Cellular Signalling 28 (2016) 1833–1841

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

E3 ubiquitin ligase NKLAM ubiquitinates STAT1 and positively regulates STAT1-mediated transcriptional activity

Donald W. Lawrence [a], Jacki Kornbluth [a,b,*]

[a] Department of Pathology, Saint Louis University School of Medicine, St. Louis, MO 63104, United States
[b] VA St. Louis Health Care System, St. Louis, MO 63106, United States

ARTICLE INFO

Article history:
Received 7 June 2016
Received in revised form 19 August 2016
Accepted 24 August 2016
Available online 26 August 2016

Keywords:
Innate immunity
Interferon gamma
Macrophage
NKLAM
STAT1
Ubiquitination

ABSTRACT

Signal transducer and activator of transcription 1 (STAT1) is critically important for the transcription of a large number of immunologically relevant genes. In macrophages, interferon gamma (IFNγ) signal transduction occurs via the JAK/STAT pathway and ends with the transcription of a number of genes necessary for a successful host immune response. The predominant mechanism of regulation of STAT1 is phosphorylation; however, there is a growing body of evidence that demonstrates STAT1 is also regulated by ubiquitination. In this report we show that JAK1 and STAT1 in macrophages deficient in an E3 ubiquitin ligase termed Natural Killer Lytic-Associated Molecule (NKLAM) are hyperphosphorylated following IFNγ stimulation. We found NKLAM was transiently localized to the IFNγ receptor complex during stimulation with IFNγ, where it bound to and mediated K63-linked ubiquitination of STAT1. In vitro nucleofection studies demonstrated that STAT1-mediated transcription was significantly reduced in NKLAM-KO macrophages. There was no obvious defect in STAT1 nuclear translocation; however, STAT1 from NKLAM-KO macrophages had a reduced ability to bind a functional gamma activation DNA sequence. There was also less mRNA expression of STAT1-mediated genes in NKLAM-KO macrophages treated with IFNγ. Our results demonstrate for the first time that NKLAM is a positive regulator of STAT1-mediated transcriptional activity and is an important component of the innate immune response.

1. Introduction

Natural killer lytic-associated molecule (NKLAM) is a transmembrane E3 ubiquitin ligase and a member of the RING-in between RING-RING (RBR) family of proteins. Originally discovered in NK cells, NKLAM is expressed in other hematopoetic cells, including T cells, monocytes and macrophages [1,2]. NKLAM, like its closely related family member Dorfin, contains two centrally located transmembrane domains. The N-terminal end of the molecule contains a really interesting new gene (RING) domain followed by an in-between RING domain (IBR) and another RING domain [3]. The C-terminal portion of NKLAM is devoid of any known domains. Studies from our laboratory have shown that NKLAM colocalizes with NK cell granules and bacteria-containing phagosomes in macrophages [1,4]. NKLAM is weakly expressed in unstimulated cells and upregulated by cytokines (e.g. IFNγ) and bacterial products such as lipopolysaccharide (LPS) [4]. Studies from our laboratory have demonstrated that NK cells from NKLAM-knock out (NKLAM-KO) mice have diminished anti-tumor activity [5]. Additionally, we have shown that NKLAM plays a role in controlling tumor metastasis [6]. As a family, RBR members have been shown to play a role in neurological diseases as well as innate immunity. Mutations in the *parkin* gene (PARK2) are associated with early-onset Parkinsonism and recessive juvenile Parkinsonism [7,8]. Dorfin overexpression has been shown to lessen deleterious neurological effects in a mouse model of amylotrophic lateral sclerosis [9]. Polymorphisms in PARK2 are also associated with increased susceptibility to infection by *Salmonella typhi*, *Salmonella paratyphi*, and certain *Mycobacterium* strains [10–13]. Our laboratory found that NKLAM colocalized with internalized *Escherichia coli* in phagosomes and that macrophages from NKLAM-KO mice have a diminished bacteria killing response [4]. Precisely how NKLAM affects bacterial killing is currently under investigation in our laboratory. However, we have shown that NKLAM-KO macrophages have dysregulated NF-κB signaling that includes attenuated p65 phosphorylation, delayed nuclear translocation and diminished NF-κB transcriptional activation in response to LPS [4]. This leads to

Abbreviations: NKLAM, natural killer lytic-associated molecule; STAT1, signal transducer and activator of transcription 1; RBR, RING in between RING; JAK1, Janus kinase 1; IFNγ, interferon gamma; IFNGR, interferon gamma receptor; GAS, gamma interferon activation sequence; BMDM, bone marrow-derived macrophage; LPS, lipopolysaccharide; Ub, ubiquitin; WT, wild type; KO, knock out; iNOS, inducible nitric oxide synthase; ISRE, interferon stimulated response element; IRF-1, interferon regulatory factor 1.

* Corresponding author at: Department of Pathology, Edward Doisy Research Center, Saint Louis University School of Medicine, 1100 South Grand Blvd., St. Louis, MO 63104, United States.
E-mail address: kornblut@slu.edu (J. Kornbluth).

http://dx.doi.org/10.1016/j.cellsig.2016.08.014
0898-6568/

reduction in inducible nitric oxide synthase and less nitric oxide production. Therefore, one mechanism by which NKLAM affects innate immunity is by regulating the cellular transcriptional response.

STAT1 is a member of a family of seven signal transducing transcription factors that transduce signals from growth factors, interleukins, and cytokines [14]. Receptor ligation induces a cascade of phosphorylation events that culminate in gene transcription. In the canonical IFNγ signaling pathway, IFNγ ligation to its cognate receptor IFNGR initiates the transactivation of constitutively associated JAK1 and JAK2 tyrosine kinases. Phosphorylation of IFNGR1 on a tyrosine residue by JAK1 provides an SH2-mediated binding site for STAT1. STAT1 is then activated via phosphorylation at tyrosine residue 701 by JAK1. This tyrosine phosphorylation event stabilizes a "parallel" STAT1 dimer confirmation, enhances nuclear trafficking and promotes DNA binding [15].

Although phosphorylation is the predominant and most studied post-translational regulatory mechanism, members of the STAT family can also be regulated by ubiquitination. Kim and Maniatis were the first to demonstrate that STAT1 was ubiquitinated and degraded by the proteasome [16]. More recent studies further support ubiquitin-mediated STAT degradation. Nuclear E3 ubiquitin ligase STAT–interacting LIM protein (SLIM), ubiquitinates STAT1 and STAT4, causing their degradation in vitro and in vivo [17]. Similarly, E3 ligase Smad ubiquitination regulation factor 1 (Smurf1) was shown to bind a PY domain in STAT1 via a WW domain in Smurf1and facilitate STAT1 proteasomal degradation [18]. Additionally, Sendai virus C proteins monoubiquitinate STAT1 causing its degradation in order to circumvent the innate immune response [19]. There are also reports that suggest ubiquitination positively regulates transcription factor activation. Leidner et al. demonstrated that cotransfection of plasmids encoding ubiquitin and NFκB subunit RelB into HEK 293 cells increased the transcriptional activity of RelB in an ubiquitin concentration dependent manner [20]. Similarly, transcription factor FOXO is monoubiquitinated in response to cellular oxidative stress, which promotes its nuclear localization and increased transcriptional activity [21].

These studies indicate that transcription factor ubiquitination may positively or negatively regulate transcriptional activity. It is likely that the outcome of transcription factor ubiquitination is cell type and stimulation specific. In this present study, we provide novel data that RBR family member NKLAM ubiquitinates STAT1. Our results show for the first time that E3 ubiquitin ligase NKLAM is a transient component of the IFNGR complex and serves to enhance STAT1-dependent transcription.

2. Material and methods

2.1. Macrophage culture

All experiments on mice were approved by the Institutional Animal Care and Use Committees at Saint Louis University and the VA St. Louis Health Care System. This study was carried out in strict accordance with the provisions of the USDA Animal Welfare Act Regulations and Standards, PHS policy, recommendations in the Guide for the Care and Use of Laboratory Animals and VA policy. Wild type (WT) C57BL/6 and corresponding age-matched NKLAM-KO mice were used in all studies. For isolation of bone marrow, euthanized mice were sprayed with 70% ethanol and the femurs and tibias were dissected. The bones were flushed with DMEM and the collected marrow was resuspended in BM20 media (DMEM supplemented with 20% fetal bovine serum (FBS), 20% L929-cell conditioned media, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 1 mM sodium pyruvate). The bone marrow cells were cultured for 7 days in non-tissue culture petri dishes with a partial media change on day 3. RAW 264.7 (ATCC#TIB-71) and HEK 293 (ATCC#CRL-1573) cells were acquired from ATCC. AG129 bone marrow was a kind gift from Dr. Mark Buller (Saint Louis University).

2.2. Plasmid constructs and transfection

STAT1 alpha Flag pRc/CMV was a gift from Jim Darnell (Addgene plasmid # 8691). The pGAS-luc plasmid was obtained from Agilent Technologies. Human NKLAM and myc-tagged NKLAM plasmids have been described elsewhere [3]. For transient transfection of plasmids into HEK 293 cells Lipofectamine 3000 was used according to the manufacturer's instructions.

2.3. Immunoblotting

Whole cell protein lysates were separated using SDS-PAGE then transferred to PVDF membrane. Membranes were blocked with 1% (wt/vol) BSA in Tris-buffered saline plus 0.1% Tween-20 (TBS-T) then incubated in primary antibody with rocking overnight at 4 °C. The antibodies for STAT1, pSTAT1 (Tyr701), JAK1, ubiquitin K63 linkage, and pJAK1 were purchased from Cell Signaling. The anti-flag tag (M2) and beta actin antibodies were from Sigma-Aldrich. The anti-NKLAM antibody has been described previously [1] and the anti-IFNGR1 was purchased from Leinco. After three washes in TBS-T, the blots were probed with HRP-conjugated secondary antibodies and the proteins were visualized with BioRad Immun-Star Western C chemiluminescence kit. Images were captured and analyzed using a BioRad Chemidoc XRS + imager (BioRad).

2.4. Flow cytometry with biotinylated IFNγ

Murine IFNγ (25 μg) was biotinylated as described by Brooks et al. [22]. Briefly, 3 μL of biotin-X-NHS (10 mg/mL in DMSO) was added to 25 μg of murine IFNγ for 4 h at room temperature. The biotinylated IFNγ was then purified from reaction components using Zeba spin columns (Life Technologies). The biotinylated IFNγ retained its full biologic activity as determined by phosphorylation of macrophage STAT1 (data not shown). Biotinylated IFNγ was then used to assess the surface expression of IFNGR1. Briefly, WT and NKLAM-KO (2×10^5) bone marrow-derived macrophages (BMDM) were stimulated with 100 U/mL biotinylated IFNγ for 30 or 60 min at 37 °C. An aliquot of cells (2×10^5) was also treated with biotinylated IFNγ and kept on ice (60 min) to prohibit internalization of the receptor during stimulation. The treated cells were washed once in cold PBS and streptavidin-PE was used to detect the bound IFNγ by flow cytometry.

2.5. Cell surface IFNGR1 immunoprecipitation

BMDM were grown to confluence on 100 mm petri dishes and then stimulated with 100 U/mL IFNγ for 30 or 60 min. The monolayers were washed 2 times with ice cold PBS then collected with the addition of 1.7 mM EDTA. After pelleting, the cells were resuspended in 100 μL PBS/3% BSA and 2 μg of anti-IFNGR1 antibody was added. After incubation on ice for 1 h, the cells were washed twice in 5 mL of ice cold PBS to remove any unbound anti-IFNGR1 then lysed in 500 μL of lysis buffer (1% Triton X-100, 65 mM Tris; pH 7.5, 137 mM NaCl, 10% glycerol). Total protein concentration was determined by BCA. Immunocomplexes were isolated using protein G magnetic beads (Bio-Rad). Additionally, IFNGR complexes were isolated using bound biotinylated-IFNγ. Briefly, WT or NKLAM-KO macrophages were incubated with 100 U/mL biotinylated IFNγ for 30 or 60 min. The cells were washed with cold PBS then lysed in lysis buffer. Streptavidin-agarose beads were then added to equivalent amounts of protein and incubated for 3 h. The isolated immune complexes were washed three times with lysis buffer and then solubilized in sample buffer. Western blots were then performed for the desired proteins.