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The Mechanism of CIRP in Regulation of STAT3 Phosphorylation and Bag-1/S Expression Upon UVB Radiation

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Abstract

Cold-inducible RNA binding protein (CIRP) is a stress-inducible protein, which could be activated by various cellular stresses, such as hypothermia, hypoxia and UV irradiation. Our previous study indicated that UVB (3 mJ/cm²) induces CIRP expression, which promotes keratinocytes growth, survival and eventually transformation via activation of STAT3-Bag-1/S signaling cascade. However, the mechanism(s) of CIRP in regulating p-STAT3 activation and Bag-1/S expression have not been fully elucidated. In this study, we demonstrate that repeated exposure of UVB (3 mJ/cm²) or overexpression of CIRP could lead to an elevation of the phosphorylation of Janus kinase (JAK) family proteins (JAK2 and JAK3) in HaCaT cells. The increased phosphorylation of the JAKs correlates to an increased phosphorylation of STAT3 (p-STAT3) in the cells; inhibiting JAKs using JAK inhibitor I lead to a reduction of STAT3 phosphorylation and Bag-1/S expression in the HaCaT and CIRP stably transfected HaCaT cells with or without UVB exposure. Furthermore, our data indicated that inhibiting the downstream factor of CIRP, NF- κ B, using BAY11–7085 could also decrease the p-STAT3. These results lead us to propose that CIRP mediates the activation of STAT3-Bag-1/S signaling cascade via activating the JAKs and NF- κ B signaling pathways.

Graphic Abstract

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Conflict of Interest

There is no conflict of interest.

Supporting Information

Additional Supporting Information is available in the online version of this article:

Cold-inducible RNA binding protein (CIRP) is a stress-inducible protein, which could be activated by UV irradiation. In this study, we demonstrate that repeated exposure of UVB (3 mJ/cm²) or overexpression of CIRP could lead to an elevation of the phosphorylation of JAK family proteins in HaCaT cells. The increased phosphorylation of the JAKs correlates to an increased phosphorylation of STAT3 (p-STAT3) in the cells. In addition, inhibiting NF-κB could also decrease p-STAT3. These results lead us to propose that CIRP mediates the activation of STAT3-Bag-1/S signaling cascade via activating the JAKs and NF-κB signaling pathways.

Introduction

Cold-inducible RNA binding protein (CIRP), also named A18 hnRNP or CIRBP, belongs to a family of cold-shock proteins. It contains one N-terminal RNA recognition motif (RRM) domain and one C-terminal glycine-rich domain (1, 2). It is a stress-induced protein that responds to various cellular stresses, including hypoxia (3), hypothermal stress (4), and UV radiation (5, 6). CIRP is generally considered as a cytoprotective protein, which accelerates recovery of cells from stresses (7, 8). CIRP has also been reported to be associated with various types of cancers such as breast (9), brain (10), prostate (11) and liver cancers (12).

Overexposure to ultraviolet (UV) radiation is widely accepted as one of the carcinogens to cause the development of skin cancers including basal cell carcinoma, squamous cell

carcinoma and cutaneous malignant melanoma (13, 14). Upon UV radiation, the expression level of CIRP increases; and it translocates to the cytoplasm from the nucleus in the cells (5, 15). In the nucleus, CIRP can bind to the 3'UTR region of mRNA of replicate protein A (RPA2) and thioredoxin and facilitate their cytoplasm translocation. Both RPA2 and thioredoxin are known to be multifunctional proteins which help cell survival upon UV radiation (16).

In our previous study, we demonstrated that chronic low-dose UVB radiation can induce the CIRP expression and CIRP is involved in reducing growth arrest and apoptosis (15). We also established the correlation between induction of CIRP and UVB-induced keratinocytes transformation. By Overexpressing CIRP in keratinocytes, it desensitizes cells to UVB-induced cell death (15). However, the detailed mechanism of how CIRP is regulated upon UVB radiation remains unclear. In the manuscript, we further investigated the UVB-induced CIRP signaling pathway.

Materials and methods

Cell culture.

Immortalized human keratinocyte HaCaT (kindly generated and provided by Dr. N. E. Fusenig), were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin-Streptomycin (Hyclone). For cells stably expressing CIRP, the medium was supplemented with 1 mg/ml puromycin (Sigma-Aldrich). All cells were incubated at 37 °C in 5% CO₂.

Plasmid.

Human cDNA was amplified by PCR using mRNA prepared from HaCaT cells. The CIRP-CMV-GFP-puro plasmid was generated as described previously (15). The oligonucleotide primers used for the plasmid construction were as follows:

CIRP sense 5' CCCAAGCTTATGGCATCAGATGAAGGCAAAC 3'

CIRP Anti-sense 5' ACGCGTCGACGTCAGGCTGGGTTTGACAAGA3'

Stable cell Line generation.

The CIRP-CMV-GFP-puro plasmid, lentiviral helper vectors pCMV-VSV-G and pCMV-dR8.2-dvpr were transfected into HEK-293T cells using Effectene Transfection Reagent (QIAGEN). The cells were incubated for 48 hours, then the viral supernatant fraction was collected. HaCaT cells were infected with lentiviral particles for 24 hours and then selected with puromycin for more than 1 week.

UVB-transformed cell line and cell transformation assay.

HaCaT cell was irradiated with 3 mJ/cm² UVB once every other day for three weeks to generate UVB-induced transformed HaCaT cell line. The success of cell transformation was determined by 96-well cell transformation assay following the manufacture protocol (Cell Biolabs, Inc.). Briefly, base agar layer was prepared using 1.2% agar solution with 2XDMEM/20%FBS and solidified at 4 °C for 30 minutes. The cell agar layer was prepared

similarly, with 5000 cells/well seeded in each well of a 96-well plate. 100 μ L of cell culture medium was added into each well, and the plate was kept in the incubator for 10 days at 37 °C and 5% CO₂. For cell harvest, 50 μ L of agar solubilization solution was added to each well and incubate for 1 h at 37 °C, followed by 25 μ L lysis buffer to each well. Cells were stained with CyQuant GR Dye and the fluorescent was read at 485/520 nm.

Drug treatment.

JAK inhibitor I (Santa Cruz Biotechnology) and BAY-11-7085 (Santa Cruz Biotechnology) were dissolved in dimethyl sulfoxide (DMSO) as stock solution. JAK inhibitor (JAK inhibitor I) was added to the cells 1 hour before UVB irradiation, and then kept in the medium for up to 24 hours until protein extraction. NF- κ B inhibitor (Bay-11-7085) was added to cells immediately after UVB exposure and was kept in the medium until harvest.

UVB irradiation.

UVB was generated from a Bench XX-Series UV Lamp (UVP Inc.) equipped with one 15-watt UVB lamp (UVP Inc.). UVP model UVX digital radiometer (UVP Inc.) was used to calibrate the UVB intensity to maintain a dose rate of 0.8 mW/s. Before UVB radiation, cells were washed with PBS for three times. Fresh medium was added immediately to cells after UVB exposure. 3 mJ/cm² of UVB was used for all the experiments. The cells were harvested at 24 hours post UVB radiation.

Western blot.

Cells were washed with PBS three times before protein extraction. Cells were lysed using ice-cold NP-40 buffer (100 mM Tris, pH 7.4, 80 mM NaCl, 10mM EDTA, 0.5% NP-40, 0.1% SDS) with proteinase inhibitor mixture (Sigma–Aldrich). Cell lysates were incubated on ice for 30 minutes with a brief vortex every 5 minutes. Cell lysate was centrifuged at 13,000 rpm for 15 minutes at 4 °C. Protein concentration was measured by DC protein assay (Bio-Rad Laboratories). Protein samples were resolved on SDS-PAGE and transferred to nitrocellulose membrane (Invitrogen). Membranes were blocked in TBST with 5% nonfat milk at room temperature for 1 hour and then incubated with primary antibodies at 4 °C overnight. After washing with three times with TBST, the membranes were incubated with corresponding secondary antibodies for 1h at room temperature. The membrane was then washed three-times with TBST followed by three-times wash with TBS, and developed by West Pico Super Signal chemiluminescent substrate (Pierce). Primary antibodies used: anti-JAK2, anti-JAK3, anti-STAT3 and anti- β actin (Santa Cruz Biotechnology), anti-Bag-1, anti-p-JAK2, anti-p-JAK3, anti-CIRP and anti-p-STAT3 (Cell Signaling Technology). For relative quantification, the integrated optical density (IOD) was estimated using ImageJ (NIH).

Statistics.

The data were expressed as the mean $\pm$ SD and each experiment was repeated three times. The statistical significance of differences for the mean values between groups was determined with Student's t-test. Differences with a *p*-value less than 0.05 were considered statistically significant.

Results

The p-JAK2 and p-JAK3 induction in transformed HaCaT cells

Previously, we reported that CIRP expression was increased in skin cancer cell lines and transformed HaCaT (HaCaTt) cells compared to normal keratinocytes; and the increased CIRP expression is involved in regulating STAT3 phosphorylation (p-STAT3) (15). However, the mechanism for how CIRP regulates STAT3 phosphorylation is not known. Because STAT3 is phosphorylated by Janus kinase (JAK) family kinases, we further determined the correlation between the activity of JAK family proteins and STAT3 phosphorylation in HaCaT and HaCaTt cells. Our data showed that the phosphorylated JAK2 (p-JAK2) were increased approximately 2-fold without significant change of JAK2 expression, while phosphorylated JAK3 (p-JAK3) and JAK3 were increased approximately 1.3-fold in HaCaTt cells compared to HaCaT cells (Fig. 1). JAK1 was expressed in HaCaT cells but its phosphorylation was not detectable in both cell lines (data is not shown). Meanwhile, the STAT3 phosphorylation was also increased approximately 2-fold without significant changes in HaCaTt cells compared to HaCaT cells (Fig. 1). These results suggested that JAK2 and JAK3 might mediate the CIRP-regulated STAT3 phosphorylation upon UVB radiation.

p-JAK2 and p-JAK3 induction in HaCaT^{CIRP} cell and upon UVB radiation

To determine whether the JAKs are playing a role in regulation of CIRP signaling, we analyzed the JAK2 and JAK3 phosphorylation in HaCaT and HaCaT with CIRP stably over expressing (HaCaT^{CIRP}) with and without UVB exposure (3 mJ/cm²). Our data showed that overexpression of CIRP alone could increase p-JAK2 and p-JAK3 level for approximately 2.5 and 2-fold respectively and increase of p-STAT3 approximately 2-fold (Fig. 2). At 24 hours post-UVB, the p-JAK2 p-JAK3 and p-STAT3 were increased approximately 1.8, 3.1 and 1.8-fold respectively in HaCaT cells; while increased approximately 3.3, 5 and 2.8-fold respectively in HaCaT^{CIRP} cells (Fig. 2). These results confirmed that JAK family kinases are involved in CIRP signaling pathways.

JAKs mediated the signal transduction from CIRP to p-STAT3

To further confirm the role of JAKs in mediating CIRP to p-STAT3 signaling cascade, we determined the effect of JAK inhibitor, JAK inhibitor I, on p-STAT3 level in HaCaT cells and HaCaT^{CIRP} cells post-UVB. In both cell lines, UVB exposure increased the phosphorylation of STAT3 by approximately 1-fold; and JAK inhibitor treatment could totally inhibit the phosphorylation of STAT3 in regardless of UV radiation (Figs. 3A, 3B). Furthermore, our data showed that overexpressing CIRP alone induced the background expression of Bag-1/S, a downstream gene of STAT3 upon UVB radiation (15), by approximately 1.3-fold; and UVB radiation induced Bag-1/S expression by 1.5-fold in HaCaT cells and by 1.8-fold in HaCaT^{CIRP} cells (Figs. 3C, 3D). Treatment of JAK inhibitor reduced the expression of Bag-1/S in both HaCaT and HaCaT^{CIRP} cells to slightly below background and near background expressions respectively. However, the treatment of JAK inhibitor could significantly reduce the UVB-induced Bag-1/S expression in HaCaT cells and HaCaT^{CIRP} cells to 50% and 70% of the background expressions respectively (Figs. 3C, 3D). These results indicated that while the JAKs mediate CIRP-regulated STAT3

phosphorylation and Bag-1/S expression, there might be other pathway(s) that regulates Bag-1/S expression in a JAK-STAT3 independent manner.

NF- κ B involved in the signal transduction from CIRP to p-STAT3

NF- κ B has been shown to be activated by CIRP (17) and mediate CIRP-regulated signaling pathways including inducing the expression of cytokines (18) and regulating activities of STAT family proteins (19). Thus, we further investigated whether NF- κ B also involves in the activation of CIRP/JAK/STAT3 signaling pathway upon UVB radiation. Phosphorylation and expression of STAT3 were studied in HaCaT and HaCaT^{CIRP} cells treated with NF- κ B inhibitor (Bay-11–7085) in the absence or presence of UVB radiation.

In HaCaT cells, while p-STAT3 was increased 0.8-fold post-UVB, the treatment of Bay-11–7085 reduced p-STAT3 below background by 26% or 48% in the absence or presence of UVB respectively (Fig. 4). In HaCaT^{CIRP} cells, the treatment of Bay-11–7085 reduced p-STAT3 from 1-fold to 0.5-fold or from 2-fold to 0.2-fold above background in the absence or presence of UVB (Fig. 4). The total STAT3 expression level remained unchanged for all treatments. These results suggest that NF- κ B is also involved in CIRP-induced phosphorylation of STAT3 upon UVB irradiation.

Discussion

CIRP is highly expressed in cancer cells compared to their normal counterparts (20) (21). Our previous report also demonstrated that CIRP expression level is higher in skin cancer cell lines compared to normal keratinocytes (15). The induction of CIRP is believed to be related to cell growth, cell survival and anti-apoptosis pathways (21). We previously demonstrated that low dose UVB-induced activation of CIRP is involved in phosphorylation of STAT3, and in turn its downstream Bag-1 protein expression. As both STAT3 and Bag-1 are involved in anti-apoptosis pathways, we suggested that the induction of CIRP could desensitize the cell upon UVB radiation (15). In here, we further investigated the role of CIRP and its related pathways. We first confirmed the transformation of normal keratinocytes to cancerous keratinocytes by repeated low-dose UVB radiation by soft agar assay and checked the cancerous marker proteins such as cyclin D1, c-Myc, MMP-9 alone with CIRP. The induction of these protein levels indicated a successful transformation to cancerous cells (Supporting Information, Figure S1). We further showed that JAKs family proteins, JAK2 and JAK3, in along with CIRP, are activated in UV-transformed keratinocytes (Fig. 1). JAK family is tyrosine kinases and is known for direct phosphorylation and activation of STATs. We proved here that the JAK family kinases are critical in mediating UV-induced CIRP mediated activation of STAT3, with no significance discrimination of JAK2 or JAK3 (Figs. 2, 3). The activity of STAT3 is critical in regulating Bag-1 expression, which has various isoforms (Bag-1/S, Bag-1/m, Bag-1/L) by alternative splicing (22). The Bag-1 binds to Bcl-2 and Raf and mediating the process in anti-apoptosis (23) (23). We revealed here that unlike the total inhibition of p-STAT3, Bag-1/S remained background expressing with JAK inhibitor treatment. This indicated that the baseline level of Bag-1/S is not regulated by CIRP nor JAK. One possible mechanism is that CIRP may directly involve in Bag-1 alternative splicing process, and the background expression level of

Bag-1/S related to the equilibrium alternative splicing with Bag-1/m or/and Bag-1/L. In addition, in both HaCaT and HaCaT^{CIRP} cells, while UVB radiation alone induced Bag-1/S expression and JAK inhibitor slightly reduce Bag-1/S expression, the combined treatment further reduced the Bag-1/S expression level compared to JAK inhibitor treatment alone (Fig. 3C). It suggested that upon UVB radiation, multiple pathways, including both activated and inhibitory ones, may exist in regulating Bag-1/S expression. It is possible that one of the pathways that activate Bag-1/S is mediated by JAK-STAT3, thus by UVB radiation alone, the activation of JAK-STAT3 pathways will overcome the inhibitory ones. On the other hand, when UVB radiation is coupled with JAK inhibitor, the inhibitory pathways will become predominant which causes the significant reduction of Bag-1/S expression.

Previous report demonstrated that CIRP could directly bind to toll like receptor 4 (TLR4) (17). The activated TLR4 can then activate NF- κ B to release cytokines which activates JAKs (24). However, there are controversial reports in regard to whether human keratinocytes express TLR4 (25) (26). The activation of TLR4 has also been reported to inhibit the keratinocytes growth which is contradictory to the growth facilitating role of CIRP (27). On the other hand, the activated NF- κ B can also directly regulate STAT3 activity (28). Our data indicated that NF- κ B plays an important role in regulating STAT3 activity and is involved in CIRP/STAT3 signaling pathways (Fig. 4). However, whether the signal is transduced through TLR4, or whether NF- κ B is upstream of JAKs or coordinately control STAT3 with JAKs remains unclear. In conclusion, we provided evidence in this report that CIRP activates both JAKs and NF- κ B, and both proteins regulate STAT3 phosphorylation and protect cell death upon UVB radiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

JAKs was activated in transformed HaCaT Keratinocytes cells. (A) Western blotting analyses of p-STAT3 and JAKs family member expression in HaCaT and HaCaTt cell lines. (B) Quantitative analysis of band density in western blot using ImageJ (NIH), (n=3, *p<0.05 versus HaCaT cells).

Figure 2.

CIRP overexpression causes the up-regulation of JAKs. (A) Western blotting analyses of p-STAT3 and JAKs family member expression in HaCaT and HaCaT^{CIRP} cell lines with or without UVB radiation (3 mJ/cm²). (B-D) Quantitative analysis of band density in western blot using ImageJ (NIH), (n=3, *p<0.05, **p<0.01 versus HaCaT no UVB, #p<0.05, ##p<0.01 means total JAK3 expression versus HaCaT no UVB).

Figure 3.

JAKs mediated the signal transduction from CIRP to p-STAT3. (A)(C) Western blotting analyses of p-STAT3 or Bag-1/S expression in HaCaT and HaCaT^{CIRP} cell lines with or without 3 mJ/cm² UVB and 5 μm JAKs inhibitor. (B)(D) The densities of p-STAT3 or Bag-1/S expression in the western blot were quantitative analyzed using ImageJ (NIH), (n=3, *p<0.05, **p<0.01 versus HaCaT control, #p<0.05, ##p<0.01 versus HaCaT^{CIRP} control).

Figure 4.

NF-κB signaling pathway was involved in the signal transduction from CIRP to p-STAT3. (A) Western blotting analyses of p-STAT3 expression in HaCaT and HaCaT^{CIRP} cell lines with or without 3 mJ/cm² UVB and 5 μM NF-κB inhibitor. (B) Quantitative analysis of band density in western blot using ImageJ (NIH), (n=3, *p<0.05, **p<0.01 versus HaCaT control, #p<0.05, ##p<0.01 versus HaCaT^{CIRP} control).