Cytokine-inducible SH2 protein (CIS3) and JAK2 binding protein (JAB) abolish prolactin receptor-mediated STAT5 signaling

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Abstract The ability of five members of the cytokine-inducible SH2 protein family (CIS1–4) and JAK2 binding (JAB) protein to affect prolactin receptor (PRLR)-mediated activity was tested in human 293 embryonic kidney fibroblasts transiently transfected with rat PRLR, five concentrations of CIS/JAB Myc-tagged cDNAs and a STAT5-responsive reporter gene encoding luciferase. The protein expressions of CIS1, CIS2, CIS3 and JAB were comparable, whereas the level of CIS4 was slightly lower. PRLR-mediated luciferase activity was abolished in a dose-dependent manner in cells transfected with cDNA of CIS3 or JAB, even at concentrations below the level of protein detection by anti-Myc antibody. In contrast, CIS1, CIS2 and CIS4 had little or no effect, despite similar levels of expression. CIS1 expression in postpartum mouse mammary glands was high and changed little in the course of 3 days. CIS2 and CIS3 expression was also high and increased further, whereas JAB expression was very low. These results hint that at least in mammary gland CIS3 is likely the main physiological negative regulator of the PRLR-mediated JAK2/STAT5 pathway.

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Key words: Cytokine-inducible SH2 protein; JAK2 binding protein; Prolactin receptor; Inhibition; Cytokine signaling

1. Introduction

Prolactin receptors (PRLRs) belong to the cytokine receptor family, which consists of transmembrane single-chain proteins with considerable similarity in their extracellular domains, also termed cytokine homology domains [1,2]. The mechanism of prolactin (PRL) signaling has been intensively investigated over the last decade, leading to a well-established paradigm, namely: the signal is initiated by hormone-induced receptor homodimerization, which leads to immediate transphosphorylation of the associated tyrosine kinase JAK2, followed by activation of STAT5 or MAP kinase pathways [3–5]. Although this paradigm is in general correct for other cytokines, we have recently documented that unlike growth hormones (GHs), the interaction of PRLs with their homologous receptors is extremely transient, albeit sufficient to activate the signal transduction [6]. One possible reason for this could be related to the finding that JAK2 kinase, which serves as a first mediator of both receptors, is already associated with lactogenic receptors prior to hormone-binding-induced receptor dimerization [7,8]. We suggested that once the signal is initiated, the homodimer is no longer required and the activated tyrosine-phosphorylated JAK2 continues its enzymatic activity and is capable of docking and/or phosphorylating downstream proteins [9,10]. Little is known, however, about how this signal pathway is terminated or attenuated. In the case of erythropoietin (EPO) receptor the phosphorylated intracellular domain of the receptor is capable of binding the protein tyrosine phosphatase SHP-1, which in turn dephosphorylates the activated JAK2 [11]. Whether this or a similar mechanism also exists in other members of the cytokine family, or whether the signal is only attenuated by receptor internalization, is not clear. Indirect evidence hints at the involvement of tyrosine phosphatase in PRLR-mediated pathways [12,13], although conflicting results suggest that the protein tyrosine phosphatase SHP-2 acts as a positive rather than negative regulator [14]. In the last three years, a new family of proteins termed cytokine-inducible SH2-containing proteins (CIS) [15,16], JAK2 binding protein (JAB) [17], suppressors of cytokine signaling (SOCS) [18] or STAT-induced STAT inhibitors (SSI) [19], which are involved in attenuating cytokine signaling, has been cloned and partially characterized. At present, this family consists of seven (CIS1–6 and JAB) [16] or eight members, though it may be larger [20]. The inhibitory activity of these proteins results from their ability to interact with JAK family members, with tyrosine-phosphorylated STATs or with cytokine receptors [15–21]. Whether this interaction leads to dephosphorylation of these proteins via the recruitment of phosphatases, or by blocking phosphotyrosine docking sites, and what their specificity is toward different cytokines, is only partially understood. Involvement of these proteins in PRLR-mediated signaling has not yet been reported, though it has been suggested that some of them are likely to be involved in its regulation [5]. In order to extend this knowledge, we tested the ability of five members of the CIS/JAB family to affect PRLR-mediated STAT5-dependent activity, using human 293 embryonic kidney (HEK) fibroblast cells transiently transfected with rat (r) PRLR and a reporter gene as a model [22,23]. In parallel, we checked the expression of CIS/JAB in postpartum mouse mammary glands.

2. Materials and methods

2.1. Materials

Recombinant bovine placental lactogen (bPL) was prepared as described previously [24]. Molecular mass markers for SDS-PAGE, DMEM and DMEM/F12 media were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated

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antibodies for Western blot analysis were purchased from Enco Co. (Jerusalem, Israel), SDS-PAGE reagents from Bio-Rad Laboratories (Richmond, CA, USA), enhanced chemiluminescence (ECL) reagents for Western blot analysis from Amersham (Buckinghamshire, UK), fetal calf serum (FCS) from Bet Haemek Co. (Jerusalem, Israel) and luciferin from Promega Inc. (Madison, WI, USA). Vectors encoding the full-size rPRLR in pRc/CMV and luciferase were obtained from Drs. P.A. Kelly and V. Goffin [22], and preparation of Myc-tagged (repeated five times) CIS was as previously reported [16]. All other chemicals were of analytical grade.

2.2. In vitro bioassays in transiently transfected 293 HEK cells

The effect of five CIS/JAB-encoding constructs on lactogen-inducible activity was assayed in 293 cells transiently co-transfected with vectors encoding rPRLR and the luciferase reporter gene; the latter is controlled by a six-repeat sequence of LHRE (lactogenic hormone response element with a STAT5 binding sequence) fused to a minimal TK promoter. The transfection and bioassay were carried out as described previously [22,23]. The experiments were performed in six-well plates. Each pair of wells was co-transfected with identical amounts of rPRLR and LHRE-luciferase (0.1 μg DNA) and different concentrations of CIS1–4 or JAB (for the specific concentrations see the legend to Fig. 1) using the calcium-phosphate method. The initial concentrations of CIS1–4 and JAB DNAs were chosen to achieve similar levels of expression [16]. After 24 h, one of the two wells was activated by adding 400 ng bPL. After a further 24 h, the cells from each well were lysed in 0.5 ml lysis buffer and luciferase activity was determined in a 50 μl aliquot using a Biocounter M 2500 apparatus (Lumac, Celsis, The Netherlands). The protein concentration in each lysate was determined with Bradford reagent (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). The protein concentrations were in general close to 1 μg/μl (the maximal range was 0.6–1.3 μg/μl) and the activity was normalized per 50 μg protein. Then the relative lactogen-induced luciferase activity for each well pair was calculated as follows: (activity in the well activated with bPL)/(activity in the non-activated well). The six values obtained from two plates transfected with each of the CIS or JAB constructs were normalized using the following equation: (activity in cells transfected with CIS or JAB−1)/(activity in non-transfected cells−1)×100. The final results are presented as the mean ± S.E.M. of four experiments.

2.3. Expression of CIS/JAB in postpartum mammary glands

Two mice were killed at days 1, 2 and 3 postpartum and total RNA was prepared. For Northern blotting, 5 μg of total RNA of mouse mammary gland at 1, 2 and 3 days postpartum was separated on 1.0% agarose gels containing 2.4% formaldehyde, then transferred to positively charged nylon membranes. Preparation of RNA, probe cDNAs and hybridization were as described previously [16] and α-lactalbumin was used as a control [25]. The density of the band was determined with a UMAX scanner, using the NIH program for MacIntosh computer.

3. Results and discussion

Our previous results indicated that the efficiency of transfection with rPRLR and reporter gene constructs shows little variation [23]. Therefore, instead of assaying the efficiency of transfection by an unrelated vector, we detected it directly by measuring the expression of the transfected CIS/JAB constructs, using Western blotting with anti-Myc antibodies. As shown in Fig. 1 (lane 5) the expressions of CIS1, CIS2, CIS3 and JAB proteins in cells transfected with 10, 10, 5 and 0.5 μg of cDNA, respectively, were comparable, whereas the level of CIS4 in cells transfected with 5 μg was slightly lower. The proteins were clearly detected in cells transfected with the highest cDNA concentration (lane 5) or with the first 1:8 cDNA dilution (lane 4), but were barely visible or below the level of detection at lower DNA concentrations (lanes 1–3).

To assay the effect of CIS/JAB on PRLR-mediated signaling, we chose bPL, which activates all PRLRs [24,26]. The concentration of bPL was chosen to achieve a sub-maximal

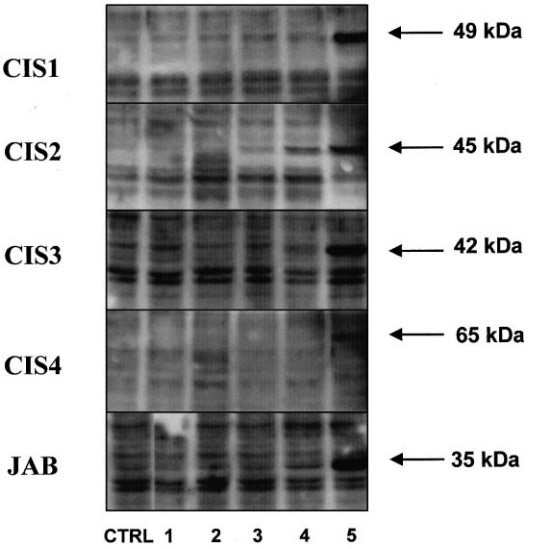

Fig. 1. Expression of CIS1–4 and JAB in cells co-transfected with constructs of rPRLR (0.1 μg), LHRE-luciferase (0.1 μg) and various concentrations of CIS1–4 and JAB. The concentrations of the transfected DNAs of the latter were: CTRL: none; lanes 5–1: consecutive eightfold dilutions from the highest to the lowest concentrations. The highest concentrations of the respective DNAs per well (lane 5) were: CIS1 10 μg, CIS2 10 μg, CIS3 5 μg, CIS4 5 μg, JAB 0.5 μg. The cell lysates (30 μl) were fractionated by 10% SDS-PAGE in minigels, blotted and developed with anti-Myc serum. For more details see text.

response, as determined by preliminary experiments (not shown). The results shown in Fig. 2 clearly indicate that bPL-inducible luciferase activity is abolished in a dose-dependent manner in 293 cells transiently transfected with cDNAs of CIS3 or JAB. Partial inhibition was already observed in cells transfected with as little as 1.2 ng of CIS3 or 0.12 ng of JAB constructs (bar 1) (13% and 35% respectively, only the latter difference being statistically significant, $P < 0.05$). At eightfold higher construct concentrations (9.6 ng of CIS3 or 0.96 ng of JAB) 50% and 70% of inhibition was observed (bar 2) and the respective differences were statistically significant ($P < 0.05$ and $P < 0.01$). At still higher cDNA concentrations, the inhibition was complete (bars 4 and 5) or nearly so (bar 3). It seems, therefore, that bPL-inducible activity can be attenuated by very low concentrations of both CIS3 and JAB proteins, i.e. those below the level of anti-Myc antibody detection. Our results are similar to those reported by others, in cells transfected with receptors of EPO or leukemia inhibitory factor (LIF) [16,27], interleukin-6 (IL6) [18], GH [28], interferon-γ [29], and in cells expressing an intact long form of leptin receptors [30], thus indicating that CIS3 and JAB are major negative regulators in cytokine signaling.

In contrast to our findings with CIS3 and JAB, transfection of 293 cells with CIS1, CIS2 and CIS4 cDNA had little or no effect on bPL-inducible activity (Fig. 2), although their levels of expression (see above) were similar to those of CIS3 and JAB. Some statistically significant decrease ($P < 0.05$) was observed, in cells transfected with 10 μg CIS2 or 5 μg of CIS4 (bar 5). Thus, it seems that the inhibition of lactogen-inducible signal transduction is at least 500–4000 times less sensitive to CIS2 and CIS4 than to CIS3 and JAB, suggesting that the former proteins do not interfere with PRLR-mediated STAT5

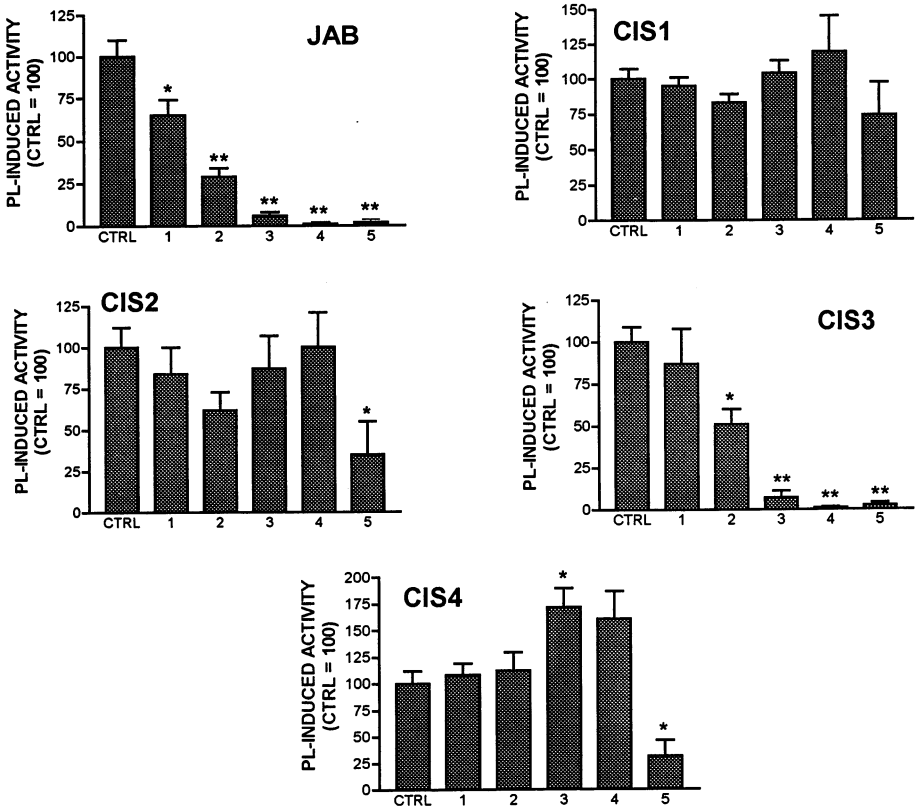

Fig. 2. Effect of CIS1–4 and JAB on bPL-inducible luciferase activity in 293 cells that were co-transfected with constructs of rPRLR (0.1 μg), LHRE-luciferase (0.1 μg) and various concentrations of CIS1–4 and JAB. For the concentrations of the transfected constructs see the legend to Fig. 1. Each of the wells in a six-well plate was transfected with identical amounts of rPRLR and LHRE-luciferase and different concentrations of CIS1–4 or JAB. One of the two wells was activated by bPL as described in Section 2. After the results were normalized to the same protein level in lysates the relative lactogen-induced luciferase activity for each couple of wells was calculated as follows: (activity in the well activated with bPL)/(activity in the non-activated well) and the values obtained from each pair of wells were normalized using the following equation: (activity in cells transfected with CIS or JAB−1)/(activity in non-transfected cells−1)×100. The final results are presented as the mean ± S.E.M. of the four experiments. Results significantly different from the control (as analyzed by two-way *t*-test) are marked with * ($P<0.05$) or ** ($P<0.01$).

signaling. Interestingly, in cells transfected with lower concentrations of CIS4, bPL-inducible activity was elevated by 60–70% (Fig. 2, bars 3 and 4). Though one of these increases was significantly different from the control the nature of this increase is not clear. It should also be noted that the transfected CIS/JAB constructs did not affect the basal luciferase activity in cells that were not stimulated with bPL, except in cells transfected with the highest concentration of JAB (0.5 μg), in which the basal activity was elevated two- to threefold (not shown).

Previous studies have shown that both CIS3 and JAB have the in vitro and in vivo capacity to interact with the JAK2-JH1 domain, with JAB's interaction being considerably stronger [16]. However, the present results indicate that the inhibition pattern of PRLR-mediated luciferase activity is almost identical for both CIS3 and JAB (see Figs. 1 and 2). One

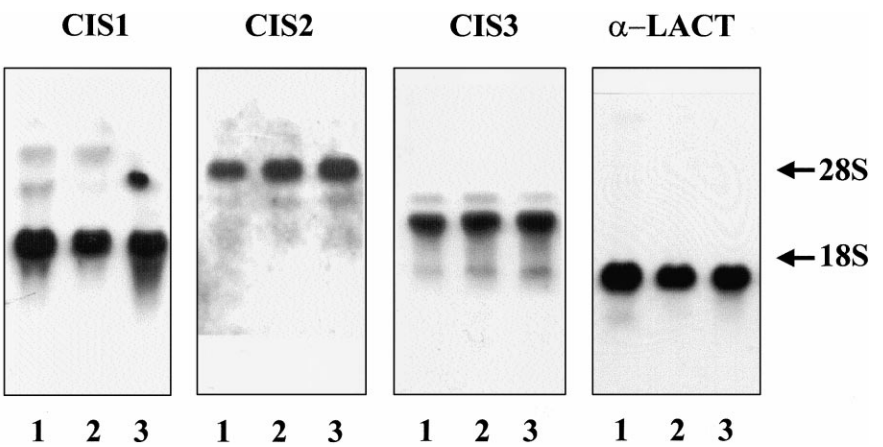

Fig. 3. Northern analysis of CIS1–3 expression in postpartum mouse mammary gland. Total RNA prepared from 1, 2 and 3 days postpartum mammary gland (5 μg) was analyzed. Probing of α-lactalbumin was used as a control.

possible explanation for this discrepancy is that CIS3 may have additional inhibitory functions unrelated to JAK2 binding, the nature of which is at present unknown. Indeed, whereas the only function related to JAB activity to date is its interaction with either JAK2 [21], or other related kinases, such as JAK1, JAK3 and Tyk [17], and with a non-receptor Tec protein-tyrosine kinase [31], CIS3 has been shown to interact with lck [16]. Though participation of lck in PRLR signaling has not been reported [5], another src-like kinase, Fyn, was activated by PRLR stimulation in a Nb2 rat T-lymphoma cell line [32] and an association of PRLR with c-*srk* kinase in rat liver has also been suggested [33]. Therefore, the inhibitory effect of CIS3 in PRLR-transfected 293 cells may not be limited to an interaction with JAK2. Since transactivation of luciferase is controlled by LHRE promoter, which also requires phosphorylation of tyrosines 473, 479 and, in particular, 580 in long PRLRs [34], we should consider them as possible target sites for CIS3. CIS1 has been shown to interact with the phosphorylated erythropoietin receptor (EPOR) [16] or interleukin-3Rβ [35], and CIS1 and CIS2 with insulin-like growth factor-I receptor [36]. The lack of direct evidence for the effects of CIS1, CIS2 and CIS4 does not, therefore, preclude their participation in downregulation of PRL signaling. In fact indirect evidence, namely the hormone-dependent expression of various members of this family [15–19,27–31], hints at their playing a regulatory role. For instance, the results shown for a GHR which is closely related to PRLR indicate GH-dependent upregulation in expression not only of CIS3 and JAB, but also of CIS1 and CIS2, though the kinetics and specificity differ in various tissues or cell lines bearing GHRs [28].

To assess the physiological relevance of our results, we tested the expression of CIS/JAB in postpartum lactating mouse mammary glands. The results shown in Fig. 3 show results obtained from three randomly chosen mice. The figure is a representative of two gels that showed similar results. The relative densities of each band were related to that of α-lactalbumin at the same day. As shown in Fig. 3, CIS1 is highly expressed on day 1 postpartum, and the level remains high on days 2 and 3 (relative densities 1.13, 1.09, 1.14). CIS1 has been shown to be a target of STAT5 [35], but CIS1 expression was undetectable in STAT5A,B double-knockout mice [37]. Therefore, CIS1 may serve as a target gene of PRL signaling. However, overexpression of CIS1 did not inhibit PRL-dependent STAT5 activation in 293 cells (Fig. 2), while it partially inhibited EPO-dependent STAT5 activation [16]. Furthermore we did not obtain any direct evidence indicating the interaction between the PRL receptor and CIS1 (data not shown). Thus, further study is necessary to clarify the function of CIS1 in mammary gland. In contrast, CIS2 and CIS3 expression gradually increased in postpartum mammary gland (Fig. 3) and the following relative densities were obtained for days 1, 2 and 3: CIS2 0.36, 0.67, 0.77 and CIS3 0.52, 0.78, 0.85. In contrast, JAB expression was below our detection limit using Northern blots, even after lactation (not shown). As an increase in circulating prolactin in response to suckling is well-documented [38], our results (Figs. 2 and 3) hint that CIS3 may be the physiological inhibitor of the PRLR system in mammary gland.

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