RESEARCH COMMUNICATION

Cytoplasmic STAT proteins associate prior to activation

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The commonly accepted model of STAT factor activation at the cytoplasmic part of the receptor assumes that signal transducers and activators of transcription (STATs) are recruited from a cytoplasmic pool of monomeric STAT proteins. Based on a previous observation that non-phosphorylated STAT3–Src homology 2 domains dimerize *in vitro*, we investigated whether the observed dimerization is of physiological relevance within the cellular context. We show that STAT1 and STAT3 are pre-

associated in non-stimulated cells. Apparently, these complexes are not able to translocate into the nucleus. We provide evidence that the event of STAT activation is more complex than previously assumed.

Key words: DNA binding, HepG2 hepatoma cells, interleukin-6, A375 melanoma cells, nuclear translocation.

INTRODUCTION

Extensive studies have established the central role of signal transducers and activators of transcription (STATs) in cytokine signalling and have led to a widely accepted model of cytokine action via the Janus kinase/STAT pathway (for recent reviews see [1–3]). Activation of the STAT family members requires the transient association of the STATs with cytokine receptors [4,5]. The STATs are thought to be recruited from a cytoplasmic pool of monomeric STATs and have been shown to interact via their Src homology 2 (SH2) domains with specific phosphotyrosine motifs within the cytoplasmic parts of the activated receptors [6,7]. Subsequent to receptor binding, the STAT factors are phosphorylated on a single tyrosine residue by receptor-associated tyrosine kinases of the Janus kinase family [8–10]. This activation of the STAT factors by, for example, interleukin (IL)-6 leads to their homo- and/or heterodimerization and translocation to the nucleus where they bind to enhancers of IL-6-inducible genes, resulting in the transcriptional activation of, for example, acute-phase protein genes [11–13]. The dimerization of activated STAT factors has also been shown to be mediated by the SH2 domains [10], which has been confirmed by X-ray structures of the STAT1 and STAT3 dimers bound to DNA [14,15].

Previous experiments have shown that the SH2 domain is also the sole determinant of specific STAT factor activation via gp130 and the interferon- γ receptor [16,17]. The mechanism for the binding of STATs to phosphotyrosine-containing recruitment sites of the cytoplasmic region of signal transducing receptor subunits still needs to be elucidated. We have found that isolated STAT3–SH2 domains dimerize at pH 7.6 and that the monomer/dimer transition is accompanied by a conformational change within the SH2 domain [18]. On the basis of this observation, we set out to investigate whether such an association of STATs can be found in the cellular context.

Here we show that STATs form stable complexes in hepatoma and melanoma cells before stimulation and that IL-6 stimulation has no appreciable effect on the extent of STAT association. This pre-association seems to be limited to cytoplasmic STATs and has no apparent effect on STAT–DNA binding.

EXPERIMENTAL

Cell culture and materials

Human melanoma (A375), human hepatoma (HepG2) and monkey kidney (COS7) cells were purchased from A.T.C.C. (Rockville, MD, U.S.A.) (CRL 1619, HB 8065 and CRL 1651 respectively). Cell culture media and antibiotics were obtained from Life Technologies (Rockville, MD, U.S.A.) and fetal-calf serum was from Seromed (Berlin, Germany). Recombinant human IL-6 was prepared as described [19]. Soluble human IL-6 receptor (sIL-6R) was expressed in baculovirus-infected insect cells [20]. HepG2, A375 and COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12-Mix, RPMI 1640 medium and DMEM respectively. All media were supplemented with fetal-calf serum [10% (v/v) for HepG2 and COS7 cells, 5% for A375 cells], 50 μ g/ml penicillin and 100 μ g/ml streptomycin. For stimulation, HepG2 and COS7 cells were incubated with 20 ng/ml IL-6 for 15 min at 37 °C. COS7 and A375 cells were starved of serum for 6 h before stimulation. A375 cells were stimulated with 20 ng/ml IL-6 and 1000 ng/ml sIL-6R. Antibodies were purchased from Transduction Laboratories (Lexington, KY, U.S.A.), New England Biolabs (Beverly, MA, U.S.A.), Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) or BabCO (Richmond, CA, U.S.A.) and are listed in Table 1. Polyclonal monospecific antisera against STAT3 α (STAT3-ME) and STAT3 β were generated by immunization of rabbits with peptides corresponding to specific C-terminal amino acids of STAT3 α and STAT3 β . pCAGGS/haemagglutinin (HA)–STAT3 α

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; IL, interleukin; sIL-6R, soluble human IL-6 receptor; SH2, Src homology 2; STAT, signal transducer and activator of transcription; STAT3-ME, monospecific antisera against STAT3 α ; HA, haemagglutinin; HA-Ab, HA-antibody; EMSA, electrophoretic-mobility-shift assay.

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Table 1 Antibodies used in the present study

Designation	Manufacturer/Catalogue number	Characteristics
STAT1-TLp	Transduction Laboratories/G16930	Polyclonal, rabbit
STAT1-TLm	Transduction Laboratories/S21120	Monoclonal, mouse
STAT1-BL	New England Biolabs/7172	Polyclonal, rabbit
STAT1-E23	Santa Cruz Biotechnology/SC-346X	Polyclonal, rabbit
STAT1-pY	New England Biolabs/91715	Polyclonal, rabbit
STAT3-C20	Santa Cruz Biotechnology/SC-482	Monoclonal, mouse
STAT3-pY	New England Biolabs/91315	Polyclonal, rabbit
STAT3-TL	Transduction Laboratories/S21320	Monoclonal, mouse
STAT3-ME	Directed against the 33 C-terminal amino acids of murine STAT3 α (GNNGEGAEPSSAGGQFESLTFDMDLTSECATSPM)	Polyclonal, rabbit
STAT3- β	Directed against the seven C-terminal amino acids of murine STAT3 β (FIDAVWK)	Polyclonal, rabbit
HA-Ab	BAbCO/MMS101R	Monoclonal, mouse

was kindly provided by Dr T. Hirano (Osaka University, Osaka, Japan) [21] and the construction of pSVL/STAT3 β plasmid has been reported previously [22].

Preparation of transfectants

For the generation of stably transfected A375 cells, cells were electroporated in the presence of plasmid DNA (pCAGGS/HA-STAT3 α). Clones expressing exogenous protein were selected by Western-blot analysis with a monoclonal antibody against the HA tag from BabCO (Richmond, CA, U.S.A.) [23].

COS7 cells were transiently transfected with pCAGGS/HA-STAT3 α and pSVL/STAT3 β by using the DEAE-dextran method. Cells were incubated in 5 ml DMEM supplemented with 4 μ l of chloroquine (100 mM), 40 μ l of DEAE-dextran (50 mg/ml) and 10 μ g of each plasmid DNA for 80 min at 37 °C, avoiding gas exchange. Cells were then treated with PBS containing 10% (v/v) DMSO for 1 min. The cells were harvested after a further 48–72 h in culture medium.

Cell fractionation and preparation of nuclear extracts

The following lysis buffers were used for cell fractionation: (a) hypotonic buffer (10 mM Hepes/KOH, pH 7.5/2 mM EDTA); (b) Brij buffer [20 mM Tris/HCl, pH 7.5/150 mM NaCl/1% (v/v) Brij 96/2 mM EDTA]; (c) Triton buffer [20 mM Tris/HCl, pH 7.5/150 mM NaCl/1% (v/v) Triton X-100/2 mM EDTA]. All buffers were supplemented with NaF (50 mM), pepstatin A (2 μ g/ml), leupeptin (5 μ g/ml), aprotinin (5 μ g/ml), PMSF (1 mM) and Na₃VO₄ (1 mM). For lysis, the harvested cells were resuspended in 500 μ l of lysis buffer and incubated at 0 °C for 30 min. After centrifugation at 14000 *g* for 10 min at 4 °C, the supernatants were used for immunoprecipitation. Nuclear extracts were prepared as described [11], with modifications. Cells were lysed in hypotonic buffer A [10 mM Hepes/KOH pH 7.9/1.5 mM MgCl₂/10 mM KCl/1 mM Na₃VO₄/0.2 mM PMSF/0.5 mM dithiothreitol (DTT)] for 10 min at 0 °C. After centrifugation at 14000 *g* at 4 °C, supernatants were collected as cytoplasmic extracts. For preparation of nuclear extracts, the crude nuclei were washed twice by resuspension in buffer A and subsequent centrifugation. Rupture of the nuclei with buffer C [20 mM Hepes/KOH, pH 7.9/420 mM NaCl/1.5 mM MgCl₂/0.2 mM EDTA/25% (v/v) glycerol/1 mM Na₃VO₄/0.2 mM PMSF/0.5 mM DTT] for 20 min at 4 °C and centrifugation at 14000 *g* for 2 min yielded nuclear extracts.

Immunoprecipitation and Western-blot analysis

Immunoabsorption was performed by incubating 500 μ l of the extracts with 1 μ g of antibody overnight at 4 °C. Immuno-complexes were precipitated by the addition of Protein A-Sepharose (1 h, 4 °C) and washed three times with lysis buffer. Immunoabsorbed proteins were subjected to SDS/PAGE, transferred to an Immobilon PVDF membrane (Millipore, Eschborn, Germany) using a semi-dry electroblotting apparatus and probed with the respective antibody. Polyclonal goat anti-mouse or goat anti-rabbit horseradish-peroxidase-conjugated secondary antibodies (DAKO, Hamburg, Germany) were used to reveal the immunoreactive bands using the ECL[®] system (Amersham-Pharmacia, Freiburg, Germany).

Electrophoretic-mobility-shift assay (EMSA)

Protein concentration of nuclear extracts was measured with the Bio-Rad protein assay. A double-stranded mutated SIE-oligonucleotide from the *c-fos* promoter (m67SIE: 5'-GAT CCG GGA GGG ATT TAC GGG AAA TGC TG-3') was labelled by filling in 5' protruding ends with the Klenow enzyme, using α -[³²P]dATP (10 mCi/ml, 3000 Ci/nmol). Nuclear extracts containing 10 μ g of protein were incubated with about 10 fmol of probe in gel-shift incubation buffer [10 mM Hepes, pH 7.8/1 mM EDTA/5 mM MgCl₂/10% (v/v) glycerol/5 μ M DTT/0.7 μ M PMSF] containing 0.1 mg/ml poly(dI-C) and 1 mg/ml BSA for 10 min at room temperature. The protein-DNA complexes were resolved on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE [20 mM Tris/HCl (pH 8.0)/20 mM boric acid/0.5 mM EDTA] at 20 V/cm for 4 h. Gels were fixed in methanol/acetic acid/water (1:1:8, by vol.) for 30 min, dried and autoradiographed.

RESULTS

To investigate whether STAT factors associate prior to activation, we performed co-immunoprecipitation experiments with endogenous STAT1 and STAT3. Cellular extracts from IL-6-stimulated or untreated human hepatoma cells (HepG2) were subjected to immunoprecipitation with an antibody raised against the C-terminus of STAT3 (see Table 1). Interestingly, Western-blot analysis showed that the precipitates from stimulated as well as untreated cells contain both STAT3 and STAT1 (Figure 1A). Stimulation with IL-6 had no significant effect on the amount of co-precipitated STAT1. To analyse whether the STAT3-STAT1

Figure 1 Co-precipitation of STAT3 and STAT1 in human hepatoma cells

(A) HepG2 cells were stimulated with 20 ng/ml of IL-6 or left untreated. Cellular extracts were prepared using hypotonic, Brij 96 or Triton X-100 lysis buffer. After immunoprecipitation (IP) with STAT3-ME, proteins were resolved by SDS/PAGE and subjected to Western-blot analysis (WB) with antibodies to STAT1 (STAT1-TLp) or STAT3 (STAT3-TL). Lanes 1–4 show possible non-specific binding to Protein A–Sepharose. (B) As in (A) except that the STAT3 antibody, STAT3-C20, was used for precipitation. Stimulation was checked with a STAT3 phosphotyrosine antibody (STAT3-pY), which recognized the phosphotyrosine residue Tyr-705. (C) Specificity of the detecting STAT1 antibody (lane 2) was confirmed by resolving HepG2 lysates on a 7% polyacrylamide gel and detecting the blotted proteins with antibodies to STAT1 or STAT3 (lane 1, STAT3-TL; lane 2, STAT1-TLp; lane 3, STAT1-TLm; lane 4, STAT1-BL). The positions of molecular-mass markers are shown on the right.

complex persisted under different lysis conditions, co-immunoadsorption was performed in hypotonic buffer and in buffers containing Brij 96 or Triton X-100. The pre-association of STAT1 and STAT3 was found to be independent of buffer conditions. Control experiments lacking STAT3 antibodies clearly excluded non-specific binding to Sepharose as being responsible for the observed effect (Figure 1A, lanes 1–4).

In order to exclude the possibility that STAT3-ME shows cross-reactivity, and therefore might directly precipitate STAT1, we repeated the experiment with the STAT3-C20 antibody (Figure 1B). Activation of STAT1 and STAT3 was checked by the use of antibodies specific for tyrosine phosphorylated STAT1 or STAT3 respectively. Activated STAT3 was only found in extracts of IL-6-treated cells (Figure 1B, WB: STAT3-pY). Similar results were obtained for STAT1 (results not shown). Again, activation of the STAT factors had no effect on the co-precipitation of STAT1 and STAT3.

To rule out cross-reactivity of the STAT1-detecting antibody, STAT1-TLp, STAT1 and STAT3 were separated by SDS/

PAGE, immunoblotted and detected with a set of antibodies to STAT1 (Figure 1C, lanes 2–4) and STAT3 (lane 1). The STAT1 antibodies, STAT1-BL (lane 4), STAT1-TLm (lane 3) and STAT1-TLp (lane 2) specifically recognized STAT1 α and STAT1 β but not STAT3.

As phosphorylated STAT dimers readily translocate into the nucleus and bind to enhancer elements of target genes, we checked the subcellular localization of the preformed STAT dimers. Cytoplasmic and nuclear extracts from IL-6-stimulated or untreated HepG2 cells were prepared and probed for STAT1 and STAT3 by Western-blot analysis (Figures 2A and 2B, lanes 7 and 8). Similar amounts of STAT1 and STAT3 were found in the cytoplasmic fractions of both stimulated and untreated cells. As for Figure 1, immunoprecipitation was performed with different antibodies to STAT3. Co-precipitated STAT1 was detected in the cytoplasmic extracts of stimulated as well as untreated cells (Figure 2A, lanes 3–6). As observed for total cell extracts, STAT1 and STAT3 form stable complexes before activation (Figure 2A, lanes 4 and 6). Stimulation was controlled by monitoring the phosphorylation of Tyr-705 of STAT3 (Figure 2A; WB: STAT3-pY). In contrast, pre-associated STAT complexes were barely detectable in the nuclear extracts of untreated cells (Figure 2B, lanes 4 and 6). Association of STAT1 and STAT3 was shown for stimulated HepG2 cells (Figure 2B, lanes 3 and 5). Furthermore, we monitored the total amounts of STAT1 and STAT3 in nuclear extracts (Figure 2B, lanes 7 and 8). Although STAT1 could be found to the same extent in the nucleus of stimulated and untreated cells, only marginal amounts of nuclear STAT3 were detected prior to stimulation. Thus the observed stimulation-independent association of STAT1 and STAT3 occurs primarily in the cytoplasm.

In addition, we performed an EMSA (Figure 2C) with the cytoplasmic (lanes 1 and 2) and nuclear extracts (lanes 3–8) used for the precipitation experiments in Figures 2(A) and 2(B). The DNA binding activity of STAT1–3 heterodimers, as well as that of STAT1–1 and STAT3–3 homodimers, appeared only after IL-6 stimulation (lanes 1 and 3). The identity of STAT1 and STAT3 was confirmed by the use of antibodies to STAT1 (lanes 5 and 6) and STAT3 (lanes 7 and 8). Both antibodies failed to produce significant supershifting, rather they led to an inhibition of STAT–DNA binding. This shows that dimerization of STATs is necessary, but not sufficient, for DNA-binding activity.

Co-precipitation of STAT1 and STAT3 was also performed with extracts of human A375 melanoma cells. Endogenous STAT1 and STAT3 were co-precipitated with the STAT3-ME antibody from extracts of IL-6-stimulated and untreated cells (Figure 3, lanes 5 and 6). In addition, we utilized A375 cells stably transfected with STAT3 and carrying an N-terminal HA tag [23]. Precipitates obtained with an HA antibody (HA-Ab) were probed for co-immunoadsorbed STAT1. As observed for the parental A375 cells, equal amounts of STAT1 were co-precipitated with STAT3 independently of tyrosine phosphorylation of STAT (lanes 3 and 4).

In order to investigate whether a similar pre-association could be found for STAT3, we co-transfected COS7 cells with expression vectors for HA-STAT3 α and STAT3 β . Due to their different molecular masses, these forms are readily resolved by SDS/PAGE. After immunoadsorption with HA-Ab, precipitates were probed for co-precipitated STAT3 β (Figure 4). Detection with STAT3-TL, raised against the N-terminus of STAT3 and thus recognizing both STAT3 and STAT3 β , shows that STAT3 β associates with HA-STAT3 α (lanes 5 and 6). The identity of HA-STAT3 α and STAT3 β was confirmed by reprobing the blot with HA-Ab (lanes 7 and 8) and a STAT3 β -specific polyclonal antibody (results not shown).

Figure 2 Co-precipitation of STAT3 and STAT1 from nuclear extracts

HepG2 cells were stimulated as described in the legend of Figure 1. Cytosolic (A) and nuclear extracts (B) were prepared and immunoprecipitation (IP) with STAT3-ME (lanes 3 and 4) and STAT3-C20 (lanes 5 and 6) was carried out in Triton X-100 lysis buffer. Lysates (lanes 7 and 8) and precipitates (lanes 3–6) were subjected to Western-blot (WB) analysis with antibodies to STAT1 or STAT3 (STAT1-TLp, STAT3-pY and STAT3-TL). (C) Cytosolic and nuclear extracts (lanes 1, 2 and 3–8 respectively) were analysed by EMSA with a STAT1–STAT3 specific DNA probe (m67-SIE). Supershifts were performed with specific antibodies to STAT1 (STAT1-E23; lanes 5 and 6) or STAT3 (STAT3-ME; lanes 7 and 8).

DISCUSSION

STAT factors play a key role in cytokine signal transduction. They are recruited to activated tyrosine phosphorylated cytokine receptors via their SH2 domain. Phosphorylation at a conserved C-terminally located tyrosine residue leads to dissociation from the receptor and subsequent homo- and/or hetero-dimerization of the STATs. These activated STAT dimers translocate to the nucleus and exert their function as activators of transcription (for reviews see [1–3]).

Although the activation of STAT factors has been extensively studied, data on STAT factors prior to their activation is scarce. The still widely accepted model postulates that STATs are recruited from a cytoplasmic pool of monomeric STATs [10]. We recently reported that isolated STAT3-SH2 domains are dimerized at pH 7.6 in a phosphotyrosine-independent manner and that conformational differences existed between the monomeric and the dimeric SH2 domains [18]. This observation prompted us to investigate whether such an association might be relevant for the full-length STAT factors in the cellular context.

Figure 3 Co-precipitation of STAT3 and STAT1 in human melanoma cells

A375 cells (lanes 5 and 6) and A375 cells stably over-expressing HA-STAT3 α (lanes 1–4) were stimulated with 20 ng/ml of IL-6 and 1000 ng/ml sIL-6R or left untreated. Cellular extracts were prepared using Triton X-100 lysis buffer. After immunoprecipitation (IP) with antibodies to the HA tag (HA-Ab; lanes 3 and 4) or STAT3 (STAT3-ME; lanes 5 and 6), proteins were resolved by SDS/PAGE and subjected to Western-blot analysis with antibodies to STAT1 or STAT3 (STAT1-TLp, STAT3-TL). Stimulation was checked with the STAT3-pY antibody. Lanes 1 and 2 show controls for non-specific binding to Protein A–Sepharose.

Figure 4 Co-precipitation of HA-STAT3 α and STAT3 β in transfected COS7 cells

COS7 cells were transiently co-transfected with expression plasmids for STAT3 β and HA-tagged STAT3 α . Cellular extracts were prepared using Triton X-100 lysis buffer and subjected to immunoadsorption with an antibody to the HA tag of STAT3 α (HA-Ab; lanes 5–8). Western-blot analysis (WB) was performed with STAT3-TL (lanes 1–6) and HA-Ab (lanes 7 and 8). Lanes 1 and 2 show controls for non-specific binding to Protein A–Sepharose.

In order to address this question, we performed co-precipitation experiments with STAT1 and STAT3. We have shown that endogenous STAT3 and STAT1 form complexes before activation (Figures 1 and 3). This pre-association was found in both HepG2 and A375 cells indicating that non-phosphorylated STATs might not generally exist as monomers but as preformed complexes. In order to investigate whether the observed complexes can also be found in the nucleus, we analysed nuclear extracts for the presence of STAT factors and performed immunoprecipitation experiments (Figure 2B). In contrast to cytoplasmic extracts (Figure 2A), nuclear STAT1 co-precipitates with STAT3 only after cytokine stimulation. Furthermore, although similar amounts of STAT1 were detected in nuclear fractions of stimulated and untreated cells, only very small amounts of STAT3 could be found in the nucleus of untreated cells (Figure 2B, lane 8). We conclude that pre-association does

not render nuclear translocation of the STAT1–STAT3 complex possible. Thus it seems that the pre-association occurs only within the cytoplasmic environment.

Furthermore, we tested whether a stimulation-independent interaction also occurred between STAT3 molecules (Figure 4). We were able to show that the two splice variants STAT3 α and STAT3 β are associated in extracts of unstimulated cells. A recent report by Novak et al. [24] demonstrated the existence of stable STAT3 homodimers in the presence of bivalent cations before activation. A truncated STAT3 construct lacking 85 amino acids at the C-terminus failed to interact with the full-length STAT3. The complexes observed by these authors were disrupted by the addition of 0.5 mM EDTA. The association reported in the present work did not depend on EDTA concentration within the range tested (1–40 mM; results not shown).

Stancato et al. [25] reported that STAT1 and STAT3 could readily be co-precipitated in extracts from 3T3-F442A mouse fibroblasts under hypotonic conditions. Although, in the present work, buffer conditions had little effect on the amount of co-precipitated STAT1 in human hepatoma and melanoma cells, the trend of the results was similar. As the pre-association of STAT1 and STAT3 is found in different cell types, we feel that the reported pre-association is of general relevance and that monomeric STATs represent only a fraction of the pool of cytoplasmic STAT proteins in untreated cells. This is in line with an observation made by Ndubuisi et al. [26] in a very recent report. These authors found that only a small pool of monomeric STAT3 exists in the cytoplasm, whereas most STAT3 appeared in high-molecular-mass complexes of 200–400 kDa and 1–2 MDa, which they named 'statosome I' and 'statosome II' respectively. Although some of the proteins participating in these complexes have been identified, their exact composition remains to be elucidated. The molecular mass of homo- or hetero-dimeric STATs, like the STAT1–STAT3 or STAT3–STAT3 complexes reported in the present study, would fit into the size range of the 'statosome I' [26].

Taken together, we provide evidence that the event of STAT recruitment is more complex than previously assumed, and that some aspects of the widely accepted model of STAT activation need to be revised.

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