The transcription factor STAT3 is most important for the signal transduction of interleukin-6 and related cytokines. Upon stimulation cytoplasmic STAT3 is phosphorylated at tyrosine 705, translocates into the nucleus, and induces target genes. Notably, STAT proteins are also detectable in the nuclei of unstimulated cells. In this report we introduce a new method for the real time analysis of STAT3 nucleocytoplasmic shuttling in living cells which is based on the recently established fluorescence localization after photobleaching (FLAP) approach. STAT3 was C-terminally fused with the cyan (CFP) and yellow (YFP) variants of the green fluorescent protein. In the resulting STAT3-CFP-YFP (STAT3-CY) fusion protein the YFP can be selectively bleached using the 514-nm laser of a confocal microscope. This setting allows studies on the dynamics of STAT3 nucleocytoplasmic transport by monitoring the subcellular distribution of fluorescently labeled and selectively bleached STAT3-CY. By this means we demonstrate that STAT3-CY shuttles continuously between the cytosol and the nucleus in unstimulated cells. This constitutive shuttling does not depend on the phosphorylation of tyrosine 705 because a STAT3(Y705F)-CY mutant shuttles to the same extent as STAT3-CY. Experiments with deletion mutants reveal that the N-terminal moiety of STAT3 is essential for shuttling. Further studies suggest that a decrease in STAT3 nuclear export contributes to the nuclear accumulation of STAT3 in response to cytokine stimulation. The new approach presented in this study is generally applicable to any protein of interest for analyzing nucleocytoplasmic transport mechanisms in real time.

The Janus kinase/STAT pathway plays a major role in cytokine and growth factor signaling. In particular, the family members of the α-helix bundle cytokines comprising the hematopoietins and interferons exert their biological effects by the activation of STAT transcription factors (for review, see Ref. 1). According to the canonical model (2), signaling through the Janus kinase family. The activated Janus kinases phosphorylate the receptor on tyrosine residues followed by the recruitment of STAT monomers to these phosphorylated tyrosine motifs. While bound to the receptor, STATs are phosphorylated at a single tyrosine residue and subsequently form dimers by intermolecular phosphotyrosine-SH2 domain interactions. The STAT dimers freely diffuse through the cytosol (3), associate with importin-α (4), and translocate via the nuclear pore complex to the nuclear compartment to transactivate target genes.

This canonical model has been challenged by accumulating data suggesting that STATs dimerize prior to activation. The preformation of STAT dimers seems to be independent of tyrosine phosphorylation (5–10). Furthermore, recent observations from several laboratories suggest that a subpopulation of STAT proteins is located in the nucleus of unstimulated cells (8, 10–15). The latter finding is either the result of a static subcellular distribution or a continuous dynamic shuttling of STAT molecules between the cytosol and the nucleus.

STAT3 is one of the seven mammalian STAT proteins. It acts as a signal transducer for many cytokines and growth factors (16) and is of particular importance for the family of IL-6-type cytokines (17). STAT3 participates in a variety of biological processes such as the induction of acute phase protein synthesis in hepatocytes (18), the regulation of hematopoiesis and the immune response (16, 19), embryo implantation (20, 21), and development (22, 23). As a consequence of these diverse functions, STAT3 plays a crucial role in inflammatory, autoimmune, and certain neoplastic diseases (24). Mice having a targeted deletion of STAT3 die early during embryonic development (22).

STAT proteins consist of six domains: an N-terminal domain involved in cooperative DNA binding, a coiled coil domain, a DNA binding domain, a linker domain, an SH2 domain, and a C-terminal transactivation domain. The structure of a truncated, tyrosine-phosphorylated, dimeric STAT3 bound to DNA has been solved by X-ray crystallography (25). The dimerization of STAT3 molecules is enforced by reciprocal binding of the phosphotyrosine-containing regions to the SH2 domains, which is a prerequisite for nuclear accumulation and DNA binding of STAT3. The activity of the C-terminal transactivation domain is modulated by phosphorylation at serine 727 (26).

The major part of the data concerning the nucleocytoplasmic transport mechanisms of STAT proteins was obtained for STAT1 (27) and STAT5 (12, 28). Only recently, some reports concentrated on the structural requirements for the nuclear translocation of STAT3. Several amino acid residues in the coiled coil domain and DNA binding domain were identified to be essential for nuclear translocation of STAT3 (29). Furthermore, three export signals were defined in the C-terminal part...
of the coiled coil domain, the DNA binding domain, and the linker domain (15).

In the present paper, we focus on the nuclear translocation of STAT3 in unstimulated as well as in IL-6-stimulated HepG2 cells. We established a novel real time method for the analysis of nucleocytoplasmic shuttling in living cells which is based on the recently described FLAP (fluorescence localization after photoactivation) approach (30, 31). Our application involves a fluorescent fusion protein consisting of STAT3 connected to two independent fluorophores (STAT3-CFP-YFP) combined with pulse bleach techniques. The new method allowed us to investigate the dynamics of the nucleocytoplasmic transport of STAT3 by monitoring the general subcellular distribution of fluorescently labeled STAT3 and synchronous tracing a selectively bleached subpopulation. Applying this novel approach we found that (i) STAT3 constitutively shuttles between the cytoplasmic and nuclear compartments in unstimulated cells; (ii) constitutive shuttling of STAT3 is independent of tyrosine phosphorylation; (iii) balanced shuttling of STAT3 requires the interplay of export and import signals; and (iv) reduced nuclear export contributes to IL-6-induced nuclear accumulation of STAT3.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

All experiments were carried out using the human hepatoma cell line HepG2 and the simian monkey kidney cell line COS7 (both purchased from ATCC, Rockville, MD). Dulbecco’s modified Eagle’s medium/Ham’s F-12 1:1 mix with 15 mM Hepes and l-glutamine (Cambrex Corp., East Rutherford, NJ) supplemented with 10% heat-inactivated fetal calf serum was employed for culturing of HepG2 cells, whereas COS7 cells were grown in Dulbecco’s modified Eagle’s medium with Glutamax-I (Invitrogen) containing 10% fetal calf serum. For starvation conditions HepG2 and the simian monkey kidney cell line COS7 (both purchased from DAKO, Hamburg, Germany).

**DNA Transfection**

Transient transfection of plasmids into HepG2 cells was performed by using FuGENE 6 (Roche Applied Science) according to the instructions of the manufacturer. COS7 cells were transiently transfected utilizing the DEAE-dextran method.

**Cloning of STAT3-CY**

pSVL-STAT3-CY was cloned on the basis of pSVL-EcoRI gp130-ET- CFP-YFP and pSVL-EcoRI STAT3-YFP (32). These plasmids encode fluorescent fusion proteins that consist of the N-terminal signal protein STAT3 or gp130 and the C-terminal fluorescent YFP or CFP-YFP tag, respectively. All of these plasmids contain a single XhoI and a single BstEI restriction site. The XhoI site is located in the multiple cloning site upstream of the start codon. The BstEI site connects the STAT3 or gp130 with the C-terminal fluorescent YFP or CFP-YFP tag. After cloning into the TOPO-vector (Invitrogen) the DNA was sequenced. The verified plasmid was digested with XbaI (Roche Applied Science) and cloned into pSVL-STAT3-CY vector that included the DNA sequence for the CFP-YFP tag.

**Cloning of STAT3(1–320)-CY, STAT3(321–771)-CY, and STAT3(1–705)-CY**

Cloning of STAT3(1–320)-CY—A fragment corresponding to amino acids 113–320 of STAT3 was amplified by PCR introducing a BstEI site at the C terminus with the antisense primer (5’-ggtgaggtc accatcgg ggpact-3’). The product was analyzed as described above, cut with XhoI and Xmal (New England Biolabs), and cloned into pSVL-STAT3-CY digested with the same enzymes.

Cloning of STAT3(1–705)-CY—Using pCAG GS Neo HA STAT3(Y705F) (33) as a template, a fragment corresponding to amino acids 596–711 of STAT3(Y705F) was created by PCR introducing a BstEI site at the C terminus with the antisense primer (5’-gttgaggtc accatcgg ggpact-3’). The product was analyzed as described above, digested with Xmal and BstEI, and ligated into pSVL-STAT3-CY cut with the same enzymes.

**Antibodies**

Antibodies against STAT3 (BD Biosciences), phosphorylated STAT3 (Sigma), and GFP (Rockland Immunochemicals, Gilbertsville, PA) were used for immunoprecipitation (3 µl lysate) and for Western blot analysis (11,000 dilution). Secondary antibodies were purchased from DAKO (Hamburg, Germany).

**Preparation of Cell Lysates, Immunoprecipitation, SDS-PAGE, and Western Blotting**

HepG2 cells were cultured on 10-nl dishes, starved for 16 h, stimulated with 20 ng/ml recombinant human IL-6 for the indicated periods of time, and lysed by treatment with radioimmune precipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM NaF, 15% glycerol, 20 mM β-glycerophosphate, 1 mM Na3VO4, 0.25 mM phenylmethylsulfonyl fluoride, 5 µg/ml apstatin, 1 µg/ml leupeptin). The lysates were incubated with the specific antibody for 16 h at 4 °C, which was previously immobilized to protein A-Sepharose for immunoprecipitation. After two washing steps with ice-cold lysis buffer the proteins were eluted with 4× Laemmli buffer separated by SDS-PAGE, and subsequently transferred to a polyvinylidene difluoride membrane by semidry blot technique. For detection the membrane was blocked with 10% bovine serum albumin in TBS-N (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Nonidet P-40) for 1 h, rinsed three times with TBS-N, incubated with the appropriate antibody (1:1,000 dilution in TBS-N) for 16 h at 4 °C, washed twice with TBS-N for 10 min, treated with a suitable horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution) for 30 min, again washed twice with TBS-N for 10 min, and finally exposed to enhanced chemiluminescence (Amersham Biosciences) followed by fluorography. The membrane was stripped in stripping buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.7, 78 µl of 0.1 M mercaptoethanol/10 ml) for 35 min at 70 °C, washed twice with TBS-N for 10 min, and treated with the primary and secondary antibodies as indicated in the figure.

**Reporter Gene Assays**

HepG2 cells were cultured on 6-well plates (9.6 cm²/well), transiently transfected with pGL3-e2 M Luc (luciferase construct with α-galactosidase promoter), pCAG™ Lu2g (galactosidase construct with a constitutively active promoter) (Amersham Biosciences) and the indicated plasmid by using FuGENE 6 (Roche Applied Science), starved with serum-free medium for 16 h, and stimulated with 20 ng/ml IL-6 for 16 h. The preparation of the lysate and the luciferase measurements were carried out according to the instructions that were supplied with the luciferase kit (Promega, Madison, WI). Luciferase activity values were normalized to the transfection efficiency that was measured as β-galactosidase activity. The experiments were done in triplicate, and the mean ± S.D. values were calculated.

**Confocal Real Time Imaging Studies and Pulse Bleach Analysis in Living Cells**

The imaging and bleaching studies were performed by using a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) equipped with an argon and a helium-neon laser, a 63× water-corrected objective, and a perfusion chamber (manufactured in house) in which living adherent cells could be studied microscopically. HepG2 cells were transiently transfected with the indicated plasmid and grown on glass coverslips for later transfer to the perfusion chamber. Prior to the experiments the cells were starved for 16 h. The perfusion chamber was loaded with the coverslip and filled with cell culture medium. A constant temperature of 37 °C was maintained by a thermostat.

For real time imaging studies cyan fluorescence was excited at λ = 458 nm and detected by using a bandpass filter BP 470–490. Yellow fluorescence was excited at λ = 514 nm and a bandpass filter BP 530–560 was used. The experiments were carried out by stimulation with 20 ng/ml IL-6 at time point 0, and thereafter pictures were taken every 30 s or at the indicated time points. For the pulse bleach experiments in living cells the microscope settings are shown in detail in Tables I and II.
RESULTS

Characterization of STAT3-CY Fluorescent Fusion Proteins—The subcellular concentrations of fluorescent fusion proteins and their variations over time can be monitored using confocal laser-scanning microscopy. In terms of studying transport processes it is helpful to label a population of fluorescent signal proteins using a bleaching approach as done in fluorescence loss in photobleaching experiments (34). The problem that GFP labeling of cytoplasmic signal proteins using a single fluorophore is that as soon as bleaching has been performed it is impossible to follow their concentration because they have lost their fluorescence. To overcome this problem, the protein of interest can be fused to two distinct fluorophores as in the recently described FLAP method (30, 31). As a modification of this approach, we have constructed a STAT3-CFP-YFP fusion protein (STAT3-CY) as a functional variant of STAT3 with two fluorescent tags (Fig. 1A). The STAT3 part consisting of 771 amino acids is identical with wild type murine STAT3 (Fig. 1B, top panel). Tyrosine phosphorylation in response to cytokine stimulation is observed only for STAT3-CY and not for the deletion mutants (Fig. 1B, bottom panel). An unspecific band appears in all panels (Fig. 1, middle panel). The mobilities of the fusion proteins in SDS-PAGE correspond well with the calculated molecular masses of 149, 93, and 110 kDa for STAT3-CY, STAT3(1–320)-CY, and STAT3(321–771)-CY, respectively. An antibody directed against an N-terminal epitope of STAT3 recognizes STAT3-CY and STAT3(1–320)-CY but not STAT3(321–771)-CY (Fig. 1B).

After lysis of stimulated and unstimulated cells immunoprecipitations were performed with a GFP antibody that also recognizes YFP and CFP. Subsequently, the recombinant proteins were detected in Western blot with the GFP antibody (Fig. 1B, top panel). The mobilities of the fusion proteins in SDS-PAGE correspond well with the calculated molecular masses of 149, 93, and 110 kDa for STAT3-CY, STAT3(1–320)-CY, and STAT3(321–771)-CY, respectively. An antibody directed against an N-terminal epitope of STAT3 recognizes STAT3-CY and STAT3(1–320)-CY but not STAT3(321–771)-CY (Fig. 1B, middle panel). Tyrosine phosphorylation in response to cytokine stimulation is observed only for STAT3-CY and not for the deletion mutants (Fig. 1B, bottom panel). An unspecific band appears in all lanes at the position of STAT3-CY. Nevertheless, stimulation-induced phosphorylation of STAT3-CY is clearly detectable. Nontagged STAT3 is not detected because it has not been precipitated by the GFP antibody.

The following experiments were carried out to demonstrate that STAT3-CY is as functional as wild type STAT3 with respect to transactivation and nuclear translocation. The transactivation capability of STAT3-CY was tested in a reporter gene assay (Fig. 1C). IL-6 stimulation of mock-transfected HepG2 cells leads to a pronounced induction of the a2-macroglubulin promoter mediated by endogenous STAT3. A significantly enhanced induction was observed in cells transfected with wild type STAT3. Transfection of HepG2 cells with the fluorescent constructs STAT3-YFP and STAT3-CY led to results similar to those shown for wild type STAT3. Real time

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**TABLE I**

| Microscope settings for the pulse bleach experiments presented in Fig. 3 |
| --- |
| **Laser configuration** |
| Argon laser output | 100% |
| **Multi-track mode** |
| Track 1 (YFP) | Track 2 (CFP) |
| Excitation | 514 nm | 458 nm |
| Transmission | 0.05% | 5.0% |
| HFT | 458/514 | 458/514 |
| Filter | BP* 530-600 | BP 470-490 |
| **Scan settings** |
| Resolution | 256 x 256 |
| Data depth | 12 bit |
| Scan speed | Max |
| Zoom | 5.0 |
| Scan direction | One way |
| Mode | Line |
| Number | 1 |
| Method | Mean |
| YFP channel | CFP channel |
| Pinhole | 262 μm | 262 μm |
| Detector gain | 700 | 1000 |
| Amplifier offset | 0.0 | 0.0 |
| Amplifier gain | 1.0 | 1.0 |
| **ROI and bleach settings** |
| Detection ROI diameter | 20 pixel |
| Bleach ROI diameter | 40 pixel |
| Bleach laser line | 514 nm |
| Transmission | 100% |
| Bleach iterations |
| First bleach pulse | 100 |
| Continuous pulse | 1 iteration every 3.3 s |
| or 180 s (as indicated) |
| **Time series settings** |
| Measurement interval | 3.3 s |

* BP, bandpass.

**TABLE II**

| Microscope settings for the pulse bleach experiments presented in Fig. 4 |
| --- |
| **Laser configuration** |
| Argon laser output | 35% |
| **Multi-track mode** |
| Track 1 (YFP) | Track 2 (CFP) |
| Excitation | 514 nm | 458 nm |
| Transmission | 0.1% | 5.0% |
| HFT | 458/514 | 458/514 |
| Filter | BP* 530-600 | BP 470-490 |
| **Scan settings** |
| Resolution | 256 x 256 |
| Data depth | 12 bit |
| Scan speed | Max |
| Zoom | 5.0 |
| Scan direction | One way |
| Mode | Line |
| Number | 1 |
| Method | Mean |
| YFP channel | CFP channel |
| Pinhole | 262 μm | 262 μm |
| Detector gain | 750 | 1000 |
| Amplifier offset | −0.025 | −0.05 |
| Amplifier gain | 1.0 | 1.0 |
| **ROI and bleach settings** |
| Detection ROI diameter | 20 pixel |
| Bleach ROI diameter | 40 pixel |
| Bleach laser line | 514 nm |
| Transmission | 100% |
| Bleach iterations |
| First bleach pulse | 345 or 1,300 (as indicated) |
| Continuous pulse | 30 iterations every 10 s |
| or bleeding |
| **Time series settings** |
| Measurement interval | 3 s |

* BP, bandpass.
imaging studies with IL-6-stimulated HepG2 cells that were transfected with STAT3-CY (Fig. 2A) demonstrate identical kinetics with regard to nuclear translocation as observed in studies with wild type STAT3 and single-tagged STAT3-YFP (32). The highest concentrations of fluorescent STAT3 within the nucleus were found between 15 and 40 min after IL-6 stimulation. From these findings we conclude that the fluorescent tags do not interfere with the basic functions of STAT3.

IL-6 stimulation did not affect the subcellular distribution of STAT3(Y705F)-CY (Fig. 2B) or the deletion mutants (not shown). In unstimulated cells the distribution of STAT3(Y705F)-CY was indistinguishable from STAT3-CY. Notably, the subcellular distributions of the two deletion mutants differed markedly. Whereas STAT3(1–320)-CY was almost equally distributed between the cytoplasm and the nucleus, STAT3(321–771)-CY was largely excluded from the nucleus (Fig. 2C).

The YFP Fluorophore in STAT3-CY Can Be Bleached Selectively—STAT3-CY was designed to have two independent fluorophores that can be bleached selectively. The yellow fluorescent fluorophore is bleached efficiently and selectively using intense laser light at a wavelength of 514 nm. According to the excitation spectrum of CFP the cyan fluorophore should not be affected. This prediction has been tested by bleaching STAT3-CY nuclear foci that are often formed upon IL-6 stimulation of transfected HepG2 cells (Fig. 2, A and D). The formation of these subnuclear structures can also be observed in the case of wild type STAT3 and single-tagged STAT3-YFP (32). Application of a 514-nm laser pulse to a single dot (Fig. 2D, upper panels, arrows) by using the laser of the confocal microscope leads to a strong decrease in YFP fluorescence, whereas CFP fluorescence remains largely unaffected (Fig. 2D, lower panels, arrows). The selective bleaching of YFP enabled marking of a subpopulation of STAT3-CY and following its fate by determining the ratio of YFP to CFP fluorescence.

Fig. 3A shows a general setup of the pulse bleach experiments in which we studied the nuclear import and export processes of STAT3-CY in living cells. For this purpose one “bleach ROI” and three “detection ROIs” were defined. Within the area of the bleach ROI the YFP moiety of STAT3-CY can be bleached with 514-nm laser pulses. The detection ROIs monitored cytosolic, nuclear, and background fluorescence intensities. In the following figures the data are presented in diagrams that show background-corrected fluorescence intensities of CFP and YFP within the indicated cytosolic or nuclear ROIs.

A Dynamic Equilibrium between Nuclear Import and Export Results in Constant Subcellular Concentrations of STAT3-CY in Unstimulated Cells—Fig. 3A and the first image in Fig. 2A show the subcellular distribution of STAT3-CY in unstimulated HepG2 cells. Interestingly, STAT3-CY is detectable within the nucleus. Monitoring of fluorescence intensities within the cytosolic and nuclear detection ROIs without any bleaching proves that subcellular concentrations of STAT3-CY remain constant in unstimulated cells for at least 20 min (Fig. 3A).
We asked whether the observed constant subcellular distribution of STAT3-CY fusion proteins is caused by the lack of any transport between cytosol and nucleus or whether it is a result of a dynamic equilibrium between nuclear import and export processes. For this reason, we examined the nuclear transport process by pulse bleach experiments. In addition to the three detection ROIs, a bleach ROI was defined in the cytosol as outlined above. Yellow and cyan fluorescence intensities in the cytosol and the nucleus were measured for 20 min. At time 3B). Slight intensity variations are caused by random cellular movements, small shifts in the focus plane, or little bleaching because of data recording.

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We asked whether the observed constant subcellular distribution of STAT3-CY is caused by the lack of any transport between cytosol and nucleus or whether it is a result of a dynamic equilibrium between nuclear import and export processes. For this reason, we examined the nuclear transport process by pulse bleach experiments. In addition to the three detection ROIs, a bleach ROI was defined in the cytosol as outlined above. Yellow and cyan fluorescence intensities in the cytosol and the nucleus were measured for 20 min. At time
point 30 s a strong bleach pulse at a wavelength of 514 nm was applied to the cytosol of the cell followed by further bleach pulses every 3.3 s for 2 or 3 min as described in the legends to the figures. These bleach pulses greatly reduced the amount of functional yellow fluorophores in the cytosol leading to a decrease in fluorescence intensity in the cytoplasmic detection ROI from 1,150 to about 300 units (Fig. 3C). Most interestingly, the nuclear YFP fluorescence also decreased over time from
550 to 350 although no bleach pulses were applied to the nucleus. This loss of 36% of the initial nuclear fluorescence is indicative of an export process of nonbleached STAT3-CY from the nucleus to the cytosol.

The total amount of nuclear as well as cytosolic STAT3-CY remained almost constant as confirmed by the mainly invariable CFP fluorescence in both monitored cellular compartments. The increase in cytosolic CFP fluorescence after YFP bleaching from 1,000 to 1,100 is caused by the loss of fluorescence resonance energy transfer between the two fluorophores. The constant nuclear CFP fluorescence implies that the export of nonbleached STAT3-CY from the nucleus to the cytosol is compensated by an adequate import of YFP-bleached STAT3-CY. Thus, we draw the important conclusion that STAT3 permanently shuttles between the cytosol and the nucleus in unstimulated cells. This reflects a steady state that is based on a dynamic equilibrium between nuclear import and export of STAT3.

Balanced Constitutive Shuttling of STAT3 Does Not Depend on Tyrosine Phosphorylation but Requires Structural Motifs of the N- and C-terminal Moieties—Next the potential role of STAT3 tyrosine phosphorylation in constitutive nucleocytoplasmic shuttling was investigated. Therefore, STAT3(Y705F)-CY-transfected HepG2 cells were analyzed as described above. As observed in STAT3-CY-transfected cells, the fluorescence monitored in the cytosolic ROI is considerably higher than the fluorescence in the nuclear ROI and remains constant for the observation period of 20 min (Fig. 3D). Upon cytosolic pulse bleaching the nuclear YFP fluorescence decreases, whereas the nuclear CFP fluorescence remains constant (Fig. 3E). As outlined above, this phenomenon is indicative of nucleocytoplasmic shuttling. Within 20 min the nuclear YFP fluorescence decreases by 37%. Thus, the mutation of Tyr705 to Phe does not affect the stimulation-independent shuttling of STAT3, showing that constitutive nucleocytoplasmic shuttling does not require tyrosine phosphorylation or dephosphorylation.

To characterize the structural requirements of STAT3 shuttling the two fluorescent STAT3 deletion mutants, STAT3(1–320)-CY and STAT3(321–771)-CY were studied in the shuttling assay. In HepG2 cells transfected with STAT3(1–320)-CY (Fig. 3F) the initial cytosolic fluorescence before bleaching is lower than the nuclear fluorescence, which is in agreement with the general subcellular distribution observed in the microscopic images (Fig. 2C). Upon cytosolic pulse bleaching the nuclear YFP fluorescence decreases rapidly and equilibrates with the cytoplasmic YFP fluorescence within 3–4 min, indicating a much faster shuttling of this mutant compared with STAT3-CY and STAT3(Y705F)-CY. Nuclear and cytoplasmic CFP fluorescence remain constant with the exception of the increase in cytoplasmic CFP fluorescence after pulse bleaching because of the loss of fluorescence resonance energy transfer.

The distribution of the deletion mutant STAT3(321–771)-CY is completely different (Figs. 2C and 3G). In the nucleus of transfected HepG2 cells only weak YFP fluorescence is detectable. The nuclear CFP fluorescence only marginally exceeds background fluorescence. After pulse bleaching of cytoplasmic YFP a change in nuclear fluorescence is hardly detectable because of the low initial levels of nuclear fluorescence. Of note, the slow but strong recovery of cytoplasmic fluorescence after bleaching indicates that STAT3(321–771)-CY exhibits an decreased mobility in the cytoplasmic compartment.

We conclude that the structural integrity of the protein is required for a well balanced nucleocytoplasmic shuttling of STAT3. The N-terminal moiety favors nuclear accumulation and rapid shuttling whereas the C-terminal moiety drives exclusion from the nucleus so that no shuttling can be measured with the assay established in this study.

A Reduction of Nuclear Export Contributes to the IL-6-dependent Nuclear Accumulation of STAT3-CY—During the first 20 min after IL-6 stimulation of HepG2 cells that were transiently transfected with STAT3-CY a nuclear accumulation of the fluorescent fusion protein was observed as shown in Fig. 2A. This accumulation could be monitored using the experimental setup as described in the previous section. One min after IL-6 stimulation CFP and YFP fluorescence were measured within a cytosolic and a nuclear detection ROI for about 20 min (Fig. 4A). As expected, fluorescence intensities increased in the nuclear and decreased in the cytosolic compartment. Highly fluorescent nuclear STAT3-CY bodies appear in a subpopulation of cells upon stimulation (see Fig. 2, A and D). A variability of the measured nuclear fluorescence (time point 500 s) occurs if nuclear STAT3-CY bodies enter or leave the nuclear detection ROI (Fig. 4A).

In principle, the change from the dynamic steady state to the nuclear accumulation of STAT3-CY can be caused by an increase of nuclear import, a decrease of nuclear export, or a process that includes both mechanisms. Thus, we performed pulse bleach experiments to determine the relative contribution of nuclear export. Transiently transfected HepG2 cells were stimulated with IL-6, and 1 min later measurements were started as indicated by time point 0 s in Fig. 4B. At a defined time point a strong bleach pulse was applied to the cytosol, and short pulse bleaches were applied thereafter every 10 s during the whole observation period of 20 min. This procedure resulted in the nearly complete cytosolic depletion of yellow fluorescent STAT3-CY molecules. For that reason, imported STAT3-CY could not contribute substantially to nuclear YFP fluorescence any longer. This enabled us to follow the fate of initially present nuclear STAT3-CY. Interestingly, in contrast to the experiment in unstimulated cells (Fig. 3C) the nuclear yellow fluorescence did not decrease. It remained at a constant level, which indicates an extremely reduced nuclear export process of STAT3-CY during nuclear accumulation. Again, the overall redistribution of STAT3-CY from the cytosol to the nucleus upon IL-6 stimulation is evident from the monitored cytosolic and nuclear CFP fluorescence intensities.

After Nuclear Accumulation of STAT3-CY Nuclear Import and Export Are Both Greatly Reduced So That Hardly Any Nucleocytoplasmic Transport Occurs—In the following experiments the situation after the nuclear accumulation of STAT3-CY was analyzed. About 15 min after IL-6 stimulation of transiently transfected HepG2 cells fluorescence measurements revealed constant subcellular concentrations of STAT3-CY with low cytosolic and high nuclear levels (Fig. 4C). We examined whether this situation was again based on a steady state with permanent nuclear import and export of STAT3-CY. In a further pulse bleach experiment that was performed 15 min after IL-6 stimulation and executed as described above, we could not observe any decrease in nuclear yellow fluorescence despite the cytosolic depletion of the remaining yellow fluorophores (Fig. 4D). This finding suggests that accumulated nuclear STAT3-CY was not replaced by YFP-bleached cytosolic STAT3-CY. The constant cytosolic and nuclear CFP fluorescence indicates a nearly invariable subcellular distribution of STAT3-CY. Thus, we conclude that both nuclear import and export of STAT3 were greatly reduced upon nuclear accumulation.

DISCUSSION

According to the canonical model of the Janus kinase/STAT pathway the STATs are latent cytoplasmic proteins that enter the nucleus in response to cytokine stimulation. Phosphorylation of the STATs at a single tyrosine residue is the major trigger for their nuclear accumulation. Besides this cytokine-
induced nuclear accumulation of STATs, more recently a stimulation-independent nuclear localization of a considerable fraction of cellular STAT proteins has been described (27). In the case of STAT3, a stimulation-independent nuclear localization has been observed for endogenous STAT3 (8, 14) as well as for fluorescent STAT3 fusion proteins (10, 29) in different cell types including HepG2 cells. HepG2 cells are well suited to study STAT3 because they physiologically respond to IL-6 stimulation with the STAT3-mediated induction of acute phase proteins (35, 36).

The question arises as to whether the constitutive nuclear localization of a subpopulation of STAT molecules reflects a static distribution in which no nuclear translocation occurs or a dynamic equilibrium in which nuclear import equals nuclear export. The first evidence for a constitutive nucleocytoplasmic shuttling of STAT1 (13) and STAT5 (12) has been provided recently. Bhattacharya and Schindler (15) observed that treatment of cells with the nuclear export inhibitor leptomycin leads to a nuclear accumulation of STAT3 after several hours. This finding was interpreted as a first hint on cytokine-independent nucleocytoplasmic shuttling.

In the present study we set out to investigate nucleocytoplasmic shuttling of STAT3 in HepG2 cells in real time. In two recent publications (30, 31), Dunn and co-workers described the FLAP approach, which is based on the labeling of a protein of interest with the two different fluorophores, CFP and YFP. Transfection of cells with CFP-actin and YFP-actin enabled selective bleaching by YFP-actin and permitted following the fate of the bleached region by monitoring the ratio of CFP and YFP fluorescence (30, 31). We used this approach in the analysis of STAT3 nucleocytoplasmic shuttling. A STAT3-CFP-YFP (STAT3-CY) construct was generated instead of two separate STAT3-CFP and STAT3-YFP constructs. Transient transfection of STAT3-CY guarantees equimolar concentrations of the two fluorophores, whereas cotransfection of STAT3-CFP and STAT3-YFP would result in varying ratios of the basal CFP and YFP fluorescence. The fluorescent fusion protein is an appropriate model for endogenous STAT3 because STAT3-CY shows a ligand-induced nuclear accumulation and a transactivation activity similar to wild type STAT3. Detection of fluorescence within small ROIs instead of taking whole cell images strongly reduces the bleaching of the fluorophores during data recording. Moreover, the small ROIs can be measured in short time intervals. Because STATs diffuse freely in the cytoplasm and in the nucleus (3), the fluorescence intensity within a ROI is representative of the entire cellular compartment.

Using transfected HepG2 cells combined with nuclear and cytoplasmic detection ROIs and a cytoplasmic bleach ROI, shuttling of STAT3-CY in living cells has been clearly demonstrated. Intriguingly, although the decrease in cytosolic YFP fluorescence upon cytosolic bleach pulse application is rapid, the decrease in nuclear YFP fluorescence is slow. Therefore, nuclear shuttling of STAT3-CY seems to be limited by the
Nucleocytoplasmic Shuttling of STAT3

transport capacity of the nuclear pore complexes. The apparently static subcellular distribution of STAT3 in unstimulated cells is in fact based on a dynamic equilibrium between nuclear import and nuclear export.

Constitutive nucleocytoplasmic shuttling could arise from a futile cycle of STAT3 phosphorylation in the cytoplasm which results in nuclear import and dephosphorylation in the nucleus, which triggers export. The fact that STAT3-Y705F, in which the tyrosine phosphorylation site of STAT3 is mutated, shuttles virtually indistinguishably from STAT3-CY excludes a role of tyrosine phosphorylation in shuttling. Similar results were obtained for STAT1 and STAT5. Although tyrosine phosphorylation is mandatory for ligand-induced nuclear accumulation, constitutive shuttling is independent of phosphorylation (12, 13).

Experiments with N- and C-terminal deletion mutants of STAT3-CY clearly show that balanced nucleocytoplasmic shuttling is an integrated property of STAT3 and leads to a steady state of high cytoplasmic and low nuclear concentrations. The nuclear localization and rapid shuttling of STAT3(1–320)-CY are driven by the N-terminal moiety of STAT3, whereas the C-terminal part of STAT3 in STAT3(321–771)-CY causes exclusion from the nucleus. The subcellular distribution of the deletion mutants can be explained in the light of two recent publications dealing with cytokine-induced nuclear accumulation of STAT3. In these studies two STAT3 nuclear localization signals (29) and three nuclear export signals (15) were identified. The construct STAT3(1–320)-CY contains one nuclear localization signal (Arg214/Arg215) that triggers nuclear accumulation independently from stimulation. This nuclear localization signal is compensated by only a single nuclear export signal (amino acids 306–318). Therefore, the shuttling equilibrium of this mutant is shifted toward nuclear accumulation. The construct STAT3(321–771)-CY lacks the stimulation-independent nuclear localization signal but contains two nuclear export signals (amino acids 404–414 and 524–535). As a consequence nuclear export is not counterbalanced by adequate import leading to nuclear exclusion of this mutant. From these findings we draw the conclusion that the balanced nucleocytoplasmic shuttling in unstimulated cells stems from an interplay between diverse constitutively active export and import signals and results in a preferentially cytoplasmic localization of STAT3. The presence of a small fraction of STAT3 in the nucleus of unstimulated cells allows the participation of this transcription factor as a transcriptional coactivator in enhancerosomes independent of tyrosine phosphorylation and DNA binding. A similar function has been recently suggested for STAT1 (11).

Although the lack of tyrosine phosphorylation in response to IL-6 stimulation was predictable for STAT3(1–320)-CY, which does not contain Tyr705, it is intriguing in the case of STAT3(321–771)-CY. The latter mutant contains the SH2 domain required for the recognition of phosphorylated receptor peptides as well as Tyr705 as the phosphorylation site. The activity of STAT3(321–771)-CY is in agreement with a recent study of Zhang et al. (37) suggesting an essential role of the coiled coil domain (amino acids 130–320) for receptor recruitment and subsequent phosphorylation of STAT3. We also applied the FLAP approach to the analysis of IL-6-induced nuclear accumulation of STAT3. For these studies only STAT3-CY was employed because none of the mutants did respond to cytokine stimulation underlining the essential role of tyrosine phosphorylation for nuclear accumulation of STAT3. IL-6 treatment of HepG2 cells leads to a nuclear concentration of STAT3 within 15 min. A switch from steady state to accumulation in the nuclear compartment occurs if the import exceeds the export. We found evidence for an extreme reduction in the export rate of STAT3-CY during the accumulation phase. The constitutive shuttling of STAT3 is slow compared with the rapid cytokine-induced nuclear accumulation. Therefore, the reduced export must be accompanied by an increased import to achieve the rapid nuclear accumulation of STAT3. About 15 min after stimulation our measurements reveal a phase with constant, low cytosolic and high nuclear STAT3-CY concentrations in parallel with a sustained reduction of the export of nuclear STAT3-CY. Because the subcellular distribution was constant the import also had to be reduced to the same extent. During this stage, nucleocytoplasmic shuttling is very limited, and tyrosine-phosphorylated STAT3 is largely trapped in the nuclear compartment.

In this study the recently described FLAP approach has been applied for the first time to investigate nucleocytoplasmic transport. By this means shuttling of STAT3 in unstimulated living cells has been demonstrated in real time. This shuttling is independent of tyrosine phosphorylation and requires the balanced action of constitutively active import and export signals. Nuclear accumulation of STAT3 in response to cytokine stimulation is strongly dependent on tyrosine phosphorylation and is a result of reduced nuclear export and enhanced import. The approach presented here can be exploited further to determine the structural requirements for STAT3 shuttling in more detail but can also be adapted to the study of nucleocytoplasmic shuttling of any protein of interest.

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