STAT3 is enriched in nuclear bodies

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Summary

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is involved in a variety of biological functions. It is essential for the signal transduction of interleukin-6 (IL-6) and related cytokines. In response to IL-6 stimulation STAT3 becomes phosphorylated and translocates into the nucleus where it binds to enhancer sequences of target genes. We found that activated STAT3 is enriched in dot-like structures within the nucleus, which we termed STAT3 nuclear bodies. To examine the dynamics of STAT3 nuclear body formation, a fusion protein of STAT3 and yellow fluorescent protein (YFP) was constructed. Studies in living cells have shown that the appearance of STAT3 nuclear bodies is transient, correlating with the timecourse of tyrosine-phosphorylation of STAT3. Furthermore, we show by fluorescence recovery after photobleaching (FRAP) analysis that STAT3 within nuclear bodies consists of a highly mobile and an immobile fraction. Colocalization studies provided evidence that these bodies are accompanied with CREB binding protein (CBP) and acetylated histone H4, which are markers for transcriptionally active chromatin. Moreover, STAT3 nuclear bodies in HepG2 cells are not colocalized with promyelocytic leukemia oncoprotein (PML)-containing bodies; neither is a sumoylation of activated STAT3 detectable. Taken together, our data suggest that STAT3 nuclear bodies are either directly involved in active gene transcription or they serve as reservoirs of activated STAT3.

Key words: STAT3, Nuclear bodies, FRAP, PML, CBP, Acetylated histones

Introduction

Signal transducer and activator of transcription 3 (STAT3), formerly known as acute phase response factor, was originally identified as the main transcription factor involved in the interleukin 6 (IL-6)-dependent induction of acute phase protein synthesis in hepatocytes (Wegenka et al., 1993). Today, seven different genes are known that encode STAT transcription factors (STATs 1, 2, 3, 4, 5a, 5b and 6). Although the other STATs have rather specific functions in response to a limited number of cytokines, STAT3 is involved in a wide spectrum of biological functions, as it is activated by various cytokines and growth factors (Levy and Darnell, 2002). Consequently, deletion of STAT3 by gene targeting has the most severe phenotype of all STAT-knockout mice. STAT3-deficient mice die in utero at the onset of gastrulation (Takeda et al., 1997), indicating an important role for STAT3 in embryonal development. Besides acute phase protein induction in hepatocytes, STAT3 is essential for the anti-inflammatory activities of IL-10 in macrophages (Takeda et al., 1999), the differentiation and proliferation of lymphocytes and the regulation of other immune functions (Levy and Lee, 2002). Moreover, STAT3 is required for heart development during embryogenesis (Yoshida et al., 1996) and is involved in fertility, having a role in blastocyst implantation and decidualization (Robb et al., 1998; Stewart et al., 1992). Owing to these diverse functions, STAT3 plays a key role in the development of several diseases such as chronic inflammation, autoimmune diseases and certain types of cancer (Akira et al., 1993).

STAT3 is essential for the signal transduction of the family of IL-6-type cytokines consisting of IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1) and cardiotrophin-like cytokine (CLC). The mechanism of STAT3 activation by IL-6-type cytokines has been investigated in detail and is therefore well understood (Heinrich et al., 1998). Gp130 is the common signal-transducing receptor component of all IL-6-type cytokines. Like all cytokine receptors, gp130 has no intrinsic kinase activity but is constitutively associated with Janus kinases (Jaks). Among the four known Jaks, Jak1, Jak2 and Tyk2 but not Jak3 have been shown to associate with gp130 (Lütticken et al., 1994). For gp130-mediated signal transduction, Jak1 seems to be the most important (Guschin et al., 1995; Rodig et al., 1998). On stimulation of gp130 by one of the IL-6-type cytokines, associated Jak1 is activated by phosphorylation. The activated Jak in turn phosphorylates tyrosine residues in the cytoplasmic tail of gp130. These phosphotyrosines serve as docking sites for STAT3, which binds via its SH2-domain to the receptor (Lütticken et al., 1994; Stahl et al., 1994). The recruited STAT3 becomes phosphorylated on a single tyrosine residue (Y705), most probably also by Jak1. Phosphorylated STAT3 is released from the receptor, dimerizes by intermolecular phosphotyrosine SH2-domain interactions and translocates into the nucleus. Nuclear tyrosine phosphorylated STAT3 dimers specifically bind to enhancer sequences and induce gene expression (Wegenka et al., 1994). The transactivation activity of STAT3

is augmented by an additional serine phosphorylation (Lütticken et al., 1995; Wen et al., 1995). The Jak/STAT-pathway from the plasma membrane to the nucleus has been delineated in detail, but the distribution and dynamics of STAT3 within the nuclear compartment are less well defined.

The nucleoplasm is far from being homogeneous, but rather it is divided into several subcompartments (Dundr and Misteli, 2001; Hendzel et al., 2001). In contrast to cytosolic organelles, these subcompartments are not confined by a membrane but are believed to arise and be maintained by self organization (Misteli, 2001). The best-characterized nuclear structures are chromatin and the nucleolus. Chromatin consists of DNA and DNA-bound proteins, most prominently histone proteins. It is subdivided into several chromosome territories occupied by DNA from distinct chromosomes. Chromatin can also be functionally discriminated in dense transcriptionally inactive heterochromatin and less condensed transcriptionally active euchromatin. Active chromatin is characterized by the presence of acetylated histones. The nucleolus, where transcription of ribosomal RNA takes place, is readily visible in most microscopic images of the nucleus. Besides chromatin and the nucleolus, less well defined nuclear compartments have been described in the interchromatin space. There, the nuclear speckles or splicing factor compartments (SFCs) are located in which splicing factors are enriched. These factors are recruited from the SFCs to the sites where splicing occurs (Misteli, 2000). SFCs vary in size from a diameter of about 0.5-3 μm. Even smaller compartments (0.5-1 μm diameter) have been characterized and are referred to as nuclear bodies. Here, Cajal bodies (Gall, 2000) and PML-nuclear bodies (Zhong et al., 2000) have been described. Cajal bodies are functionally involved in small nuclear ribonucleoprotein (snRNP) biogenesis. PML-nuclear bodies are dot-like structures to which several transcription factors and cofactors such as histone acetyl transferases [CREB binding protein (CBP)/p300] have been localized (Boisvert et al., 2001). Its characteristic component is the PML-protein that is mutated in promyelocytic leukemia (Zhong et al., 2000).

In the study presented here we set out to define the localization of STAT3 within these nuclear compartments. To achieve this, immunostaining of STAT3 and other nuclear components was performed. Most interestingly, activated nuclear STAT3 was found to be enriched within dot-like structures resembling nuclear bodies. To investigate the dynamics of biogenesis and the steady-state of these STAT3-enriched structures, a fusion protein of STAT3 and the yellow fluorescent protein (YFP) was constructed (STAT3-YFP). After biochemical characterization of STAT3-YFP, the fusion protein was used for live cell imaging and fluorescence recovery after photobleaching (FRAP) studies.

Materials and Methods

Cell culture and cell transfection

COS-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), HepG2 cells in DMEM/F12, both supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin and 100 μg/ml streptomycin (BIO-Whittaker, Verviers, Belgium). Transient transfections were performed by using the DEAE-dextrane method for COS-7 cells or the calcium-phosphate method for HepG2 cells as described. Alternatively, FuGENE 6 (Roche, Mannheim, Germany) transfection reagent was used according to the manufacturer's instruction.

Cloning of STAT3-YFP

To construct STAT3 fused to YFP, STAT3 cDNA was amplified using the primers 5′-CAAGCTCGAG ATGGCTCAGT GGAACCAGCT GCAG-3′ (sense) and 5′-GTGTGAGGTG ACCACATGGG GGAGGT-3′ (antisense). A *Xho*I restriction site at the 5′ end and a *BstE*II site at the 3′ end were introduced with the primers. The stop codon was deleted. The PCR product was introduced into pSVL-gp130-YFP using the restriction enzymes *Xho*I and *BstE*II to substitute the gp130 insert with STAT3. The resulting expression vector encodes full-length murine STAT3 fused by a linker of three amino acids (WSP) to YFP.

Electrophoretic mobility shift assay (EMSA)

Cells were transfected 48 hours before stimulation. Nuclear extracts were prepared as described previously (Wegenka et al., 1993). Protein concentrations were measured with the Bio-Rad protein assay (Bio-Rad, Richmond, USA). A double-stranded mutated *sis*-inducible element (SIE) oligonuleotide from the *c-fos* promotor (m67SIE: 5′-GAT CCG GGA GGG ATT TAC GGG AAA TGC TG-3′) was labeled by filling in 5′ protruding ends with the Klenow enzyme using [α-^{32}P]dATP (3000 Ci/mmol). Nuclear extracts containing 5-10 μg protein were incubated with about 10 fmol (10,000 cpm) of labeled oligonucleotides in gel shift incubation buffer [10 mM HEPES (pH 7.8), 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 5 μM DTT, 0.7 μM PMSF, 0.1 mg/ml of poly(dI-dC), and 1 mg/ml BSA] for 10 minutes at room temperature. For supershifts, the radioactively labeled probe and nuclear extracts were incubated with 1 μg α-STAT3 antibody (St Cruz, C-20) or 1 μg α-green fluorescent protein (GFP) antibody (Rockland, Gilbertsville, USA) for 10 minutes. The DNA/protein complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25% TBE (200 mM Tris, 166 mM boric acid, 2 mM EDTA, adjusted to pH 8.3) at 20 V/cm for 4 hours. Gels were fixed in a water solution of 10% methanol and 10% acetic acid for 30 minutes, dried and autoradiographed.

Reporter gene assay

m67-SIE-TK-Luc contains a STAT3 binding site upstream of the luciferase-encoding sequence. For reporter gene assays, HepG2 cells were transfected with 6 μg of luciferase reporter construct, 2 μg of β-galactosidase control plasmid pCH110 (Pharmacia, Uppsala, Sweden) and 4 μg of the respective expression vector. Twentyfour hours after transfection, cultures were subdivided, and after a 12 hour recovery period they were treated for 24 hours with 20 ng/ml IL-6. Luciferase assays were performed using a luciferase assay kit (Promega, Madison, USA). The values were normalized to β-galactosidase activity.

Live cell imaging

HepG2 cells were transfected with STAT3-YFP using the calcium phosphate method. Fortyeight hours after transfection, cells were placed into a perfusion chamber. Stimulation was performed at 37°C with 20 ng/ml IL-6. Ten minutes after stimulation a single cell was scanned every 30 seconds for 65 minutes by confocal laser-scanning microscopy using microscope settings as described below.

Photobleaching analysis (FRAP)

HepG2 cells transiently transfected with pSVL STAT3-YFP were grown on glass coverslips. Fortyeight hours after transfection living cells were studied at the confocal microscope Zeiss LSM510 (Carl

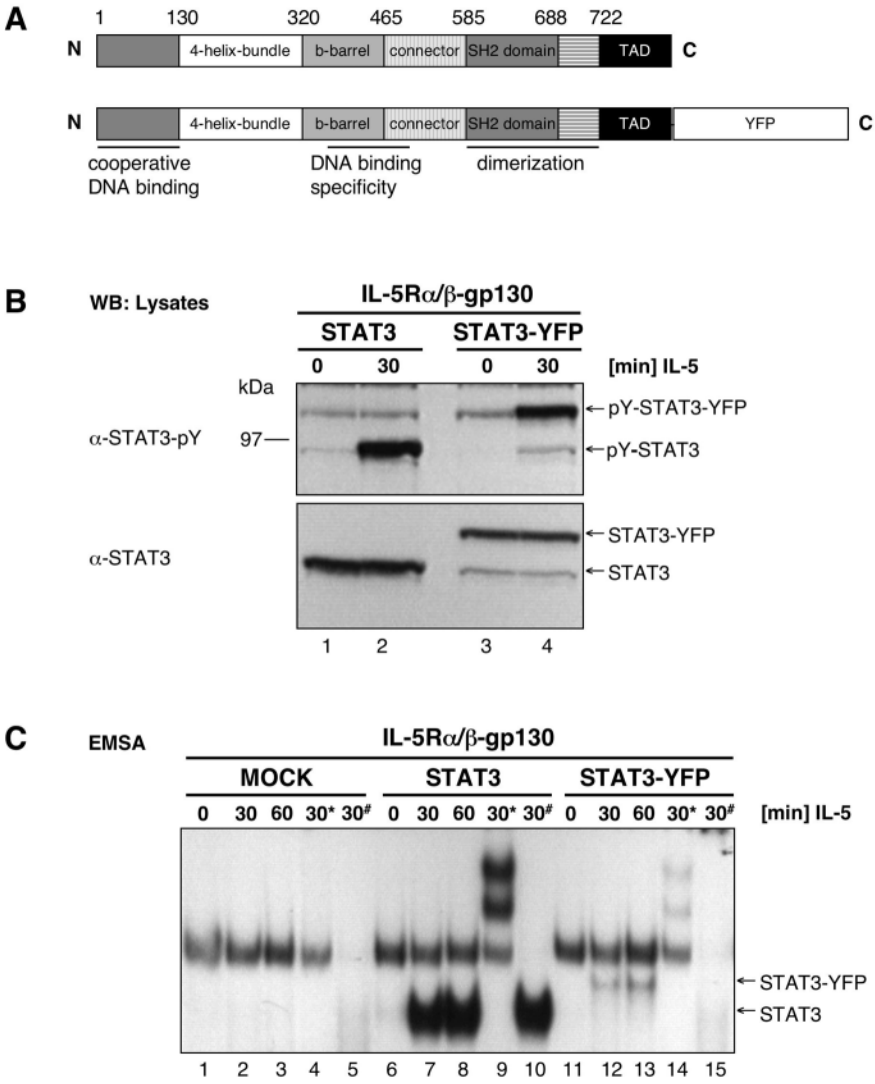

Fig. 1. Biochemical and functional characterization of STAT3-YFP. (A) Domain structures of STAT3 and STAT3-YFP. Domain borders were chosen according to Becker et al. (Becker et al., 1998). (B) The fusion protein STAT3-YFP is a functional player. COS-7 cells were cotransfected with expression vectors encoding STAT3 or STAT3-YFP and chimeric IL-5R/gp130 receptor chains as indicated. Cells were stimulated with IL-5 (20 ng/ml) for 30 minutes or left unstimulated. Lysates were analyzed by western blotting (WB) using a STAT3 phosphotyrosine-specific antibody (upper panel). After stripping, the blot was reprobed with a STAT3 antibody (lower panel). (C) Cells transfected as described above were stimulated with IL-5 (20 ng/ml) for various time periods as indicated. STAT3 DNA-binding activity was monitored by an EMSA using the *sis*-inducible element (SIE)-probe. Supershifts were performed using STAT3 (*) or GFP (#) antibodies. (D) HepG2 cells were cotransfected with a STAT3-specific luciferase-reporter gene plasmid (m67-SIE-TK-luc), a plasmid encoding β-gal for determination of transfection efficiency, and mock vector, STAT3, or STAT3-YFP as indicated. Cells were stimulated for 16 hours with 20 ng/ml IL-6 (dark gray bars) or left untreated (light gray bars). Relative luciferase activities were normalized with β-gal activities. Mean values of experiments performed in triplicate are depicted.

Zeiss, Jena, Germany) at 37°C in DMEM by using a homemade perfusion chamber. YFP was excited by the 514 nm line of the argon laser and emission was detected using a 530-600 nm bandpass filter. A 63× water corrected immersion lens (numerical aperture 1.2) was used and a pinhole adjustment resulting in an optical slice of 2 μm in width was chosen. Fifteen minutes after stimulation with 20 ng/ml IL-6, pictures were taken every second. After the first five seconds, a selected nuclear body was bleached using the 514 nm laser at 100% intensity with 100 iterations. For quantitative FRAP analysis the overall setup remained largely unchanged. The optical slice was reduced to 1 μm. In this case only the fluorescence intensity within a region of interest (ROI) of 10 pixels (resolution 256×256, zoom factor 5.0) in diameter was measured every 1/50 seconds. Bleaching was performed with 25 iterations after 2 seconds of initial data recording.

Immunoprecipitation and immunoblotting

After stimulation for 30 minutes with 20 ng/ml IL-6 or IL-5, respectively, transiently transfected cells were lysed on ice using a Triton lysis buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 10 mM NaF, 1% Triton and supplemented with protease inhibitors. Cell lysates were centrifuged at 18,700 ***g*** for 10 minutes. The supernatants were used for precipitation of STAT3 and STAT3-YFP with α-STAT3 antibody (C-20, St Cruz Biotechnology, St Cruz, USA) or α-GFP antibody (Rockland, Gilbertsville, USA). After overnight incubation at 4°C, immune complexes were precipitated with protein A-Sepharose for 1 hour, washed three times with lysis buffer and boiled in Lämmli buffer for 5 minutes at 95°C. Proteins were separated by 10% SDS-PAGE, followed by electroblotting onto a PVDF (polyvinylidene difluoride) membrane (PALL, Dreieich, Germany). Western blot analysis was performed with the indicated antibodies and the enhanced chemiluminescence kit (Amersham, Arlington Heights, USA) according to the manufacturer's instructions.

Indirect immunofluorescence

HepG2 cells were transiently transfected with plasmids encoding the indicated fusion proteins, grown on coverslips and stimulated with 20 ng/ml IL-6 48 hours after transfection. Bromouridine

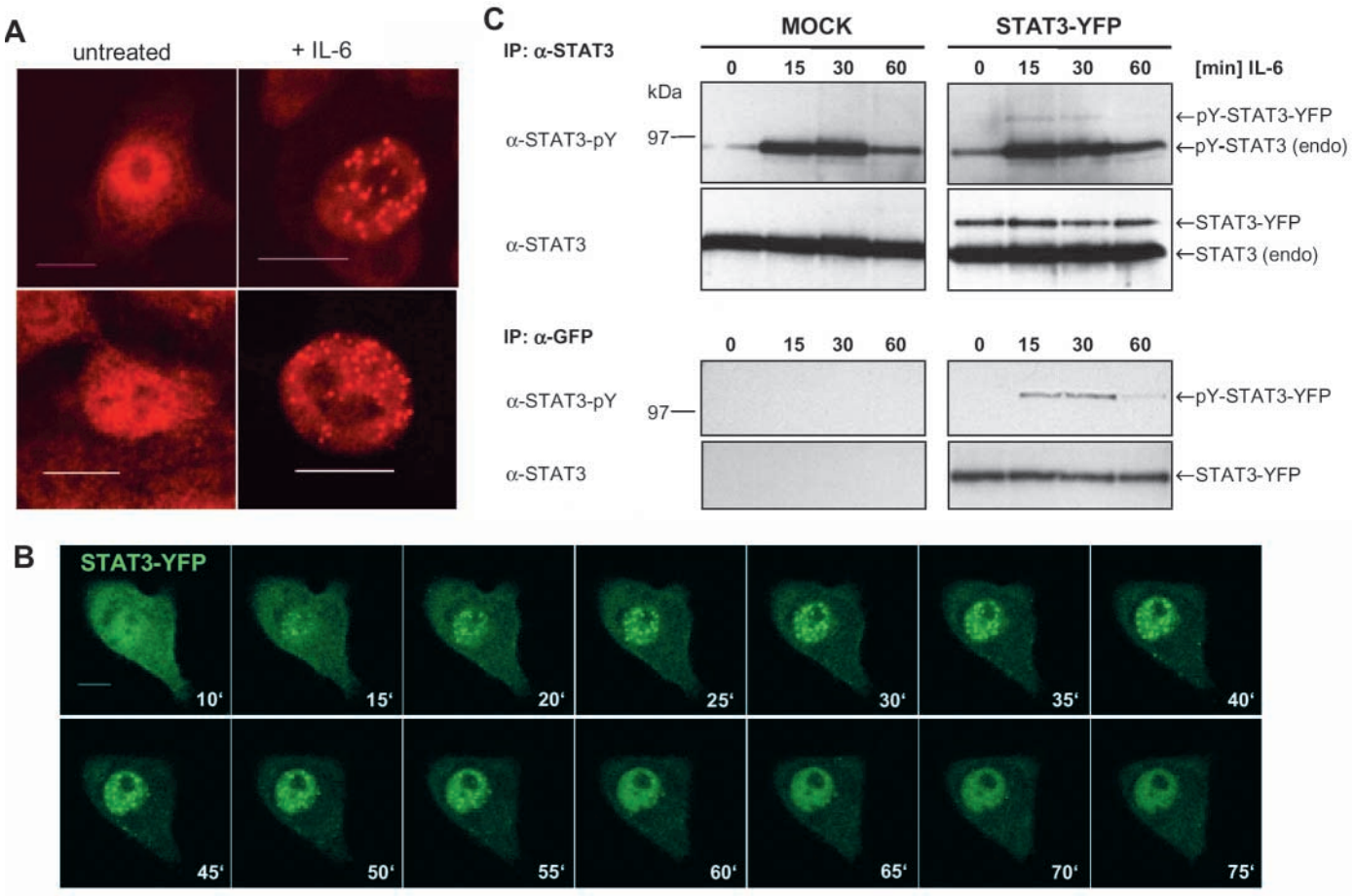

Fig. 2. STAT3 in nuclear bodies. (A) Untransfected HepG2 cells (upper panels) or HepG2 cells transfected with STAT3 (lower panels) were stimulated with 20 ng/ml IL-6 for 15 minutes or left unstimulated as indicated. Cells were fixed and STAT3 was immunostained with a STAT3-specific antibody and a TRITC-conjugated secondary antibody. Images were taken by confocal microscopy. Endogenous (upper panels) as well as transfected (lower panels) STAT3 was detected. Bars, 10 μm. (B) STAT3-YFP nuclear translocation analyzed by live cell imaging. HepG2 cells transfected with STAT3-YFP were placed in a perfusion chamber at 37°C and analyzed by confocal laser scanning microscopy. Cells were stimulated with 20 ng/ml IL-6 and pictures of a single cell were taken every 30 seconds. Images taken at the timepoints indicated are depicted. Bar, 10 μm. A time-lapse movie can be downloaded as supplementary data on the homepage of the *Journal of Cell Science* (jcs.biologists.org). (C) Kinetics of STAT3 and STAT3-YFP phosphorylation. HepG2 cells were transfected with mock vector or STAT3-YFP. Lysates were prepared after stimulation with 20 ng/ml IL-6 for the timepoints indicated. Endogeneous STAT3 (endo) and STAT3-YFP were precipitated by a STAT3-specific antibody (upper panels). STAT3-YFP was precipitated by a GFP-specific antibody (lower panels). Tyrosine phosphorylation of STAT3 and STAT3-YFP was detected by western blot analysis using a STAT3 phosphotyrosine-specific antibody. After stripping the blots were reprobed with a STAT3 antibody for loading control.

incorporation was performed according to the protocol by Hendzel et al. (Hendzel et al., 1998). Briefly, cells were pre-incubated with 1 mM bromouridine (Sigma-Aldrich, Germany) for 1 hour before fixation. Cells were washed twice with phosphate-buffered saline (PBS) containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS^{++}). Subsequently, cells were fixed with 2% paraformaldehyde for 20 minutes, permeabilized with PBS^{++} containing 0.1% Triton X-100 (PBS^{T++}) for 5 minutes, quenched with 50 mM NH_4Cl in PBS^{T++} and blocked with PBS^{T++} containing 1% BSA (SERVA, Heidelberg, Germany). Immunostaining was performed using the antibodies as indicated, i.e. the St Cruz antibodies STAT3 (C-20, sc-482), PML (PG-M3, sc-966) and CBP (C-20, sc-583), a bromodeoxyuridine antibody that also recognizes bromouridine (Roche Molecular Biochemicals, USA), the Cell Signaling (Beverly, USA) antibodies pY-STAT3 (#9131) and pS-STAT3 (#9134), an acetylated histone H4 antibody (#06-866) from Upstate Biotechnology (Lake Placid, USA) and the SC-35 antibody (S4045) from Sigma-Aldrich (Taufkirchen, Germany). Antibodies were diluted 1:100 in PBS^{T++} containing 0.2% BSA. Cells were incubated for 45 minutes with the specific first antibody raised against the protein of interest and then with the secondary antibody conjugated with TRITC (Dako, Hamburg, Germany). Cells were mounted with Mowiol (Calbiochem-Novabiochem, La Jolla, USA). All stainings were performed at room temperature. Treatment of cells with 4 μM actinomycin D in DMSO or an equal volume of DMSO as a control was performed 2 hours before stimulation.

Results

Functional characterization of STAT3-YFP

To study STAT3 nuclear translocation by live cell imaging and to determine the mobilities of nuclear STAT3 by FRAP, a fusion protein of full-length STAT3 and the yellow fluorescent protein (YFP) was generated (STAT3-YFP) by fusing YFP to the C-terminus of STAT3 (Fig. 1A). To compare the functional

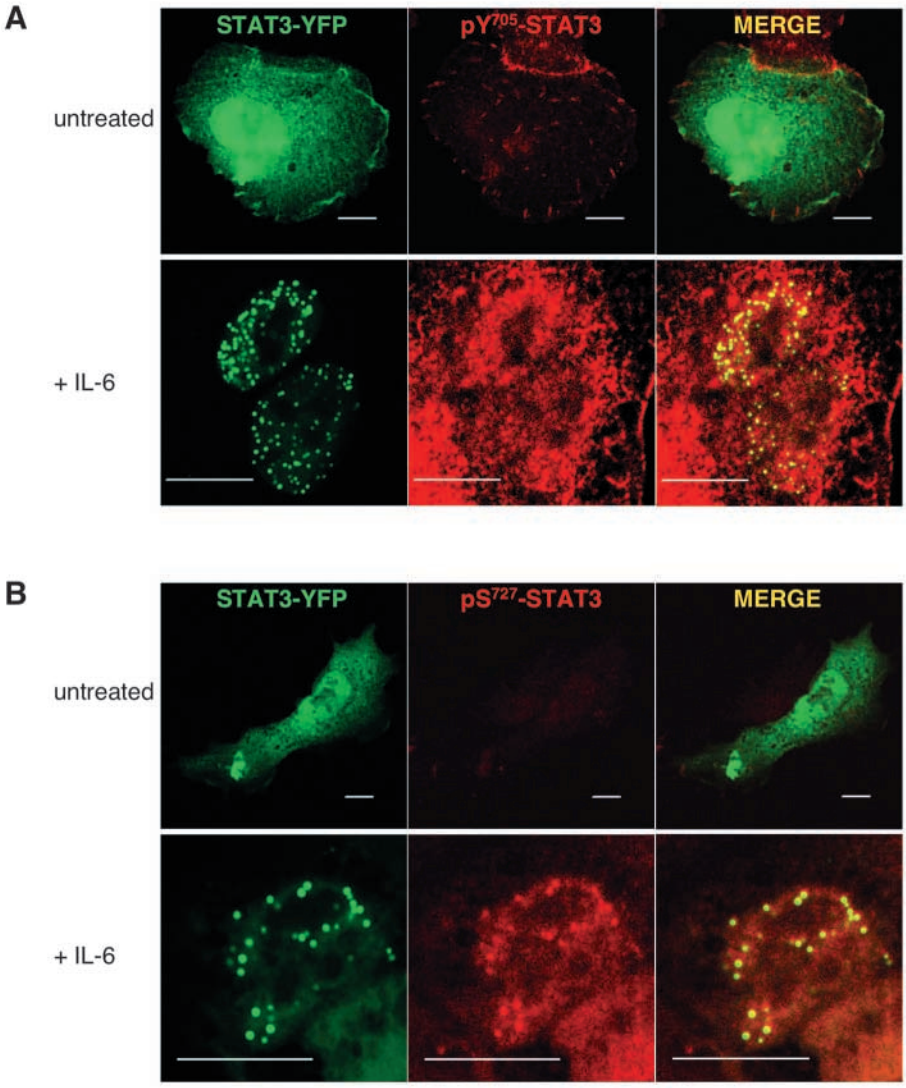

Fig. 3. Phosphorylation of STAT3 in nuclear bodies. HepG2 cells transiently transfected with STAT3-YFP were stimulated with IL-6 (20 ng/ml) for 30 minutes or left unstimulated as indicated. Subsequently, cells were fixed and incubated with (A) STAT3 phosphotyrosine-specific antibodies or (B) STAT3 phosphoserine-specific antibodies. A TRITC-conjugated secondary antibody was used for immunostaining. Cells were analyzed by confocal microscopy. Bars, 10 μm.

properties of STAT3 and STAT3-YFP, COS-7 cells were transfected with STAT3 or STAT3-YFP together with IL-5Rα/β-gp130 chimeric receptors to achieve stimulation of transfected cells only (Behrmann et al., 1997). By immunoblotting, transfected as well as endogenous STAT3 was detected (Fig. 1B, lower panel). Compared with STAT3, the electrophoretic mobility of STAT3-YFP was lower because of a higher molecular mass of about 25 kDa, which was due to the YFP moiety of the fusion protein (Fig. 1B, lower panel, lanes 3 and 4). On IL-5 stimulation, both STAT3 and STAT3-YFP become tyrosine phosphorylated (Fig. 1B, upper panel), indicating that fusion of YFP does not interfere with phosphorylation of STAT3.

To study DNA binding, nuclear extracts of the transfected COS-7 cells were prepared after different times of IL-5 stimulation. In an electrophoretic mobility shift assay (EMSA) no STAT3 DNA-binding activity was detectable in cells transfected with chimeric receptors alone (Fig. 1C, lanes 1-5). This is because of the known impaired response of endogenous STAT3 in these cells (Haan et al., 2002). The STAT3/oligonucleotide band resulting from lysates of STAT3 cotransfected cells (Fig. 1C, lanes 6-10) is shifted by the addition of STAT3 antibodies (lane 9) but not by YFP antibodies (lane 10). Also, DNA-binding of STAT3-YFP in response to IL-5 is detectable, albeit with reduced intensity (lanes 12 and 13). As expected, this band is shifted by both STAT3 and YFP antibodies (lanes 14 and 15). Throughout the EMSA an additional band is observed that is shifted by the GFP-antibody. Because this band is also visible in the EMSA from lysates of untransfected cells it cannot contain a GFP fusion protein. Moreover, the occurence of this band does not depend on stimulation. The nature of this DNA/protein complex is not understood.

To test the activity of STAT3-YFP with respect to gene induction, a reporter gene assay was performed in HepG2 cells. Transfection of STAT3 significantly increases reporter gene induction compared with mock transfected cells that contain only endogenous STAT3 proteins (Fig. 1D). A similar increase in reporter gene activity is observed with transfection of STAT3-YFP. Taken together, with respect to tyrosine phosphorylation, DNA-binding and gene induction, STAT3-YFP is a functional transcription factor.

STAT3 is enriched in nuclear-body-like structures

To study the distribution of STAT3 within the nucleus, HepG2 cells were stained with a STAT3 antibody before and after IL-6 stimulation (Fig. 2A, upper panels). Even in unstimulated cells STAT3 is found within the nucleus in considerable amounts (Meyer et al., 2002). STAT3 appears heterogeneously distributed within the nucleus, with the exclusion of the nucleolus. After IL-6 stimulation for 15 minutes STAT3 is found concentrated within the nucleus. In many stimulated cells small dot-like structures were observed. These dots were not visible in unstimulated cells. When cells were transfected with STAT3, the dot-like structures appeared with increased intensity but again only in stimulated cells (Fig. 2A, lower panel).

After these initial observations, we were interested in whether the formation of these structures could be studied by live cell imaging using STAT3-YFP. For this purpose living HepG2 cells expressing STAT3-YFP were stimulated with IL-

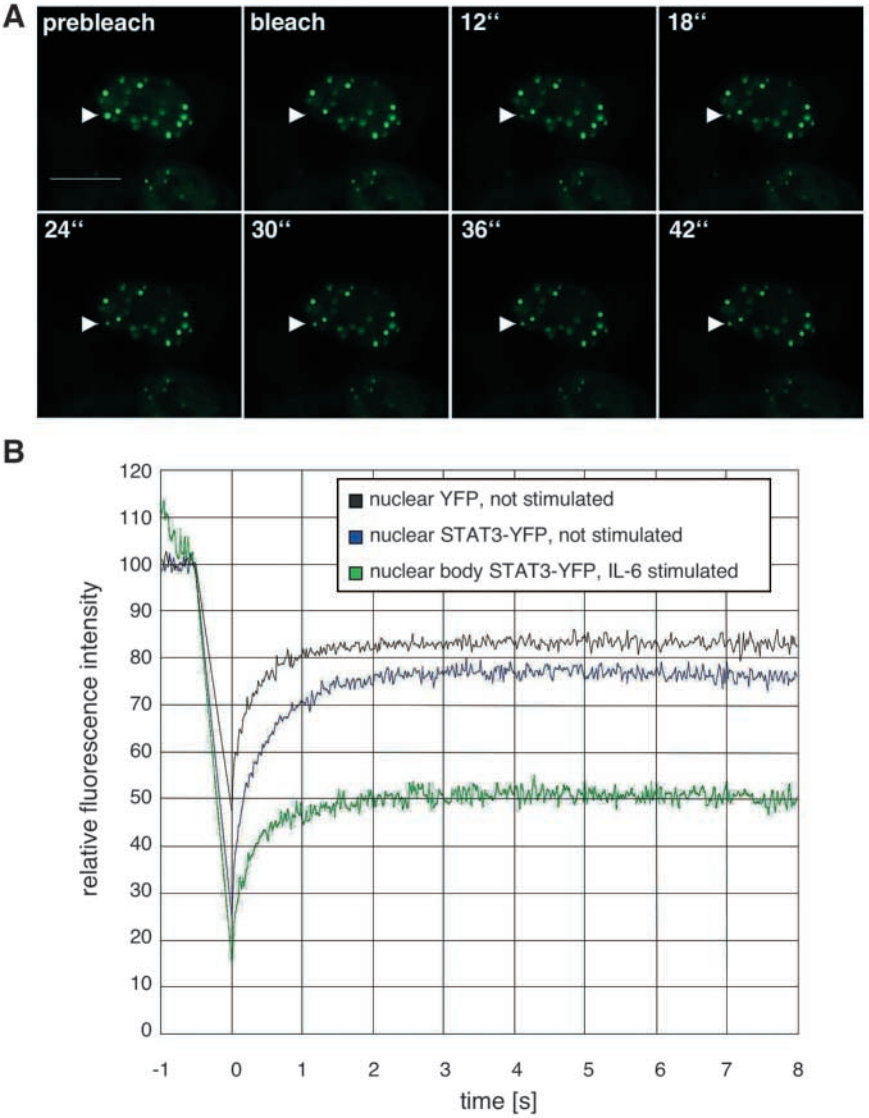

Fig. 4. FRAP analysis of STAT3-YFP mobility in the nucleus. HepG2 cells were transfected with STAT3-YFP and living cells were stimulated with 20 ng/ml IL-6. (A) 15 minutes after stimulation, a single STAT3-YFP nuclear body was bleached using the 514 nm laser of the laser-scanning microscope. Subsequently, images were taken at the timepoints indicated. Bar, 10 μm. (B) For quantitative FRAP analysis, ROIs of 1 μm in diameter within the nuclei of STAT3-YFP-transfected HepG2 cells before (blue graph) and 15 minutes after IL-6 stimulation (green graph) were bleached and subsequently the recovery of fluorescence was monitored. Fluorescence before bleaching was normalized to 100. YFP was transfected into HepG2 cells as a control for a freely mobile protein. Unstimulated cells were analyzed as described above (black graph). Each graph represents the mean values of five independent experiments.

6 and confocal images of a single cell were taken every 30 seconds. In Fig. 2B selected pictures are shown. STAT3 appears in nuclear bodies between 10 and 13 minutes after stimulation. The fluorescence intensity of the nuclear bodies increases until about 25 minutes of stimulation. Subsequently, the intensity decreases until the nuclear bodies disappear after about 50-55 minutes. The timecourse of nuclear translocation in live cell imaging corresponds to the tyrosine phosphorylation of transfected STAT3-YFP as well as of endogenous STAT3 (Fig. 2C). Tyrosine phosphorylation of endogenous STAT3 (Fig. 2C, upper panels) and STAT3-YFP (best visible in Fig. 2C, lower right panels) occurs with comparable kinetics.

STAT3 in nuclear bodies is phosphorylated at tyrosine 705 and serine 727

To analyze the phosphorylation status of STAT3 within nuclear bodies, HepG2 cells transfected with STAT3-YFP were immunostained with pY705-STAT3 antibodies (Fig. 3A) or pS727-STAT3 antibodies (Fig. 3B) before and after IL-6 stimulation. In unstimulated cells STAT3 phosphotyrosine staining resulted in only a weak background signal. The latent nuclear STAT3 is not phosphorylated (Fig. 3A, upper panels). On stimulation, the amount of tyrosine-phosphorylated STAT3 increases throughout the cell. A strong colocalization is observed for STAT3-YFP in nuclear bodies and STAT3-specific phosphotyrosine staining (Fig. 3A, lower panels), showing that STAT3 in nuclear bodies is tyrosine phosphorylated.

Similarly, phosphorylation of STAT3 at serine 727 was analyzed. In unstimulated cells STAT3 serine phosphorylation was not detected in the cytosol or in the nucleus (Fig. 3B, upper panels). Interestingly, on stimulation serine-phosphorylated STAT3-YFP was detected within the nucleus but hardly in the cytosol, indicating that serine-phosphorylated STAT3 is present predominantly in the nucleus of stimulated cells (Fig. 3B, lower panels). Again, a strong colocalization is observed between STAT3-YFP in nuclear bodies and STAT3-specific phosphoserine staining. Thus, STAT3 in nuclear bodies is phosphorylated at tyrosine 705 and serine 727.

STAT3 in nuclear bodies consists of a highly mobile and an immobile fraction

The mobility of STAT3-YFP within nuclear bodies was analyzed by FRAP-imaging using living HepG2 cells transfected with STAT3-YFP. Fifteen minutes after IL-6-stimulation, a single STAT3-YFP nuclear body was selected and the YFP moiety of the fusion protein was bleached using the 514 nm laser of the confocal microscope. Subsequently, recovery of the fluorescence was observed by live cell imaging (Fig. 4A). Recovery of fluorescence at the location of the original dot was observed within a few seconds after bleaching. Subsequently, the intensity of the recovered dot remained constant but did not reach the intensity of the dot before bleaching. These data indicate that a considerable portion of the STAT3-YFP within the nuclear body is of high mobility and rapidly exchanges with the STAT3-YFP pool in the

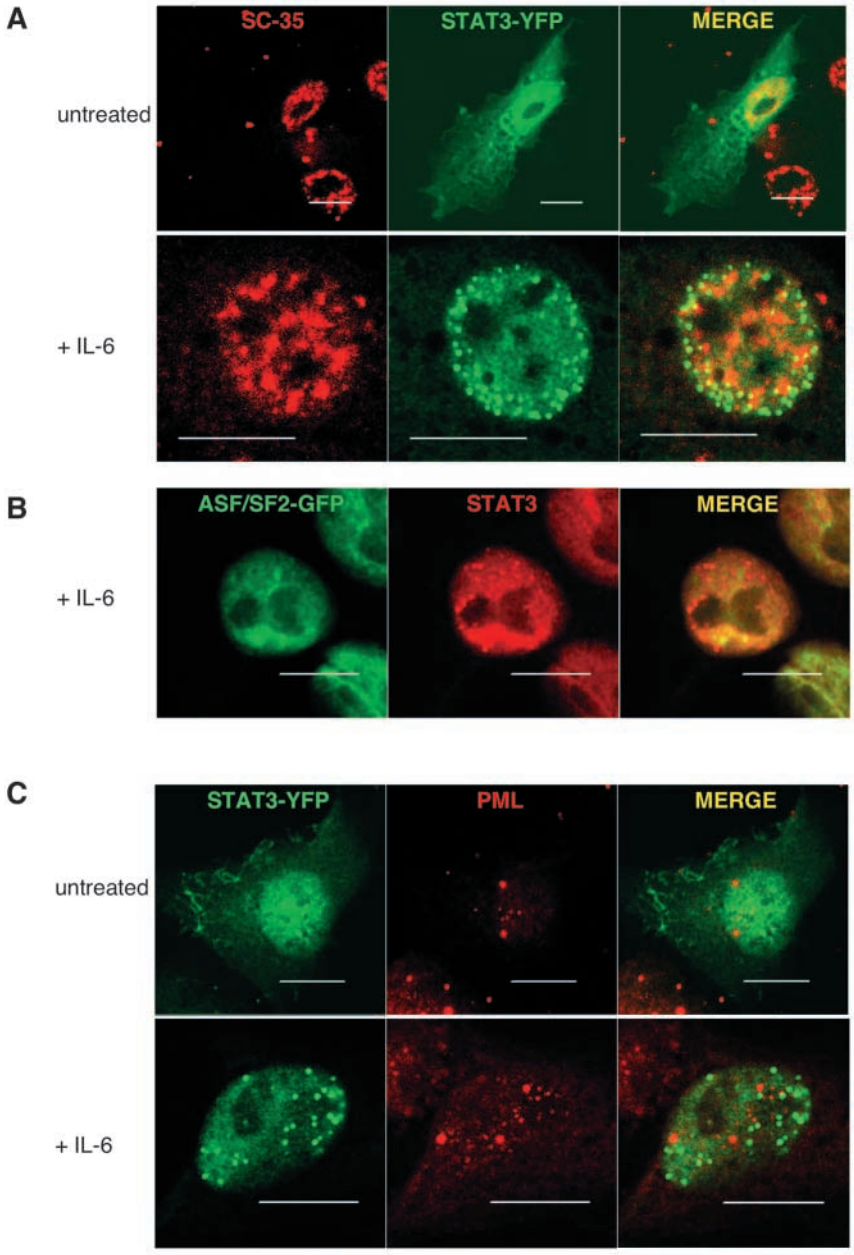

Fig. 5. Localization of SC-35, GFP-SF2/ASF, PML and STAT3. (A) HepG2 cells were transfected with STAT3-YFP. Cells were stimulated with IL-6 for 30 minutes or left unstimulated as indictated. Subsequently, cells were fixed and SC-35 was immunostained with a SC-35-specific antibody and a TRITC-conjugated secondary antibody. Images were taken by confocal microscopy. Bars, 10 μm. (B) HepG2 cells were cotransfected with the splicing factor fusion protein GFP-SF2/ASF and STAT3. After IL-6 treatment for 15 minutes cells were fixed and STAT3 was immunostained with a STAT3-specific antibody and a TRITC-conjugated secondary antibody. Images were taken by confocal microscopy. Bars, 10 μm. (C) HepG2 cells were transfected with STAT3-YFP. Cells were stimulated with IL-6 for 30 minutes or left unstimulated. Subsequently, cells were fixed and PML was immunostained with a PML-specific antibody and a TRITC-conjugated secondary antibody. Images were taken by confocal microscopy. Bars, 10 μm.

nucleus. A very similar behaviour of STAT3-YFP was observed in FRAP-imaging experiments with transfected COS-7 cells (data not shown).

To compare the mobilities of STAT3-YFP in the nucleus of unstimulated cells and within nuclear bodies of stimulated cells, more quantitative FRAP measurements were performed in transfected HepG2 cells. As a reference for a freely diffusing protein, cells expressing YFP alone were also analyzed. Small circular regions of interest (ROI) were bleached so that recovery of fluorescence could be continuously monitored immediately after bleaching. The graphs in Fig. 4B show that the mobility of nuclear STAT3-YFP in unstimulated cells is similar to the mobility of YFP. The rapid increases in fluorescence intensities immediately after the bleaches are characteristic of freely diffusing proteins. The values of the mobile fractions of YFP (83.2±5.5%) and STAT3-YFP are similar (76.6±4.1%). After photobleaching, STAT3-YFP fluorescence within nuclear bodies also recovers rapidly but reaches only half of the initial value (50.7±7.6%). Thus, STAT3 within nuclear bodies consists of a highly mobile and an immobile fraction.

STAT3 nuclear bodies are distinct from splicing factor compartments (SFCs) and PML nuclear bodies

From the experiments presented so far it can be concluded that activated STAT3 concentrates within nuclear bodies. Within this structure the mobility of STAT3 is restricted, but still a rapid exchange with the nuclear environment occurs. Such properties have been described for proteins located within SFCs or PML bodies (Boisvert et al., 2001). Therefore, we performed costainings of STAT3 with proteins resident in these subnuclear structures, i.e. SC-35, SF2/ASF and PML. HepG2 cells were transfected with STAT3-YFP and immunostained with a SC-35 antibody before and after stimulation (Fig. 5A). In a further experiment, HepG2 cells were transfected with the splicing factor SF2/ASF fused to GFP (GFP-SF2/ASF). STAT3 was immunostained 15 minutes after IL-6 stimulation (Fig. 5B). The confocal images show a speckled organization of both SC-35 and GFP-SF2/ASF within the nucleus. These SFCs appear more diffuse than the dot-like STAT3 nuclear bodies. From the overlay it is obvious that the STAT3 nuclear bodies do not colocalize with the SFCs. However, it should be noted that the STAT3 nuclear bodies are concentrated at the periphery of the SFCs.

To investigate whether STAT3 nuclear bodies are identical with or distinct from PML nuclear bodies, HepG2 cells were transfected with STAT3-YFP and immunostained with a PML antibody before and after stimulation (Fig. 5C). PML bodies appear in unstimulated as well as in stimulated cells, whereas STAT3 bodies are only observed after IL-6 stimulation. PML bodies and STAT3-YFP nuclear bodies clearly do not

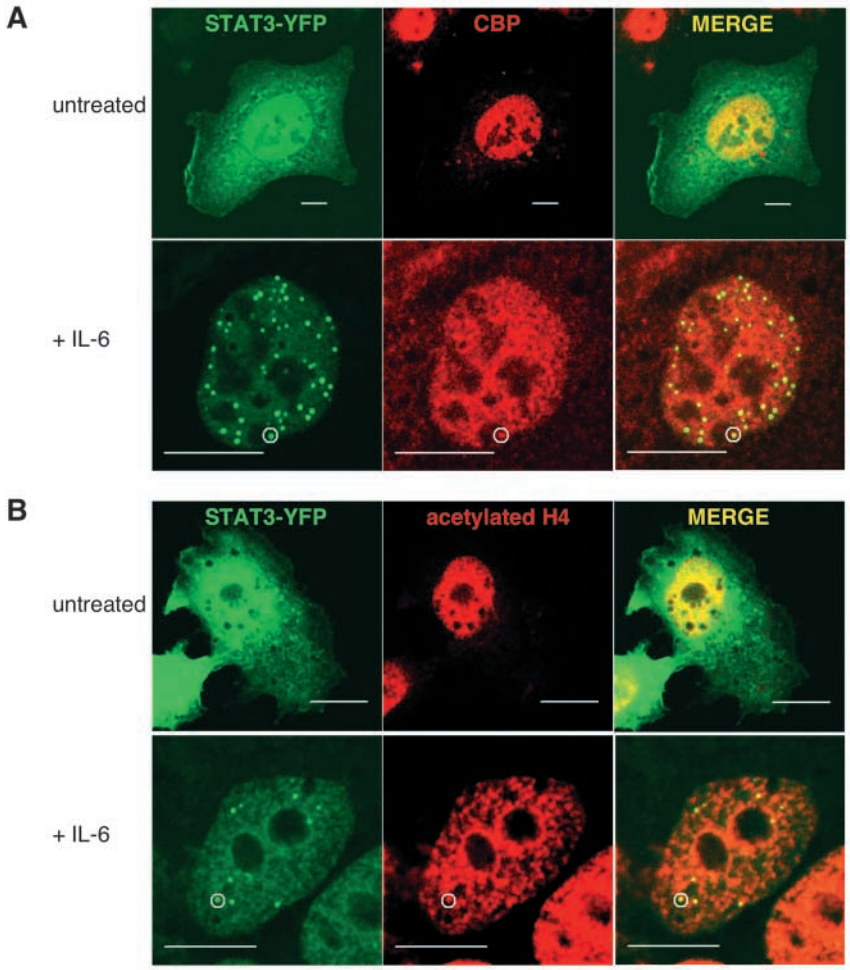

Fig. 6. Localization of acetylated histone H4, CBP and STAT3. HepG2 cells were transfected with STAT3-YFP. Cells were stimulated with 20 ng/ml IL-6 for 30 minutes or left unstimulated. Subsequently, cells were fixed and (A) CBP or (B) acetylated histone H4 was immunostained using the respective specific antibodies and a TRITC-conjugated secondary antibody. Images were taken by confocal microscopy. White circles mark selected STAT3 bodies that colocalize with domains of CBP or acetylated histone H4, respectively. Bars, 10 μm.

colocalize. From these data we conclude that STAT3 nuclear bodies are distinct from SFCs and PML nuclear bodies.

Previous studies have shown that post-translational modification of proteins by sumoylation can lead to a change in subnuclear localization of the modified protein (Chakrabarti et al., 2000). Furthermore, the STAT3 interacting protein PIAS3 exhibits SUMO-ligase activity (Kotaja et al., 2002). Immunofluorescence and western blot analysis failed to detect any sumoylation of STAT3 (data not shown). Therefore, we conclude that STAT3 in nuclear bodies is not a primary target of sumoylation.

STAT3 nuclear bodies are associated with transcriptionally active chromatin

STAT3 was identified as a transcription factor that induces acute phase protein genes in response to IL-6 (Wegenka et al., 1993). Therefore, one would expect that nuclear STAT3 is localized at sites of active gene transcription. To provide a possible link of STAT3 bodies with sites of transcription, HepG2 cells expressing STAT3-YFP were stained with markers for active chromatin such as the histone acetyltransferase and coactivator of transcription CBP or acetylated histone H4. As expected, CBP (Fig. 6A) and acetylated H4 (Fig. 6B) appear predominantly in the nucleus of HepG2 cells. Interestingly, the STAT3 bodies that form after IL-6 stimulation are preferentially located at nuclear regions marked by CBP or acetylated H4. At some sites, STAT3 nuclear bodies colocalize with similar structures stained by CBP or acetylated H4 specific antibodies (Fig. 6, white circles). Similar observations were made when nascent RNA was labeled by bromouridine incorporation. Bromouridine labeling and staining was performed in HepG2 cells expressing STAT3-YFP. The bromouridine staining shows newly synthesized RNA concentrated in diffuse dot-like structures. These structures colocalize to a large extent with STAT3 bodies (Fig. 7, upper panel). Regions of increased concentrations of nascent RNA are also enriched in STAT3 bodies (Fig. 7, lower panel, white circle). These results suggest that STAT3 nuclear bodies are associated with sites of active transcription.

There is no requirement for active transcription for the formation of STAT3 bodies

Is active transcription required for the formation of STAT3 nuclear bodies? To answer this question, STAT3-YFP nuclear localization was analyzed in the presence of actinomycin D, a potent inhibitor of DNA-dependent RNA synthesis. Compared with untreated cells, pretreatment of STAT3-YFP-transfected HepG2 cells with actinomycin D or the solvent DMSO does not influence IL-6-induced nuclear translocation or nuclear body formation of STAT3-YFP (Fig. 8). Thus, although STAT3 nuclear bodies are associated with transcriptionally active chromatin, transcription itself is not required for STAT3 body formation.

Discussion

In the present study we analyzed the nuclear distribution of STAT3. Interestingly, on IL-6-induced translocation, activated STAT3 accumulated in nuclear body-like structures. These structures appeared more prominent in cells transfected with STAT3 or STAT3-YFP. The formation of these structures designated as STAT3 nuclear bodies was transient and strongly correlated with tyrosine phosphorylation. An immunostaining revealed that STAT3 nuclear bodies consist of activated STAT3, which is phosphorylated on tyrosine and serine residues. Moreover, on stimulation, STAT3 tyrosine phosphorylation

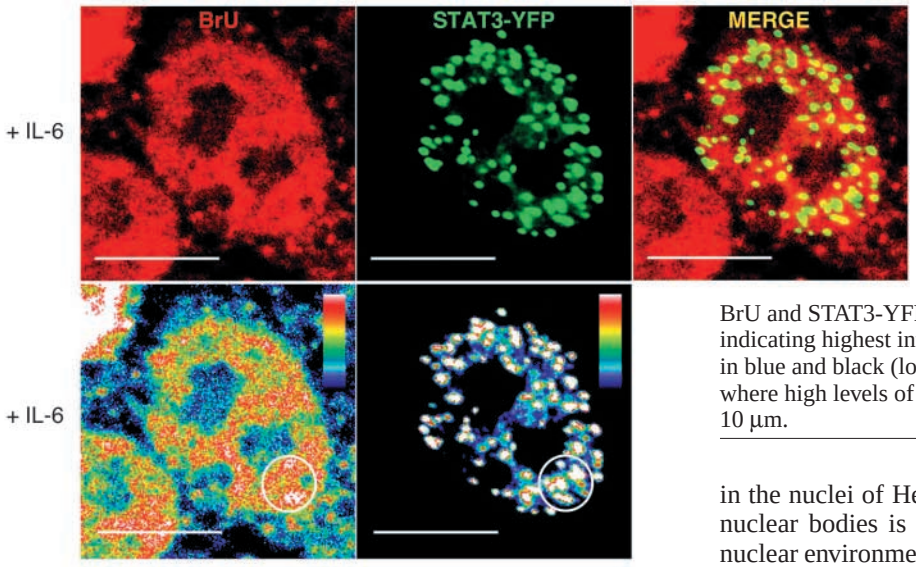

Fig. 7. Localization of nascent RNA and STAT3 nuclear bodies. HepG2 cells were transfected with STAT3-YFP. Fortyeight hours after transfection cells were pre-incubated in medium supplemented with 1 mM bromouridine for 30 minutes. Subsequently, cells were stimulated with 20 ng/ml IL-6 for another 30 minutes in the presence of bromouridine. Images of fixed cells were taken by confocal laser-scanning microscopy (upper panel). Intensities of the BrU and STAT3-YFP signals are also shown in false-color mode, indicating highest intensities in white and red and lowest intensities in blue and black (lower panel). White circles mark a selected area where high levels of STAT3 and nascent RNA were detected. Bars, 10 μm.

could be detected throughout the cytoplasm and the nucleus, whereas the serine phosphorylation was detected only in the nucleus. This picture supports the known mechanism of tyrosine phosphorylation of STATs outside the nucleus triggering nuclear translocation and the subsequent serine phosphorylation of tyrosine phosphorylated STAT3 in the nucleus (Decker and Kovarik, 2000).

It has been shown that other transcription factors such as proximal sequence element (PSE)-binding transcription factor (PTF) and octamer binding transcription factor 1 Oct1 also accumulate in nuclear domains. In these cases, body formation is observed preferentially in late G1 and S phase of the cell cycle (Pombo et al., 1998). The formation of nuclear bodies has also been described for TEL, which is a strong repressor of transcription. TEL bodies appear during S phase, and the TEL body formation depends on sumoylation (Chakrabarti et al., 2000).

From our observations we became interested in the properties of STAT3 bodies. It was apparent from the microscopic images that STAT3 bodies have a diameter of about 0.2-1 μm. We found between 10 and 70 STAT3 bodies in the nuclei of HepG2 cells. To prove whether STAT3 within nuclear bodies is in equilibrium with the STAT3-pool in the nuclear environment, a FRAP analysis was performed. The live cell imaging (Fig. 4A) revealed that bleached molecules of STAT3-YFP within a nuclear body are rapidly replaced by unbleached STAT3-YFP. This suggests an assembly of STAT3 into nuclear bodies at a defined site. The rapid exchange of the STAT3 molecules in nuclear bodies points to a particle that is formed by self organization.

Moreover, we found that STAT3 in nuclear bodies consists of two different subpopulations. About half of the STAT3 molecules are highly mobile, whereas the remaining molecules are strongly restricted in their mobility (Fig. 4B). Half-lives of fluorescence recovery of rapidly diffusing proteins cannot be exactly determined using a conventional confocal microscope. Nevertheless, from the recovery curves we estimate that the $t_{1/2}$ of recovery of YFP, nuclear STAT3-YFP in unstimulated cells and STAT3-YFP in nuclear bodies is in the range of 0.1-0.2 seconds. Thus, the mobile fraction of STAT3-YFP in nuclear bodies is as mobile as freely diffusing YFP. However, in nuclear bodies about half of the STAT molecules are limited in their mobility, suggesting that this subpopulation of STAT3 is bound to an interaction partner that restricts its mobility. Possible partners are DNA, components of the nuclear matrix, or other constituent residents that assemble the dot-like structures. It would be interesting to determine the portion of the STAT3 protein that mediates this hypothetical interaction and therefore directs STAT3 into nuclear bodies.

In a recent publication by Lillemeier et al. (Lillemeier et al., 2001) the mobility of STAT1-GFP was studied by FRAP analysis in human fibrosarcoma cell lines. STAT1-GFP is like STAT3-YFP – a functional protein that becomes tyrosine phosphorylated on cytokine stimulation,

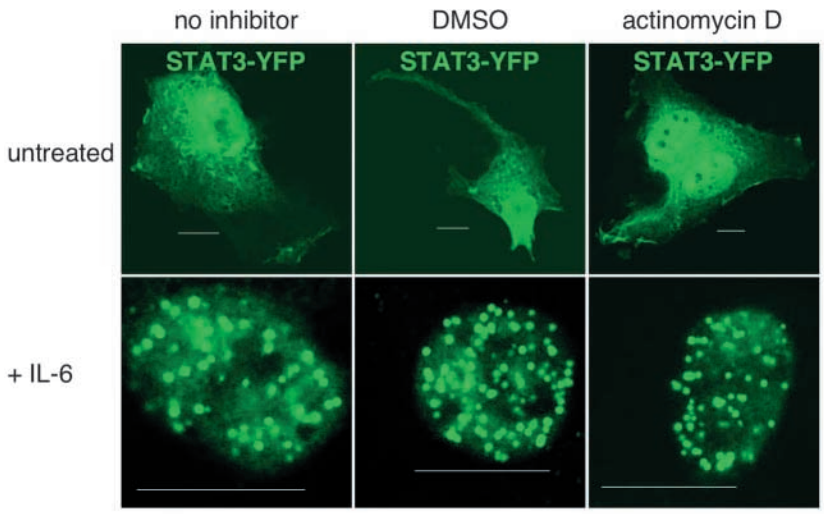

Fig. 8. Actinomycin D has no effect on STAT3-YFP nuclear body formation. HepG2 cells were transfected with STAT3-YFP, preincubated with 4 μM actinomycin D in DMSO for 2 hours or left without inhibitor and were subsequently treated for 30 minutes with 20 ng/ml IL-6 or left untreated as indicated in the figure. Images of fixed cells were taken by confocal laser-scanning microscopy. DMSO treatment alone was taken as a control. Bars, 10 μm.

translocates into the nucleus and binds to DNA. Like STAT3-YFP, the mobile fraction of STAT1-GFP recovers comparably to freely diffusing GFP. The subnuclear distribution of STAT1-GFP was not in the focus of that study. However, in FLIP (fluorescence loss in photobleaching) experiments no immobile nuclear STAT1-GFP has been observed after interferon-γ stimulation. Thus, at the moment it is not clear whether STAT nuclear body formation is a general phenomenon or whether it depends on the STAT protein investigated and the cellular context.

We evaluated the functional relevance of STAT3 bodies by colocalization studies. Therefore, STAT3 bodies were compared with known subnuclear structures such as splicing factor compartments (SFCs) and PML bodies. Interestingly, STAT3 nuclear bodies appear at the periphery of SFCs. It was shown in previous studies that sites of active gene transcription are also located at the periphery of SFCs, suggesting that STAT3 nuclear bodies might be somehow involved in gene transcription (Xing et al., 1995; Zirbel et al., 1993). Costaining of STAT3 with markers of transcriptionally active chromatin such as CBP and acetylated H4 revealed a colocalization with STAT3 bodies. Moreover, STAT3 bodies are enriched in areas that harbour increased levels of nascent RNA. We conclude that STAT3 nuclear bodies accumulate in subnuclear regions where active gene transcription occurs. However, actinomycin D does not block STAT3 nuclear body formation, indicating that their assembly does not require active gene transcription.

Another well-established nuclear structure resembling STAT3 nuclear bodies are PML bodies (Zhong et al., 2000). Transcription factors within PML bodies are often modified by sumoylation and are thought to be transcriptionally repressed (Zhong et al., 2000). In this context it is interesting that the STAT3 interacting protein PIAS3 (protein inhibitor of activated STAT3) has SUMO-ligase activity (Kotaja et al., 2002). One could imagine that the STAT3 inhibitor PIAS3 acts via sumoylation of STAT3, leading to its accumulation in PML nuclear bodies. However, STAT3 nuclear bodies do not colocalize with PML nuclear bodies; nor is a sumoylation of STAT3 detectable. Thus, there is no evidence that STAT3 is transcriptionally repressed by recruitment into PML nuclear bodies, supporting the above notion that STAT3 bodies contain transcriptionally active STAT proteins.

From our findings we propose two possible functions of STAT3 nuclear bodies. First, once activated, STAT3 translocates into the nucleus and accumulates in nuclear bodies at sites of active gene transcription. Second, STAT3 nuclear bodies might be reservoirs containing activators and coactivators of transcription. STAT3 would be recruited from this reservoir to bind to DNA. A similar model is thought to be relevant for the assembly of splicing factors in speckles (Dundr and Misteli, 2001). Further studies are required to understand the function of these newly described nuclear structures.

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