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MgcRacGAP inhibition stimulates JAK-dependent STAT3 activity

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ABSTRACT

Male germ cell Rac GTPase-activating protein (MgcRacGAP) is a core regulator of cytokinesis. Furthermore, it appears to be involved in human oncogenesis through cytokinesis-independent mechanisms and has been reported to be essential for nuclear translocation of signal transducer and activator of transcription (STAT) proteins, including the oncoprotein STAT3. Here we utilized MgcRacGAP inhibitor compound 1 (MINC1), a small molecule inhibitor of MgcRacGAP, to further investigate how MgcRacGAP regulates STAT3. Surprisingly, both MINC1 treatment and small interference RNA (siRNA)-mediated gene silencing of MgcRacGAP resulted in increased STAT3 phosphorylation and STAT3-driven transcriptional activity in our experimental systems. Finally, we demonstrated that MINC1-induced STAT3 activation likely is due to increased STAT3 phosphorylation caused by a Rac1-PAR3-IL6-IL6R-JAK2 mediated autocrine/paracrine mechanism.

1. Introduction

Male germ cell Rac GTPase Activating Protein (MgcRacGAP) functions as a GAP for Rac1 and other Rho family guanosine triphosphatases (GTPases) [1,2], meaning that it stimulates the intrinsic GTPase activity of the target Rho proteins switching them from their active GTP-bound form to inactive GDP-bound form [3]. Together with the kinesin MKLP1, MgcRacGAP forms the evolutionary conserved heterotetrameric complex centralspindlin that controls cytokinesis [1,2,4]. The complex binds to the microtubule-based mitotic spindle, initiating the formation of the central spindle. Subsequently, the associated Rho guanine nucleotide exchange factor Ect2 is recruited to the central spindle to help orchestrate cytokinesis [5], which involves both the activation and inactivation of two GTPases. During the contractile ring constriction, centralspindlin inactivates Rac1 and in parallel, RhoA is activated in the equatorial region to initiate and progress through furrow formation [6,7]. Without the components of centralspindlin (MgcRacGAP/MKLP1) or Ect2, cleavage furrow ingression will not complete [2,4,5,8–10]. Thus, the centralspindlin complex is crucial for initiation and completion of cytokinesis.

In addition to being a cytokinetic regulator, MgcRacGAP and the other members of the MgcRacGAP/MKLP1/Ect2 complex have been linked to human oncogenesis by controlling cell polarity [11] and driving epithelial-to-mesenchymal transition [12]. Overexpression of the complex members has also been correlated to a poor clinical prognosis in numerous types of cancers, including high-risk early breast cancer [13], hepatocellular carcinoma [14], gastric cancer [15], colorectal cancer [16] and melanoma for MgcRacGAP [17].

Signal transducer and activator of transcription (STAT) proteins are transcription factors that are activated by cytokine and growth factor signaling via tyrosine phosphorylation, dimerization and translocation to the nucleus. The family of STATs consists of 7 members, of which STAT3 is a well-known oncoprotein and its activation plays a key role in cell signaling in many types of cancer [18–21].

While tyrosine phosphorylation, most often by JAK family kinases, represents the major activation mechanism of STAT proteins, other co-regulatory events such as regulation of STAT nuclear traffic and alternative activation pathways appear to play

Abbreviations: GAP, GTPase Activating Protein; GTPase, guanosine triphosphatase; HEK293 SIE-Luc, HEK293 GloResponse SIE Luc2P Hygro cells; IL-6, interleukin-6; MgcRacGAP, male germ cell Rac GTPase-activating protein; MINC1, MgcRacGAP inhibitor compound 1; STAT3, signal transducer and activator of transcription 3

Author contributions: AA and KW conceived the study and designed experiments; AA performed experiments; KW supervised the study; AA and KW analyzed the data and wrote the manuscript.

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important roles [20–22]. More specifically, the small GTPase Rac1 has been proposed to regulate STAT3 activity in different ways. First, Rac1 activation was shown to induce STAT3 activity through stimulating autocrine IL-6 secretion [23]. Second, it has been proposed that activated, but not inactive variants of Rac1 form a complex with STAT3 and can regulate STAT3 phosphorylation and activity [24]. Further along those findings, Kitamura and coworkers have described the seemingly contradictory interactions between MgcRacGAP, activated (GTP-bound) Rac1 and STAT family members 3 and 5A [25–27]. They showed that the MgcRacGAP/Rac-GTP complex acts both as a mediator of STAT tyrosine phosphorylation and as a chaperone for nuclear translocation of STAT transcription factors [27].

We recently described the discovery of MINC1, a small molecule that acts on the Rac1-MgcRacGAP complex and inhibits the GAP activity of MgcRacGAP [28]. Because MgcRacGAP has been proposed to play a vital role in STAT3 activation through mediating phosphorylation and nuclear translocation [27], we hypothesized that MINC1-mediated inhibition of MgcRacGAP could inhibit STAT signaling. According to this model, inhibition could happen either by reduced phosphorylation of the STAT proteins or, if STAT phosphorylation at the cytokine receptor occurred and dimers are formed, by causing impaired nuclear translocation of the phosphorylated and activated STAT dimers. However, when we treated cells with MINC1, we instead detected increased STAT signaling. Furthermore, small interference RNA (siRNA) mediated knockdown of MgcRacGAP expression caused a similar effect in our experimental settings. Using both small compounds and siRNA-mediated gene silencing, we conclude that STAT3 activation by MINC1 likely was an effect of stimulation of a autocrine/paracrine Rac1-PAR3-IL6-IL6R-JAK2 signal resulting in STAT3 phosphorylation.

2. Materials and methods

2.1. Cell culture

HEK293 cells stably expressing a STAT3 firefly luciferase reporter were obtained from Promega (HEK293 GloResponse SIE Luc2P Hygro cells, hereafter HEK293 SIE-Luc) and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 200 μg/ml Hygromycin B (Life Technologies) as recommended by the supplier. HeLa (CCL-2) and HEK293 (CRL-1573) cells were obtained from ATCC and cultured in Eagle's minimal essential medium (EMEM) and DMEM, respectively, with 10% fetal bovine serum. Cells were frozen down in small aliquots, which allowed us to perform and repeat all experiments with cells in the same passage number.

2.2. Luciferase reporter assays

HEK293 SIE-Luc cells were plated in 96-well plates at 14,000 cells/well and left overnight to adhere. The following day, cells were starved in DMEM with 0.5% fetal bovine serum for 3 h in the presence of DMSO, 10 μM Stattic (CID 2779853, Tocris Bioscience 2798) or 12.5–25–50 μM of MINC1 (CID 744230) [28] and finally induced with or without IL-6 (100 ng/mL, AH Diagnostics 14-8069-62) for 3 h. One-Glo luciferase detection reagent (Promega) was used to determine luciferase activity according to the manufacturer's recommendations. To verify whether MINC1 induced STAT3 phosphorylation, we obtained whole cell lysates of parallel treated cells and performed western blot analysis using mouse anti-STAT3 (Cell Signaling, 9139), rabbit anti-phospho-STAT3 (Cell Signaling, 9131). Secondary antibodies goat anti-mouse IRDye 680 (Li-cor Odyssey, 926-32220) and goat anti-rabbit IRDye 800 (Li-cor Odyssey, 926-32211) were used to visualize the primary antibodies with an Odyssey infrared imaging system (Li-cor).

Gene silencing was achieved by RNA interference using or target sequence of STAT3 siRNA (Qiagen, SI02662898), MgcRacGAP siRNA (pooled Qiagen SI00101178 and SI04954880), Rac1 siRNA (Qiagen SI02655051, SI03040884 and SI03065531) or a non-targeting negative control siRNA (Qiagen, 1027280). HEK293 SIE-Luc cells were bulk transfected using 12.5 nM siRNA and Lipofectamine 2000 (Life Technologies). After 48–72 h, the cells were trypsinized and subjected to the reporter assay as described above. We performed western blots on whole cell lysates to verify knockdown using mouse anti-α-tubulin (Sigma Aldrich, T9026), mouse anti-MgcRacGAP (Santa Cruz Biotechnology, 166477), as well as mouse anti-STAT3, rabbit anti-phospho-STAT3 and secondary antibodies.

To study the effects of MgcRacGAP regulation of STAT3 in HeLa cells, these were plated at 15,000 cells/well and subsequently forward transfected with STAT3 reporter plasmid (pGL4.47[luc2P/SIE/Hygro], Promega). The following day, cells were starved in EMEM with 0.5% fetal bovine serum supplemented with DMSO, 10 μM Stattic or MINC1 (12.5, 25 or 50 μM). After 6 h, luciferase activity was determined as specified above.

To explore the role of MgcRacGAP in regulating STAT5, HEK293 cells were plated at 15,000 cells/well and transfected with STAT5 reporter plasmid (pGL4[Luc2P/STAT5/Hygro], Promega). The subsequent treatment and readout was similar as the HEK293 SIE-Luc cells, except IFNα (100 ng/mL, Immuno diagnostic Oy, 11350-1) was used to induce the STAT5 activity instead of IL-6.

To determine whether JAK kinases are involved in MINC1-mediated STAT3 phosphorylation, HEK293 SIE-Luc cells were plated in 384-well plates at 500 cells/well and after three days the cell culture medium was supplemented with 25 or 50 μM of MINC1 followed by 3 h incubation. The cells were induced with IL-6 (100 ng/mL) in the presence of DMSO, 300 nM ruxolitinib (ChemieTek, CT-INCB) or 10 μM Stattic for 3 h and after which One-Glo luciferase detection reagent was used to determine luciferase activity. In parallel, HEK293 SIE-Luc cells plated in 6-well plates were treated in similar fashion and lysed to conduct western blot analysis for STAT3 phosphorylation status.

To test the hypothesis that MINC1-mediated STAT3 activation occurs through the PAR3-IL6-IL6R-JAK2 feedback loop, an Echo 550 Liquid Handler (Labcyte) was used to transfer the following siRNAs in a 384-well plate; PAR3 (F2RL2) siRNA (Ambion, s4928, s4929 and s4930), JAK1/2/3 siRNA (Ambion, s7646, s7647, s7648, s7649, s7650, s7651, s7652, s7653 and s7654), IL6 siRNA (Ambion, s7311, s7312 and s7313), IL6R siRNA (Ambion, s7314, s7315 and s7316), STAT3 siRNA (Ambion, s744, s745 and s746) and Rac1 siRNA (Ambion, s11711, s11712 and s11713). The siRNAs were reconstituted using 5 μL OptiMEM and 15 nL Lipofectamine RNAiMAX (Life Technologies) per well. Next, HEK293 SIE-Luc cells were plated at 500 cells/well and after three days the cell culture medium was supplemented with either DMSO or 25 μM MINC1 followed by 3 h incubation. The cells were induced with IL-6 (100 ng/mL) and after 3 h CellTiter Fluor (Promega) and One-Glo luciferase detection reagents were used to determine cell viability and luciferase activity, respectively. For each siRNA paired treatment, the fold change between the DMSO and MINC1 treated condition was calculated to determine the change in STAT3 activation signal for each target.

2.3. Immunofluorescence

HEK293 SIE-Luc cells were grown in an 8-well chamber slide system (Nunc Lab-Tek, 154534) and treated with either DMSO or 25 μM MINC1, as described for the luciferase assays. Next, the chambers were removed and the slide as a whole was fixed with