Suppressors of cytokine signaling (SOCS) 1 and SOCS3 interact with and modulate fibroblast growth factor receptor signaling

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Summary

Fibroblast growth factor receptor (FGFR) signaling is transduced by the mitogen-activated protein kinase (MAPK) cascade and the signal transducers and activators of transcription (STATs). Suppressors of cytokine signaling (SOCS) proteins are expressed in response to cytokine-inducible stimulation of STAT phosphorylation, acting in a negative-feedback mechanism to hinder the activities of these receptors. However, there are no data concerning the role of SOCS proteins in the regulation of fibroblast growth factor receptor (FGFR) signaling. In the present study, we show that activation of FGFR in chondrocytes induces the expression of SOCS1 and SOCS3 mRNA, and that these proteins are constitutively associated with FGFR3, as demonstrated by co-immunoprecipitation studies. Transfection of cells with FGFR3-GFP and SOCS1-CFP revealed their colocalization, clustered prominently in the perinuclear cytosolic part of the cell. The effect of the interaction between FGFR3 and SOCS1 on receptor activity was investigated in a chondrocytic cell line overexpressing SOCS1. In these cells, STAT1 phosphorylation is repressed, MAPK phosphorylation is elevated and prolonged, and FGFR3 downregulation is attenuated. Expression of osteopontin (OPN), which is directly upregulated by FGF in chondrocytes, was stimulated by lower levels of FGF in cells expressing SOCS1 compared with parental cells. Blocking of MAPK phosphorylation by PD98059 decreased OPN expression in both cell types, but this decrease was more marked in cells expressing SOCS1. The presented results suggest a novel interaction between the SOCS1 and SOCS3 proteins and the FGFR3 signaling pathway.

Key words: Achondroplasia, Chondrocytes, MAPK

Introduction

Fibroblast growth factor receptor 3 (FGFR3) is one of four members of the tyrosine kinase high-affinity receptors for at least 22 different FGFs (Basilico and Moscatelli, 1992; Givol and Yayon, 1992; Ornitz and Itoh, 2001). Binding of the FGF ligand in concert with a heparan sulfate (Ibrahimi et al., 2005; Rapraeger et al., 1991; Schlessinger et al., 2000; Yayon et al., 1991) induces receptor dimerization, trans-phosphorylation and activation, followed by receptor downregulation. These lead to the controlled activation of specific signal transduction pathways and the expression of FGF target genes, which are critically required during embryogenesis, tissue repair, angiogenesis and bone elongation (Basilico and Moscatelli, 1992; Givol and Yayon, 1992; Goldfarb, 2001; Schlessinger, 2000). Three inherited human dwarfism syndromes – hypochondroplasia, achondroplasia and thanatophoric dysplasia (TD) – are caused by missense mutations in the gene encoding FGFR3 (Bellus et al., 1995; Francomano, 1995; Rousseau et al., 1994; Rousseau et al., 1995; Tavormina et al., 1995). The molecular mechanisms underlying these syndromes involve constitutive, mostly ligand-independent, overexpression and activation of the mutant receptors and their downstream signaling (Monsonego-Ornan et al., 2000; Naski et al., 1996; Webster and Donoghue, 1997).

The phosphorylated tyrosine kinase receptor functions as a binding site for the Src-homology 2 (SH2) domain and as phosphotyrosine-binding domains for a variety of downstream signaling enzymes and adaptor proteins (Goldfarb, 2001; Powers et al., 2000). A key component of FGF signaling is the docking protein FGFR substrate 2 (FRS2) (Lax et al., 2002), which recruits several signal-transducing molecules, leading to activation of the mitogen-activated protein kinase (MAPK) cascade. This cascade regulates the activities of downstream kinases or transcription factors, as well as the phosphoinositide 3-kinase (PI 3-kinase)-AKT anti-apoptotic pathway (Boilly et al., 2000). In mammalian cells, the Ras-Raf-MEK1/2-ERK1/2 cascade is activated by growth factors and has been implicated in cell proliferation, differentiation and survival. Another cascade activated by FGFR3 results in the phosphorylation of signal transducers and activators of transcription (STAT) proteins (Hart et al., 2000), originally identified in several cytokine signaling pathways. STAT proteins are subsequently dimerized and translocated to the nucleus, where they serve as transcription factors (Darnell, 1997). STAT1 has been detected

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in the nuclei of hypertrophic TD chondrocytes, suggesting that *FGFR3* mutations in TD alter chondrocyte differentiation through activation of the STAT signaling pathway (Legeai-Mallet et al., 1998). FGF signaling inhibits chondrocyte proliferation both in vitro (Rozenblatt-Rosen et al., 2002) and in vivo through STAT1 function (Sahni et al., 2001). Chen et al. suggested that an expanded resting zone, and narrowed proliferating and hypertrophic zones, as seen in achondroplasia, are correlated with the activation of STAT proteins (Chen et al., 1999). Ebong et al. showed that STAT activation by different growth factors induces the expression of suppressor of cytokine signaling (SOCS) proteins in lens cells (Ebong et al., 2004).

The SOCS family of proteins contains eight members (SOCS1-SOCS7, and CIS) that share a central SH2 domain and a C-terminal SOCS box (Masuhara et al., 1997). Accumulating evidence indicates that CIS, SOCS1, SOCS2 and SOCS3 participate in a classical negative-feedback loop modulating cytokine-mediated signaling pathways (Greenhalgh and Hilton, 2001; Hanada et al., 2003; Yoshimura, 1998) by several different mechanisms: SOCS1 inhibits cytokine signaling by interaction of its SH2 domain with phosphorylated Tyr1007 of JAK2 (Yasukawa et al., 1999), whereas SOCS3 binds to activated cytokine receptors through its SH2 domain and inhibits JAK2 phosphorylation (Sasaki et al., 1999); CIS competes with signaling proteins, such as STAT, for phosphotyrosine-binding sites (Yoshimura et al., 1995). SOCS family members target proteins for degradation through the binding of elongin B, cullin and Rbx1 to form an E3 ubiquitin ligase complex, which tags the proteins with polyubiquitin chains, leading to their degradation by the proteasome (Johnston, 2004; Kile et al., 2002). Ubiquitylation of tyrosine kinase receptors is directly involved in receptor downregulation. In the activating mutations of FGFR3, such as the achondroplasia mutation, downregulation is dissociated from receptor internalization, resulting in the accumulation and activation of membrane-associated FGFR3 (Monsonego-Ornan et al., 2000).

It has also been suggested that SOCS proteins play a role in the regulation of tyrosine kinase receptor signaling; Yoshimura et al. showed an interaction between FGFR1 and SOCS1 and SOCS3 in a yeast two-hybrid system, but concluded that it had no effect on receptor activity in HEK-293T cells (Yoshimura, 1998). Others have shown that SOCS1 and SOCS3 bind to the epidermal growth factor receptor (EGFR) and facilitate its proteasomal degradation (Xia et al., 2002). Both SOCS1 and SOCS3 associate with insulin receptor substrate 1 (IRS1) and IRS2 in response to insulin stimulation (Rui et al., 2002). It has also been suggested that SOCS1, SOCS3 and SOCS6 block insulin signaling by blocking the access of IRS1 and STAT5b to the receptor (Emanuelli et al., 2001; Mooney et al., 2001).

The present study was conducted to examine the interactions between FGFR3 and the SOCS proteins. In addition, we investigated the regulatory role of the SOCS proteins in FGFR activity by testing the effect of receptor activation on SOCS transcription, the localization of the interacting partners and the consequent changes in FGFR signaling pathways.

Results

FGF upregulates SOCS1 and SOCS3 expression in chondrocytes

Because SOCS transcription is under the control of phosphorylated STAT (Yoshimura, 1998), and FGFR induction also results in STAT phosphorylation (Sahni et al., 1999), we examined the effect of FGF on the transcription of SOCS1-SOCS4, in two cell lines: HEK-293T cells, which were used for the expression studies and are known to express different FGFRs (Adar et al., 2002); and rat chondrosarcoma (RCS) cells, which are known for their relevance to cartilage-disorder-linked mutations in FGFRs (Rozenblatt-Rosen et al., 2002). Furthermore, RCS cells have been shown to exert FGF-dependent STAT signaling (Sahni et al., 2001). Combined FGF and heparin treatment resulted in upregulation of SOCS1 and SOCS3 expression in a time-dependent manner in RCS but not HEK-293T cells (Fig. 1). Neither SOCS2 nor SOCS4 were expressed in either cell line, and SOCS3 was upregulated in primary cultured articular chondrocytes (not shown). These results indicate that FGF regulates the expression of SOCS1 and SOCS3 in chondrocytes.

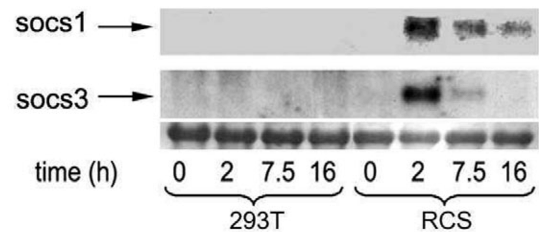

Fig. 1. Expression of SOCS1 and SOCS3 mRNA in HEK-293T and RCS cells. The cells were stimulated with 50 ng/ml FGF and 5 μg/ml heparin for different periods of time (as indicated) after 8 hours in serum-free medium. Northern analysis was carried out on 2 μg mRNA hybridized with SOSC1 (socs1) or SOCS3 (socs3) probes.

SOCS1 and SOCS3 bind differentially and constitutively to FGFR3

To determine the possible interactions between FGFR3 and SOCS1 or SOCS3, we cotransfected HEK-293T cells with constructs encoding WT hFGFR3 (R3-WT), and SOCS1 (S1) or SOCS3 (S3) fused to a Myc tag, or empty vector. After the transfections, cells were incubated with or without FGF and heparin, their lysates were precipitated with anti-Myc agarose-conjugated antibody, and putative coprecipitation was followed by western immunoblot analysis using anti-hFGFR3 antibody. As shown in Fig. 2A, FGFR3 was constitutively coprecipitated with both SOCS1 and SOCS3 in a ligand-independent manner; however, the nature of the interaction differed between SOCS 1 and SOCS3, as shown by the different FGFR3 band patterns. SOCS1 was mainly bound to the mature glycosylated 130 kDa form of the receptor, whereas SOCS3 precipitated primarily with the lower 100 kDa form, which is less processed, and is localized in the Golgi and endoplasmic reticulum (ER) (Keegan et al., 1991). To examine the importance of receptor phosphorylation in the FGFR3-SOCS interaction, we used a kinase-dead (KD) mutant (K508A) of FGFR3, which does not undergo phosphorylation (Monsonego-Ornan et al., 2002). HEK-293T cells were transiently cotransfected with R3-WT, KD or with empty vector, S1 or S3 (Fig. 2B). Total cell lysates were subjected to immunoprecipitation with anti-Myc agarose-conjugated antibody. Immunoprecipitated proteins were electroblotted with anti-Myc or anti-hFGFR3 antibodies. As

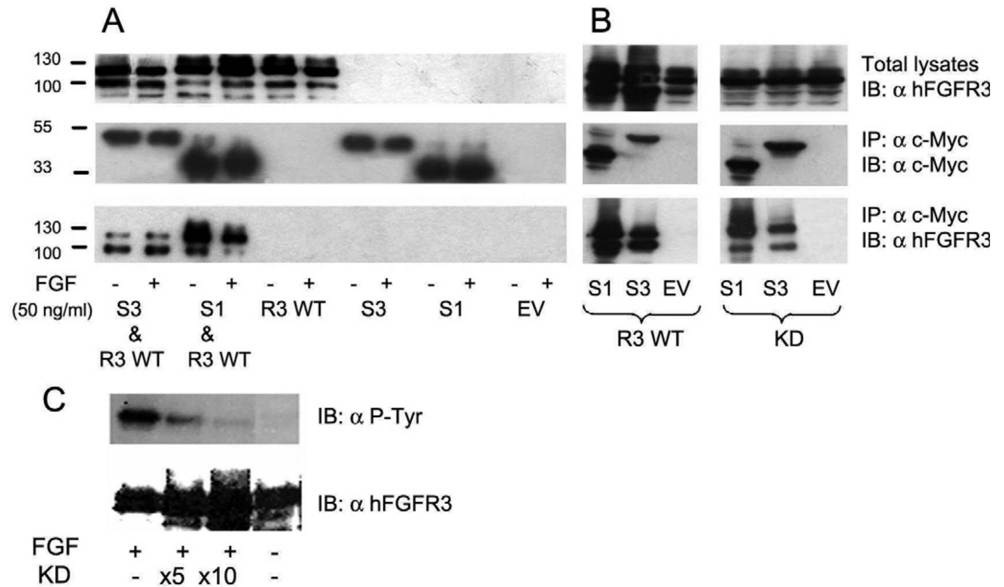

Fig. 2. hFGFR3 recruits SOCS1 and SOCS3 proteins. (A) HEK-293T cells transiently cotransfected with wild-type hFGFR3 (R3 WT), Myc-tagged SOCS1 (S1), or Myc-tagged SOCS3 (S3), as indicated. The amount of DNA was equalized with empty vector (EV). After 8 hours in serum-free medium, cells were treated with or without 50 ng/ml FGF and 5 μg/ml heparin for 5 minutes. (B) HEK-293 cells were transiently cotransfected with R3 WT or kinase-dead (KD) mutant and with EV, S1 or S3, as indicated. In both experiments, cells were dissolved in lysis buffer and total cell lysates were subjected to 7.5% SDS-PAGE followed by western blotting with anti-hFGFR3 antibody (Total lysates, IB: α hFGFR3). Cellular lysate protein (1 mg) was incubated overnight with anti-Myc agarose-conjugated antibody. Immunoprecipitated proteins were separated on 10% SDS-PAGE followed by western blotting with anti-Myc (IP: α cMyc, IB: α cMyc) or anti-hFGFR3 (IP: α cMyc, IB: α hFGFR3) antibodies. (C) HEK-293 cells were transiently transfected with R3 WT and five or ten times excess amount of KD mutant, as indicated. The DNA amount was equalized with empty vector. After 8 hours in serum-free medium, cells were stimulated with FGF9 for 5 minutes, lysed and subjected to IP with anti-hFGFR3 antibody. Immunoprecipitated proteins were separated on 10% SDS-PAGE followed by western blotting with anti-phosphotyrosine (IB: α P-Tyr) or anti hFGFR3 (IB: α hFGFR3) antibodies.

shown in Fig. 2B, both WT and KD FGFR3 coprecipitated with both SOCS1 and SOCS3 in a similar pattern, confirming that both SOCS proteins constitutively associate with FGFR3, independently of receptor activation. To confirm the dominant-negative action of the KD mutant on the WT FGFR3, we cotransfected HEK-293T cells with constructs encoding WT hFGFR3 (R3-WT), and five or ten times excess of the construct encoding KD FGFR3. Following immunoprecipitation with anti-hFGFR3 antibodies, receptor tyrosine phosphorylation levels were checked in the presence or absence of FGF with anti-phosphotyrosine antibodies. As shown in Fig. 2C, FGFR3 was phosphorylated only in the presence of the ligand; in cells cotransfected with KD receptor, its phosphorylation was diminished in a dose-dependent manner.

To reveal the cellular localization of the FGFR-SOCS complex, we used constructs encoding: hFGFR3 C-terminally tagged with green fluorescent protein (R3-GFP); SOCS1 N-terminally tagged with Myc (S1-Myc) or cyan fluorescent protein (S1-CFP); and a C-terminal fusion of prolactin receptor (PRLR) to yellow fluorescent protein (PRLR-YFP). In the absence of ligand, the GFP-tagged FGFR3 localized to the cell membrane (Fig. 3A) (Lievens et al., 2004). Addition of FGF induced internalization of the receptor, as can be seen by its localization in the cytosol in diffused and granular form, and its clustering prominently in the perinuclear region (Fig. 3B). Similar localization of FGFR3-GFP was also observed when cotransfected with non-fluorescent SOCS1 in the absence of ligand (Fig. 3C). Expression of the CFP-tagged SOCS1 in HEK-293T cells in the absence of FGF was localized to the nucleus, and could not be detected in the membrane, cytosol or nucleolus (Fig. 3D), confirming the former results (Ben-Yair et al., 2002). Addition of the ligand induced a minor shift in the localization of SOCS1 and, although most of it could still be detected in the nucleus, some fluorescence was also found in the perinuclear cytosolic part of the cell (Fig. 3E). Transfection of cells with both FGFR3-GFP and SOCS1-CFP followed by exposure to FGF demonstrated colocalization of these proteins only in granular form clustered in the perinuclear fractions (Fig. 3F). As an additional control, we checked the colocalization of SOCS1-CFP with PRLR-YFP, a receptor from the cytokine family that is known to associate with SOCS1 (Helman et al., 1998; Pezet et al., 1999). As in the case of FGFR3, most of the SOCS was detected in the nucleus, whereas the PRLR was localized in the perinuclear cytosolic part of the cell, and the only site of colocalization was clustered in the perinuclear fractions (Fig. 3G). Although this might represent the actual site of interactions of these proteins, we cannot exclude the possibility that this localization in the Golgi and ER was owing to overloading of the translation machinery in the overexpressing cells, and the resultant insufficient vesicular trafficking of the membrane protein.

Constitutive activation of FGFR3, as in Achondroplasia, results in cellular accumulation of mature, undegraded receptor protein. To further study the effect of SOCS1 on FGFR3 recycling, we conducted a pulse-chase experiment comparing RCS cells that either do or do not express SOCS1. Both cell lines were pulse-labeled with [^{35}S]methionine and treated with FGF (20 ng/ml) and heparin (5 μg/ml) for the indicated times. Cell lysates were subjected to immunoprecipitation with anti-hFGFR3 antibodies, separated on 10% SDS-PAGE followed by fluorography. Fig. 3 IV shows a prolonged half-life of FGFR3 in the presence of SOCS1. In the parental RCS cells, the receptor declined dramatically after 60 minutes and almost completely disappeared after 120 minutes; by contrast, in the SOCS1-expressing cells, the metabolically labeled FGFR3 was still highly expressed even after two hours. These results

support the idea that, in the presence of SOCS1, FGFR3 is re-routed to recycling endosomes from its natural course of downregulation and degradation.

SOCS1 inhibits STAT1 phosphorylation and elevates MAPK phosphorylation in RCS cells

To investigate the consequences of the FGFR3-SOCS1 interaction on receptor activity, we used RCS cells capable of upregulating SOCS1 and SOCS3 following FGF treatment (Fig. 1). SOCS1 fused to Myc-encoding plasmid was introduced into an RCS chondrocytic cell line (RCS-S1) and pools of transfected cells expressing RCS-S1 were obtained and analyzed. To study phosphorylation of STAT1, WT (RCS-WT) and SOCS1-transfected RCS (RCS-S1) cells were treated with FGF. FGF induced STAT phosphorylation in a time-dependent manner, the strongest effect being observed at 2.5 minutes; this effect was partially attenuated in RCS-S1 cells (Fig. 4A). In an additional control experiment, interferon-α-induced STAT1 phosphorylation was also abolished in RCS-S1 cells but not in RCS-WT cells. Conversely, FGF-induced MAPK phosphorylation was markedly higher at the peak of expression (12 minutes) and was sustained for a longer period in the cells expressing SOCS1 (Fig. 4B), demonstrating the reciprocal effect of SOCS1 on these two FGFR3 pathways (i.e. inhibition of the STAT1 cascade and activation of the MAPK cascade).

SOCS1 elevates OPN mRNA levels in RCS cells through the MAPK pathway

To study the effect of SOCS1 on FGF-dependent OPN expression and MAPK activity, we first compared the effect of FGF on two cell lines. As the effect on OPN expression was detected only in RCS but not in HEK-293T cells (Fig. 5A), the subsequent experiment was carried out in RCS-WT and RCS-S1 cells treated with different concentrations of FGF and heparin. The expression level of OPN was upregulated by 10, and even more by 20 ng FGF/ml in RCS-WT cells, whereas the effect was much stronger in the RCS-S1 cells and could even be detected at 2.5 ng/ml FGF (Fig. 5B).

Although OPN is a known differentiation-sensitive marker in chondrocytes, the results presented in this work show that its expression is regulated by FGFR3, downstream of the MAPK cascade. Recently, we have shown that FGF signaling directly activates OPN expression independently of chondrocyte differentiation (Weizmann et al., 2005). As this was also demonstrated in RCS cells, which are a chondrocytic cell line that express type II collagen and do not differentiate in culture (Mukhopadhyay et al., 1995), we checked the differentiation status of RCS and RCS-S1 cells. As no changes or differences were detected using chondrocytic markers such as collagen types II and X expression and alkaline phosphatase activity (not shown), we conclude that, in RCS cells, SOCS altered FGF signaling and its downstream expression of the gene encoding OPN, but did not affect the differentiation state.

To check whether this effect of SOCS1 is also linked to MAPK phosphorylation, we used the MAPK kinase (MAPKK) inhibitor PD98059 (PD). RCS-WT and RCS-S1 cells were treated with PD for 30 minutes and then MAPK phosphorylation was tested in cell lysates 15 minutes after exposure to FGF (Fig. 6A). PD completely inhibited the FGF-induced phosphorylation of MAPK in RCS-WT cells; by contrast, in the RCS-S1 cells, traces of pMAPK bands were

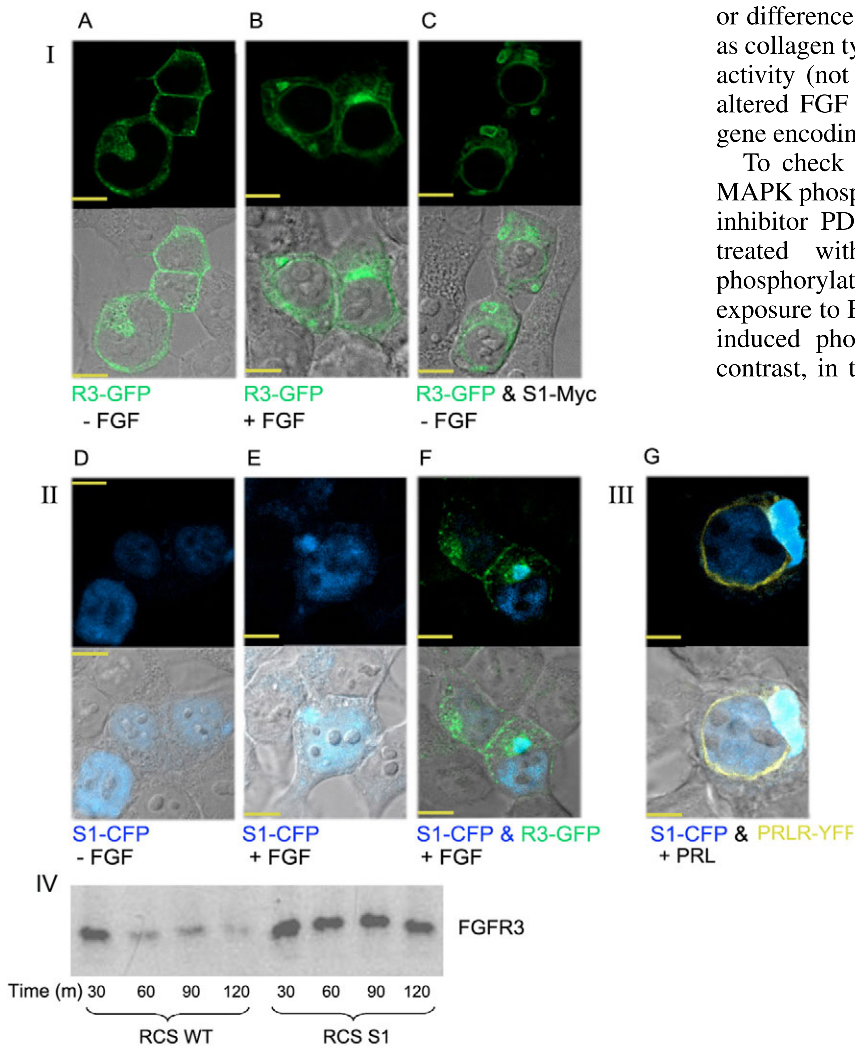

Fig. 3. Expression patterns of the fluorescently tagged proteins. Confocal images (top) and confocal images merged with transmitted-light images (bottom). (I) HEK-293T cells transiently transfected with GFP-tagged hFGR3 (R3-GFP) together with Myc-tagged SOCS1 (S1-Myc) (C) or empty vector (A,B). (II) HEK-293T cells transiently transfected with CFP-tagged SOCS1 (S1-CFP) together with R3-GFP (F) or empty vector (D,E). After 8 hours in serum-free medium, cells treated with or without 50 ng/ml FGF and 5 μg/ml heparin for 1 hour. (III) HEK-293T cells transiently transfected with S1-CFP together with PRLR (G). After 8 hours in serum-free medium, cells were treated with ovine prolactin (PRL) for 1 hour. (IV) RCS WT and RCS S1 cells were pulsed with [^{35}S]methionine for 30 minutes and treated with 20 ng/ml FGF and 5 μg/ml heparin. In the indicated periods of time, cells were lysed and subjected to immunoprecipitation with anti-hFGFR3 antibodies. Immunoprecipitated proteins were separated on 10% SDS-PAGE followed by fluorography. Bars, 10 μm.

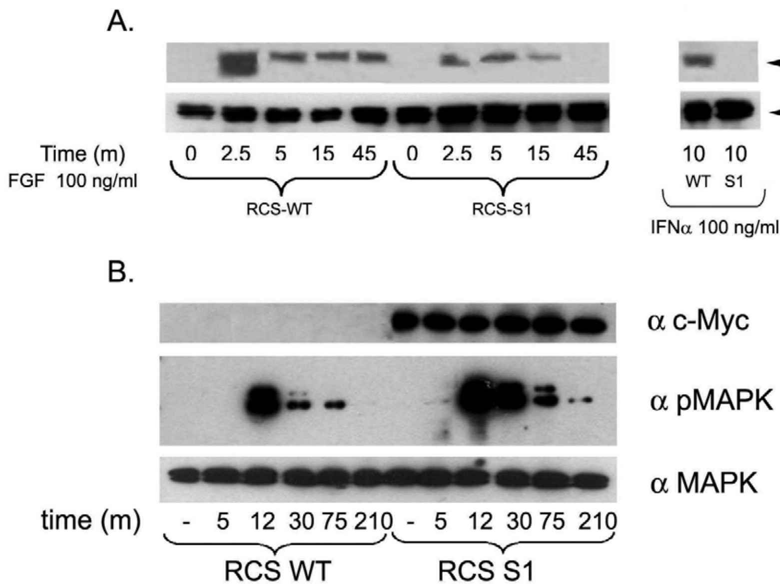

Fig. 4. SOCS1 inhibits STAT1 phosphorylation but elevates phosphorylated (p)MAPK levels in RCS cells. (A) RCS-WT and RCS-S1 cells were treated with 100 ng/ml FGF and 5 μg/ml heparin for different periods of time (as indicated) or with interferon-α (IFNα) after 8 hours in serum-free medium. Total cell lysates were subjected to 10% SDS-PAGE followed by western blotting with anti-pSTAT1 (α p-STAT1) and anti-STAT1 (α STAT1) antibodies. (B) RCS-WT and RCS-S1 cells were treated with 10 ng/ml FGF and 5 μg/ml heparin for different periods of time (as indicated) after 8 hours in serum-free medium. Total cell lysates were subjected to 10% SDS-PAGE followed by western blotting with anti-Myc (α c-Myc), anti-pMAPK (α pMAPK) and anti-MAPK (α MAPK) antibodies.

still visible in the presence of the inhibitor (Fig. 6A). Blocking of MAPK phosphorylation decreased OPN expression (Fig. 6B) in both cell types, although the effect was more pronounced in the cells expressing SOCS1, resulting in stronger inhibition despite the higher levels of OPN mRNA in these cells. Taken together, these results suggest that RCS cells expressing SOCS1 are more sensitive to FGFR signaling through the MAPK pathway.

Discussion

The negative control of cytokine- or FGFR-mediated signaling in chondrocytes by SOCS proteins has not yet been investigated. This report is the first to demonstrate the induction of SOCS1 and SOCS3 (but not SOCS2 or SOCS4) as a result of activation of FGFR in a chondrocytic cell line (RCS), which shows phenotypic stability and typical chondrocytic markers (Sahni et al., 1999). By contrast, no such expression was observed in the embryonic kidney HEK-293T cells despite the fact that both cell lines express FGFR. As SOCS1 and SOCS3 expression is mediated by the STAT pathway, our results confirm the existence of such a pathway in chondrocytes and its activation by FGFR (Hart et al., 2001; Legeai-Mallet et al., 1998; Xiao et al., 2004).

SOCS1 and SOCS3 proteins are structurally comparable: both are solitary SOCS proteins and contain the KIR sequence, which enables the binding and inhibition of JAK proteins in the cytokine signaling cascade (Fujimoto and Naka, 2003). In contrast to binding to cytokine receptors, which is ligand dependent (Nicola et al., 1999), both SOCS1 and SOCS3 are constitutively bound to FGFR3, independent of the activation state of the receptor, as also shown by their binding to the KD variant of FGFR3. Similar findings have been published for their interaction with EGFR, where these SOCS proteins bind to a YXDP motif through their SH2 domain even when the tyrosine residue is not phosphorylated (Xia et al., 2002). Furthermore, the SH2 domain of SOCS1 has been found to bind non-phosphorylated VAV protein through a YXDL motif that is also found on the cytosolic domain of FGFR3 (De Sepulveda et al., 2000; De Sepulveda et al., 1999). This YXDL motif includes residue Y760 of FGFR3, which is phosphorylated upon FGF-dependent receptor activation and, together with Y724, is required for maximal activation and translocation of STAT1 and STAT3 to the nucleus (Hart et al., 2000). However, whereas SOCS1 co-immunoprecipitated with the mature 130 kDa form of the receptor, SOCS3 co-immunoprecipitated with the 100 kDa non-glycosylated form. The physiological significance of this finding is not clear and requires additional investigation, especially since we could not find any such differential binding of SOCS1 and SOCS3 to insulin or EGF receptors (Johnston et al., 2003; Rui et al., 2002).

Exposure to FGF changed the localization of FGFR3 by shifting it from the membrane to the cytosol, confirming previous reports. Surprisingly, the expression of SOCS1 in the

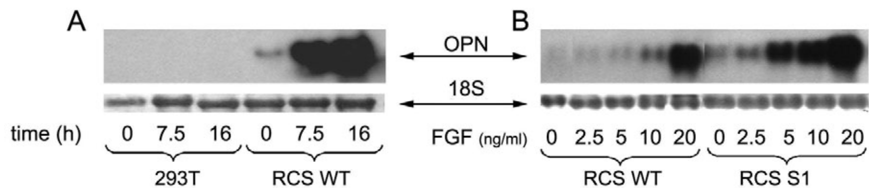

Fig. 5. SOCS1 elevate OPN mRNA in RCS cells. (A) RCS WT and HEK-293T cells were stimulated with 50 ng/ml FGF and 5 μg/ml heparin for different periods of time (as indicated) after 8 hours of starvation with serum-free medium. Northern analysis of 2 μg mRNA hybridized with the OPN probe. (B) RCS WT cells and RCS cells stably transfected with Myc-tagged SOCS1 (RCS S1) were starved for 8 hours in serum-free medium and then treated as indicated with different concentration of FGF and heparin for 24 hours. Northern analysis of 10 μg total RNA hybridized with the OPN probe. The amount of RNA on the membrane was visualized by Methylene-Blue staining of the 18S ribosomal RNA.

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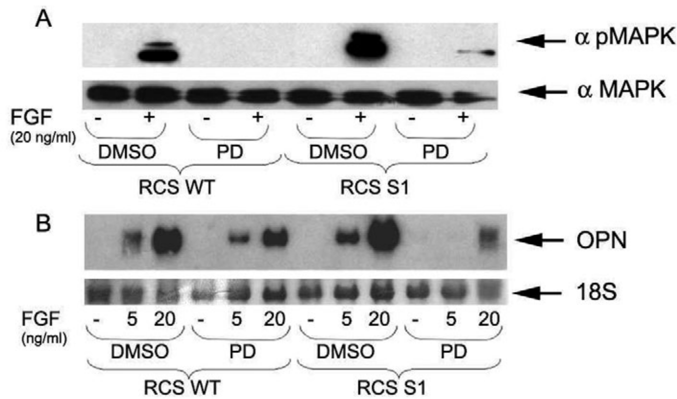

Fig. 6. PD decreases OPN level more dramatically in RCS SOCS1 than RCS WT cells. (A) RCS WT and RCS S1 cells were starved for 8 hours in serum-free medium, treated with the MAPKK inhibitor PD98059 (PD) or DMSO for 30 minutes and then treated with or without 20 ng/ml FGF and 5 μg/ml heparin for 15 minutes. Cell lysates were subjected to 10% SDS-PAGE followed by western blotting with anti-pMAPK (α pMAPK) and anti-MAPK (α MAPK) antibodies. (B) RCS WT and RCS S1 cells were starved for 8 hours in serum-free medium, treated with PD or DMSO for 30 minutes, and then treated as indicated with different concentration of FGF and heparin for 16 hours. Northern analysis of 10 μg total RNA hybridized with OPN probe. The amount of RNA on the membrane was visualized by Methylene-Blue staining of the 18S ribosomal RNA.

cells affected the FGFR3 localization in a similar way. Because, as shown in Fig. 3A-C, the overall amount of the receptor remained constant, we propose that, following internalization, FGFR3 was not ubiquitylated and degraded but rather recycled to the membrane. This is in contrast to the situation in which internalization of activated FGFR is followed by ubiquitylation and degradation, mediated by the association of Cbl with the Grb2 that is bound to FGFR through FRS2 (Cho et al., 2004; Monsonego-Ornan et al., 2002; Wong et al., 2002). Interestingly, mutations in FGFR3 that cause achondroplasia and TD, which have been found to obstruct the recruitment of Cbl to the receptor, shift FGFR3 from degradation to recycling and prolong its signaling. These mutated FGFR3 have been found in the recycling endosomes localized to the perinuclear fractions (Cho et al., 2004). In the present work, we found that FGFR3-GFP and SOCS1-CFP colocalized in granular form clustered in the perinuclear fractions. We further demonstrated that ectopic overexpression of SOCS1 in RCS cells prolonged the half-life of FGFR3, suggesting that SOCS1 inhibited FGFR3 degradation, possibly by re-directing the receptor to recycling endosomes; this is most probably a result of the inhibition of FGFR3-FRS2-Grb-Cbl complex formation.

Stimulation of FGFR3 enhances two major pathways: the STAT pathway, which inhibits chondrocyte proliferation (Sahni et al., 1999; Sahni et al., 2001); and the MAPK cascade, which contributes to matrix synthesis and inhibition of chondrocyte differentiation (Murakami et al., 2004; Yasoda et al., 2004). In the present work, we demonstrate that overexpression of SOCS1 in RCS cells attenuates FGF-induced STAT1 phosphorylation, similar to the activity of STAT in cytokine receptors; however, in parallel, it elevates the MAPK pathway by extending the duration of its signal. Data reporting extended TKR signaling owing to the inhibition of receptor degradation following internalization (Clague and Urbe, 2001; Waterman and Yarden, 2001) support the enhanced and prolonged MAPK phosphorylation, suggesting that the current dogma depicting the SOCS proteins exclusively as signal inhibitors (Johnston, 2004) is only partially correct. We conclude that the effects of SOCS proteins might be highly cell and receptor specific, as demonstrated by the binding of SOCS3 to RasGAP, which prolongs MAPK signaling (Cacalano et al., 2001), in contrast to the binding of SOCS1, -3 and -6 to insulin receptor, which results in the blockage of its MAPK, inositol (1,4,5)-trisphosphate 3-kinase, STAT5 and IRS signaling (Emanuelli et al., 2001; Mooney et al., 2001). Induction of the MAPK cascade by SOCS1 might also imply that the latter serves as a docking protein that binds to FGFR3, becomes phosphorylated, and subsequently recruits PTB and SH2-containing proteins, such as Shc and SHP2. These transduce the signal further downstream in the MAPK cascade (Kanai et al., 1997). Because FGF-inducible OPN expression was elevated in RCS cells transfected with SOCS1 relative to non-transfected cells, we tested the effect of MEK inhibitor on both MAPK activation and OPN expression in the RCS cell line. The inhibitor completely abolished MAPK activation and partially attenuated OPN expression, indicating a link between these activities, as shown previously in vascular endothelial cells (Li et al., 2002). The difference between the full and partial inhibition, along with the extensive inhibition of OPN expression in the presence of SOCS1, suggests that FGFR-signaling cascades other than MAPK are also involved in the regulation of FGF-induced OPN expression. By contrast, this might simply be attributable to the different time frames of FGF exposure (15 minutes versus 16 hours).

In conclusion, our work indicates that FGF-inducible SOCS1 (and possibly also SOCS3) in RCS cells binds constitutively to FGFR3, resulting in a change in balance between the STAT1 and MAPK pathways, attenuating the former and enhancing the latter. As SOCS1 expression can also be enhanced by growth hormone (Fasshauer et al., 2004; Garzon et al., 2004; Larsen and Ropke, 2002), our findings suggest a novel way of cross-talking between cytokine and RTK receptors.

Overactivation resulting from FGFR3 mutations during endochondral ossification has been shown to result in shorter stature (Bellus et al., 1995; Francomano, 1995; Rousseau et al., 1994). In view of the present results, we speculate that the SOCS-dependent enhancement of FGFR3-MAPK signaling, along with the simultaneous inhibition of growth hormone receptor signaling, might both enhance the short-bone phenotype.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM) was from GIBCO Invitrogen, and fetal calf serum (FCS) was from Biochemical Industries. Rabbit anti-human FGFR (hFGFR3) antibody, anti-STAT antibody, anti-Myc antibody and anti-Myc agarose-conjugated antibody were from Santa Cruz Biotechnology. Mouse anti-phospho-p44/42 MAPK antibody and PD98059 (PD), a selective and cell-permeable inhibitor of MAPKK activation, were obtained from Sigma-Aldrich. PD was dissolved in DMSO and diluted in serum-free medium before application to prevent its precipitation. Rabbit anti-p44/42 MAPK antibody was obtained from Cell Signaling Technology. Mouse anti-pSTAT antibody, and goat anti-rabbit and goat anti-mouse secondary antibodies were from Jackson ImmunoResearch Laboratories. FGF9 was obtained from ProChon Biotech.

Cell lines and transfections

Rat chondrosarcoma (RCS) cells, human embryonal kidney cells expressing large T-antigen (HEK-293T cells) and human articular chondrocyte cells were cultured in DMEM containing 10% FCS at 37°C in a 5% CO_2-enriched, humidified atmosphere. Transient transfection was performed with HEK-293T cells at ~60% confluence using the calcium phosphate method: DNA was mixed with $CaCl_2$ and 2× HBS (16 mg/ml NaCl, 0.74 mg/ml KCl, 0.25 mg/ml Na_2HPO_4, 2 mg/ml glucose, 10 mg/ml Hepes) on a vortex and the mixture was dripped on the cells. After transfection (36-48 hours), cells were harvested. Stable transfection was performed with RCS cells at ~60% confluence using the calcium phosphate method and, 36-48 hours after transfection, specific selection was performed using Geneticin Selective Antibiotic (G418 sulfate; GIBCO Invitrogen). After 4-6 weeks in selective medium, cells were pooled and used for the experiments.

Constructs

cDNAs of wild-type (WT) FGFR3, kinase-dead (KD; K508A) mutant FGFR3 (Monsonego-Ornan et al., 2002), Myc-tagged SOCS1, Myc-tagged SOCS3, green fluorescent protein (GFP)-tagged hFGFR3, cyan fluorescent protein (CFP)-tagged SOCS1 and yellow fluorescent protein (YFP)-tagged prolactin receptor (PRLR) were inserted into the pcDNA3 expression vector (Ben-Yair et al., 2002) (Invitrogen). In all experiments, DNA amounts were equalized with empty vector (pcDNA3).

Probe preparation

The probe for rat osteopontin (OPN) was prepared by PCR amplification of cDNA from a rat chondrocyte cell line using the forward and backward primers 5′-GACCATGAGATTGGCAGTGA-3′ and 5′-CTTGTCCTTGTGGCTGTGAA-3′, respectively. Probes for human SOCS3 and mouse SOCS1 were prepared by digestion of ~1000 bp fragments from pcDNA3 carrying Myc-SOCS1/SOCS3, using *Xho*I/*Hin*dIII for SOCS1 and *Mcs*I/*Hin*dIII for SOCS3. The inserts were used as probes for northern blot analysis.

RNA isolation and northern blot analysis

Cells were plated in 10 cm culture dishes, grown to confluence and dissolved in 1 ml of Trizol. Total RNA was extracted and mRNA was isolated from total RNA when needed using an mRNA isolation kit (Roche Diagnostics). Total RNA (10 μg) or mRNA (2 μg) was denatured, electrophoresed on a 1% agarose-formaldehyde gel, and transferred to 0.2 μm Nytran membranes. The RNA blots were hybridized with ^{32}P-labeled cDNA probes (Tong et al., 2003).

Imaging

HEK-293T cells were plated on polylysine-coated cover slips in 3.5 cm tissue-culture dishes and cultured in DMEM containing 10% FCS. After reaching ~60% confluence, the cells were transfected with 0.5 μg DNA using the calcium phosphate method and, 36-48 hours after transfection, cells were fixed on cover slips using 4% paraformaldehyde. Cell images were acquired using a confocal laser-scanning inverted microscope system (CLSM; OLYMPUS IX 81), with a 60× water-immersion objective lens. The cells transfected with CFP constructs were imaged at an excitation wavelength of 458 nm with a 480-495 nm emission filter; the GFP constructs were imaged separately at an excitation wavelength of 488 nm with a 515-525 nm emission filter; and the YFP constructs were imaged at an excitation wavelength of 515 nm with a 535-565 nm emission filter. Transmitted-light images were acquired using Nomarski differential interference contrast. Cell images were acquired using a CLSM system (CLSM 510, Zeiss), including a Zeiss Axiovert-100M microscope with a 63× water-immersion objective lens (or as indicated).

Immunoprecipitation and western blot analysis

HEK-293T cells were plated in 10 cm culture dishes. After reaching ~60% confluence, the cells were transfected with a total of 4 μg DNA using the calcium phosphate method and, 36-48 hours after transfection, the cells were dissolved in 1 ml of lysis buffer (1 mM EGTA, 50 mM Tris 7.5, 150 mM NaCl, 10% glycerol, 1% NP40) and clarified by centrifugation at 12,000 ***g*** for 15 minutes. Protein concentration was measured using a BCA protein assay reagent kit (Pierce Biotechnology). Cellular lysate (1 mg protein) was incubated overnight with 20 μg of rabbit anti-Myc agarose-conjugated antibody at 4°C, washed four times (50 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and resuspended in sample buffer. Immunoprecipitated proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Blots were processed using rabbit anti-Myc or rabbit anti-hFGFR3 antibodies, and goat anti-rabbit secondary antibody. Protein bands were visualized using horseradish peroxidase and an EZ kit (Biochemical Industries) according to the manufacturer's instructions.

Pulse-chase labeling experiments

RCS cells were cultured in methionine-depleted medium for 3 hours, after which [^{35}S]methionine (150 mCi/ml) was added for 30 minutes. Cells were washed extensively with DMEM and incubated at 37°C for various times. Then the cells were extracted, and lysates were precipitated with immobilized anti-FGFR3 antibodies. Proteins were separated by 10% SDS-PAGE and visualized by autoradiography.

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