most potent and a complete carcinogen.[3,4)]

UV causes sunburn reactions, immunosuppression, aging, cancer, and it is considered to be an important environmental hazard for humans.[5)] At cellular levels, UV radiation has been shown to trigger cytokine production, affect cellular mitosis, and induce apoptotic cell death.[6～8)] With respect to molecular mechanisms, UV radiation is known to alter cellular function via DNA damage,[9)] generation of reactive

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Received : November 20, 2004, Accepted : December 15, 2004

oxygen species (ROS),[10] and activation of selected transcriptional factors, including AP-1, AP-2 and nuclear factor kappa B (NF-κB).[11]

NF-κB has emerged as a central component of the cellular signaling machinery that serves as an important regulatory role in inflammation, immunity, cell proliferation, and oncogenesis.[12] The NF-κB is composed of homodimers and heterodimers of Rel protein that is also involved in regulating the transcription of various genes.[13] According to recent reports, during UV exposure, a positive correlation between NF-κB/Rel activation and its translocation to the nucleus, and phosphorylation and degradation of I-κB protein have been known.[14] In *Drosophila,* three Rel/NF-κB transcription factors have been identified: Dorsal, Dif and Relish. Like their vertebrate counterparts, these fly proteins contain multifunctional and conserved Rel homology (RH) domain and an inhibitory I-κB domain.[15]

While the mechanisms involved in NF-κB activation upon inflammatory response are well documented both in insects and mammals, little is known that UV-induced NF-κB activation plays any functional roles in *Drosophila*. Therefore, we examined the role of NF-κB in response to UV-irradiation using a null mutant of Relish (Rel^{E20}). The Rel^{E20} strain described previously is a strong and null allele of *Relish.*[16] We found that *Drosophila* NF-κB protein also plays roles in immune response and apoptosis against UV irradiation.

MATERIALS AND METHODS

1) *Drosophila* strains and culture

We used *Oregon-R* as a wild-type strain and *Relish* mutant strain (Rel^{E20}) described previously[16] as a *Drosophila* NF-κB mutant strain. Flies were kept on standard culture media at 25°C and 60% humidity.

2) Oligonucleotides

All oligonucleotides were chemically synthesized. The sequences of double-stranded oligonucleotides containing *k*B sites of *defensin* promoter region were as follows: *defensin-kB,* 5'-gattaaatATGGATTCCC-ctacatc-3', 3'-ctaatttaTACCTAAGGGgatgtag-5'. *k*B-binding sequences are underlined, and lower case letters indicate the linker sequences.

3) UV irradiation

Irradiation was carried out using a UV-lighting box which contained an ultraviolet light source in a light-tight box. The UV light sources were as follows: (1) UVB lamp (UVP, Japan) emitting in the range of 290～320 nm (maximum at 312 nm); and (2) UVC lamp (UVP, Japan) emitting at 254 nm. UVB and UVC radiation were measured by a radiometer (UVB; UVX-31, UVP Inc. USA and UVC; DM-254H, Spectroline Co. USA). The intensity was multiplied by the time of exposure to obtain the UV dose (J/m^2). Third instar larvae of wild-type and *Relish* mutant strain were exposed to UVB or UVC in open Petri-dish (60 mm) with approximately 50 individuals. After the irradiation, larvae were transferred onto culture medium. This process was carried out under a red lamp to avoid the photo-repair. The larvae were kept in the dark at 25°C.

4) Preparation of nuclear extract *in vivo*

Twenty third instar larvae were homogenized in 100μl of 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM dithiothreitol (DTT). The homogenate was kept on ice for 15 min before being lysed by addition of 0.6% (v/v) NP-40. Nuclei were pelleted by centrifugation at 13,000 rpm for 5 min at 4°C in a centrifuge. The nuclear pellet was resuspended in 50μl of 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 10% (v/v) glycerol. The nuclei were lysed by shaking for 30 min at 4°C. The lysates were centrifuged at 13,000 rpm at 4°C for 15 min to remove cell debris. Protease inhibitor cocktail (Sigma) was added to all buffers according to the manufacturer's instructions.

5) Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described earlier.[17] Third instar larval nuclear extracts were incubated for 10 min in 20μl of reaction mixture containing 10 mM HEPES (pH 7.6), 50 mM KCl, 1 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol (DTT), 1μg of sonicated herring sperm DNA and 500 ng of poly (dI-dC). After that, the [γ-^{32}P]-end-labelled *defense-κB* oligonucleotides (1×10^5 c.p.m.) were added and the mixture was further incubated for 20 min at room temperature. The retarded bands were electrophoretically resolved on a 6% non-denaturating Tris-borate-EDTA polyacrylamide gel. The gels were dried and autoradiographed on X-ray film or analyzed with a BAS 2000 imaging analyzer.

6) Acridine orange staining

Larval hemocytes at 24 h after UV irradiation were collected in Ringer's solution and then incubated in PBS containing 5μg/ml of acridine orange for 5 min in the dark. Consequently they were washed for 5 min

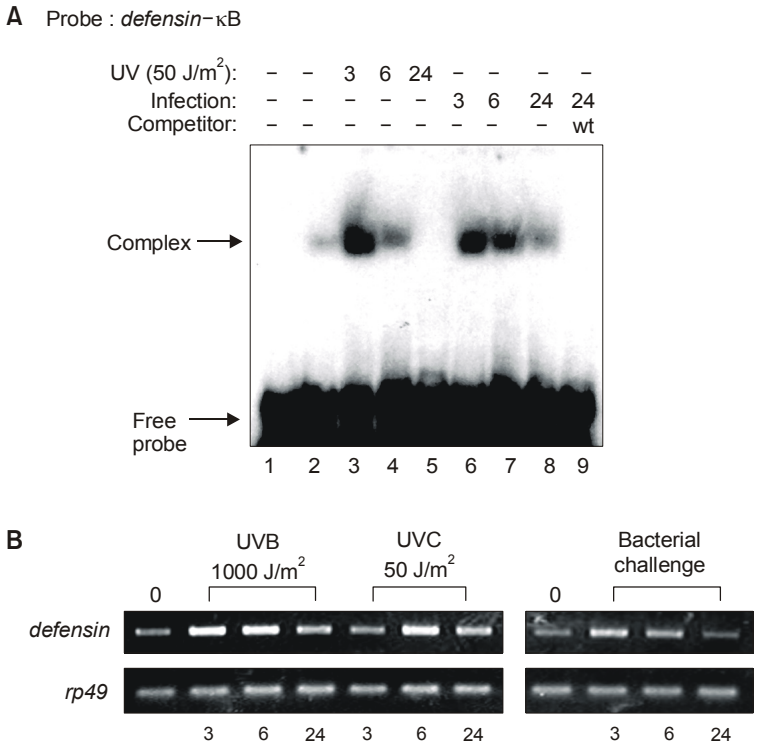

Fig. 1. UV irradiation increased NF-κB-binding activity in *Drosophila* larvae and induced *defensin* mRNA expression. (A) NF-κB binds to the κB binding sequence o*f defensin* promoter region in response to UV irradiation. Radiolabelled double stranded *k*B oligonucleotides were incubated with nuclear extracts of *Drosophila* third larvae after exposure (50 J/m^2 UVC). Lane 1, no extract. Lane 2, non-irradiated larval extract. Lanes 3-5, extract containing 3, 6, and 24 h after UV irradiation. Lanes 6-8, extract containing 3, 6, and 24 h after bacterial infection. Lane 9, competitor added. (B) The expressions of *defensin* were significantly increased in response to UV. *Drosophila* third larvae were irradiated with 1,000 J/m^2 UVB or 50 J/m^2 UVC. After 3, 6 and 24 h, total RNA was prepared, and then RT-PCR was performed to measure *defensin* mRNA levels. Wounded larvae were used as a positive control of *defensin* expression and the ribosomal protein rp49 was used as an internal control of RT-PCR experiment.

in PBS and immediately mounted onto slide glass in fresh PBS. Elapsed time from dissection to the end of the viewing was restricted to 20 min. Samples were observed with a Zeiss fluorescent microscope using the appropriate filters.

7) Scanning electron microscopy (SEM)

Third larvae were sputter-coated with platinum and observed under a Hitachi S-3500N scanning electron microscope in the low-vaccum mode.

RESULTS

1) *Drosophila* NF-κB binds to the *k*B binding sequence of *defensin* promoter region in response to UV irradiation

Defensins, a major family of antimicrobial peptides, promote a rapid cellular immune response to infection both in flies and mammals.[18] In mammal, in addition to their antimicrobial actions, *defensin* mRNA is increased in response to UVB irradiation.[19] To investigate whether *Drosophila* NF-κB protein is activated by UV, we examined NF-κB binding activity to the *k*B binding sequences of *defensin* promoter region by electrophoretic mobility shift assay. Bacterial infection was carried out as positive control. As shown in Fig. 1A, NF-κB binding activity was detected in the nuclear extracts from the third *Drosophila* larvae at 3 and 6 h after bacterial infection. We identified that NF-κB binding activity was strongly detected at 3 and 6 h after UV irradiation. Whether NF-κB contributes to the UV-induced expression of *defensin* mRNA was analyzed by RT-PCR. As shown in Fig. 1B, the expressions of *defensin* mRNA were significantly increased in response to both UVB irradiation with 1,000 J/m^2 and UVC irradiation with 50 J/m^2. This result indicates that *Drosophila* NF-κB binds to the κB binding sequence o*f defensin* promoter region in response to UV irradiation.

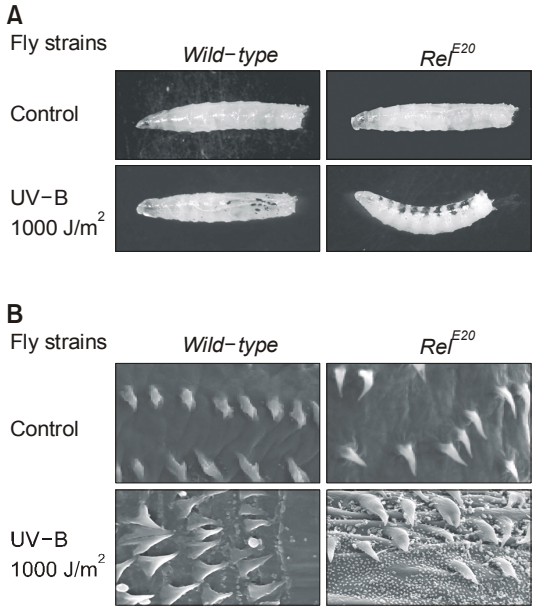

Fig. 2. *Relish* mutant was more sensitive to UV-induced epidermal pigmentation and immunosuppression than wild-type flies. The third larvae of wild-type and *Relish* mutants were irradiated with 1,000 J/m^2 UVB, and after 24 h, *Drosophila* epidermis were observed by stereomicroscopy (A) and scanning electron microscopy (SEM) (B). UV-induced epidermal pigmentation and immunosuppression were more severe in *Relish* mutant than wild-type.

2) Role of Relish in UV-induced immunosuppression

Exposure of UV radiation to the skin results in increased melanin production and thus increased pigmentation, generally described as tanning. In order to investigate the role of Relish, a *Drosophila* NF-κB protein, in response to UV irradiation, third instar larvae of *Relish* mutant and wild-type strains were irradiated to UVB (1,000 J/m^2). UV induced pigmentation in both wild-type and *Relish* null mutant larval epidermis (Fig. 2A). Interestingly, in UV-induced epidermal pigmentation *Relish* mutant was more sensitive than wild-type. The UV-induced phenotype in epidermis was observed by using scanning electron microscopy (SEM) and showed that exposure

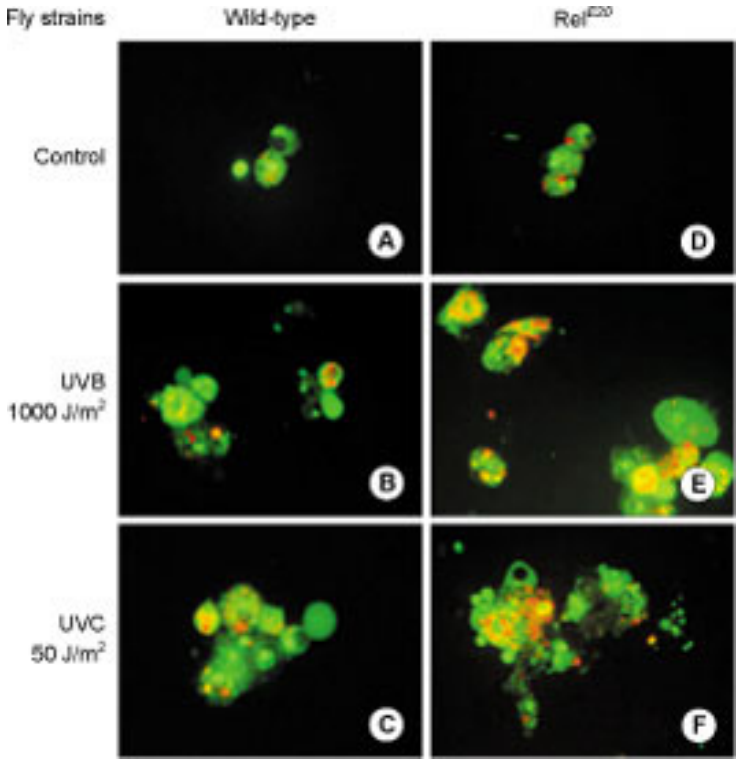

Fig. 3. *Relish* mutant was more susceptible to UV-induced apoptosis in hemocytes than wild-type. The third larvae of wild-type and Relish mutants were irradiated with 1,000 J/m^2 UVB or 100 J/m^2 UVC. After 24 h, larval hemocytes were collected in Ringer's solution, stained with acridine orange for detection of apoptotic cells, and examined by Zeiss Axioskop fluorescent microscope. We observed substantial increased apoptosis in response to UVB or UVC in hemocytes of wild- type (B and C) and *Relish* mutant strains (E and F). And we observed more severe UV-induced apoptosis in hemocytes of *Relish* mutants than in those of wild-type.

of UV caused immunosuppression in larval epidermis (Fig. 2B). *Relish* mutant was sensitive to UV-induced immunosuppression. This result indicates that NF-κB activation plays a defense role to UV-caused immunosuppression.

3) Role of Relish in UV-induced apoptosis

Since UV is known to induce apoptosis in mammalian lymphoid cells[20] and accelerate neutrophil apoptosis,[21] we examined whether UV induced apoptosis in hemocytes, *Drosophila* blood cells, of wild-type and *Relish* mutant third instar larvae using acridine orange staining methods. Acridine orange is a vital dye that provides a rapid and accurate indicator of apoptosis in insect tissues and has been used to identify apoptotic cells in *Drosophila.*[22] As shown in Fig. 3, apoptosis was induced by UV irradiation in hemocytes. We observed that Relish null mutation sensitized hemocytes to UV-induced apoptosis. Morphological changes of swelling type by UV were also observed in hemocytic. These results show that Relish may serve as an anti-apoptotic factor in response to UV irradiation in hemocytes.

DISCUSSION

The NF-κB transcription factor family induces the expression of many genes that play critical roles for responses to environmental stress. Inappropriate regulation of NF-κB activity has been implicated in the pathogenesis of several diseases: cardiovascular disease, chronic inflammation, cancer and CNS-related disease condition.[23~26] NF-κB factors can be activated by a wide variety of stimuli such as proinflammatory cytokines, bacterial lipopolysaccharide (LPS), and various stress factors such as ionizing radiation, toxins and oxidative stress.[27]

In the present study, we showed that UV irradiation resulted in a significant increase in expression of *defensin* mRNA and NF-κB binding activity in

Drosophila. Defensins, a major family of antimicrobial peptides, promote a rapid cellular immune response to infection.[18] It was reported that NF-κB contributes to the increased expression of defensin mRNA to UVB irradiation in human keratinocyte cell lines.[19]

The melanization reaction is considered as an important facet of the insect host defense. In immune challenge, plasmatocytes become stimulated, increased in number and engage in phagocytosis or differentiation into lamellocytes.[28] It is known that lamellocytes aggregate around self-tissue and these are melanized to form melanotic capsules.[29] We found that *Relish* null mutant strain was showed more severe epidermal pigmentation than wild-type (Fig. 2A). We also observed increase of the number of lamellocaytes in UVB-irradiated larvae. The number of lamellocytes in UV-irradiated *Relish* mutant larvae predominantly increased in compared with UV-irradiated wild-type (data not shown). In mammal, it was reported that selective inhibition of NF-κB signaling in skin disrupts normal epidermal homeostasis and increases the number of keratinocytes undergoing apoptosis.[30] It was also reported that the mutation of NF-κB essential modulator (NEMO)/IKKγ protein causes defective NF-κB activation, which can lead to incontinentia pigmenti, a disease marked by skin lesions, and changes in skin pigmentation.[31]

We also showed that UV irradiation caused immunpsuppression in *Drosophila* larval epidermis. *Relish* mutant was more sensitive to UV-induced immunosuppression than wild-type. These results indicate that *Drosophila* Relish plays a certain role in immune response after exposure to UV.

It is well known that UV light induce apoptosis in mammalian lymphoid cells[20] and neutrophils.[21] In this study, we also observed that UV irradiation induced apoptosis in larval hemocytes, *Drosophila* blood cells. Hemocytes of *Relish* null mutant strain were more susceptible to both UVB and UVC-induced apoptosis than wild-type (Fig. 3). Our result is coincided with that suppression of NF-κB in acute leukemia cells inhibits proliferation, causes cell cycle arrest and leads to apoptosis.[32,33]

Taken together, the present results indicate that Relish, a *Drosophila* NF-κB, is involved in immune response and serves as an anti-apoptotic factor against UV exposure.

ACKNOWLEDGEMENT

This work was supported by Korea Research Foundation Grant (KRF-2003-005-C00032).

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