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Preparation and expression of biologically active prolactin and growth hormone receptors and suppressor of cytokine signaling proteins 1, 2, 3, and 6 tagged with cyan and yellow fluorescent proteins

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Abstract

To prepare reagents for a study of the interactions of prolactin (PRL) and growth hormone (GH) receptors (Rs) with suppressor of cytokine signaling (SOCS) proteins in living cells by fluorescence resonance energy transfer methodology, the respective proteins were tagged with cyan (CFP) or yellow (YFP) fluorescent protein. Constructs encoding ovine (o)PRLR-YFP, oPRLR-CFP, oGHR-YFP, and oGHR-CFP tagged downstream of the receptor DNA were prepared in the plasmid pcDNA plasmid and tested for biological activity in HEK 293T cells transiently cotransfected with those constructs and the reporter gene encoding luciferase. All four constructs were biologically active and as potent as their untagged counterparts. Cells transfected with those proteins exhibited fluorescence in the cytoplasm and the membrane. Constructs encoding DNA tagged with YFP or CFP upstream of SOCS1, SOCS2, SOCS3, and SOCS6 were prepared in pECFP-C1 and pEYFP-C1 plasmids. The biological activities of SOCS1 and SOCS3 tagged at their amino termini were assayed by their ability to inhibit placental lactogen (PL)- or GH-induced activation of JAK2/STAT5-mediated luciferase transcription in HEK 293T cells; the activity of SOCS2 was assayed by its ability to abolish SOCS1-induced inhibition. The tagged proteins exhibited biological activity that was equal to or even more potent than their untagged counterparts. The biological activities of CFP-SOCS2 and YFP-SOCS2 were also assayed using GST–GHR binding assay. Their interaction with the cytosolic domain of GHR was equivalent to their respective untagged counterparts. The biological activity of the construct encoding SOCS6 was not tested because of lack of a suitable assay. Cells transfected with eight of these tagged constructs expressed the fluorescent proteins in both the nucleus and cytosol; the tagged SOCS2 was localized mostly in the latter compartment.

Keywords: SOCS proteins; Prolactin receptor; Growth hormone receptor; Cyan fluorescent protein; Yellow fluorescent protein

Cellular differentiation is regulated by a variety of stimuli, which act through tightly controlled signal-transduction pathways. Regulation of these pathways ensures that the correct transcription factors are activated and the appropriate target genes are induced. One such pathway is based on receptor-induced phosphorylation of Janus kinases (JAK), which results in the activation of members of the signal transducers and activators of transcription (STAT) family of transcription factors [1]. Maintaining the balance between acti-

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vation and suppression of this pathway becomes a logistical problem for the cell if it is to maintain tight regulation of the signal. This balance is central to the diverse group of important biological functions induced by cytokines, some growth factors, and specific hormones, such as prolactin (PRL) and growth hormone (GH) (for review see Refs. [2,3]).

Until recently, our understanding of the JAK/STAT pathway had been focused on its activation, while little was known about how the signal was turned off. Activation of this pathway is reversible, fast, and transient [2,3]. Deactivation is achieved by several mechanisms, including: inactivation of positive regulators by protein tyrosine phosphatases [4]; activation of negative regulators such as suppressors of cytokine signaling (SOCS),[1] a protein inhibitor family of activated STAT and STAT-dimer proteolytic degradation [2,3]; and cross talk with other signaling pathways [5]. A further complexity is the involvement of STAT proteins in the transcriptional activation of the genes encoding some of these negative regulators. Thus, JAK/STAT signaling appears to consist of a balance between activation of the transduction pathway and concomitant activation of its inhibitors. Emerging evidence suggests that SOCS proteins are key players in the suppression of JAK/STAT signaling [2,3]. The SOCS group of proteins has been alternatively called cytokine-inducible SH2-containing proteins (CIS) [6] or STAT-induced STAT inhibitor (SSI) proteins [7]. We have adopted the most commonly used terminology, i.e., SOCS. The SOCS proteins have a conserved SH2 domain and a homology box in the carboxy-terminal region [2,3]. The SH2 domain enables interaction with the JAK tyrosine-kinase domain [8]. Although essential, this is not sufficient for inhibition, implying that sequences within the conserved carboxy-terminal region, the SOCS box, are crucial for SOCS protein function [9]. It may also be that different SOCS proteins interact at different stages in the JAK/STAT pathway. For example: SOCS1 and SOCS3 may bind to and inhibit the catalytic activity of activated JAK [8,10] and SOCS2 may compete with SOCS1 for receptor binding [11] while SOCS3 competes with STAT-receptor docking [12]. The roles of SOCS6 and SOCS7 are not known.

Our previous studies have indicated that 'timing effects' play an important role in controlling molecular events subsequent to the interaction of PRL with its cell surface receptor (R). Specifically, we studied the interaction between PRL and PRLR using surface plasmon resonance. This approach indicated that a 1:2 transient complex is formed, which rapidly dissociates to a 1:1 complex [13]. However, we showed that transient complex formation was sufficient to initiate the biological signal [14,15].

The classical approach to studying association of proteins in living cells, which generated our current level of understanding of the PRLR- and GHR–STAT signaling pathways [16,17], was mainly based on co-immunoprecipitation. However, this methodology necessitated the destruction of cellular and tissue architectures, did not provide information on the biological relevance of the interaction, representing a semi-steady-state situation, and cannot be used to study the kinetics of the interaction or protein–protein interactions with short half-lives. In addition, they do not provide information on cellular localization or trafficking. This latter deficiency is becoming more and more important as it is becomes clear that biochemical processes (signal-transduction pathways in particular) occur in a spatially sensitive manner in cells. Novel techniques such as fluorescence resonance energy transfer (FRET) in intact cells can overcome most of the drawbacks and can provide information that is not attainable by other experimental strategies [18]. We have recently adopted the technique of FRET using mutant green fluorescent proteins (GFPs) to monitor endogenous protein–protein interactions in single living cells [19,20]. In the present paper, we describe the preparation of plasmids encoding biologically active GH and PRL receptors and SOCS proteins tagged with cyan or yellow fluorescent protein (CFP and YFP, respectively). These plasmids will enable studies of these proteins' interactions in living cells.

Experimental procedures

Materials

Recombinant ovine placental lactogen (oPL), growth hormone (oGH) [21], and prolactin (oPRL) [22] were prepared as described previously. Plasmids encoding full-size oPRL receptor (oPRLR) in pcDNA3 expression vectors (Invitrogen, Leek, The Netherlands) were constructed as described previously [23] and oGH receptor (oGHR) was a gift of Dr. Adams from the Centre for Animal Biotechnology, School of Veterinary Science, Victoria 3010, Australia, [24]. The expression vectors encoding cyan and yellow fluorescent proteins (pECFP-N1, pEYFP-N1, pECFP-C1, and pEYFP-C1) were obtained from Clontech (Palo Alto, CA). Plasmids (pcDNA) encoding for myc-tagged (five repeats) suppressor of cytokine signaling (SOCS) proteins 1, 2, 3, and 6 (formerly designated as JAK2 binding protein (JAB) and inducible SH2 (CIS) proteins 2, 3, and 4, respectively) were prepared in the laboratory of Dr. Yoshimura as reported previously [25]. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and kits for DNA purification (PCR-

[1] *Abbreviations used:* SOCS, suppressor of cytokine signaling; GHR, growth hormone receptor; PRLR, prolactin receptor; PL, placental lactogen; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; GST, glutathione-*S*-transferase; LUC, luciferase.

quick DNA clean-up system and QIAwell miniprep kit for preparation of high-quality DNA) were purchased from Qiagen (Valencia, CA). Fetal calf serum (FCS) and horse serum (HS) were purchased from Labotal (Jerusalem, Israel). Horseradish peroxidase (HRP)-conjugated antibodies for Western blot analysis were purchased from Enco (Jerusalem, Israel), SDS–PAGE reagents were from BioRad Laboratories (Richmond, CA), and enhanced chemiluminescence (ECL) reagents for Western blot analysis were from Amersham (Buckinghamshire, UK).

Construction of oPRLR expression plasmids tagged with YFP and CFP

To prepare oPRLR tagged at its carboxy-terminus, the pcDNA3+ vector with an oPRLR cDNA insert (GenBank Accession No. AAB96795) was digested with *Not*I and *Nhe*I and purified from an agarose preparative gel. This procedure linearized the plasmid and removed a small (168 bp) fragment along with the stop codon. The linearized plasmid was then ligated to the *Nhe*I/*Not*I 800-bp fragment encoding YFP, which was prepared similarly by digesting the pEYFP-N1 plasmid, using the Rapid DNA ligation kit (Roche Molecular Biochemicals, Basel, Switzerland). The fused plasmid (poPRLR-YFP) DNA was prepared in *Escherichia coli* cells (strain DH 10B, Life Technologies, Carlsbad, CA). To prepare an oPRLR expression plasmid tagged downstream with CFP, poPRLR-YFP, and the pECFP-N1 plasmids were digested with *Age*I (overnight), followed by *Not*I. An ~5600-bp fragment from the former and an 800-bp fragment (encoding CFP) from the latter were isolated from agarose preparative gels. The larger fragment was dephosphorylated with calf alkaline phosphatase (Roche Molecular Biochemicals) and both fragments were ligated using Roche's Rapid DNA ligation kit. The fused plasmid (poPRLR-CFP) was then prepared in *E. coli* cells, strain DH 10B.

Construction of oGHR expression plasmids tagged with YFP and CFP

A pcDNA plasmid containing a cDNA insert encoding oGH [24] was mutated to create a new *Age*I restriction site, 141 bp upstream of the stop codon. The mutation was carried out, using the primer cca agt tct gag ata ccg gtc cca gat tat ac, with a Gene-Editor mutagenesis kit (Promega, Madison, WI) according to manufacturer's directions. After ensuring that the plasmid had been correctly mutated by restriction-site analysis, it was also tested for biological activity by transient transfection in HEK 293T cells as described further on. Its activity was found to be identical to that of the non-mutated plasmid (not shown). The mutated plasmid was then digested with *Age*I (overnight), followed by digestion with *Kpn*I, and the ~1500-bp fragment encoding oGHR (−150 bp from the 3′ site) was isolated. In parallel, poPRLR-YFP and poPRLR-CFP were digested with *Kpn*I/*Age*I and the ~5500 bp pcDNA fragments containing the sequences of YFP and CFP, respectively, were isolated and dephosphorylated. Subsequently, each of those fragments was ligated to the ~1500-bp *Kpn*I/*Age*I fragment of oGHR, yielding poGHR-YFP and poGHR-CFP, respectively. Both plasmids were then prepared in *E. coli* cells, strain DH 10B.

Construction of SOCS 1-, 2-, 3-, and 6-encoding expression plasmids tagged with YFP and CFP

E. coli bacteria (strain DM-1, Life Technologies) were transformed with pECFP-C1 and pEYFP-C1 plasmids to produce a non-methylated *Xba*I restriction site in the multiple cloning site (MCS) and the respective plasmid DNAs were prepared. The plasmids were then digested with *Bam*HI and *Xba*I, purified using a PCRquick kit (Qiagen), and dephosphorylated. In parallel, fragments encoding myc-tagged (five repeats) SOCS1 (~800 bp), SOCS2 (~1000 bp), SOCS3 (~1500 bp), and SOCS6 (~2000 bp) were prepared from the respective plasmids by digestion with *Bam*HI and *Xba*I, and purification from agarose gels. The isolated fragments were then ligated using the Rapid DNA ligation kit and the fused plasmids (pCFP- SOCS1, pYFP-SOCS1, pCFP-SOCS2, pYFP-SOCS2, pCFP-SOCS3, pYFP-SOCS3, pCFP-SOCS6, and pYFP-SOCS6) were prepared in *E. coli* cells, strain DH 10B.

In vitro bioassays in transiently transfected HEK 293T cells

To compare the biological activities of non-tagged and FP-tagged receptor proteins, HEK 293T cells were transiently transfected with either oPRL or oGH receptors or with JAK2- or JH1-encoding plasmids and cotransfected with a plasmid that carries the luciferase reporter gene under the control of a six-repeat sequence of LHRE (lactogenic hormone response element with a Stat5 binding sequence) fused to a minimal thymidine kinase (TK) promoter. The transfection and bioassay were carried out as described previously [26]. Cells transfected with a constant amount of receptor DNA (100 ng/well) were induced with increasing concentrations of human (h)GH, oPRL or oPL. Luciferase (LUC) activity was measured using a luminometer (Lumac Biocounter—M2500) and β-galactosidase activity was monitored as absorbance at 405 nm. The relative activity was calculated as follows: induction ratio = (luciferase activity with hormone/absorbance at 405 nm)/(luciferase activity without hormone/absorbance at 405 nm). To assay the biological activity of the fluorescent protein

(FP)-tagged SOCS proteins, HEK 293T cells were transfected with non-fluorescent receptors and cotransfected with increasing amounts of the respective plasmids encoding these proteins.

GST–GHR binding assay

The biological activities of the FP-tagged SOCS1 and SOCS2 proteins were also tested by their ability to bind a tyrosine-phosphorylated GHR [10]. The GST–GHR fragment, which was composed of amino acids 455–638 located in the cytosolic domain of hGHR [10] and fused downstream to GST in pGEX-5X-3 expression vector, and the *E. coli* strain TKX1, which harbors a plasmid-encoded inducible tyrosine kinase gene, were provided by Dr. Nils Billestrup from the Hagedorn Institute. The protein was expressed and purified as recommended by the manufacturer (Stratagene, La Jolla, CA). For each assay, 20 μg GST–GHR was incubated for 2 h at 4 °C with 20 μl glutathione–Sepharose (GS) beads (50% v/v) in the presence of 1 ml lysis buffer, pH 8, composed of: 50 mM Hepes, 250 mM NaCl, 10% (w/v) glycerol, 2 mM EDTA, 2 mM EGTA, 0.1% (v/v) NP-40, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate. The GS pellets were washed three times with the lysis buffer to remove any excess of GST–GHR and 30 μl lysate prepared from cells transiently transfected with the respective construct (see earlier) was added. An extract of HEK 293T cells transfected with pcDNA3 expression plasmid encoding for 6 X myc-tagged SOCS1 was used as a positive control. After overnight incubation at 4 °C with rotation in the presence of lysis buffer, the GS pellets were washed five times with ice-cold lysis buffer and eluted with 30 μl of 5 mM reduced glutathione in 50 mM Tris–HCl buffer, pH 8. The eluates were then separated by SDS–PAGE and analyzed by Western blot using a 1:1000 dilution of anti-myc antibodies. Identically treated tubes with GS but without GST–GHR were used as negative controls.

Imaging

HEK 293T cells from ATCC (American Type Cell Culture) were plated at a density of 5×10^5 cells/well on polylysine-coated coverslips in 3.5-cm tissue culture dishes (NUNC, Denmark) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg/L glucose, 10% (v/v) bovine calf serum (BCS), and antibiotic-antimycotic solution (Biolab, Jerusalem, Israel) at 37 °C in a 5% CO_2-enriched, humidified atmosphere. After 8 h, having reached ~50% confluence, the cells were transfected using calcium phosphate with 0.25–1.0 μg DNA of YFP- or CFP-tagged PRLRs, GHRs or SOCSs. Cell images were acquired 36–48 h after transfection using a confocal laser-scanning microscope system (CLSM 510, Zeiss, Jena, Germany), including a Zeiss Axiovert-100M microscope with a 63× water-immersion objective lens (or as indicated). The CFP-construct-transfected cells were imaged at an excitation wavelength of 458 nm and a 475 LP emission filter, while the YFP constructs were imaged separately at an excitation wavelength of 488 nm and a 505 LP emission

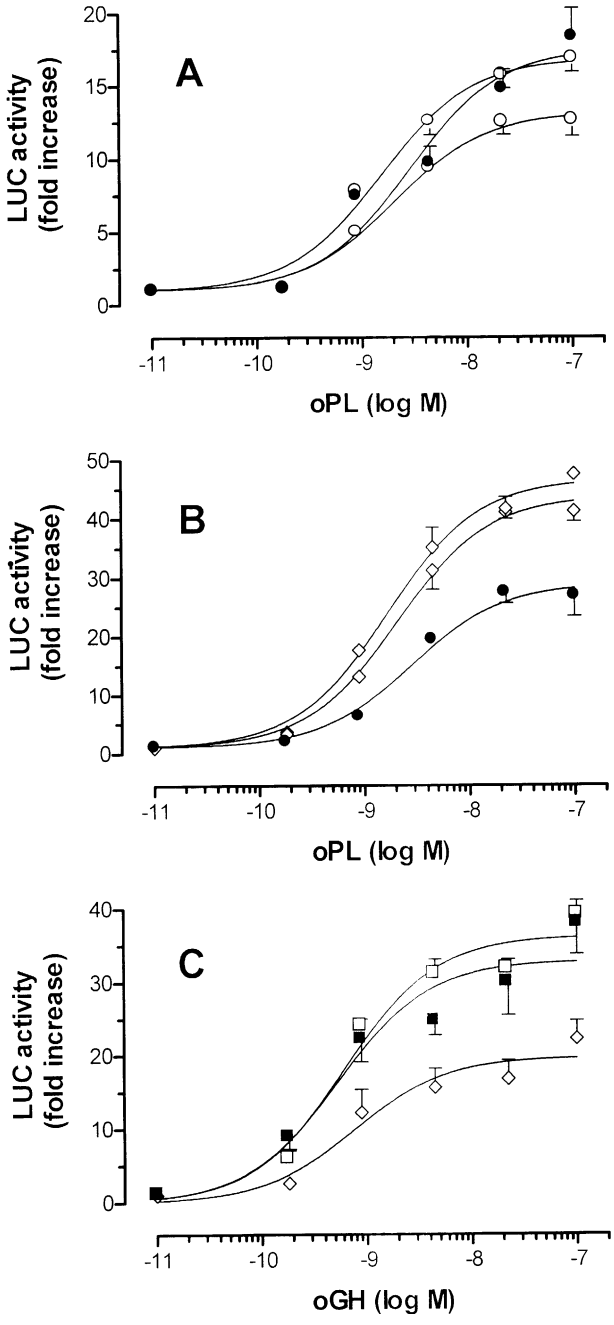

Fig. 1. Ovine PL- and oGH-induced luciferase (LUC) activity in HEK 293T cells transiently transfected with plasmids encoding oPRLR or its YFP- (A) or CFP-tagged analogs (B), or in HEK 293T cells transiently transfected with oGHR or its YFP- or CFP-tagged analogs (C). oPRLR (●), YFP-tagged oPRLR (○, two clones), CFP-tagged oPRLR (◇, two clones), oGHR (■),YFP-tagged oGHR (□), CFP-tagged oGHR (◇). (For more details, see text.)

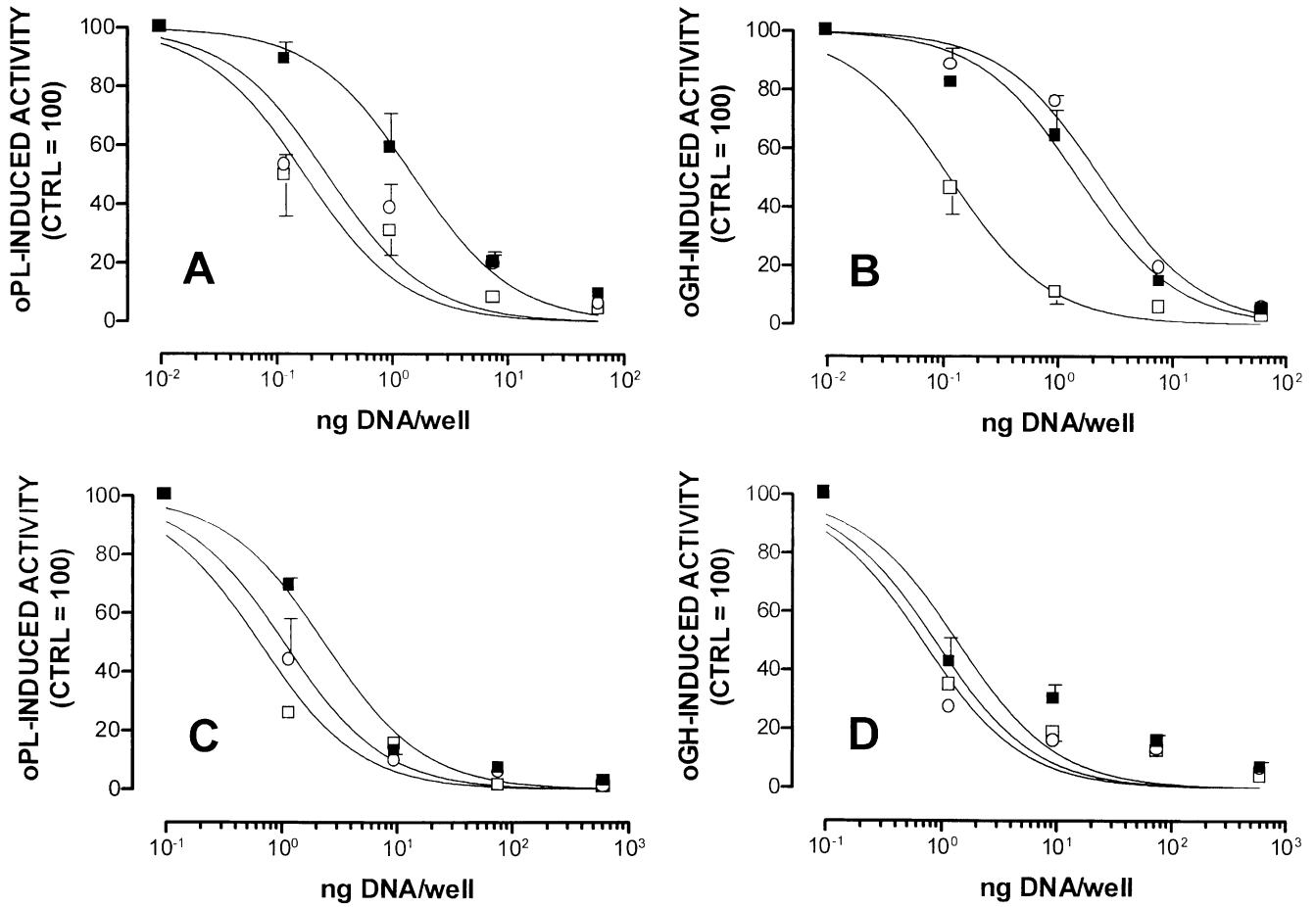

Fig. 2. Inhibition of oPL- and oGH-induced LUC activity by SOCS1 (A and B) and SOCS3 (C and D) and their YFP- and GFP-tagged analogs in HEK 293T cells transiently transfected with plasmids encoding oPRLR (A and C) or oGHR (B and D). Cells also cotransfected with plasmids encoding non-tagged SOCSs (■), YFP-tagged SOCSs (○), and CFP-tagged SOCSs (□). LUC activity was normalized to β-galactosidase activity. (For more details, see text.)

filter. Transmitted-light images were acquired using Nomarski differential interference contrast.

Results and discussion

Biological activities of CFP- and YFP-tagged PRL and GH receptors

The biological activities of oPRLR-YFP and oPRLR-CFP (two bacterial colonies each) were tested using in vitro bioassays in HEK 293T cells. As shown in Fig. 1A, oPL-induced (LUC) activities in cells transfected with oPRLR-YFP were similar to those obtained with wild-type oPRLR. The maximal response was similar and the respective EC_{50} values of oPRLR and the two tested clones of oPRLR-YFP were (in nM) 2.83, 1.54, and 2.04. The corresponding 95% confidence intervals were (in nM) 1.45–5.54, 1.05–2.26, and 1.46–2.84, respectively, showing no statistically significant ($p < 0.05$) differences. Similar results were obtained with oPRLR-CFP activity (Fig. 1B), although in that case both tagged oPRLRs exhibited a higher LUC activity. However, the respective EC_{50} values of oPRLR and the two tested bacterial colonies of oPRLR-CFP were (in nM) 2.78, 2.04, and 1.65 and the 95% confidence intervals were (in nM) 1.84–4.19, 1.56–2.67, and 1.23–2.20, respectively, indicating that the higher activity of the tagged oPRLRs could not be statistically significant. Tagging with either YFP or CFP did not influence the activity of oGHR (Fig. 1C). Though the maximal activity in cells transfected with oGHR-CFP was lower than in cells transfected with oGHR or oGHR-YFP, the respective EC_{50} values (in nM) were similar (0.75, 0.53, and 0.61). Furthermore, the respective 95% confidence intervals were (in nM) 0.35–1.64, 0.25–1.11, and 0.38–0.97, again showing no statistically significant differences.

We could thus conclude that the removal of 56 amino acids from the carboxy-terminus of oPRLR or 47 amino acids from the carboxy-terminus of oGHR does not affect their ability to transduce the agonist-dependent

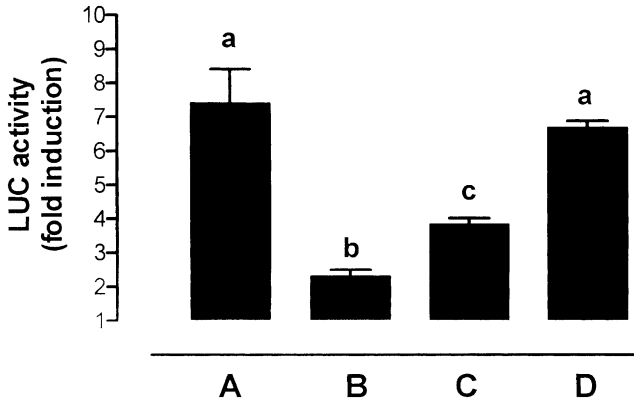

Fig. 3. Abolishment of SOCS1-induced inhibition of GHR signaling by cotransfection with plasmids encoding CFP-SOCS2 or YFP-SOCS2. HEK 293T cells were transiently transfected with plasmids encoding oGHR (A), oGHR and SOCS1 (B), oGHR, SOCS1, and CFP-SOCS2 (C), and oGHR, SOCS1, and YFP-SOCS2 (D). After stimulation with 400 ng oGH/well, the LUC activity was measured and normalized to β-galactosidase activity. The results are presented as means ± SEM and bars marked with different letters differ significantly ($p < 0.05$). (For more details, see text.)

signal through the JAK2/STAT5 pathway. These results confirm the previously published ones pertaining to the limited change in the activity of rat (r)PRLR mutated at Tyr 580 [25]. Deletion of the C-terminal half (amino acids 455–638) of the GHR ablated GH-dependent tyrosyl phosphorylation of p97 but only slightly affected the binding and phosphorylation of JAK2 [26–28]. It should also be noted that bovine and ovine PRLRs naturally lack this C-terminal tyrosine [29,30] and that truncation of rbPRL did not affect its ability to activate JAK2/STAT5 pathway [31]. Moreover, extension of the carboxy-terminus by ~270 amino acids with YFP or CFP, which likely refolds as a separate domain [32], also did not affect this activity as shown afore.

Biological activities of CFP- and YFP-tagged SOCS proteins

The biological activities of SOCS1 and SOCS3 tagged at their amino termini were assayed by their ability to inhibit PL- or GH-induced activation of JAK2/STAT5-mediated LUC transcription in HEK 293T cells, as shown previously by us [33] and others [11]. HEK 293T cells were transiently transfected with plasmids encoding oPRLR or oGHR, LUC, and β-galactosidase, and with different amounts of plasmids encoding SOCS1 (0.01–64 ng DNA/well) or SOCS3 (0.1–640 ng/well). Forty to 48 hours after transfection, followed by 24 h hormone induction (for details see Experimental procedures), the LUC activity and the extent of inhibition were determined. As shown in Fig. 2, in cells transfected with oGHRs or oPRLRs, both SOCS1 and SOCS3 tagged upstream with either CFP or YFP were at least as inhibitory as the non-tagged SOCS. Due to the fact that

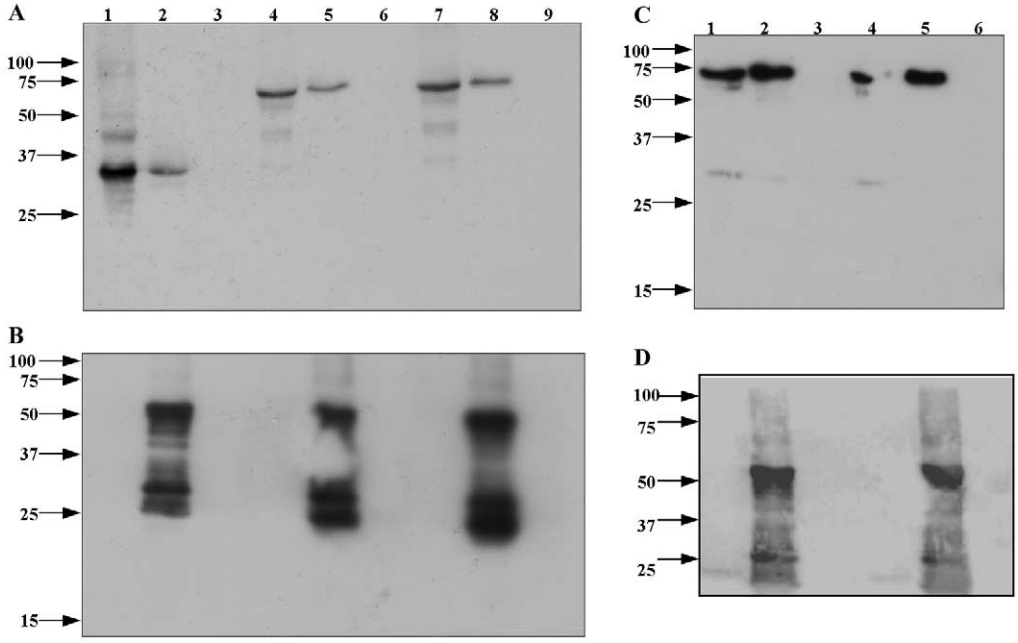

Fig. 4. Binding of SOCS1, CFP-SOCS1, YFP-SOCS1 (A) and CFP-SOCS2, YFP-SOCS2 (C) to GST-GHR (aa 455–638) prebound to glutathione–Sepharose (GS) resin. Lysates of HEK 293T cells transfected with SOCS1 (A, lanes 1–3), CFP-SOCS1 (A, lanes 4–6), YFP-SOCS1 (A, lanes 7–9), YFP-SOCS2 (C, lanes 1–3), or CFP-SOCS2 (C, lanes 4–6) were added to GS beads, (A, lanes 3, 6, and 9) and (C, lanes 3, 6) or to GST–GHR pre-attached to GS beads (A, lanes 2, 5, and 8, and C, lanes 2, 5). Cell lysates were prepared from, respectively, transfected HEK 293T cells as positve controls. After overnight incubation, the beads were washed, eluted with reduced glutathione, analyzed by Western blot analysis using anti-myc antibody (A, C), and subsequently reblotted with anti-GST antibody (B, D).

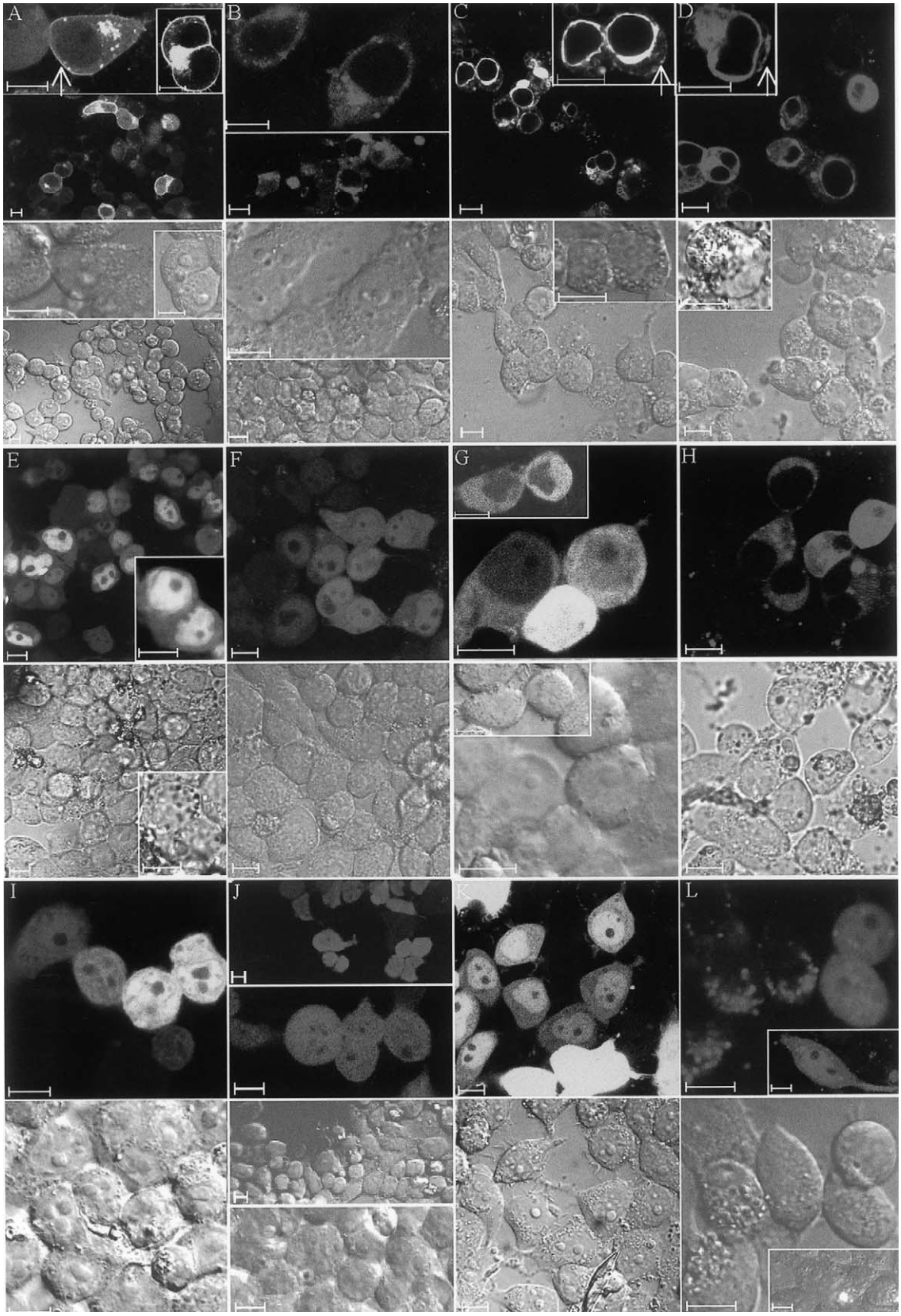

Fig. 5. Expression patterns of the fluorescently tagged proteins in HEK 293T cells. Confocal images (top) and the corresponding transmitted-light images (bottom) of cells over-expressing YFP-tagged or CFP-tagged variants of GHR (A, B), PRLR (C, D), SOCS1 (E, F), SOCS2 (G, H), SOCS3 (I, J) and SOCS6 (K, L). Bars: 10 μm. Arrows: apparent membrane localization of the tagged receptors. CFP-SOCS2 was imaged using a 40× oil-immersion objective.

SOCS2 is not active as an inhibitor of either PRLR- or GHR-mediated signaling but is able to abolish SOCS1 inhibition [11], we tested whether this property is also shared by CFP- and YFP-tagged SOCS2 (Fig. 3). Transfection of 10 ng SOCS1-encoding plasmid resulted in ~80% inhibition of LUC activity. This inhibition could be partially or fully abolished with cotransfection of 500 ng plasmids encoding, respectively, CFP- or YFP-SOCS2.

The biological activities of the tagged and wt SOCS2 were compared by binding to GST–GHR (455–638). To validate the method, similar binding experiments were also performed using tagged and non-tagged SOCS1. The results clearly showed that non-tagged SOCS1 and CFP- and YFP-tagged SOCS1 and 2 bind to GST–GHR previously adsorbed to GS beads (Figs. 4A, lanes 2, 5, and 8 and C, lanes 2 and 5), whereas no binding was found to GS alone (Figs. 4A, lanes 3, 6, and 9 and C, lanes 3 and 6). The presence of GST–GHR in the respective glutathione eluates was verified by reblotting (Figs. 4B and D). It should be noted that the molecular masses of the glutathione-eluted SOCS1 and CFP- and YFP-tagged SOCS1 and 2 were identical to those of applied proteins in respective non-treated lysates (Figs. 4A, lanes 1, 4, and 7 and C, lanes 1 and 4). Furthermore, the molecular masses of the tagged SOCS1 and 2 were, respectively, 65 and 70 kDa, as predicted from the open frame of the constructs. The biological activity of the construct encoding SOCS6 was not tested because of the lack of an appropriate assay. In conclusion, it was clear that tagging SOCS1–3 with YFP and CFP does not abolish their biological activity.

Visual analysis of CFP- and YFP-tagged GHRs, PRLRs, and SOCS proteins

The CFP- and YFP-tagged GHRs and PRLRs were observed in the cytosol in diffuse, as well as in granular form, but were absent from the nucleus, irrespective of the type of chromophore (Figs. 5A–D). The receptors also appeared in the membrane, as indicated particularly well by the fluorescent contours of the cells transfected with the YFP-GHR (see arrow in Fig. 5A). This was also observed in cells transfected with both PRLR fluorescent variants (see arrows, Figs. 5C and D). PRLR (both variants) appeared also clustered prominently in the perinuclear region, as if the usually membrane-destined protein were "locked" in the perinuclear ER. Such apparent "locking" may be attributed to the overloading of the translation machinery in the over-expressing cells, and to the resulting insufficiency, or even disruption, of vesicular trafficking of the membrane protein [34]. None of the tagged SOCS proteins could be found in the membrane or in the nucleolus and they were usually diffuse in appearance (Figs. 5E–L). The tagged SOCS1, SOCS3, and SOCS6 proteins appeared somewhat more abundantly in the nucleus than in the cytoplasm, in both their YFP- and CFP-tagged forms (Figs. 5E, F, I–L); although distribution of the CFP-SOCS6 protein appeared to be more variable; it also showed a granular appearance and was prevalent in the cytoplasm (Fig. 5L). The SOCS2 proteins were generally more abundant in the cytoplasm than in the nucleus, but in contrast to the YFP-tagged proteins that were distributed diffusely (Fig. 5G), the CFP-tagged protein was additionally accumulated in cytoplasmic granules (Fig. 5H). The granular appearance of the fluorescence in some of the cells may have been due to the accumulation of over-expressed foreign proteins in inclusion bodies [35]. The relatively higher incidence of such granularity in cells expressing the CFP constructs may reflect the relatively higher amount of DNA used for transfection with these constructs, tactics intended to compensate for the relatively lower signal intensity from their CFP-tagged products as compared to the fluorescence of their YFP-tagged counterparts [36,37]. The appearance of SOCS protein in the nucleus is a novel, as yet non-described finding but whether it results from over-expression or represents a true physiological phenomenon requires further analysis.

Acknowledgments

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