Co-activator SRC-1 is dispensable for transcriptional control by STAT3

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INTRODUCTION

IL (interleukin)-6 is a multifunctional cytokine that is able to modulate various physiological events such as cell differentiation, proliferation, survival and apoptosis in several organs and biological responses. As an early-response cytokine expressed during an acute inflammatory reaction, IL-6 is the major inducer of the hepatic production of most acute-phase proteins. We could identify STAT (signal transducer and activator of transcription) 3 as the central component of IL-6 signal transduction that is initiated through gp (glycoprotein) 130, the signal-transducing subunit of the IL-6 receptor complex [1,2]. Following binding of IL-6 to its receptor, STAT3 is recruited to phosphorytosine motifs of gp130, becomes phosphorylated at Tyr705 by gp130-associated Janus kinases, and translocates to the nucleus, where it binds to regulatory DNA elements of target genes [1–3]. Besides its contribution to cytokine signalling, STAT3 is also known to participate in cellular transformation and oncogenesis [4,5]. For an increasing number of tumour cell types, including multiple myeloma, prostate and colon carcinoma, STAT3 has been shown to control growth and cell survival [6].

Many studies indicate the involvement of various co-activators in the transcriptional action of STAT3 [7–10]. Co-activators act as bridging factors between DNA-bound transcription factors and the basal transcriptional machinery, recruiting additional co-activators and chromatin-remodelling complexes, and initiating acetylation and other modifications of nucleosomal histones as well as transcription factors. Among the first co-activators identified, CBP [CREB (cAMP-response element-binding protein)-binding protein]/p300 family proved functionally important for the transactivation potential of STAT3 and bound inducibly to STAT3 target regions. This recruitment did not depend on the presence of SRC-1. Altogether, this suggests that functional impairment of STAT3 is not involved in the induction of myeloma cell apoptosis by SRC-1 silencing. We therefore conclude that STAT3 transactivates its target genes by the recruitment of CBP/p300 co-activators and that this process generally does not require the contribution of SRC-1.

Key words: acute-phase reaction, interleukin-6 (IL-6), signal transducer and activator of transcription 3 (STAT3), steroid receptor co-activator-1 (SRC-1), transcription control.

Abbreviations used: ACT, α1-antichymotrypsin; ChIP, chromatin immunoprecipitation; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; Crf1, CRF6-interacting factor 1; DMEM, Dulbecco’s modified minimal essential medium; EGFP, enhanced green fluorescent protein; ERK, extracellular-signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; gp, glycoprotein; GRIP-1, glutamate receptor-interacting protein 1; HEK, human embryonic kidney; IL, interleukin; IκBα, inhibitor of nuclear factor κB α; NCoA, nuclear receptor co-activator; NFκB, nuclear factor κB; p/CAF, p300/CBP-associated factor; RNAi, RNA interference; shRNA, small hairpin RNA; SOCS3, suppressor of cytokine signalling 3; SRC, steroid receptor co-activator; STAT, signal transducer and activator of transcription; TRAP220, thyroid hormone receptor-associated polypeptide 220.

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Data obtained for steroid receptor co-activator-1 by microarray analysis have been deposited in the Gene Expression Omnibus (GEO) database at NCBI under accession number GSE4885.
SRC-1 was reported to interact with STAT3, bind to STAT3 target promoters and to enhance the STAT3-dependent transcription of the p21\superscript{waf}, junB, c-Myc and cyclin D1 genes [7,19–21]. Furthermore, knockdown of SRC-1 in mammary carcinoma cells interfered with the leptin- and STAT3-mediated proliferative response of these cells [19,21]. Consequently, SRC-1 was regarded as an important, if not essential, co-activator for STAT3. Although, however, targeted disruption of the \(\text{Stat3}\) gene in mice is known to be lethal [22], SRC-1/− mice display a phenotype correlated to thyroid hormone resistance [23], but are viable and do not seem to indicate defects in STAT3-related functions. This apparent contradiction prompted us to revisit the contribution of SRC-1 to STAT3-induced transcriptional and (patho)physiological responses. As we show in the present study, initial experiments with IL-6-dependent myeloma cells indeed demonstrated that silencing of SRC-1 expression by RNAi (RNA interference) caused a rapid onset of apoptosis in these cells, equivalent to the induction of apoptosis when STAT3 expression was knocked down. However, we failed to observe inducible binding of SRC-1 to STAT3 target genes in myeloma cells. We hence initiated a broader study on the potential involvement of SRC-1 in the transcriptional control of STAT3 target genes in the HepG2 human hepatocellular carcinoma cell line, for which an involvement of SRC-1 had been reported. SRC-1 was not required for the transcriptional response of STAT3 target genes or for the recruitment of CBP and p300 to STAT3-dependent promoters. Furthermore, microarray studies revealed no significant difference in IL-6 responses between normal HepG2 cells and cells depleted of SRC-1. We therefore conclude that, in general, p160 family co-activators are not essential co-activators for the transactivating potential of STAT3 and that the reported interaction between STAT3 and SRC-1 might serve other functions.

**EXPERIMENTAL**

**Reporter and expression vectors**

The luciferase reporter vector pACT-359Luc containing the promoter region (-359/+25, relative to the transcription start site) of the human \(\text{ACT}\) (\(\alpha\)-antichymotrypsin) gene was constructed by subcloning the promoter fragment from pACT-359CAT [24] into pGL3-Basic (Promega). A 543-bp fragment of the human haptoglobin promoter was amplified by PCR from HepG2 genomic DNA and inserted into the NheI and BglII sites of pGL3-Basic. SRC-1 expression vectors were gifts from Dr Eric Kalkhoven (University Medical Center Utrecht, Utrecht, The Netherlands) and Dr Edith Pfitzner (Institute for Biochemistry and Biophysics, Friedrich-Schiller-University Jena, Jena, Germany), pGAL4-CBP-(1–451) and pGAL4-CBP-(451–721), coding for fusion products of the respective CBP fragments and the DNA-binding domain of GAL4, were a gift from Dr Bernhard Lüscher (Department of Biochemistry and Molecular Biology, University Hospital Aachen, Aachen, Germany). Murine STAT3 expression vector pRC/CMV-Stat3 was a gift from Professor Christian Schindler (Department of Microbiology, Columbia University, New York, NY, U.S.A.). pRC/CMV-Stat3-LFAA was obtained by introducing mutations changing Leu\superscript{755} and Phe\superscript{757} of STAT3 into alanine residues, as described by Zhao et al. [20]. Construction of a vector encoding a STAT3 mutant resistant to silencing by pSUPER-shStat3 (see below) was achieved by introducing silent mutations into the RNAi target sequence using the QuikChange\textsuperscript{\textregistered} site-directed mutagenesis kit (Stratagene), yielding pcDNA-Stat3\textit{res}. This mutated STAT3 region was subcloned into pRC/CMV-Stat3-LFAA, yielding pRC/CMV-Stat3\textit{res-LFAA}. The vector MLV-Eg/FLAG/EGFP encoding the chimaeric receptor Eg consisting of the erythropoietin receptor extracellular domain and the gp130 intracellular domain has been described previously [25].

**RNAi vectors**

RNAi target sequences for silencing SRC-1 and STAT3 expression were chosen according to guidelines for the pSUPER RNAi vector system (OligoEngine), and the most effective ones were selected by verifying the reduction of protein levels. shRNA (small hairpin RNA) expression vectors were constructed using the method of Brummelkamp et al. [26] in the pSUPER vector (a gift from Professor Reuven Agami, Division of Gene Regulation, The Netherlands Cancer Institute, Amsterdam, The Netherlands). The following 64-bp oligonucleotides were subcloned into the BglIII and XhoI sites of pSUPER (only the sequence of the upper strands is shown; silencing target sequence and linker/loop sequences are in upper and lower case respectively): 5′-gatccccc-GATTTCCATCTAGTTCAACAGAGTCAGATCGGAAATCTTTTGGAA-3′ (pSUPER-shSRC-1) and 5′-gatccccc-CCCAAGAATGTAAACTCAACAGAGTCAGATCGGAAATCTTTTGGAA-3′ (pSUPER-shStat3). A vector encoding a scrambled sh-Stat3 sequence (pSUPER-shScrambled) was generated accordingly. To silence SRC-2 and SRC-3 expression, the target sequences were as selected and verified by Zhang et al. [27] were used with the same vector system. Oligonucleotides were obtained from MWG-Biotech. For stably silencing SRC-1 expression in HepG2 cells, the shRNA expression cassette of pSUPER-shSRC-1 was subcloned as a 315-bp XbaI/HindIII fragment into the XhoI site of the lentiviral infection vector Vir-NEO-IRES-GFP, yielding Vig-shSRC-1. Vig-NEO-IRES-GFP [containing a neomycin gene for selection and the EGFP (enhanced green fluorescent protein) gene as a marker for transduction efficiency] was generated by introducing the neomycin gene into the BamHI and SpeI sites of MLV-IRE-EGFP [25].

**Lentiviral infection**

Lentiviral supernatants for Vig-shSRC-1, Vig-shStat3 or Vig-shScrambled were obtained as described in [25]. For lentiviral infection, 2.2 ml of supernatant was added to 7 × 10\textsuperscript{6} HepG2 cells in a 60 × 15 mm tissue culture dish and incubated for 2–4 h. After adding 2.8 ml of medium, the incubation was continued for 2 days. The cells were then placed in fresh medium supplemented with 10 mg/ml G418 (genetin; Sigma–Aldrich), used for selection of stable transformants. HepG2 cells infected with empty Vig-IRES vector have been used as a control. Infection efficiency was evaluated by using flow cytometry for detection of the EGFP signal in infected cells at 3-day intervals starting from 2 days post-infection.

**Cell culture, transient transfection and reporter gene assays**

HEK (human embryonic kidney)-293 cells and HepG2 were maintained in DMEM (Dulbecco’s modified minimal essential medium) and a 1:1 (v/v) mixture of DMEM and Eagle’s F12 respectively, each containing 10% (v/v) fetal calf serum with 1% (w/v) penicillin/streptomycin (Life Technologies Gibco). Cells were transfected by the calcium phosphate co-precipitation method as described in [1]. Cells were starved in serum-free medium for 24 h before IL-6 stimulation. At 48 h after transfection, cells were seeded for 6 h with 10 ng/ml IL-6 (a gift from Professor Dr Stefan Rose-John, Department of Biochemistry, Christian-Albrechts-University Kiel, Kiel, Germany) and harvested. Luciferase activity was determined...
using the Luciferase assay system (Promega) and normalized to β-galactosidase (Roche) activity by co-transfection of the β-galactosidase expression vector pCH110 (GE Healthcare).

Human myeloma INA-6 cells were cultivated in RPMI 1640 medium (PAA Laboratories) with 1 ng/ml IL-6, 10% (v/v) fetal calf serum and 1% (w/v) penicillin/streptomycin. For transient transfection, INA-6 cells were harvested by centrifugation at 300 g for 10 min and resuspended in fresh RPMI 1640 medium without additives to a cell count of 10⁷ cells/ml. Electroporation was performed using a GenePulser™ (Bio-Rad Laboratories) and a cuvette (0.4 cm electrode, gap 50; Bio-Rad Laboratories) at settings of 950 μF and 280 V. For each experiment, 500 μl of cell suspension was used and 1.5 μg of pEGFP and 18.5 μg of pSUPER vectors. After electroporation, 500 ml of medium without additives was added and the cells were transferred to RPMI 1640 with 20% (v/v) fetal calf serum and IL-6.

**Immunoblotting**

Cell lysis and nuclear extraction were performed as described by Bellido et al. [28] and Andrews and Faller [29] respectively. Equal amounts of total protein were separated by SDS/PAGE (6% gel), blotted on to PVDF membrane (GE Healthcare) and detected by chemiluminescence (SuperSignal West Dura substrate; Pierce). Anti-SRC-1 monoclonal antibody was obtained from Upstate Biotechnology. Antibodies against SRC-2/GRIP-1 and SRC-3/NCoA-3 were from Santa Cruz Biotechnology. Anti-phospho-STAT3 (Tyr705) and anti-STAT3 monoclonal antibodies were obtained from Cell Signaling Technology and BD Transduction Laboratories respectively. Anti-ERK (extracellular-signal-regulated kinase) antibody (BD Biosciences) was used to confirm equal loading.

**Apoptosis assay**

In INA-6 cells, apoptosis was measured using the annexin V apoptosis detection kit II (BD Biosciences) using annexin V–phycoerythrin. Flow cytometric analysis was carried out using a FACScan flow cytometer using the CellQuest software (BD Biosciences). The analysis was restricted to successfully transfected cells by gating of EGFP-positive cells.

**ChIP (chromatin immunoprecipitation)**

Before ChIP, HepG2 cells were serum-starved for 2 days, and INA-6 cells withdrawn from IL-6 for 12 h. After IL-6 stimulation (10 ng/ml) for 30 min, cells (1.5 x 10⁷) were fixed by addition of formaldehyde to the medium at a final concentration of 1% and incubated for 10 min at room temperature (21°C). ChIP was performed according to the protocol of ChIP assay kit (Upstate Biotechnology).

Antibodies against the following were used for ChIP: STAT3 from R&D Systems, and SRC-1 (M-341), SRC-2/GRIP-1 (M-343), SRC-3/NcoA-3 (M-397), CBP (A-22), p300 (C-20), pCAF (p300/CREB-binding protein-associated factor) (H-369), TRAP220 (thyroid hormone receptor-associated protein 220) (M-255) and RNA polymerase II (N-20) from Santa Cruz Biotechnology.

**Gene expression analysis by DNA-oligonucleotide arrays**

Double-stranded DNA was synthesized from total RNA, amplified as cRNA, labelled and hybridized to Affymetrix HG-U133A GeneChips®, which were washed and scanned according to procedures developed by the manufacturer. Two independent sets of experiments were performed to assess IL-6-dependent expression patterns in HepG2 and HepG2-ΔSRC1 cells. Normalization of the individual arrays based on total intensity and comparative data analysis were performed with the Affymetrix software (MicroArray Suite MAS 5.0.1/MicroDB 2.0/DMT 2.0). Significance criteria for selecting genes differentially expressed in response to IL-6 were chosen as follows: the microarrays derived from INA-6 cells withdrawn from IL-6 for 12 h were set as reference chips with which microarrays obtained from INA-6 cells treated with IL-6 for 1 or 4 h were compared. Genes were regarded differentially expressed if in both experimental sets the change P-values were below 0.0025 and above 0.9975 (signal change call I and D according to the MAS5 software) for increased and decreased probe set signal intensities respectively. Alternatively, data analysis was carried out using the ArrayAssist software (Stratagenen), employing various analysis algorithms, producing equivalent results.

**RESULTS**

**Silencing of SRC-1 expression induces rapid apoptosis in INA-6 myeloma cells**

Human INA-6 multiple myeloma cells are strictly dependent on IL-6 for their survival [25, 30]. We demonstrated previously that the anti-apoptotic effect of IL-6 requires the activation of STAT3, and identified a number of target genes controlled by STAT3 in these cells [25]. We now asked whether the co-activator SRC-1, reported to be essential for STAT3 transcriptional activity towards several genes [7, 19, 20], is also required for blocking apoptosis in INA-6 cells. To this purpose, SRC-1 expression was silenced using a vector expressing a shRNA targeting human SRC-1 mRNA. This vector yielded an efficient down-regulation of SRC-1 protein when tested in human HEK-293 (Figure 1A) and HepG2 (see below) cells. In INA-6 cells transfected with this vector, apoptosis increased dramatically, whereas a vector expressing a scrambled shRNA sequence had no effect (Figure 1B). When compared with the onset of apoptosis observed in response to knockdown of STAT3, both the extent and time course of apoptosis appeared comparable (Figure 1C). In view of these experiments, we
Figure 1  SRC-1 silencing induces apoptosis in IL-6-dependent INA-6 human multiple myeloma cells as does silencing of STAT3

(A) Silencing efficiency of an shRNA targeted to the SRC-1 mRNA was verified by its expression from the vector pSUPER-shSRC-1 and transiently transfected using the calcium phosphate co-precipitation method into HEK-293 cells. At 48 h after transfection, cells were harvested, and equal aliquots of nuclear extracts or whole-cell lysates were subjected to SDS/PAGE and immunoblotted using antibodies against SRC-1 or ERK as a loading control. (B, C) Multiple myeloma INA-6 cells were transiently transfected with pSUPER-shSRC-1, pSUPER-shStat3 or a vector encoding an shRNA with scrambled sequence (sh-scrambled). After the time periods indicated, apoptosis was measured by a flow-cytometric annexin V assay. By gating to EGFP fluorescence, the analysis was restricted to successfully transfected cells. In (C), viable cell counts obtained for the sh-scrambled control were set as 100 %. Results are from a representative experiment from three independent studies.

reasoned that the apoptotic effect of SRC-1 silencing might be due to blocking the expression of survival genes that are under the control of STAT3.

SRC-1 binding to STAT3 target gene promoters is decreased rather than increased after IL-6 stimulation in both INA-6 and HepG2 cells

To substantiate this hypothesis further, we investigated whether SRC-1 is recruited to STAT3 target genes in INA-6 cells along with the transcription factor in response to the cytokine as it had been shown for p21 in HepG2 hepatocellular carcinoma cells as well as for c-Myc and cyclin D1 genes in MCF-7 mammary carcinoma cells [7,19,21]. Since INA-6 cells require the permanent presence of IL-6 for survival, the cytokine was only transiently withdrawn from the medium 12 h before the cells were restimulated with IL-6. As we have shown previously, this period of IL-6 withdrawal still allows the cells to be rescued from apoptosis, but is sufficient to entirely down-regulate STAT3 activity as well as expression of STAT3 target genes [25]. In situ binding of STAT3 and SRC-1 to promoter and enhancer regions of STAT3 target genes was analysed by ChIP. Using primers specific for the well-characterized STAT3 sites in the BCL3 intronic enhancer HS4 [31] and the promoter of the junB gene (Figure 2, upper panels), we could demonstrate inducible binding of STAT3 to both elements. Unexpectedly, however, binding of SRC-1 was evident even in untreated cells and decreased, rather than increased, in response to IL-6.

Given the reported inducible recruitment of SRC-1 for other STAT3 target genes and cell types, we asked whether binding of SRC-1 to STAT3 regulatory elements might vary in a cell-type- and/or gene-specific manner. To clarify this, we extended our study to HepG2 hepatocellular carcinoma cells. Analysing the STAT3 enhancer element of the gene encoding the acute-phase protein haptoglobin in HepG2 cells produced a similar pattern. Again, STAT3 was inducibly recruited to the promoter after IL-6 stimulation, whereas SRC-1 binding decreased (Figure 2, lower panels).
lower panels). The same response was observed for the STAT3 sites upstream of the c-fos and junB genes. To demonstrate that, under the conditions applied, transcriptional induction of IL-6 target genes effectively occurred, we investigated the presence of RNA polymerase II to the haptoglobin promoter. In fact, IL-6 strongly induced binding of the polymerase, as expected (Figure 2). Likewise, co-activator pCAF and the Mediator subunit TRAP220/Med1 were detected at the haptoglobin promoter, and slightly enhanced binding was observed upon treatment with IL-6. Inducible binding was also found for the co-activators p300 and CBP (see below). We conclude that, for the target genes studied in INA-6 and HepG2 cells, the transcriptional induction by IL-6 involves STAT3 recruitment, but reciprocally correlates with binding of SRC-1 to the promoter regions.

**SRC-1 silencing does not interfere with STAT3 transactivating activity in HepG2 cells**

Our finding seemed to challenge the view that SRC-1 is a generally required co-activator for STAT3. We therefore set out to investigate the role of the co-activator for the IL-6-induced expression of STAT3 target genes in more detail. For this purpose, we silenced SRC-1 expression by transiently transfecting the above-mentioned shRNA vector into HepG2 cells and determined the promoter activity of the ACT gene by a luciferase reporter assay. This promoter harbours two STAT3-binding sites that are essential for its induction by IL-6 [24]. As shown in Figure 3(A), ACT promoter activity did not change significantly when the cells were depleted of endogenous SRC-1. In contrast, silencing of STAT3 expression using a shRNA vector described previously [31] resulted in an almost complete inhibition of reporter activation, as expected.

In addition to the gene-silencing approach, we obstructed STAT3′s ability to interact with SRC-1. Within STAT3, the interaction site with SRC-1 has been localized to a C-terminal region of the STAT3 transactivation domain, and replacing Leu755 and Phe757 by alanine residues (STAT3-LFAA) was demonstrated to generate a STAT3 mutant unable to recruit SRC-1 [20]. We constructed an expression vector coding for this STAT3-LFAA mutant. Furthermore, by introducing silent mutations into the shRNA target region within the STAT3 cDNA, the expressed STAT3 mRNA was rendered resistant to this shRNA. Either STAT3-LFAA or wild-type STAT3 was then co-expressed with the sh-Stat3 vector, thereby knocking down endogenous STAT3 and replacing it by exogenous mutated or wild-type STAT3. STAT3 protein expression and phosphorylation was confirmed by immunoblotting (Figure 3B). Reporter studies demonstrated that the STAT3-LFAA mutant activated the ACT promoter as efficiently as wild-type STAT3. The same proved to be true for the haptoglobin promoter (results not shown). We conclude that, at least in reporter gene assays, transactivation through STAT3 of the genes studied does not require SRC-1.

Various nuclear receptors and other transcription factors are able to interact with all three members of the p160 family. We therefore asked whether SRC-2 and SRC-3 could take over the role from SRC-1 in the above-described studies and thereby conceal a functional requirement of SRC-1. To clarify this, vectors coding for shRNAs silencing SRC-2 and SRC-3 were constructed as described by Grenier et al. [32]. Successful reduction of protein levels was proven by immunoblot analysis in HEK-293 cells transfected with these vectors (Figure 4B). Silencing of SRC-2 or SRC-3 did not decrease ACT promoter activity (Figure 4A). Even the combined silencing of all three p160 members did not interfere with STAT3 activity. As with SRC-1, ChIP experiments showed the presence of SRC-2 and SRC-3 at the STAT3 target promoters of the haptoglobin and junB genes (Figure 4C). In contrast with SRC-1, however, SRC-2 binding tended to increase after IL-6 stimulation for both genes. The strong constitutive presence of SRC-3 at the haptoglobin promoter was also increased further in response to IL-6. Hence, all three p160 family members are required at STAT3 target genes, and STAT3 activation and binding to these regions might influence their ratio. In any case, however, our results demonstrate that none of the p160 members seems to be essential for the STAT3-dependent transactivation of the STAT3 target promoters tested.

**In contrast with SRC-1, CBP is required for STAT3 transcriptional activation**

Co-activators of the p300/CBP family were documented previously to interact with various STAT proteins, including STAT3 [8,33]. It has been proposed that SRC-1 is essential for the recruitment of p300 and/or CBP to STAT3 target genes [7]. Since, for the genes examined in the present study, we were unable to detect an inducible recruitment of SRC-1, the question arose whether p300 and CBP can be recruited to such genes. As shown in Figure 5(A), binding of both co-activators to the STAT3-responsive regions of the haptoglobin, junB and...
Figure 4 Silencing of SRC family members does not interfere with STAT3 transcriptional activity

(A) HepG2 cells were transfected with ACT reporter and pSUPER vectors and stimulated by IL-6 as above. Results are means ± S.D. for three experiments are shown. Significance was calculated by Student’s t test and P < 0.1 (*), P < 0.01 (**) and P < 0.001 (***) are indicated. (B) HEK-293 cells were transiently transfected with pSUPER vectors encoding shRNAs (7.75 μg) targeting SRC-2 (sh-SRC-2), SRC-3 (sh-SRC-3) or a scrambled shRNA negative control (sh-ctr). An expression vector for EGFP (0.25 μg) was co-transfected to monitor transfection efficiency. Cells were harvested 48 h after transfection, and cell lysates were analysed by immunoblotting. Results were reproduced in three independent experiments. Anti-ERK1 antibody served as a loading control. (C) The presence of SRC-2 and SRC-3 at STAT3 target regions of the haptoglobin and junB promoters was determined by ChIP analysis of HepG2 cells as described in Figure 2.

c-fos promoters was strongly induced by IL-6. Overexpression of CBP domains (fused to the GAL4 DNA-binding domain) that are known to contain STAT-interaction sites turned out to be strongly inhibitory for a STAT3-driven reporter (Figure 5B), whereas the co-transfected β-galactosidase vector, driven by the SV40 (simian virus 40) early promoter, was not affected (results not shown). Expression of the fusion proteins was confirmed by immunoblotting (Figure 5C). Similarly, when HEK-293 cells were used to monitor an endogenous STAT3 target gene, SOCS3 (suppressor of cytokine signalling 3), we observed a dramatic decrease in SOCS3 mRNA induction after transfecting the cells with the N-terminal domain of CBP (Figure 5D). In contrast, SRC-1 silencing by shRNA did not affect endogenous SOCS3 induction in this system. Therefore, in contrast with SRC-1, the activity of CBP and/or p300 is strictly required for the induction of the STAT3 target genes investigated.

Stable SRC-1 silencing does not block the induction of endogenous IL-6 target genes in HepG2 cells

Although reporter gene assays are widely used to analyse gene regulation, they represent artificial scenarios whose regulatory attributes only partially resemble the situation at endogenous promoters. Therefore we undertook to investigate a potential role of SRC-1 for the regulation of endogenous IL-6 target genes in HepG2 cells. For this purpose, HepG2 cells were infected with a lentiviral vector containing the SRC-1-directed shRNA expression cassette as well as the EGFP and neo genes for control of infection efficiency and selection respectively. By selection with neomycin, HepG2 cells with permanently down-regulated SRC-1 expression (HepG2-ΔSRC1) were derived. Immunoblotting analysis verified the efficient knockdown of SRC-1 in these cells, as shown in Figure 6(A). Reporter gene assays performed with HepG2-ΔSRC1 as well as...
with unmanipulated HepG2 cells showed that IL-6-stimulated haptoglobin promoter activity was not reduced by SRC-1 silencing (Figure 6B). The same proved to be true in the case of the ACT promoter (results not shown). Moreover, when HepG2-ΔSRC1 cells were studied by ChIP, inducible p300 and CBP binding to the haptoglobin promoter was observed in spite of the absence of SRC-1 (Figure 6C). Therefore SRC-1 is not essential for the recruitment of these co-activators to STAT3 target genes.

To extend these studies to endogenous genes as discussed above, we isolated RNA from HepG2 and HepG2-ΔSRC1 cells that were either treated with IL-6 for 1 or 4 h or left untreated, and subjected it to a microarray analysis using Affymetrix HG-U133A GeneChips®. Upon analysis of genes that were differentially expressed between unmanipulated and silenced HepG2 cells, only 11 genes were found to significantly differ in their expression levels at least 2-fold (see Supplementary Table S1 at http://www.BiochemJ.org/bj/420/bj4200123add.htm). Remarkably, the most strongly down-regulated gene turned out to be the SRC-1 gene itself verifying its successful silencing. The microarray data were then analysed for potential changes with respect to the regulation of IL-6 target genes. In control HepG2 cells, 93 and 139 genes were found to be significantly induced at least 1.5-fold, and 103 and 87 were down-regulated by 1- and 4-h IL-6 treatments respectively. Among the genes induced by IL-6, many already known IL-6 target genes were found. As shown in Figure 6(D), a comparison of data obtained for HepG2 and HepG2-ΔSRC1 cells revealed no major differences with respect to their response to IL-6. For a selected number of genes, the microarray data were verified by real-time PCR with qualitatively identical results (results not shown). Closer inspection of the data obtained for those genes with an established regulatory role of STAT3, e.g. the genes coding for SOCS3, junB, c-fos, GADD45β (growth-arrest and DNA-damage-inducible 45β), C/EBPβ (CCAAT/enhancer-binding protein-δ), IRF-1 (interferon-response factor-1) and the acute-phase protein haptoglobin clearly demonstrated that their responsiveness to IL-6 was not reduced by SRC-1 silencing (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/420/bj4200123add.htm). These findings indicated that SRC-1 is not required for the induction of endogenous IL-6 target genes in HepG2 cells.

Taken together, we conclude that recruitment of CBP/p300 to IL-6 target genes is initiated by the binding of activated STAT3 to its enhancer elements and is essential for the transcriptional initiation of these genes. In contrast, SRC-1, at least for the majority of STAT3 target genes, is not essential for either CBP/p300 recruitment or STAT3-driven transactivation.

**DISCUSSION**

Previous reports have shown that particular p160 family members play an important role in the transcriptional activation by multiple transcription factors [34–36], including several members of the STAT family [7,15,16,37,38]. In several studies dealing with STAT–co-activator interactions, a significant role was assigned to SRC-1, but not to the other two p160 family members [7,15,16,19,39]. Reports indicating an involvement of SRC-1 in STAT3 transcriptional activation were focused on the regulation of particular STAT3 target genes, i.e. p21<sup>−/−</sup>, junB, c-myc and cyclin D1 [7,19–21]. Yin et al. [19] and Saxena et al. [21] demonstrated that silencing of SRC-1 decreases leptin-stimulated and STAT3-dependent proliferation of MCF-7 mammary carcinoma cells. In the present study, we therefore addressed the question of whether SRC-1 is also an essential co-activator for STAT3-mediated physiological effects of IL-6. We and others demonstrated previously that the IL-6-dependent survival and growth of multiple myeloma cells depends on STAT3 [25,40]. Our current finding that silencing of SRC-1 induced a strong apoptotic response in INA-6 myeloma cells, and that this response was similar in extent and time course to the one provoked by STAT3 silencing, seems in line with the assumption of an essential role of SRC-1 for STAT3 function. Targeted disruption of the Stat3 gene in mice is known to be lethal [22]. Recently, evidence was provided that Crif1 (CR6-interacting factor 1) is an essential transcriptional co-activator of STAT3 [10]. In fact, murine Crif1<sup>−/−</sup>embryos die around E6.5 (embryonic day 6.5) as a result of defective proliferation and massive apoptosis, together with markedly reduced expression of STAT3 target genes in a Crif1<sup>−/−</sup>blastocyst culture [10]. In contrast, SRC-1<sup>−/−</sup> mice display a phenotype correlated to thyroid hormone resistance [23], but are viable and do not seem to indicate defects in STAT3-related functions.

This apparent contradiction led us to investigate the involvement of SRC-1 in STAT3 action in more detail. Surprisingly, our ChIP analyses revealed that SRC-1 constitutively binds to STAT3-dependent regulatory elements of the junB and BCL3 genes in INA-6 cells, with decreased binding after IL-6 addition. Likewise, when several IL-6 target genes were tested by ChIP in HepG2 cells, the cytokine again decreased constitutive SRC-1 binding to STAT3-responsive regions. These results are in contrast with previous reports from other laboratories [7,19,20]. We therefore repeated the experiments extensively employing different antibody sources and using the same experimental conditions as in published studies, but always obtained the same results. Hence, we do not know the reason for this discrepancy. The possibility exists that the STAT3 sites of the myc, cyclin D1 and p21<sup>−/−</sup>genes might behave differently [21]. In fact, we observed one STAT3 enhancer element, i.e. the 3′ enhancer of the junB gene [41], that exhibited induced SRC-1 binding (results not shown). In the case of the junB promoter, however, the ChIP primer sequences we used were identical with those published [20]. We do not have a plausible explanation for the conflicting results.

Numerous studies rely on overexpression of co-activators as an approach to examine their effects on the activity of transcription factors. For the promoters examined in the present study, an enhancement of IL-6-dependent transcriptional activation by co-expression of SRC-1 in reporter gene assays might support its co-activator function. However, in our hands, this was observed only when SRC-1 levels were raised to high, probably non-physiological, levels and when the resulting luciferase data were normalized to protein concentration (results not shown). We therefore reasoned that down-regulating SRC-1 function might provide a more reliable insight into the role of this co-activator for STAT3 transcriptional activation.

Furthermore, our data using an shRNA targeting SRC-1 expression clearly show that the STAT3 target promoters investigated do not rely on SRC-1 function. In contrast, we repeatedly observed a slight stimulatory effect on promoter activities in reporter gene assays after SRC-1 functional knockdown. To extend this study to a more global survey of IL-6 target genes, we generated HepG2 cells with permanently silenced SRC1 expression. Upon microarray analysis of these HepG2-ΔSRC1 as well as control HepG2 cells, covering more than 20000 genes, we observed an overall comparable pattern of IL-6-induced expression changes. Therefore our notion that SRC-1 is dispensable for IL-6-induced transcription as concluded from reporter assays for a limited number of genes, also applies when IL-6 target genes are studied genome-wide. We conclude further that the induction of apoptosis in myeloma cells upon SRC-1 silencing is most likely to not be due to an interference with STAT3 function, but rather indicates that SRC-1 is essential for another process in these cells. Likewise, the reduction of
Stable SRC-1 knockdown by RNAi in HEPG2 cells does not change IL-6 target gene expression patterns

To permanently silence SRC-1 expression in HEPG2 cells, an sh-SRC-1 expression vector was introduced by lentiviral infection. Selection by G418 for successfully transduced cells yielded HepG2-ΔSRC1 cells. (A) Efficiency of SRC-1 silencing was verified by immunoblots (IB) of nuclear extracts or whole-cell lysates of HepG2-ΔSRC1 cells or HepG2 cells mock-transduced with an empty vector. (B) Activities of the haptoglobin promoter in HepG2-ΔSRC1 or control HepG2 cells were determined by reporter gene assay as described above. Results derived from three independent experiments are shown as luciferase activity relative to IL-6-treated control HepG2 cells. (C) Binding of STAT3 and co-activators of the p160 and p300 families to the haptoglobin promoter in HepG2-ΔSRC1 was analysed by ChIP as described in Figure 2. β-Actin and total input chromatin controls demonstrating specificity and equal loading were performed, but are not shown. (D) HepG2-ΔSRC1 or control HepG2 cells were treated with 10 ng/ml IL-6 for 1 or 4 h or left untreated. Thereafter, total RNA was isolated and used for hybridization on Affymetrix HG-U113A GeneChips®. Two independent experiment series were carried out. Genes expressed differentially in response to IL-6 compared with the untreated controls are shown (left-hand four columns), with the colours representing the fold induction or repression as indicated beneath. For the same genes, the right-hand column compares the mean expression levels of HepG2-ΔSRC1 and control HepG2 cells.

Mammary carcinoma cell growth after knockdown of SRC-1 as observed by Yin et al. [19] might be due to functional interference with growth-controlling transcription factors other than STAT3.

There is considerable evidence for a compensatory balance between the three p160 family members [42,43]. We therefore included SRC-2 and SRC-3 in the present study. In fact, both p160 members were able to bind to STAT3 elements in various IL-6 target genes. For most elements studied, SRC-2 and SRC-3 binding was constitutive, but increased slightly after IL-6 stimulation. A number of studies have revealed that many transcription factors can interact with any of the three p160 proteins [34,44,45]. Recruitment of p160 factors does not necessarily occur synchronously. Gao et al. [46] showed that each p160 protein has a stage-specific function in the regulation of IκBα [inhibitor of NF-κB (nuclear factor κB)] gene transcription induced by NF-κB, by switching between SRC-1 and SRC-2 on IκBα gene promoter in the course of gene transcription [46]. The data of the present study might also suggest such a differential recruitment of individual p160 co-activators to regulatory elements of IL-6 target genes. However, knocking down p160 family members by shSRC expression vectors either individually or in combination did not reduce transcriptional activation by STAT3 as observed in reporter gene assays. Collectively, these data demonstrate that none of the p160 members seems to be essential for the STAT3-dependent transactivation.
Previous reports have indicated an involvement of CBP and/or p300 in STAT3 transcriptional activation [7,8,47,48]. In the present study, and in contrast with the results obtained for SRC-1, we observed an IL-6-inducible recruitment of both p300 and CBP to STAT3 target elements. Furthermore, by overexpressing dominant-negative CBP-fusion proteins we demonstrated a functional importance of these co-activators for the transactivating potential of STAT3. Our results provide evidence that neither the ability of CBP and p300 to enhance STAT3-dependent gene expression nor their recruitment to STAT3 sites are dependent on SRC-1.

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REFERENCES


SUPPLEMENTARY ONLINE DATA

Co-activator SRC-1 is dispensable for transcriptional control by STAT3

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Table S1  Genes differentially regulated between HepG2-ΔSRC1 and HepG2 cells

Table S1 lists genes differentially regulated between HepG2-ΔSRC1 and HepG2 cells. Total RNA isolated from HepG2-ΔSRC1 and HepG2 cells was used for hybridization on Affymetrix HGU113A GeneChips® arrays. Genes differentially regulated at least 2-fold are shown, with negative and positive fold change values representing genes that were down- and up-regulated in HepG2-ΔSRC1 compared with HepG2 cells respectively.

<table>
<thead>
<tr>
<th>Probe set</th>
<th>Gene name</th>
<th>Gene symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>209105_at</td>
<td>SRC-1</td>
<td>NCOA1</td>
</tr>
<tr>
<td>208669_s_at</td>
<td>CREBBP/EP300 inhibitor 1</td>
<td>CRI1</td>
</tr>
<tr>
<td>203716_s_at</td>
<td>Dipetidylpeptidase 4 (CD26)</td>
<td>DPP4</td>
</tr>
<tr>
<td>211470_s_at</td>
<td>Sulfoconjugate family, cytosolic, 1C, member 1</td>
<td>SULT1C1</td>
</tr>
<tr>
<td>212126_s_at</td>
<td>Dipeptidyl peptidase 4 (CD26)</td>
<td>CBX5</td>
</tr>
<tr>
<td>219437_s_at</td>
<td>Sulfotransferase family, cytosolic, 1C, member 1</td>
<td>SULT1C1</td>
</tr>
<tr>
<td>208998_at</td>
<td>Uncoupling protein 2 (mitochondrial, proton carrier)</td>
<td>UCP2</td>
</tr>
</tbody>
</table>

Figure S1  Stable SRC-1 knockdown by RNAi in HEPG2 cells does not change IL-6 target gene expression patterns

Figure S1 shows stable SRC-1 knockdown by RNAi in HEPG2 cells does not change IL-6 target gene expression patterns. HepG2-ΔSRC1 or control HepG2 cells were treated with 10 ng/ml IL-6 for 1 or 4 h, or left untreated. Thereafter, total RNA was isolated and used for hybridization on Affymetrix HG-U113A GeneChips®. Two independent experiment series were carried out. For six well-characterized STAT3 target genes, mean expression levels derived from the two microarray analyses are shown. The levels in control HepG2 cells treated with IL-6 for 1 h were set to 100%.

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Data obtained for steroid receptor co-activator-1 by microarray analysis have been deposited in the Gene Expression Omnibus (GEO) database at NCBI under accession number GSE4885.