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Experimental Cell Research 298 (2004) 239–248

Experimental Cell Research

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The suppressor of cytokine signaling (SOCS)-7 interacts with the actin cytoskeleton through vinexin

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Received 17 December 2003, revised version received 16 March 2004
Available online 6 May 2004

Abstract

To understand the function of the suppressor of cytokine signaling (SOCS)-7, we have looked for proteins interacting with SOCS-7 in a stringent yeast two-hybrid screen of a human leukocyte cDNA-library. We identified the cytoskeletal molecule vinexin as a partner interacting with SOCS-7. Tests with deletion mutants of SOCS-7 demonstrated that a central region of the molecule containing several proline-rich regions, N-terminal to the SH2 domain, was responsible for the binding to vinexin. It is thus likely that one of the SH3 domains of vinexin interacts with a poly-proline region of SOCS-7. The interaction with vinexin was confirmed biochemically as vinexin-α was co-precipitated with SOCS-7. Confocal laser-scanning microscopy in HEK293T, MCF-7, and 3T3-L1 cells showed that part of the transfected SOCS-7-green fluorescent protein (GFP) molecules merged with vinexin and with actin. Taken together, our data indicate that SOCS-7 interacts with vinexin and the actin cytoskeleton.

Keywords: Actin; Vinculin; Nck; Signal transduction; Vinexin

Introduction

Recently, several studies on the regulation of the cellular response to cytokines, hormones and growth factors focused on the family of suppressors of cytokine signaling or SOCS-factors. Eight SOCS-factors have been identified, namely CIS and SOCS-1 to -7, of which CIS, SOCS-1, -2, and -3 have been studied in some detail and have been described as modulators of signaling through the Jak-Stat and MAP-kinase pathways [1,2]. The analysis of the phenotypes of SOCS-knock-out mice strongly suggests that SOCS-factors serve important functions in, for example, interferon-γ, erythropoietin, growth hormone, prolactin, insulin-like growth factor I, and T-cell receptor signaling [1–4]. Important roles for SOCS-factors have been discovered in various cell types, including leukocytes, hepatocytes, and brain cells [1–8]. Some SOCS-factors act as anti-oncogenes [9].

In contrast to other SOCS-factors, SOCS-7 has been given much less attention. In 1997, Matuoka et al. [10] cloned a cDNA encoding a partial human SOCS-7 (hSOCS-7), called NAP-4, from a fetal brain cDNA-library as a partner of the adaptor protein Nck. This interaction has been confirmed by a glutathione-*S*-transferase pull-down assay. Interactions were also shown between SOCS-7 and Grb-2, phospholipase C-γ, the epidermal growth factor-receptor (EGF-R), and the insulin receptor substrates (IRS) 2 and 4. SOCS-7 is expressed in various cell types and tissues (most strongly in testis and brain), but so far, no functional activity of SOCS-7 has been reported [10,11]. To understand its function, we have initiated a search for new partners of SOCS-7. In a yeast two-hybrid screen of a

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doi:10.1016/j.yexcr.2004.04.002

human leukocyte cDNA-library, we isolated, among others, a cDNA encoding part of the cytoskeletal protein vinexin. Two isoforms of vinexin have been described, namely vinexin-α and -β, which share a common carboxyl-terminal sequence containing three SH3 domains. Vinexin localizes at cell–cell and cell–extracellular matrix junctions and is a binding partner of the cytoskeletal protein vinculin [12]. We here document the interaction of SOCS-7 with vinexin and the actin cytoskeleton and also its presence at the cell membrane, in the cytoplasm and in 3T3-L1, but not in HEK293T or MCF-7 cells, in the nucleus.

Materials and methods

Plasmids and constructs

The pGEX-2T vector containing the NAP-4 cDNA was kindly provided by K. Matuoka (University of Tokyo, Japan) [10]. The NAP-4 insert and its deletion mutants (PCR-generated; Fig. 1C) were sub-cloned next to the Gal4 DNA-binding domain (BD) between *Eco*RI and *Bam*HI restriction sites of pGBT9 using a PCR-based strategy [13,14]. In the next sections, pGBT9-NAP-4 denotes the pGBT9 vector containing the NAP-4 insert. HA-tagged hSOCS-7 cDNA was sub-cloned between *Xba*I and *Hin*dIII restriction sites of peGFP-C1 [kindly provided by C. Heirman (Free University of Brussels, Belgium)] using a PCR-based strategy. Full-length SOCS-7 cDNA has been cloned from human liver (Fig. 1A; P. Wang et al., manuscript in preparation). YFP-NAP-4 cDNA was engineered as previously described for other SOCS factors [15].

Yeast two-hybrid screening

Saccharomyces cerevisiae Y190 cells containing the two reporter genes *HIS3* and *lacZ* were transformed with pGBT9-NAP-4 and subsequently subjected to large scale transformations with 60 μg of a human leukocyte cDNA-library fused to the Gal4 activation domain (AD) in the pACT2 vector (Clontech through Becton Dickinson, Erembodegem, Belgium), according to the two-hybrid system TRAFO protocol [16]. The expression of BD-NAP-4 was verified by Western blot using anti-Gal4-BD antibody (Clontech). In total, 10.9×10^6 transformants were screened, which should have allowed to pick up all partners, as the library contained 3.5×10^6 independent clones. Double transformants were plated on synthetic dropout (SD) medium lacking uracil, tryptophan, leucine, and histidine and supplemented with 50 mM 3-amino-

A.

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   1  ATGGTGTTCCGCAACGTGGGTCGGCCGCCGGAGGAGGAGGACGTGGAGGCGGCCCCGGAG
  61  CCGGGACCCTCGGAACTGCTGTGTCCCCGGCACCGCTGTGCCCTGGACCCCAAGGCCCTG
 121  CCGCCGGGCTTGGCGCTCGAGCGGACCTGGGGCCCGGCGGCTGGACTAGAGGCGCAGTTG
 181  GCGGCTCTGGGGCTCGGGCAGCCGGCGGGGCCGGGGGTCAAGACAGTCGGTGGGGGTTGC
 241  TGCCCGTGTCCGTGTCCTTCTCAGCCGCCCCCTCCGCAGCCCCAGCCGCCTGCTGCCGCC
 301  CCGCAGGCCGGGGAGGACCCCACGGAAACGAGCGACGCGCTGCTGGTCCTGGAGGGCTTG
 361  GAATCGGAGGCCGAGAGCCTGGAGACTAACAGCTGCTCGGAAGAGGAGCTCAGCAGCCCG
 421  GGTCGCGGAGGAGGAGGGGGCGGCCGGCTTCTGCTGCAGCCCCCAGGCCCTGAATTACCT
 461  CCGGTGCCCTTCCCGCTGCAGGACTTGGTCCCTCTGGGGCGCCTGAGTAGAGGGGAGCAG
 481  CAGCAGCAGCAGCAGCAGCAACCTCCCCCGCCCCCGCCTCCTCCCGGGCCCCTCCGGCCA
 541  CTCGCGGGTCCTTCTCGGAAGGGCTCCTTCAAAATCCGCCTCAGTCGCCTCTTTCGCACC
 601  AAGAGCTGCAACGGTGGCTCCGGCGGTGGGGATGGGACCGGCAAGAGGCCTTCTGGAGAG
 661  CTGGCTGCTTCAGCTGCGAGCCTGACAGACATGGGAGGCTCTGCGGGCCGGGAGCTGGAC
 721  GCGGGGAGGAAACCCAAGTTGACAAGAACTCAAAGTGCCTTTTCTCCGGTCTCCTTCAGC
 781  CCCCTGTTCACAGGTGAAACTGTGTCGCTTGTGGATGCGGACATTTCTCAGCGGGGCCTG
 841  ACCTCTCCACACCCTCCAACTCCCCCTCCTCCTCCGAGAAGAAGCCTCAGCCTCCTAGAT
 901  GCATTTCCCCGGATTGCTCCCATCCGAGCAGCTGAATCCCTGCACAGCCAACCCCCACAG
 961  CACCTTCAGTGTCCCCTCTACCGGCCTGACTCGAGCAGCTTTGCAGCCAGCCTTCGAGAG
1021  TTGGAGAAGTGTGGTTGGTATTGGGGGCCAATGAATTGGGAAGATGCAGAGATGAAGCTG
1081  AAAGGGAAACCAGATGGTTCTTTCCTGGTACGAGACAGTTCTGATCCTCGTTACATCCTG
1141  AGCCTCAGTTCCCGATCACAGGGTATCACCCACCACACTAGAATGGAGCACTACAGAGGA
1201  ACCTTCAGCCTGTGGTGTCATCCCAAGTTTGAGGACCGCTGTCAATCTGTTGTAGAGTTT
1261  ATTAAGAGAGCCATTATGCACTCCAAGAATGGAAAGTTTCTCTATTTCTTAAGATCCAGG
1321  GTTCCAGGACTGCCACCAACTCCTGTCCAGCTGCTCTATCCAGTGTCCCGATTCAGCAAT
1381  GTCAAATCCCTCCAGCACCTTTGCAGATTCCGGATACGACAGCTCGTCAGGATAGATCAC
1441  ATCCCAGATCTCCCACTGCCTAAACCTCTGATCTCTTATATCCGAAAGTTCTACTACTAT
1501  GATCCTCAGGAAGAGGTATACCTGTCTCTAAAGGAAGCGCAGCTCATTTCCAAACAGAAG
1561  CAAGAGGTGGAACCCTCCACGTGGCGAGGGGCTCCCCGCTGGTCACCACCAAGGGCATTT
1621  GGTTGCCAAGCTCCAGCTTTGCAGAACCAAATTAAGCTACCATGA
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Fig. 1. (A) Sequence of hSOCS-7 cDNA. (B) Amino acid (AA) alignment of the deduced hSOCS-7 protein sequence with mSOCS-7. There is 96% identity at the AA level between the human and mouse SOCS-7 protein sequences (not taking into account the 34 AA missing in the N-terminal region and the 27 additional AA at the C-terminus). The putative NES, starting at AA 56, is underlined with canonical L in bold; the poly-proline stretches are boxed. The murine sequence was taken from Swissprot: locus SOCS7_MOUSE, accession Q8VHQ2. (C) Schematic representation of the protein structure of hSOCS-7 and deletion mutants used in this study. In the NAP-4 protein, 126 AA are missing at the N-terminal side. The rest of the NAP-4 protein sequence is nearly identical with that of hSOCS-7. However, the 34 AA insertion after AA 321 is found as in mice and the sequence of that part exactly matches mSOCS-7. Black bars represent proline-rich stretches; the broken line represents the putative leucine-rich nuclear export signal; the white box between AA 509 and 552 represents the SOCS-box.

B.

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hSOCS-7      1 MVFRNVGRPPEEEDVEAAPEPGPSELLCPRHRCALDPKAL
mSOCS-7      1 MVFRNVGRPPEEEDAEAAREPGPSELLCPRHRCALDPKAL
               ************** *** **********************

hSOCS-7     41 PPGLALERTWGPAAGLEAQLAALGLGQPAGPGVKTVGGGC
mSOCS-7     41 PPGLALERTWGPVAGLEAQLAALGLGQPAGPGIKTAGGGC
               ************ ******************** ** ****

hSOCS-7     81 CPCPCPSQPPPPQPQPPAAAPQAGEDPTETSDALLVLEGL
mSOCS-7     81 CPCPCPPQPPPPQPPPPAAAPQAGEDPTETSDALLVLEGL
               ****** ******* *************************

hSOCS-7    121 ESEAESLETNSCSEEELSSPGRGGGG-GGRLLLQPPGPEL
mSOCS-7    121 ESEAESLETNSCSEEELSSPGRGGGGVGGRLLLQPPGPEL
               ************************** *************

hSOCS-7    160 PPVPFPLQDLVPLGRLSRGEQQQQQQQQPPPPPPPPGPLR
mSOCS-7    161 PPVPFPLQDLVPPGRLSRGEQQQQQ---PPPPPPPPGPLR
               ************ ************   *************

hSOCS-7    200 PLAGPSRKGSFKIRLSRLFRTKSCNGGSGGGGDGTGKRPSG
mSOCS-7    198 PLAGPSRKGSFKIRLSRLFRTKSCNGGSGGGGDGTGKRPSG
               ****************************************

hSOCS-7    240 ELAASAASLTDMGGSAGRELDAGRKPKLTRTQSAFSPVSF
mSOCS-7    238 DLAASAASLTDMGGSAVRELDTGRKPRLTRTQSAFSPVSF
                ***************** **** **** *************

hSOCS-7    280 SPLFTGETVSLVDADISQRGLTSPHPPTPPPPPRRSLSLL
mSOCS-7    278 SPLFTGETVSLVDVDISQRGLTSPHPPTPPPPPRRSLSLL
               ************* ************************

hSOCS-7    320 D---------------------------------AFPRI
mSOCS-7    318 DDISGTLPTSVLVAPMGSSLQSFPLPPPPPPHAPDAFPRI
               *                                  *****

hSOCS-7    360 APIRAAGSLHSQPPQHLQCPLYRPDSSSFAASLRELEKCG
mSOCS-7    358 APIRASESLHSQPPQHLQCPLYRPDSSSFAASLRELEKCG
               *****  *********************************

hSOCS-7    400 WYWGPMNWEDAEMKLKGKPDGSFLVRDSSDPRYILSLSSR
mSOCS-7    398 WYWGPMNWEDAEMKLKGKPDGSFLVRDSSDPRYILSLSFR
               ************************************ *

hSOCS-7    440 SQGITHHTRMEHYRGTFSLWCHPKFEDRCQSVVEFIKRAI
mSOCS-7    438 SQGITHHTRMEHYRGTFSLWCHPKFEDRCQSVVEFIKRAI
               ****************************************

hSOCS-7    480 MHSKNGKFLYFLRSRVPGLPPTPVQLLYPVSRFSNVKSLQ
mSOCS-7    478 MHSKNGKFLYFLRSRVPGLPPTPVQLLYPVSRFSNVKSLQ
               ************************************

hSOCS-7    520 HLCRFRIRQLVRIDHIPDLPLPKPLISYIRKFYYYDPQEE
mSOCS-7    518 HLCRFRIRQLVRIDHIPDLPLPKPLISYIRKFYYYDPQEE
               ****************************************

hSOCS-7    560 VYLSLKEAQLISKQKQEVEPSTWRGAPRWSPPRAFGCQAP
mSOCS-7    558 VYLSLKEAQLISKQKQEVEPST
               **********************

hSOCS-7    600 ALQNQIKLP
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Fig. 1. (*continued*).

1,2,4-triazole (Sigma Aldrich, Bornem, Belgium). Plates were incubated at 30°C for 3–7 days. Resulting colonies were streaked on SD medium lacking uracil, tryptophan, and leucine. β-galactosidase production was visualized in an overlay plate assay. Plates were incubated overnight at 30°C. Blue colonies were restreaked and positive staining was verified by repeating the β-galactosidase assay.

To identify the candidate partners, yeast colonies containing interacting proteins were grown overnight at 30°C in SD medium lacking uracil, tryptophan, and leucine. Cells were lysed in equal volumes of lysis buffer (2% Triton X-100, 1%

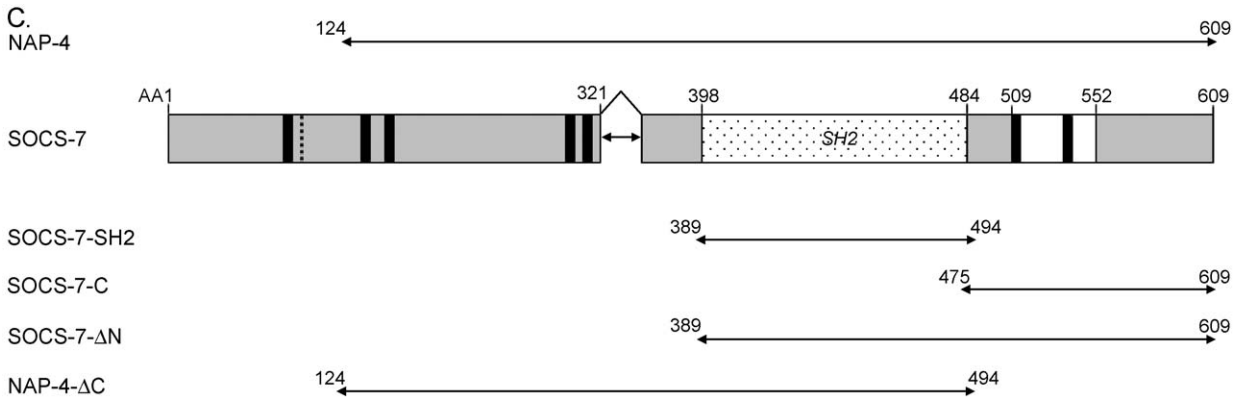

Fig. 1. (*continued*).

SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA), 100 μl glass beads (Sigma) and phenol/chloroform/isoamyl alcohol pH 6.8. Plasmids were purified using the "Concert™ Rapid plasmid miniprep system" (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions and transformed in the *leu*$^-$ *E. coli* strain KC8. Transformants were plated on LB plates containing ampicillin and grown overnight at 37°C. Cells were replica-plated on M9 minimal medium containing ampicillin and lacking leucine, thereby selecting the leucine marker on the pACT2 vectors, and grown overnight at 37°C. Resulting colonies were grown overnight at 37°C in LB medium with ampicillin and plasmids were purified as mentioned above. All cDNA-inserts encoding interacting proteins were identified by sequencing the whole insert using an Applied Biosystems sequencer. Sequence analysis was performed by online BLAST searches.

Cell culture and transfection

Human embryonic kidney (HEK) 293T and breast cancer MCF-7/6 cells (hereafter MCF/7 Ref. [17]) were obtained, respectively, from R. Beyaert and M. Mareel (University of Ghent, Belgium) and grown in DMEM/10% fetal calf serum with 1% penicillin/streptomycin/amphotericin (Invitrogen). 3T3-L1 fibroblasts were obtained from H. Heimberg (Free University of Brussels, Belgium) and grown in DMEM (4500 mg/ml glucose)/10% newborn calf serum with 1% penicillin/streptomycin/amphotericin.

For transfection of HEK293T and MCF-7 cells, 25×10^3 cells were plated on 13 mm cover slips (Thermanox 174950, VWR International, Leuven, Belgium) in 0.5 ml medium per well of a 24-well plate. Cells were grown overnight in a 37°C/5% CO_2 incubator. Plasmid DNA (200 ng) was transfected using Fugene 6 reagent (Roche, Vilvoorde, Belgium) according to the manufacturer's instructions. For immunoprecipitation experiments, 3T3-L1 cells were seeded at a density of 1.5×10^5 cells/ml per well of a 6-well plate and incubated overnight. Transfection was carried out with Lipofectamine Plus reagent (Invitrogen), as described by the manufacturer using 8 μl Lipofectamin, 8 μl Plus reagent and 2 μg DNA. For microscopy studies, 3T3-L1 cells were seeded on cover slips in 24-well plates at a density of 1×10^5 cells/ml per well, grown overnight and transfected with a mixture of 1 μl Lipofectamin, 4 μl Plus reagent and 400 ng DNA.

Production of polyclonal antibodies

Rabbit antiserum was raised against the N-terminal peptide (AA 1-13) of mouse SOCS-7 (kindly synthesized by P. Robberecht, Université Libre de Bruxelles, Belgium) coupled to keyhole limpet hemocyanin (Calbiochem, La Jolla, CA). Two rabbits were immunized. Blood was taken after the third boost and serum was subjected to ammonium sulphate precipitation. The precipitate was dissolved in the original volume water and dialyzed to yield "rabbit anti-SOCS-7 γ-globulins".

Immunoprecipitation and Western blotting

Cells were rinsed with PBS twice at room temperature, collected in microcentrifuge tubes and placed on ice. All subsequent steps were performed at 4°C. Cells were lysed for 15 min in lysis buffer (50 mM HEPES pH 7.6, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 200 μM Na_3VO_4, 10 mM NaF, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM AEBSF) and then microcentrifuged at 20000 × *g* for 10 min. For analysis of total cell extracts, aliquots of supernatants were mixed 1:1 with 2× SDS-sample buffer and incubated in boiling water for 5 min. Supernatants were mixed with 2 μg anti-HA antibodies (clone 3F10, Roche) for 1 h by rotation. Immune complexes were collected by rotation with a 50% protein A-agarose suspension (Sigma Aldrich) for 3 h followed by brief centrifugation. Pellets were washed twice with lysis buffer, twice with lysis buffer containing 1 M NaCl, and finally twice with lysis buffer. Bound proteins were eluted with SDS-sample buffer and incubated in boiling water for 5 min. Lysates and immunoprecipitates were resolved on a 10% SDS-polyacrylamide gel, blotted onto

PVDF-membranes and detected with affinity purified rabbit antibody against vinexin (kindly provided by N. Kioka) as described [12]. For detection of SOCS-7, the membrane was stripped with 0.25 M NaCl for 10 min, blocked with PBS/0.2% Tween-20/5% non-fat dry milk and incubated with rabbit anti-SOCS-7 γ-globulins (1:2000) overnight at 4°C. SOCS-7 was visualized with donkey anti-rabbit IgG horseradish peroxidase-conjugated antibody (Amersham-Pharmacia Biotech, Roosendaal, The Netherlands) and developed using an ECL kit (Perkin Elmer Life Sciences, Zaventem, Belgium).

Immunocytochemistry

To stain filamentous actin together with vinexin, the following procedure was used. After washing with PBS, fixation with paraformaldehyde 4% (10 min at room temperature) was performed. Subsequently, cells were permeabilized with acetone for 3 min at −20°C. Cells were then incubated with 2.3 μg/ml rabbit anti-vinexin. This step was omitted in control experiments. After washing, rhodamine-conjugated goat anti-rabbit antibody (1:200; Lucron, De Pinte, Belgium) was added together with Alexa Fluor 633-phalloidin solution (Molecular Probes, Leiden, The Netherlands), which stains filamentous actin, for 1 h at room temperature [18]. Slides were washed extensively with PBS and mounted with PBS/50% glycerol. The samples were viewed using confocal laser-scanning microscopy (CLSM) as described below.

CLSM

Samples were viewed and recorded with a Leica TCS-SP confocal laser-scanning microscope installed on a Leica DM-IRBE inverted microscope, using a 63× oil objective lens as described [19]. The optical system is equipped with an argon-laser and a helium/neon-laser and is running under the Leica TCS-NT image analysis software (version 1.6.587). Subsequently, images were transferred to Adobe Photoshop 5.5 software for color channel analysis and figure assembly. The magnification of the microscope was calibrated using fluoresbrite™ calibration grade microspheres (Ø 3.0 μm, Polylab, Merksem, Belgium).

Results

Human SOCS-7

Experiments were initially performed with NAP-4, a partial cDNA of hSOCS-7. Recently, we obtained a full-length hSOCS-7 cDNA clone with 378 additional nucleotides at the 5′-end (Fig. 1A; P. Wang et al., manuscript in preparation). The open reading frame encodes a protein of 64 kDa (Fig. 1B). If one excepts a 34 AA deletion (encoded by exon 4) after AA 321 and three polymorphisms (A/V at position 293, S/F at position 438 and Q/K at position 602), our clone encodes a protein identical to NAP-4 from AA 127. hSOCS-7 is also highly homologous to mSOCS-7. However, in hSOCS-7, 34 AA are missing in the N-terminal region and there are 27 additional AA at the C-terminus. For the shared regions, there is a 96% AA identity and a 99% AA identity in the SH2 domain and the SOCS-box (Figs. 1B and C). A putative nuclear export sequence (NES) is present between AA 56 and 65 (Fig. 1B).

Vinexin as a novel binding partner of SOCS-7

To get further insight into the function of SOCS-7, we first performed a yeast two-hybrid screen using NAP-4 as a bait to identify SOCS-7-binding proteins. A cDNA-library constructed from human leukocyte poly (A)+RNA was screened and 16 independent clones positive for both histidine auxotrophy and β-galactosidase activity were isolated. Sequence analysis revealed that they encoded 10 different proteins. One of these clones, NM21, contained a large fragment of the vinexin cDNA corresponding to a region common to both α- and β-isoforms of vinexin and actually encompassing the whole vinexin-β, with the exception of 120 nucleotides at the 5′ end. Clones corresponding to large fragments of the src-kinases Hck and Lck, the adaptor protein Nck-2, and the signal transducer and activator of transcription (Stat) 3 were also isolated (not shown).

To localize the vinexin-binding site in SOCS-7, various deletion mutants of NAP-4 were generated and fused to the Gal4-BD (Fig. 1C). A yeast two-hybrid assay using the deletion mutants as bait and NM21-Gal4-AD as prey was performed. Interaction with NM21 occurred only in transformants containing the N-terminal domain of NAP-4, which corresponds to a central part of SOCS-7 between the N-terminal region (missing in NAP-4) and the SH2 domain (Fig. 2). Other deletion mutants were effectively expressed, as indicated by Western blot analysis of Gal4-BD (results not shown). In addition, each deletion mutant interacted with at least one partner. The construct containing only the N-terminal domain of NAP-4 could not be used in these experiments as it resulted in transactivation of the *lacZ* reporter gene in the absence of NM21-Gal4-AD. As control, β-galactosidase staining was done on yeast cells expressing the NAP-4-encoding construct and the Gal4-AD alone (pACT2 empty) and found negative (Fig. 2).

SOCS-7 interacts with vinexin-α in mammalian cells

To investigate the in vivo association of SOCS-7 with vinexin, co-immunoprecipitation experiments were performed. Therefore, plasmids containing HA-tagged SOCS-7 cDNA were transfected into 3T3-L1 fibroblasts. In whole cell extracts, anti-vinexin antibodies detected both vinexin-α and -β, including a 75-kDa isoform of vinexin-α (Fig. 3). This lower molecular weight vinexin-α protein has been observed before [12]. As shown in Fig. 3, 82 kDa vinexin-

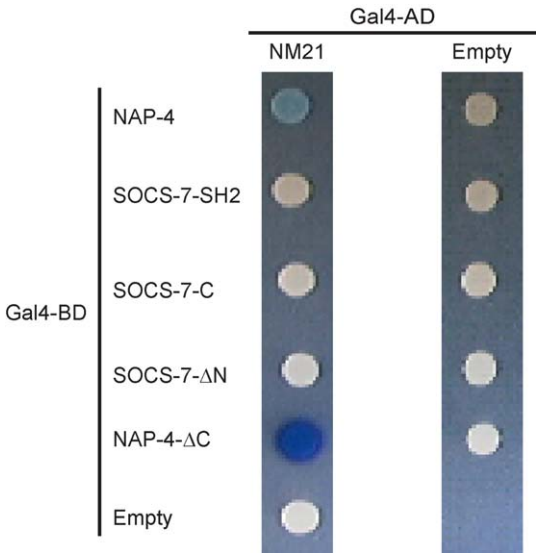

Fig. 2. Yeast two-hybrid interaction between NAP-4 and the product from clone NM21. Yeast cells expressing Gal4-AD-NM21 and Gal4-BD-NAP-4 or its deletion mutants, were assayed for β-galactosidase production. Both NAP-4 and the construct lacking the C-terminal AA sequence of NAP-4 interact with the AD-coupled insert from clone NM21. As the isolated SH2 domain does not interact with the product of NM21, the interaction occurs probably through the N-terminal region of NAP-4. The yeast two-hybrid assay was not performed with SOCS-7 (1-575) neither with the construct containing only the N-terminal domain of NAP-4 as the latter resulted in transactivation of the *lacZ* reporter gene in the absence of NM21-Gal4-AD. No interaction was detected between BD-NAP-4 or its deletion mutants and AD alone (empty).

α was co-precipitated with SOCS-7. SOCS-7 also interacted with 75 kDa vinexin-α, but to a smaller extent. Similar results were obtained after transfection of myc-SOCS-7 and immunoprecipitation with mouse anti-myc antibodies on one hand, and after transfection of NAP-4 and immunoprecipitation with goat anti-NAP-4 antibodies on the other hand (results not shown). Taken together, these observations suggest that SOCS-7 interacts with vinexin-α in mammalian cells.

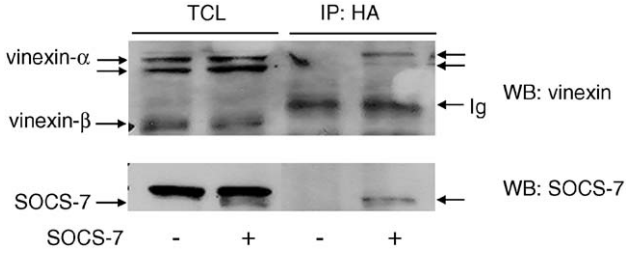

Fig. 3. Interaction between SOCS-7 and vinexin-α in 3T3-L1 fibroblasts overexpressing HA-tagged SOCS-7 as shown by immunoprecipitation using anti-HA antibodies. Cells were transfected with HA-SOCS-7 (+) or empty vector (−). Total cell lysates (TCL, left) or immunoprecipitates with anti-HA (IP:HA, right) were subjected to Western blot analysis. Upper part: although the anti-vinexin antibodies recognize both α- and β-isoforms in TCL (left), only vinexin-α was co-precipitated with SOCS-7 (right). Lower part: the blot was stripped and reprobed with rabbit anti-SOCS-7 γ-globulins to show expression and precipitation of HA-SOCS-7. In TCL, a major cross-reacting band (which is not endogenous SOCS-7) is present in both transfected and non-transfected cells.

Sub-cellular localization of SOCS-7 and co-localization with vinexin and actin

To determine whether SOCS-7 and vinexin co-localize, a plasmid construct coding for GFP-SOCS-7 was engineered and transfected into HEK293T cells. As control, a plasmid coding for GFP only was transfected. Overexpression of SOCS-7 had no clear effect on the morphology of the cell. The sub-cellular localization of SOCS-7 was observed by GFP fluorescence, and the same cells were also stained with rabbit anti-vinexin and rhodamine-coupled goat anti-rabbit antibodies. The staining for vinexin yielded the same picture whether the cells were

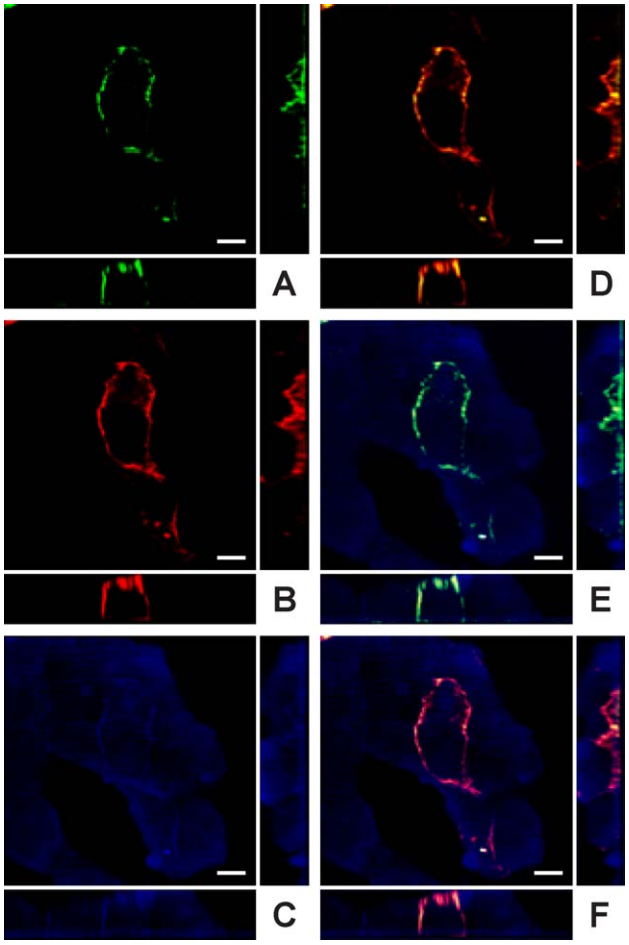

Fig. 4. Sub-cellular localization of SOCS-7 and partial co-localization of SOCS-7 with vinexin and actin in HEK293T cells. (A) HEK293T cells were transiently transfected with plasmid constructs encoding GFP-SOCS-7 and observed for GFP fluorescence. (B) Vinexin was visualized by indirect immunofluorescence using rabbit anti-vinexin and rhodamine-labeled goat anti-rabbit antibodies. (C) Filamentous actin was stained with Alexa Fluor 633-conjugated phalloidin (blue). (D) Represents the corresponding merged image of SOCS-7 and vinexin, while (E) shows the corresponding merged image of SOCS-7 and actin. (F) Represents the corresponding merged images of the three stainings. Note, the XY-images are total projections of 24 confocal sections collected at a height of 11 μm from bottom (cover slip) to top (of the cells) and are extended with the corresponding YZ-(right) and XZ (below) vertical image information at a Z-position of 5.5 μm above the cover slip (cytoplasmic region). Scale bars represent 5 μm.

left untransfected or transfected with GFP, GFP-SOCS-7, or HA-SOCS-7 (only shown for GFP-SOCS-7). There was no rhodamine staining when anti-vinexin antibody was omitted. Filamentous actin was stained using Alexa Fluor 633-phalloidin. The GPF-SOCS-7 fusion protein was mainly present near the cell membrane, including in sites where vinexin and actin were also present (Figs. 4A–C). A substantial proportion of SOCS-7 co-localized with vinexin and actin (Figs. 4D–F). In contrast, after transfection of the GFP encoding plasmid, the distribution of GFP was diffuse and there was no co-localization with vinexin (not shown). Co-localization of SOCS-7 and vinexin was observed in MCF-7 breast cancer cells, yielding a picture very similar to that observed in HEK293T cells (results not shown). As vinexin has been found in focal adhesions and these structures are not easily identified in epithelial cells, we also examined the localization of SOCS-7 in the 3T3-L1 cells. As in HEK293T and MCF-7 cells, cell morphology was not affected by overexpression of GFP-SOCS-7. In contrast, however, SOCS-7 was also seen in the nucleus (Fig. 5A). A smaller portion of SOCS-7 co-localized with vinexin and actin (Figs. 5D–F). Focal adhesions were not easily seen in untreated cells. After exposure of the cells to lysophosphatidic acid (10 μM, 5 min), staining for vinexin and actin clearly showed focal adhesions. SOCS-7, however, was clearly excluded from focal adhesions (not shown).

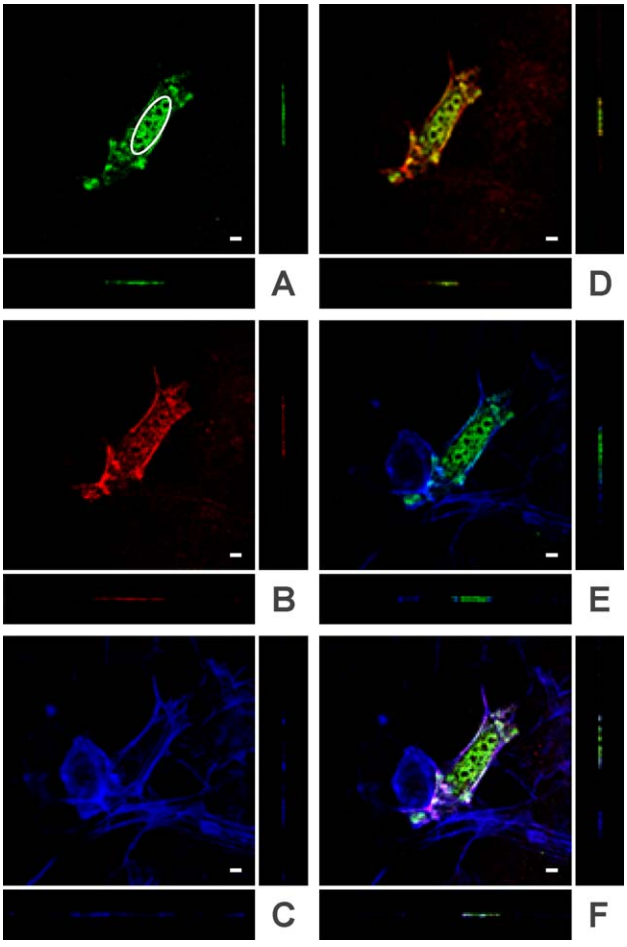

Fig. 5. Sub-cellular localization of SOCS-7 and partial co-localization of SOCS-7 with vinexin and actin in 3T3-L1 fibroblasts. (A) 3T3-L1 cells were transiently transfected with plasmid constructs encoding GFP-SOCS-7 and observed for GFP fluorescence. The position of the nucleus is marked with a white ellipse. (B) Vinexin was visualized by indirect immunofluorescence using rabbit anti-vinexin and rhodamine-labeled goat anti-rabbit antibodies. (C) Filamentous actin was stained with Alexa Fluor 633-conjugated phalloidin (blue). (D) Represents the corresponding merged image of SOCS-7 and vinexin, while (E) shows the corresponding merged image of SOCS-7 and actin. (F) Represents the corresponding merged images of the three stainings. Note, the XY-images are total projections of 12 confocal sections collected at a height of 5 μm from bottom (cover slip) to top (of the cells) and are extended with the corresponding YZ-(right) and XZ (below) vertical image information at a Z-position of 2.5 μm above the cover slip (cytoplasmic region). Scale bars represent 5 μm.

In preliminary studies to examine which part of the SOCS-7 molecule is responsible for its localization, HEK293T cells were transfected with a yellow fluorescent protein-tagged NAP-4 construct. Fluorescence was found nearly exclusively in the nucleus, indicating that the first 126 AA of SOCS-7 containing the NES are required for localization at the cell membrane (results not shown).

Discussion

Human SOCS-7

Structurally, SOCS-factors are characterized by three distinct domains: a C-terminal SOCS-box, a central SH2domain and an N-terminal domain. The length of the latter varies from 44 to 398 AA [1,2]. SOCS-7 has the longest N-terminal domain and this shows no recognizable domain structure, with the exception of several poly-proline areas and a putative, previously unrecognized, NES [1,10,20]. The 34 AA deletion corresponding to exon 4 probably results from alternative splicing. We are presently investigating the exon usage and the existence of splice variants of hSOCS-7 in various tissues and cell lines.

SOCS-7 interacts with vinexin

In a stringent yeast two-hybrid screen of a leukocyte cDNA-library, we have detected vinexin as a potential partner for SOCS-7. Indeed, the product of clone NM21, which corresponds to vinexin-β (without the first 40 AA), was shown to interact with NAP-4, a protein encoded by a partial hSOCS-7 cDNA used as bait [10]. Furthermore, neither the SOCS-box, nor the SH2 domain (alone or joined together) interacted with NM21. Thus, vinexin may interact with the N-terminal part of the NAP-4 protein, a region that comprises AA 127-364 of SOCS-7, though a contribution of the SH2-region to the interaction cannot be excluded, as the N-terminal region did not lend itself to yeast two-hybrid

screening. Remarkably, the N-terminal region has several poly-proline stretches, including a canonical PXXPXR sequence that allows interaction with class II SH3 domains [21,22]. Vinexin-α and -β have three SH3 domains which could interact with one or several of the poly-proline stretches of SOCS-7 [12]. Vinexin also has a WW domain, interacting with somewhat different proline-rich areas, though there is overlap between targets of SH3 and WW domains [21,22].

The interaction between full-length hSOCS-7 and vinexin in mammalian cells was documented by co-immunoprecipitation. All isoforms of vinexin were present in total cell lysates from 3T3-L1 fibroblasts, but only the higher molecular weight forms were co-precipitated with SOCS-7, indicating that only vinexin-α interacts with SOCS-7 in mammalian cells.

After transfection, SOCS-7 is found at the cell membrane, in the cytoplasm, and in the nucleus

In HEK293T and MCF-7 cells (but less so in 3T3-L1 cells), SOCS-7 was found close to the cell membrane. This is in line with the interaction of SOCS-7 with vinexin-α, which, through its sorbin homology domain (SoHo) is anchored to membrane flotillin, that is, to lipid rafts, and with the putative interaction with the src kinases Hck and Lck (Lck is also present in the lipid rafts) [23–25].

In 3T3-L1 cells, SOCS-7 was also abundant in the nucleus. Flow cytometry analysis of cells transfected with GFP-SOCS-7 suggested that differences in the level of expression cannot account for different localization of SOCS-7 in HEK293T and MCF-7 cells on one hand and 3T3-L1 cells on the other hand (results not shown). Matuoka et al. [10] have predicted that SOCS-7 localizes to the nucleus from a poly-Q/poly-P run similar to that found in bromodomain-containing protein 4 and other nuclear proteins [26]. NAP-4 was indeed found in the nucleus of HEK293T cells. The fact that the full-length SOCS-7 protein was not found in the nucleus of HEK293T cells is possibly due to a leucine-rich NES present in SOCS-7, but not in NAP-4 and recognized by the exportin protein Crm-1 [27]. Previously, the presence of several other SOCS-factors (as GFP-fusion proteins) in the nucleus was also reported [15]. Why this NES would be less effective in 3T3-L1 cells is not known. Matuoka et al. [10] have proposed that SOCS-7 could serve as a nuclear transporter for partner proteins.

After transfection, SOCS-7 co-localizes with vinexin and filamentous actin

Overexpression of SOCS-7 had no detectable effect on cell morphology in either 3T3-L1, MCF-7, or HEK293T cells. A CLSM study showed that a substantial proportion of SOCS-7 molecules co-localized with vinexin. SOCS-7 not only merged with vinexin, but also with polymerized actin. SOCS-7 had been initially cloned from its interaction with the adaptor protein Nck-1 [10]. Our yeast two-hybrid screen also identified Nck-2 (which is closely related to Nck-1) as a partner for SOCS-7 (not shown). Interestingly, the association of Nck-2 with the actin cytoskeleton has been known for some time and further points towards a connection between SOCS-7 and the cytoskeleton [28,29]. In addition, Hck and Lck (expressed mainly in myeloid and in lymphoid T-cells, respectively) are also potential partners for SOCS-7. The association of these kinases with the cytoskeleton is well documented [24,30]. Moreover, several IRS-proteins including IRS-2, a known partner of SOCS-7, associate with the actin cytoskeleton [11,31]. Taken together, our data indicate that SOCS-7 interacts with the actin cytoskeleton through vinexin. SOCS-7 could also be connected to actin through Nck-2, src-kinases, and/or IRS-2.

Prospects: possible functions of SOCS-7

Our data document the association of SOCS-7 with vinexin, mainly at the cell membrane but outside focal adhesions. The function of SOCS-7 is still under investigation. SOCS-factors interfere with cytokine, growth factor, and hormone signaling through different mechanisms, such as interaction with receptors, Jaks and Stats or the insulin signal transduction machinery ([1,10,11,32–35] and own unpublished results). Activation of MAP-kinases by the SOCS-factor CIS has also been documented but the mechanism is not understood [7]. Thanks to its multi-domain structure, SOCS-7 can be considered as an adaptor molecule, as is the case for the other SOCS factors. The N-terminal domain binds to vinexin and Nck. Some poly-proline stretches can interact with SH3 and WW domain proteins. The SH2 domain could bind tyrosine-phosphorylated src-kinases and activated receptors and Stats. Finally, the SOCS-box is known to bind the elongin BC-complex, involved in proteasomal degradation of target proteins. SOCS-7, like other SOCS-factors, could target its partners for degradation, a known pathway mediated through the SOCS-box [2,36]. In several systems, however, the SOCS-box has no role in the suppression of cytokine-dependent signaling [1,2]. Multi-domain proteins can compete for a given site on the same partner. For instance, SOCS-7 could compete with R-Ras or Sos, which also interact with SH3 domains of Nck and vinexin, respectively [37,38]. As vinexin is involved in the signal transduction from EGF-R to JNK and in cytoskeletal organization and cell spreading, SOCS-7 could modulate these functions and thus play a role in adhesion-dependent signaling and in cytoskeletal remodeling in normal and transformed cells [38]. Whether SOCS-7 is a bona fide suppressor of cytokine and growth factor signaling is still an open question.

Acknowledgments

Supported by GOA 97-02-04, FWO-Vlaanderen, the VUB and the Region of Brussels-Capital. F. Braet is a

postdoctoral fellow of the Fund for Scientific Research-Flanders. Many thanks to K. Matuoka for support and discussion, to E. Quartier for sequencing and Blast analysis, to N. Kioka for the anti-vinexin antibodies, to P. Robberecht for peptide synthesis, to R. Kooijman, R. Beyaert, R. Schauvliege, and V. Van Mullem for discussion.

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