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Increased cytoplasmic levels of CIS, SOCS1, SOCS2, or SOCS3 are required for nuclear translocation

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Abstract **We investigated the cellular localization of ectopically-expressed CIS, SOCS1, SOCS2 and SOCS3 proteins. We found that SOCS proteins localize to the nucleus where they reduce Stat3 proteins and that the presence of proteasome inhibitors increased SOCS nuclear localization. Our results indicate that increased nuclear localization resulted from increased levels of SOCS proteins in the cytoplasm. Finally, we demonstrate that the same effect occurs with endogenously-expressed SOCS proteins. These observations suggest that increased cytoplasmic levels of proteins in the SOCS family are regulated through nuclear translocation.**

Keywords: SOCS; Nuclei; Cytoplasm; Localization; Proteasome inhibitor

1. Introduction

Suppressors of cytokine signaling (SOCS) proteins are a group of negative feedback regulators of the Janus kinase (Jak)/signal transducer and activator of transcription (Stat), or receptor tyrosine kinase pathways [1,2]. SOCS proteins are involved in inhibiting a large variety of stimulatory proteins, including insulin, interleukin-2 (IL-2), IL-3, prolactin, growth hormone (GH), and erythropoietin [1,3]. The SOCS family of proteins consists of eight members, cytokine-inducible SH2-containing protein (CIS) and SOCS1-7 [1,4]. These proteins are composed of three structural domains: a SOCS box, a cytokine-inducible Src homology 2 domain protein (SH2) domain, and a N-terminal region of variable length [4,5]. SOCS members can be divided into two groups based on the length of their N-terminal region. SOCS4-7 are each composed of a long N-terminal region of over 200 amino acids, whereas CIS and SOCS1–3 have a short N-terminal region of less than 50 amino acids [4,5]. SOCS proteins with a short N-terminal domain have been extensively studied, and are disease-associated [6–9].

Studies of SOCS proteins show that the factors they interact with and regulate are primarily located in the cytoplasm. For example, they interact with the Jak, Stat, or other receptor tyrosine kinases via their SH2 domain in order to block signaling [2,10]. SOCS proteins also interact with E3 ubiquitin ligase, which is involved in proteasome-mediated degradation of proteins [11,12]. However, recent studies showed that SOCS members with a long N-terminal region localize to the nucleus. For example, SOCS6 localizes to the nucleus where it reduces Stat3 [13]. Furthermore, SOCS7 is a shuttling protein that transports cytoplasmic proteins into the nucleus and contributes to cell cycle arrest [14].

In the present study, we investigated whether SOCS proteins with a short N-terminal region localize to the nucleus. We found that overexpressed SOCS proteins localize to the nucleus where they reduce the Stat3 protein. Moreover, we found that SOCS proteins localize to the nucleus when their levels increase. These results suggest that SOCS proteins are regulated by translocation from the cytoplasm to the nucleus under certain biological conditions.

2. Materials and methods

2.1. Reagents

Antibodies against hSnf2H, HA tag and Lamin B1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin and anti-Flag tag antibodies were purchased from Sigma (St. Louis, MO). Antibodies against SOCS2 and SOCS3 were obtained from Abcam (Cambridge, UK). The antibody against SOCS1 was from Zymed (South San Francisco, CA). The proteasome inhibitors MG132 and Epoxomicin were purchased from Calbiochem (La Jolla, CA).

2.2. Cell culture

HEK293T, HeLa, and U87MG (ATCC, Manassas, VA) were cultured using DMEM or RPMI1640, containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (WelGENE, Seoul, Korea).

2.3. Plasmids and transfection

Plasmids pl-Pkcδ, pl-Erk1, and Jnk1 [15] were received from Dr. Gang Min Hur at Chungnam University, Korea. The pEF-Flag-CIS, pEF-Flag-SOCS1, pEF-Flag-SOCS2, pEF-Flag-SOCS3, PKBα, and Stat3-C plasmids have been described previously [16–18].

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Abbreviations: SOCS, suppressors of cytokine signaling; Stat, signal transducer and activator of transcription; Jak, Janus kinase; SH2, cytokine-inducible Src homology 2 domain protein; CIS, cytokine-inducible SH2-containing protein

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Prior to transfection, HEK293T cells were plated in 60 mm dishes at 30% confluence, and were grown for 20 h. Cells were transiently transfected according to the manufacturer's instructions (WelGENE), as previously described [13].

2.4. Isolation of cytoplasmic and nuclear protein extracts

The nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) was used to isolate the respective fractions according to the manufacturer's instructions, as previously described [13].

2.5. Immunocytochemistry

HeLa cells were plated onto chamber slides 16 h prior to transfection. They were then transiently transfected with SOCS expression vectors and incubated for 24 h, fixed for 10 min with 4% paraformaldehyde, and permeabilized for 20 min at room temperature in PBS containing Triton X-100 and BSA. They were incubated with anti-Flag tag primary antibody overnight at 4 °C. Incubation with FITC-conjugated secondary antibody (Zymed) and DAPI were performed for 1 h at 37 °C. To visualize endogenous SOCS3, U87MG cells were incubated with anti-SOCS3 primary antibody (Zymed) followed by a fluorescently-labeled secondary antibody (Alexa Fluor 568, Molecular Probes, Invitrogen, Carlsbad, CA). The stained cells were then subsequently examined with an inverted fluorescence microscope [13].

3. Results

3.1. Overexpressed CIS, SOCS1, SOCS2, and SOCS3 proteins primarily localize to the nucleus in HeLa cells

To investigate the cellular location of SOCS proteins with short N-termini, we transfected plasmids encoding Flag-CIS, Flag-SOCS1, Flag-SOCS2, and Flag-SOCS3 into HeLa cells, and observed the localization of proteins by immunocytochemical analysis. Transfected SOCS1, SOCS2, and SOCS3 proteins localized to the nucleus, while the CIS proteins were distributed both in the cytoplasm and nucleus (Fig. 1A–D).

3.2. Overexpressed SOCS proteins localize to the nucleus where they reduce Stat3

Next, we examined the levels of SOCS proteins in the nucleus and cytoplasm using Western-blot analysis. We transfected plasmids encoding Flag-CIS, Flag-SOCS1, Flag-SOCS2, and Flag-SOCS3 into HEK293T cells to confirm the localization of the SOCS proteins in a cell line other than HeLa. Cytoplasmic and nuclear protein extracts were isolated, and localization of CIS, SOCS1, SOCS2, and SOCS3 was observed using anti-Flag antibodies. As a control, we used the hSnf2H protein, which is a subunit of the SWI/SNF co-activator complex required for transcription and is primarily located in the

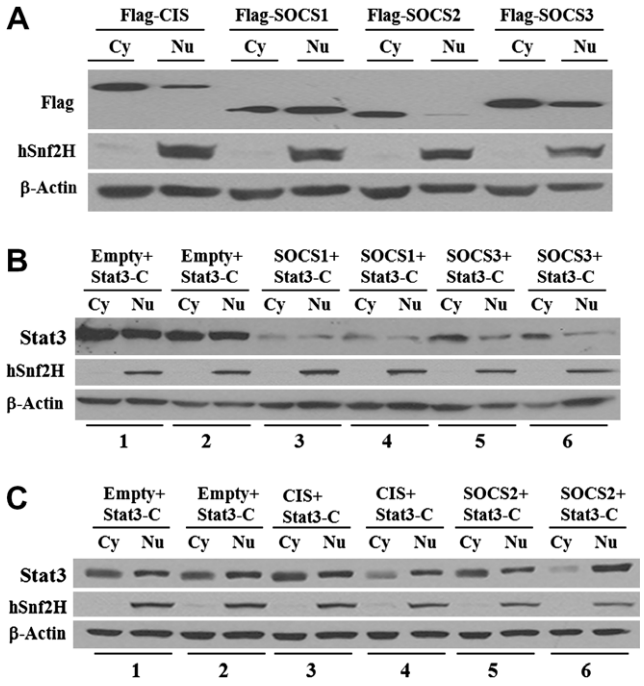

Fig. 2. Overexpressed SOCS proteins are detected in the nucleus and reduce the levels of activated Stat3 in HEK293T cells. (A) Plasmids encoding Flag-CIS, Flag-SOCS1, Flag-SOCS2, and Flag-SOCS3 were transfected into HEK293T and cytoplasmic (Cy) and nuclear (Nu) protein extracts were prepared 25 h after transfection. Western-blot analysis was then performed using antibodies against Flag, hSnf2H, and β-actin. (B, C) Plasmids encoding Flag-Stat3-C, the activated form of Stat3, were co-transfected with Flag-CIS, Flag-SOCS1, Flag-SOCS2, and Flag-SOCS3 plasmids or the empty vector (Empty+-Stat3-C), and Cy and Nu protein extracts were prepared at 25 hrs (lanes 1, 3, and 5) or 40 h (lanes 2, 4, and 6) after transfection. Western-blot analysis was then performed using antibodies against Flag, hSnf2H, and β-actin.

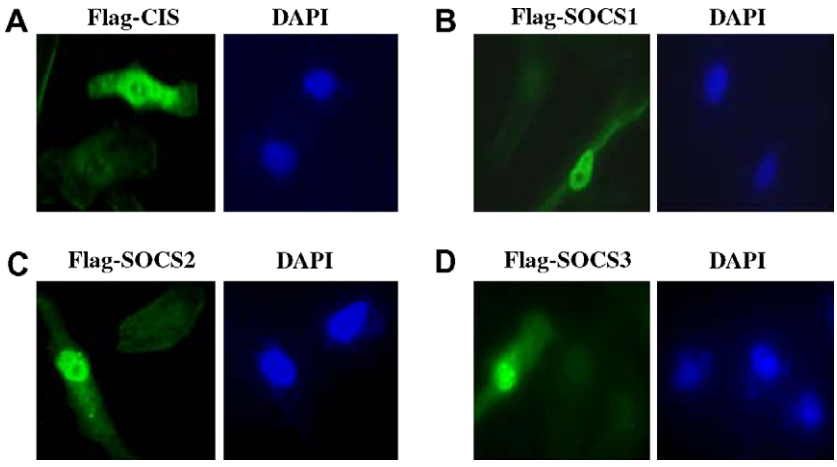

Fig. 1. Overexpressed CIS, SOCS1, SOCS2, and SOCS3 proteins are primarily localized in the nucleus of HeLa cells. Plasmids encoding (A) Flag-CIS, (B) Flag-SOCS1, (C) Flag-SOCS2, and (D) Flag-SOCS3 were transfected into HeLa cells and analyzed by immunocytochemistry. Staining for the respective proteins (green) is shown in the left panels, and DAPI nuclear staining (blue) is shown in the right panels.