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LIGHT, a member of the TNF superfamily, activates Stat3 mediated by NIK pathway

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Abstract

Stat3, a member of the signal transducers and activators of transcription (STAT) family, is a key signal transduction protein activated by numerous cytokines, growth factors and oncoproteins that controls cell proliferation, differentiation, development, survival and inflammation. Constitutive activation of Stat3 has been found frequently in a wide variety of human tumors and induces cellular transformation and tumor formation. In this study, we demonstrated that LIGHT, a member of tumor necrosis factor superfamily, activates Stat3 in cancer cells. LIGHT induces dose dependent activation of Stat3 by phosphorylation at both the tyrosine 705 and serine 727 residues. The activation of Stat3 by LIGHT appears to be mediated by NIK phosphorylation. Expression of a kinase-inactive NIK mutant abolished LIGHT induced Stat3 activation. Overexpression of an active NIK induces Stat3 activation by phosphorylation at the both tyrosine 705 and serine 727 residues. Activation of Stat3 by NIK requires NIK kinase activity as showed by kinase assays. In addition, LIGHT increases the expression of Stat3 target genes including cyclin D1, survivin, and Bcl-xL, and stimulates human LNCaP prostate cancer cell growth *in vitro* which can be blocked by expression of a dominant-negative Stat3 mutant. Taken together, these results indicate that in addition to activating NF- κ B/p52, LIGHT also activates Stat3. Activation of Stat3 together with activating non-canonical NF- κ B/p52 signaling by LIGHT may maximize its effects on cellular proliferation, survival, and inflammation.

Keywords

Stat3; LIGHT; NIK; Prostate

INTRODUCTION

LIGHT (lymphotoxin homolog, inducible and competes with HSV glycoprotein D for HveA and is expressed on T-lymphocytes) is a type II transmembrane protein belonging to the tumor necrosis factor superfamily [1]. Cytokines of TNF family member regulate various cellular responses, including proliferation, differentiation, inflammation, and cell death [2]. LIGHT forms a membrane-anchored homotrimeric complex that is capable of binding to both lymphotoxin β receptor (LT β R) and herpes simplex virus entry mediator (HVEM), resulting

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in induction of both apoptotic and non-apoptotic cell death [1;3]. LIGHT crosslinking to LT β R can lead to activation of the non-canonical NF- κ B transcription factor NF- κ B2/p52 and the mitogen-activated protein (MAP) kinase JNK [4;5;6]. The NF- κ B family of transcription factors consisting of Rel A (p65), Rel B, c-Rel, p50 and p52 plays a critical role in controlling expression of numerous genes that are involved in diverse processes, including inflammatory and immune responses, apoptosis, stress responses, malignant transformation and tumor progression [7;8;9;10]. The non-canonical NF- κ B pathways that are involved in the processing of p100 to p52 require the recruitment of NF- κ B-inducing kinase (NIK) and subsequent activation of the I κ B kinase α (IKK α). IKK α phosphorylates p100 at two c-terminal serines and following ubiquitination and degradation, the subunit p52 is released. While the processing of p105 to p50 is constitutive, the processing of p100 to p52 is a tightly controlled event in many cells and tissues [11;12;13;14]. The processing of p100 to generate p52 is an important alternative step in NF- κ B regulation and plays critical roles in lymphocyte hyperplasia, cellular transformation, and apoptotic cell death [8;15;16;17].

Stat3, a member of the signal transducers and activators of transcription (STAT) family, is a key signal transduction protein that mediates signaling by numerous cytokines, peptide growth factors, and oncoproteins [18]. Accumulating evidence demonstrates that Stat3 activation plays important roles in cell differentiation, proliferation, development, apoptosis, inflammation, and tumor cell evasion of the immune system [19]. Elevated activity of Stat3 has been found frequently in a wide variety of human tumors, including hematologic malignancies, head and neck, breast and prostate cancer [19]. The phosphorylation of Stat3 by receptor-associated Janus kinases at Tyr 705 leads to activation, hetero- or homo-dimerization and nuclear translocation of Stat3, where interactions with specific DNA-response elements lead to multiple gene transcription [20]. In addition to tyrosine phosphorylation, phosphorylation of Stat3 at serine 727 has also been demonstrated to play a regulatory role in Stat3 activation [21;22]. Several kinases have been implicated to be involved in the serine phosphorylation of Stat3 in different experimental systems [23;24;25;26;27;28]. In normal cells activation of Stat3 is transient, whereas in tumor cells constitutive activation of Stat3 has been reported. In this present study, we investigated the effects of LIGHT on Stat3 activation, and demonstrated that LIGHT induces Stat3 activation.

MATERIALS AND METHODS

Cell lines, plasmids and antibodies

The human LNCaP prostate cancer cells and HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA). The antibodies against: stat3, NF- κ B/p100/p52, NIK, phospho-NIK, cyclin D1, survivin, Bcl-xL, and β -actin were purchased from Santa Cruz biotech. Antibodies against phospho-Tyr 705-Stat3 and phospho-Ser 727-Stat3 were purchased from Cell Signaling Technologies (Beverly, MA). The LT β R agonist, LIGHT was purchased from Alexis Biochemicals (San Diego, CA).

Preparation of cell lysates and immunoblotting

Cells were lysed in a high salt buffer containing 10 mM Hepes pH 7.9, 0.25 M NaCl, 1% NP-40, 1 mM EDTA and whole cell and nuclear protein were isolated as described previously [29]. Protein concentrations in the lysates were determined with the Coomassie Blue Protein Assay Reagent (Pierce). Equal amounts of protein were electrophoresed on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked for 1 h at room temperature in 5% milk in 1X PBS+0.1% Tween-20 and incubated with primary antibody diluted in 1% BSA overnight. After washing, the membranes were incubated for 1 h in secondary antibody conjugated to HRP diluted in 5% milk in 1X PBS+0.1% Tween-20. After

washing, the membranes were incubated in 1:1 ratio of reagents A and B (ECL, Amersham) and exposed to film.

Electrophoretic mobility shift assay

Cytoplasmic and nuclear extracts were made from the cells after appropriate treatments using low salt and high salt buffers respectively as described previously [29]. Ten micrograms of nuclear protein was incubated with binding buffer containing 10 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA, 40% glycerol, and 1 µg poly (dI-dC) per reaction with 10^5 cpm of the [γ - 32 P]ATP-labeled Stat3 consensus oligonucleotides (5'-GATCCTTCTGGGAATTCCTAGATC) for 20 min at room temperature. The reactions were stopped with the addition of 6 X DNA-loading buffer and electrophoresed on a 5% non-denaturing polyacrylamide gel. The gels were dried and exposed to a phosphorimager screen.

NIK kinase assays

Equal amounts of appropriate lysates were immunoprecipitated overnight with anti-NIK antibodies and the protein A/G-agarose beads were incubated with 5 µCi (1 Ci = 37 GBq) of [γ - 32 P]ATP in the presence of 1 X kinase assay buffer (20 mM Hepes pH 7.5, 10 mM MgCl₂, 20 mM β-glycerophosphate, 50 µM Na-orthovanadate, 1 mM DTT and 20 µM ATP) for 30 min at 30°C. HEK293 cells were transfected with either pCDNA3.1-HA-IKKα, or pCDNA3.1-HA-Stat3 expression vectors and 50 µg of total protein with over expressed Stat3 or IKKα was used as the substrate in each reaction. The reaction was stopped by the addition of 20 µl of 4X SDS-PAGE sample buffer and boiling for 10 min. The reaction mixtures were electrophoresed by 10% SDS-PAGE and transferred to nitrocellulose membranes and the substrate phosphorylated by the kinase, either Stat3 or IKKα was visualized by autoradiography. The membrane was probed with anti-NIK antibody to normalize for equal amounts of kinase in each reaction.

In vitro cell growth assays

LNCaP cells were plated at 2×10^5 per well in 12 well plates in triplicate in RPMI 1640 with 10% FBS and transfected with 2 µg of dominant-negative mutant Stat3F and vector control, respectively. All cells contains equal amount of DNA. Cells were treated with or without 50 ng/ml of LIGHT as indicated. Cell growth was determined at 0, 24, 48 and 72 h time points by using erythrosine B dye exclusion.

Statistical analysis

Student's *t* test (two-tailed) was used to determine the significance between treatments and untreated controls, and *p* < 0.05 was considered significant.

RESULTS AND DISCUSSION

LIGHT induces Stat3 activation

LIGHT is a potent inducer of non-canonical NF-κB2/p52 activation via NIK. To test whether LIGHT induces Stat3 activation, we treated LNCaP cells with different doses of recombinant LIGHT and analyzed the levels of phosphorylated Stat3. LIGHT induces both tyrosine 705 and serine 727 phosphorylation of endogenous Stat3 in LNCaP cells (Fig. 1A). The phosphorylation at both tyrosine 705 and serine 727 of Stat3 by LIGHT occurs within 15 min and reached maximum level at 60 min (Fig. 1B), suggesting that LIGHT activates Stat3 at the posttranslational level. To examine whether LIGHT induces Stat3 transactivation, we tested the DNA binding ability of Stat3 activated by LIGHT in electrophoretic mobility shift assays and found that Stat3 DNA binding is indeed enhanced by stimulation with LIGHT (Fig. 1C). Similar results were observed in HEK293 cells in which LIGHT induces Stat3 phosphorylation

at both tyrosine 705 and serine 727 residues (Fig. 1D), indicating that LIGHT activation of Stat3 is not a cell-type-specific phenomenon.

Stat3 activation by LIGHT requires NIK activation

LIGHT forms a membrane anchored homotrimeric complex that is capable of binding to both lymphotoxin β receptor (LT β R) and herpes simplex virus entry mediator (HVEM). LT β R ligation by binding to LIGHT leads to activation of NF- κ B2/p52 by activation of NIK. NIK is a serine kinase, preferentially phosphorylates IKK α over IKK β , leading to the activation of IKK α kinase activity. To test whether NIK is involved in LIGHT induced Stat3 activation, we employed an expression vector containing a kinase-inactive mutant of NIK (KA), which has an alanine residue at the conserved lysine residue in its kinase domain. After transfection of the kinase-inactive mutant of NIK, cells were treated with LIGHT and cell lysates were isolated. The expression of the kinase-inactive mutant of NIK completely abolished LIGHT induced Stat3 phosphorylation (Fig. 2), suggesting that LIGHT activation of Stat3 requires NIK kinase activity.

NIK induces Stat3 phosphorylation

We next examined the ability of exogenous NIK in inducing Stat3 phosphorylation. A plasmid expressing wild type NIK was transfected into LNCaP cells which express very low levels of endogenous NIK protein and the cell lysates were probed for the presence of phospho-tyrosine and phospho-serine-Stat3 using antibodies specific to these two forms of Stat3 phosphorylation by western blot. Both tyrosine and serine phosphorylation of Stat3 were induced to a robust extent by NIK overexpression. Transfection of an expression vector containing a kinase inactive mutant of NIK abolished NIK induced Stat3 activation (Fig. 3A). These results suggested that kinase activity of NIK is required for the induction of Stat3 phosphorylation.

To examine whether NIK phosphorylation of Stat3 requires NIK kinase activity, we employed *in vitro* kinase assays with immunoprecipitated NIK enzyme. We immunoprecipitated NIK from LNCaP or HEK293 cells transfected with NIK and the enzyme-antibody complexes immobilized on agarose beads were used in the *in vitro* kinase assays with γ -³²P-ATP with incubation at 30°C for 30 min. The resultant phospho-proteins were resolved on 10% SDS-PAGE and visualized by autoradiography. Elevated levels of phospho-Stat3 were detected in reactions containing NIK, which was abolished by the use of a kinase-inactive mutant of NIK (Fig. 3B).

Effect of LIGHT on cell growth

We performed cell growth assays on cells treated with LIGHT. For the assays, similar numbers of LNCaP cells were plated and treated with 50 ng/ml of LIGHT. Cell numbers were counted over 3 days. LIGHT treatment increased LNCaP cell growth compared to the control LNCaP cells without LIGHT treatment (Fig. 4A). To determine whether LIGHT induced growth stimulation is mediated by Stat3 activation, LNCaP cells were transfected with a dominant-negative mutant of Stat3, Stat3F, and treated with LIGHT. Blocking Stat3 activation by Stat3F abolished LIGHT induced LNCaP cell growth (Fig. 4A). Levels of phosphorylated Stat3 were measured in these cells to confirm that the observed effects were due to the induction of Stat3 phosphorylation by LIGHT (Fig. 4B). These results suggest that LIGHT induced LNCaP cell growth *in vitro* is mediated by activation of Stat3.

The oncogenic potential of Stat3 is mediated by its target genes including cyclin D1, Bcl-xL, and survivin. Since LIGHT activates Stat3, we examined the effects of LIGHT treatment on the expression of Stat3 target genes. LIGHT treatment increased the expression of cyclin D1, Bcl-xL, survivin in LNCaP cells (Fig. 4B), which was abolished by expression of the dominant negative Stat3 mutant (Fig. 4B). These results suggest that LIGHT mediated growth stimulation

in LNCaP cells is through activation of the Stat3 signaling pathway, which activates Stat3 target genes and alters cellular functions.

LIGHT activates Stat3 in a dose-dependent manner within 15 min and achieved maximum levels at 60 min, suggesting that LIGHT activates Stat3 through post-translational modification. We found that LIGHT activates Stat3 via phosphorylation at both the tyrosine 705 and serine 727 residues by NIK. NIK is a serine-threonine kinase which phosphorylates IKK α leading to phosphorylation of NF- κ B p100 and production of p52 when activated through ligation of LT β receptor [30]. When we transfected NIK into LNCaP cells, NIK was able to phosphorylate Stat3 at both tyrosine 705 and serine 727 residues (Fig. 3A). Our results demonstrate that in addition to activating NF- κ B/p52, NIK also activates Stat3 signaling. NIK is a serine-threonine kinase and the fact that NIK is able to phosphorylate Stat3 at both tyrosine 705 and serine 727 residues is intriguing. It has been postulated that serine 727 phosphorylation is essential for Stat3 to reach maximal transcriptional activation potential [22;31]. However, serine 727 phosphorylation of Stat3 has been variously suggested to mediate transcriptional activation without detectable Tyr 705 phosphorylation [24;32;33].

In conclusion, we attempted to determine whether LIGHT activates Stat3, one of the most commonly activated signal pathways in human cancers. We found that LIGHT activates Stat3 by phosphorylation at both tyrosine 705 and serine 727 residues in LNCaP human prostate cancer cells and HEK293 cells. Activation of Stat3 by LIGHT requires NIK kinase activity. Taken together, our results indicate that in addition to activating NF- κ B2/p52, LIGHT also activates Stat3. Activation of Stat3 together with activating non-canonical NF- κ B2/p52 signaling by LIGHT may maximize its effect on cellular proliferation, survival, and inflammation.

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A

B

C

D

Figure 1.

LIGHT induces Stat3 activation. **A.** LNCaP cells were treated with increasing doses of LIGHT as indicated for 8 h and the whole cell lysates were isolated. Twenty micrograms of protein were subjected to Western blot analysis. LIGHT increases both tyrosine and serine phosphorylation of Stat3. **B.** LNCaP cells were treated with 50 ng/ml of LIGHT for different time as indicated, whole cell lysates were isolated and 20 μ g of protein were subjected to Western blot analysis. The phosphorylation of Stat3 at both tyrosine 705 and serine 727 by LIGHT occurs within 15 min. **C.** LNCaP cells were treated with increasing doses of LIGHT as indicated for 8 h and nuclear protein were isolated. Ten micrograms of the protein were subjected to EMSA. Stat3 activity was analyzed using radiolabeled probe containing consensus

Stat3 DNA binding sequence as described in Materials and Methods. Oct-1 DNA binding activity was used as a control. **D.** LIGHT induces Stat3 phosphorylation in HEK293 cells. HEK293 cells were treated with increasing doses of LIGHT as indicated for 8 h and the whole cell lysates were isolated. Twenty micrograms of protein were subjected to Western blot analysis.

Figure 2.

Blocking NIK kinase activity abolished LIHGT induced Stat3 phosphorylation. LNCaP cells were transfected with either a kinase-inactive mutant of NIK (KA) or vector control and treated with 50 ng/ml of LIGHT for 8 h. Whole cell lysates were isolated and subjected to Western blot analysis using antibodies against either tyrosine 705 Stat3 or serine 727 Stat3, or total Stat3 as a control.

A

B

Figure 3.

NIK induces Stat3 phosphorylation. **A.** LNCaP cells were cotransfected with increasing amounts of NIK expression plasmid and the kinase inactive mutant of NIK expression plasmid (KA) as indicated. The whole cell lysates were isolated and subjected to Western blot analysis using antibodies against either tyrosine 705 Stat3 or serine 727 Stat3, or total Stat3 as a control. NIK levels show that the transfection of NIK plasmids express NIK protein in cells. **B.** Kinase assay to show the phosphorylation of Stat3 and IKKα by NIK. Lysates from vector, NIK, and NIK plus KA transfected LNCaP cells were immunoprecipitated with anti-NIK antibody, and the immunoprecipitated enzyme was used to phosphorylate Stat3 and IKKα *in vitro*. Reactions were stopped with SDS loading buffer after 30 min, loaded on SDS/PAGE, and transferred to a nitrocellulose membrane. The phosphorylated Stat3 and IKKα were visualized by autoradiography. The membrane was reprobed with anti-NIK antibody to normalize for equal amounts of kinase in each reaction.

A

B

Figure 4.

LIGHT increases LNCaP cell growth *in vitro*. **A.** LNCaP cells were transfected with either the dominant-negative mutant Stat3 (S3F) or vector (EV) and treated with 50 ng/ml of LIGHT for a period of 72 h. The cells were counted at different time points as indicated. Each point is represented as the means $\pm$ SEM of four independent experiments. * indicates significantly different ($p < 0.05$) from the control. **B.** LNCaP cells were transfected with either the dominant-negative mutant Stat3 (Stat3F) or vector (EV) and treated with 50 ng/ml of LIGHT for 72 h. The whole cell lysates were isolated and subjected to Western blot analysis. LIGHT increases the expression of cyclin D1, survivin, Bcl-xL, and phosphorylated Stat3, which was abolished by expression of the dominant-negative Stat3 mutant (Stat3F). β -actin was used as a loading control.