Analysis of Stat3 (signal transducer and activator of transcription 3) dimerization by fluorescence resonance energy transfer in living cells

Antje K. KRETZSCHMAR*, Michaela C. DINGER†, Christian HENZE*, Katja BROCKE-HEIDRICH* and Friedemann HORN*[1]

*Institute of Clinical Immunology and Transfusion Medicine, University Hospital, Leipzig Johannisallee 30, D-04103 Leipzig, Germany, and †Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, Leipzig, Germany

Signal transducer and activator of transcription 3 (Stat3) dimerization is commonly thought to be triggered by its tyrosine phosphorylation in response to interleukin-6 (IL-6) or other cytokines. Accumulating evidence from *in vitro* studies, however, suggests that cytoplasmic Stat3 may be associated with high-molecular-mass protein complexes and/or dimerize prior to its activation. To directly study Stat3 dimerization and subcellular localization upon cytokine stimulation, we used live-cell fluorescence spectroscopy and imaging microscopy combined with fluorescence resonance energy transfer (FRET). Stat3 fusion proteins with spectral variants of green fluorescent protein (GFP), cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were constructed and expressed in human hepatoma cells (HepG2) and human embryonic kidney cells (HEK-293). Like wild-type Stat3, the fusion proteins redistributed from a preferentially cytoplasmic to nuclear localization upon IL-6 stimulation and supported IL-6-dependent target gene expression. FRET studies in cells co-expressing Stat3–CFP and Stat3–YFP demonstrated that Stat3 dimers exist in the absence of tyrosine phosphorylation. IL-6 induced a 2-fold increase of this basal FRET signal, indicating that tyrosine phosphorylation either increases the dimer/monomer ratio of Stat3 or induces a conformational change of the dimer yielding a higher FRET efficiency. Studies using a mutated Stat3 with a non-functional src-homology 2 (SH2) domain showed that the SH2 domain is essential for dimer formation of phosphorylated as well as non-phosphorylated Stat3. Furthermore, our data show that visualization of normalized FRET signals allow insights into the spatiotemporal dynamics of Stat3 signal transduction.

Key words: interleukin-6 signalling, signal transducer and activator of transcription, tyrosine phosphorylation.

INTRODUCTION

The signal transducer and activator of transcription-3 (Stat3) plays a key regulatory role in various cytokine-controlled cellular processes such as acute-phase and immune responses, differentiation, proliferation, and cell survival [1]. In the signalling pathway of the cytokine interleukin-6 (IL-6) we, and others, found Stat3 to transiently associate with the gp130 subunit of the IL-6 receptor upon IL-6 binding [2,3]. This interaction is due to a specific interaction of the SH2 (src-homology 2) domain of Stat3 with phosphotyrosine motifs in the gp130 cytoplasmic part [4,5]. Subsequently Stat3 is phosphorylated at Tyr-705 by protein tyrosine kinases of the janus kinase family that are constitutively associated with gp130 [2]. This modification triggers the factor to dimerize via reciprocal phosphotyrosine-SH2 domain interactions, and to translocate to the nucleus. There, Stat3 binds to regulatory DNA elements controlling the expression of target genes [6,7]

A commonly cited model of Stat3 activation suggests that the protein is recruited to plasma membrane receptors from a cytoplasmic pool of non-phosphorylated monomers [8]. On the basis of co-immunoprecipitation experiments, however, several laboratories provided evidence that STAT proteins exist as dimers prior to their activation [9,10]. Furthermore, a recombinant Stat3–SH2 domain exhibited a tendency to dimerize *in vitro* [11]. Ndubuisi et al. [12] reported that only a small portion of cytoplasmic Stat3 molecules are present as monomers while the bulk is found associated with high-molecular-mass complexes of 200–400 kDa and 1–2 MDa that the authors designated 'statosomes' I and II, respectively. These complexes were shown to comprise various proteins including scaffolding proteins. Rather than indicating the presence of genuine dimers of non-phosphorylated STATs, co-precipitation studies [9,10] might reflect the formation of such high-molecular-mass complexes.

Fluorescence resonance energy transfer (FRET) has made it possible to monitor real-time protein–protein interactions, protein conformational changes, or post-translational modifications *in vivo* in a non-invasive manner (for reviews, see [13,14]). The proteins of interest are labelled by fusing to fluorophores, most commonly spectral variants of the green fluorescent protein (GFP). Since FRET requires a distance of the fluorophore pair of less than 100 Å (1 Å = 0.1 nm), a significant FRET signal is observed only if the proteins are in immediate contact.

Here we report on Stat3-dimerization studies in intact cells using FRET fusion proteins containing either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). Our data indicate that Stat3 dimers do exist prior to stimulation and that dimer formation of non-phosphorylated Stat3 depends on an intact SH2 domain.

EXPERIMENTAL

Plasmid construction

Expression vectors pStat3-YFP and pStat3-CFP coding for the Stat3 fusion proteins were generated by inserting a *Bgl*II–*Bam*HI

Abbreviations used: STAT, signal transducer and activator of transcription; IL, interleukin; SH2, src-homology 2; FRET, fluorescence resonance energy transfer; FRET^c, corrected FRET; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; R609Q etc., the substitution of Arg-609 with glutamine etc.; HEK, human embryonic kidney; DsRed-Nuc, a red fluorescent protein 'DsRed' fused to a nuclear translocation signal; gp, glycoprotein; ACT, α_1-antichymotrypsin.

[1] To whom correspondence should be addressed (e-mail fhorn@medizin.uni-leipzig.de).

fragment of pRc/CMV-Stat3-HA [15], containing the full murine Stat3 cDNA without the stop codon into the *Eco*RI and *Bgl*II sites of pECFP-N1 and pEYFP-N1 (Clontech), resulting in a linker between Stat3 (or its mutant variants) and CFP or YFP of the amino acids RSILQSTVPRARDPPVAT. pStat3-R609Q-CFP (where R609Q is the substitution of Arg-609 with glutamine) and pStat3-Y705F-YFP were constructed by site-directed mutagenesis using the quick change kit (Stratagene, Heidelberg, Germany) and mutagenesis primers (sequences: 5′-CCC CCA GGC ACC TTC CTA CTG CAG TTC AGC GAG AGC-3′ and 5′-GGT AGT GCT GCC CCG TTC CTT AAG ACC AAG TTC ATC-3′, respectively). The vector pYFP-CFP, coding for the YFP–CFP fusion protein was generated by inserting the YFP cDNA obtained from pEYFP-N1 by PCR using the primers 5′-GTC GCG GCC GAT TCC CTT GTA CAG C-3′ and 5′-CCA CCG CTC GAG CCA TGG TGA GC-3′ into the *Sal*I and *Xho*I sites of pECFP-N1, resulting in a GIGRDSTVPRARDPPVAT spacer between the fluorescent proteins.

The expression vector for DsRed-Nuc (a red fluorescent protein 'DsRed' fused to a nuclear translocation signal) was purchased from Clontech (Heidelberg, Germany), T4 DNA ligase and restriction enzymes were from Roche (Mannheim, Germany).

Cell culture and transfection

Human embryonic kidney (HEK)-293 cells or human hepatoma cells (HepG2) were grown in Dulbecco's modified Eagle's F12 medium containing 10 % (v/v) foetal calf serum with 1 % (w/v) penicillin and 1 % (w/v) streptomycin [from Life Technologies (Gibco), Eggenstein-Leopoldshafen, Germany].

Transfections were carried out using the calcium phosphate coprecipitation technique as described previously [6]. For FRET studies, expression vectors coding CFP- and YFP-fusion proteins were transfected in a ratio of 1.5:1. Medium was replaced 6 h after transfection. Before stimulation (24 h), the cells were placed in medium containing 0.5 % foetal calf serum. For stimulation of HEK-293 and HepG2 cells, 7 units/ml erythropoietin and 10 ng/ml IL-6 were used, respectively. Recombinant human IL-6 and erythropoietin were gifts from Professor Stefan Rose-John (Institute of Biochemistry, University of Kiel, Germany) and Professor Martin Gramatzki (Division of Hematology/Oncology, Department of Medicine III, University of Erlangen-Nuernberg, Germany) respectively.

Immunoblot analysis

Cells were lysed as described previously [16]. Cellular proteins were separated by SDS/PAGE (7.5 % gel) and transferred to PVDF membrane (Amersham Biosciences, Frieburg, Germany) by semidry electroblotting, and immunodetection was performed by standard techniques using chemiluminescence detection (SuperSignal, West Dura Substrate; Pierce, Rockford, IL, U.S.A.). Anti-phospho-Stat3 (Y705) and Stat3 monoclonal antibodies were obtained from Cell Signalling Technology, Beverly, MA, U.S.A. and BD Transduction Laboratories, Lexington, KY, U.S.A., respectively. Monoclonal antibody to GFP was from Clontech.

Reporter gene assays

HEK-293 cells grown in 6-well tissue-culture dishes were transfected with 0.25 μg of vector pACT-Luc containing the firefly luciferase gene under the control of the human α_1-antichymotrypsin promoter, 0.25 μg of pCH110 (Pharmacia) encoding β-galactosidase as an internal standard, 1 μg Rc/CMV-Eg coding for a chimeric erythropoietin receptor/gp130 protein [5], and 1.5 μg of Stat3 expression vectors. After 48 h cells were stimulated with erythropoietin for 4 h, harvested, and luciferase and β-galactosidase activities were determined using kits from Promega and Roche, respectively.

Fluorescence microscopy

Images were captured at 37 °C on a heatable table using a Leica DM IRB microscope with an HCX PL APO 63×/1.32 oil objective (Leica) and an ORCA-ER camera (Hamamatsu Photonics, Bridgewater, NJ, U.S.A.) under the control of Openlab software (Improvision, Warwick, U.K.). Detection of CFP, YFP, FRET, and DsRed was performed using Polychrome IV (Photonics) as exitation source and filter channels specific for the fluorophores (emitter D480/40, beamsplitter 455 DCLP; emitter HQ 535/30, beamsplitter Q 515lp; emitter D535/30, beamsplitter 455 DCLP; and emitter HQ 610/75, beamsplitter Q 570lp, respectively, from AHF Analysentechnik, Tuebingen, Germany). The excitation wavelengths for CFP, YFP and DsRed were 433 nm, 510 nm and 550 nm, respectively. The mean fluorescence intensity and areas of cells and nuclei were measured using Openlab software.

FRET measurements

To obtain spectra of CFP- and YFP-fusion proteins, cells were washed twice with PBS, transferred to 1 cm quarz cuvettes and measured using a Fluorolog-3 fluorescence spectrophotometer (Jobin Yvon Spex, Longjumeau, France) with a 450 W xenon lamp. For CFP and FRET measurements, the spectrum between 460 nm and 540 nm was recorded at an excitation wavelength of 433 nm. The measurements were performed with an increment wavelength of 1 nm and an increment time of 0.75 s.

For FRET microscopy the method of sensitized FRET was used [17]. Briefly, calculation of corrected FRET ($FRET^c$) was carried out on a pixel-by-pixel basis for the entire image. The bleedthrough of CFP and YFP through the FRET filter channel was corrected applying the equation:

$$FRET^c = FRET - (\text{bleedthrough CFP} \times CFP) - (\text{bleedthrough YFP} \times YFP) \quad (1)$$

For YFP intensity-modulated images [18], normalized FRET ($FRET^c$/YFP) images were generated by dividing $FRET^c$ by YFP fluorescence signals. Images are displayed in pseudocolour mode, where red and blue areas display high and low values of FRET in the range of 0–4000 relative light units, respectively. The overall intensity of FRET was calculated using Openlab software (Improvision).

RESULTS

Characterization of Stat3–CFP and Stat3–YFP fusion proteins

In order to evaluate the dimerization status of Stat3 in living cells, a FRET-based analysis using fusion proteins of Stat3 with YFP and CFP was established [19]. To allow efficient energy transfer between the YFP and CFP domains within a Stat3 dimer, we chose to fuse the fluorescent proteins to the C-terminus of Stat3. This is based on the three-dimensional structure of a DNA-bound Stat3 dimer as published by Becker et al. [20] demonstrating a close proximity of the Stat3 carboxy termini (Figure 1A). The constructs used in this work are shown schematically in Figure 1(B).

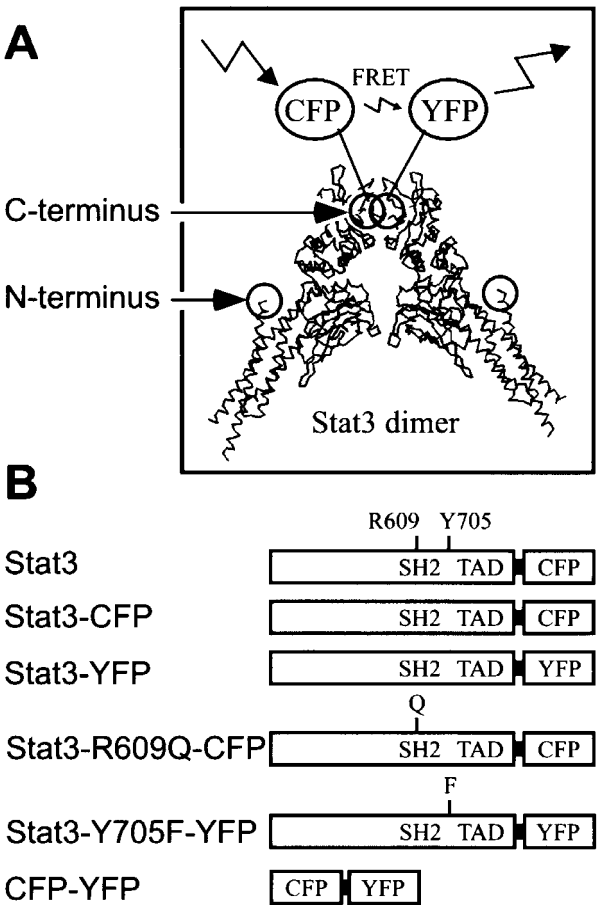


Figure 1 Schematic representation of the Stat3 constructs used in this work

(**A**) Structure of Stat3 fused to CFP and YFP, respectively, in the DNA-bound dimer form according to [20]. The C-termini of the Stat3 molecules are in close proximity, enabling FRET between fluorophore domains fused to them. (**B**) Overview of the fusion proteins used. TAD, transactivation domain.

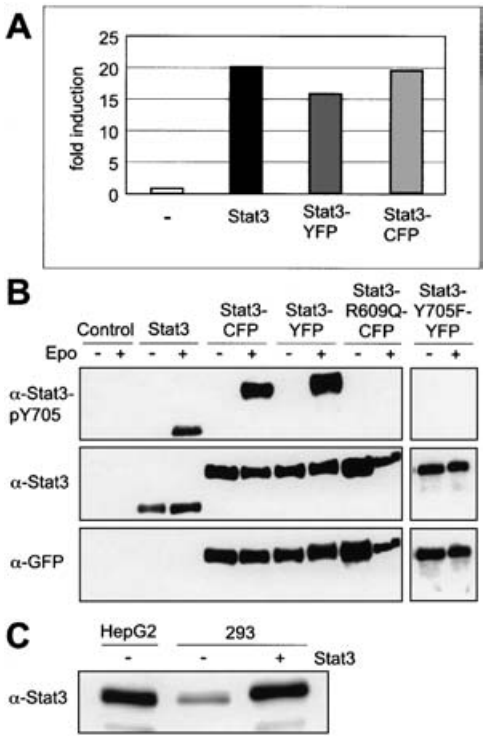


Figure 2 Stat3–CFP and Stat3–YFP fusion proteins are fully functional

(**A**) Reporter-gene assay. HEK-293 cells were transiently transfected to co-express the Stat3 fusion proteins, as indicated, with the erythropoietin receptor/gp130 hybrid Eg, luciferase under the control of the human ACT promoter, and β-galactosidase. Cells were incubated with erythropoietin for 4 h prior to harvesting. In the cell lysates, luciferase activities were measured, normalized to β-galactosidase activities as an internal standard for transfection efficiency, and the values for fold-induction by erythropoietin were calculated. (**B**) Stat3 or Stat3 fusion proteins were co-expressed with Eg in HEK-293 cells. After 30 min of stimulation with erythropoietin, cells were harvested, and cell lysates separated by SDS polyacrylamide electrophoresis. Immunoblotting was done with antisera to Stat3 phosphorylated at Tyr-705 (Stat3-pY705), to Stat3, and to GFP. (**C**) Ectopic Stat3 expression levels in HEK-293 cells were compared with those of endogenous Stat3 from non-transfected HepG2 cells by immunoblotting with anti-Stat3.

For FRET analysis two cell types were used; HepG2 human hepatoma cells have previously been used by us to dissect the IL-6 signal transduction pathway [2,6] and to express high levels of endogenous Stat3 and IL-6 receptor. In contrast, HEK-293 cells show poor endogenous expression of Stat3 (results not shown) and therefore allow the functional analysis of ectopically expressed Stat3 fusion proteins on a low background of endogenous wild-type protein. To activate Stat3 in these cells, a hybrid receptor consisting of the erythropoietin receptor extracellular and the gp130 transmembrane and cytoplasmic parts (named Eg) was co-expressed and stimulated by erythropoietin as described previously [21].

To verify that both Stat3–CFP and Stat3–YFP are fully functional we examined their expression, cytokine-dependent tyrosine phosphorylation, and transactivating potential. HEK-293 cells expressing the Stat3 fusion proteins and the Eg receptor hybrid were stimulated with erythropoietin for 30 min. Cell extracts prepared from these cells were separated by SDS/PAGE and immunoblotted using an antibody specific for Stat3 phosphorylated at Tyr-705 (Figure 2B). Equal expression of the fusion proteins was verified by immunoblotting with antibodies to Stat3 and GFP (Figure 2B, bottom panel). To avoid artifacts due to excessive abundance of the ectopically expressed proteins, the amounts of transfected vectors were chosen so that the expression levels were comparable with those observed for endogenous Stat3 in HepG2 cells (Figure 2C).

The Stat3 fusion proteins were tyrosine-phosphorylated in response to erythropoietin as was wild-type Stat3. As expected, mutating Arg-609 into glutamine (Stat3-R609Q-CFP) yielding a non-functional SH2 domain [21,22] or Tyr-705 into phenylalanine (Stat3-Y705F-YFP) abolished gp130-mediated Stat3 tyrosine phosphorylation. Transactivation by the Stat3 fusion proteins was examined by a reporter gene assay using the IL-6-dependent human α_1-antichymotrypsin (ACT) promoter driving the luciferase gene. This promoter contains an IL-6 response element comprising two adjacent Stat3 binding sites [23]. Stat3–CFP and Stat3–YFP both induced luciferase expression from the ACT promoter to an extent comparable with wild-type Stat3 (Figure 2A).

Activation-dependent nuclear translocation of Stat3–CFP and Stat3–YFP

To analyse the subcellular localization of the Stat3 fusion proteins, Stat3–YFP or Stat3–CFP were co-expressed in HEK-293 cells with DsRed-Nuc. In most transfected cells, the Stat3 fusion proteins were found distributed all over the cell in the absence of cytokines as shown in Figure 3(A). However, in a small minority of

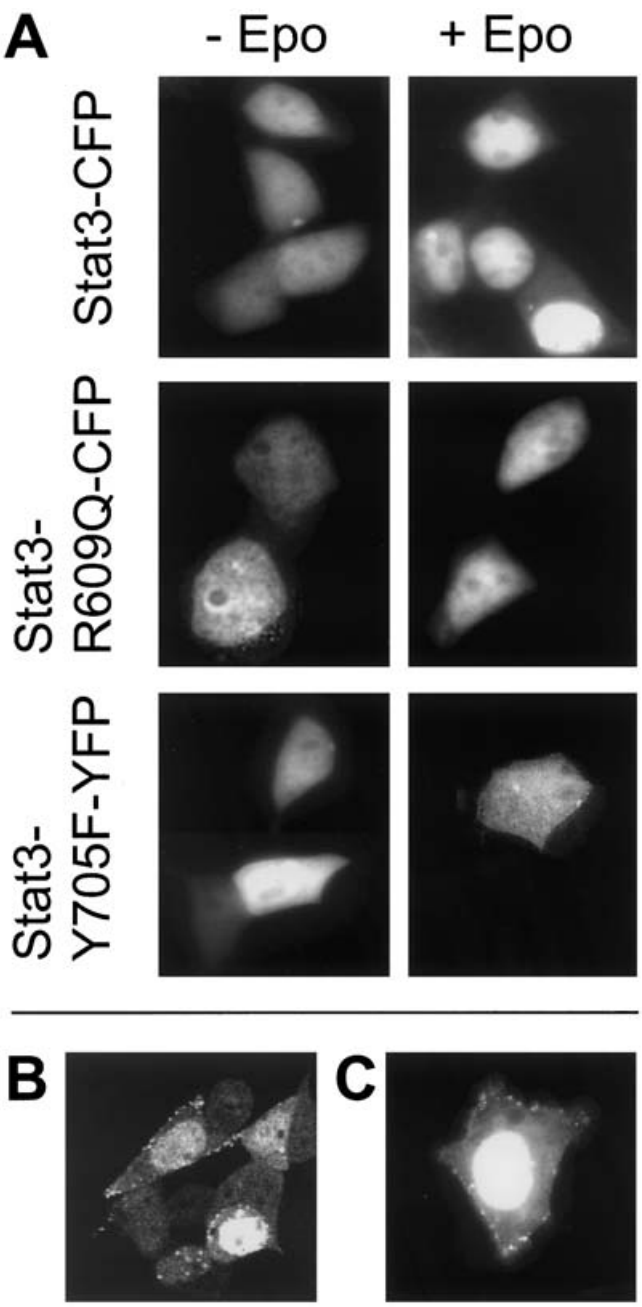


Figure 3 Nuclear translocation of Stat3–CFP analysed by fluorescence microscopy

(**A**) HEK-293 cells were transiently transfected with expression vectors coding for the erythropoietin receptor/gp130 hybrid Eg and either Stat3–CFP, Stat3-R609Q-CFP, or Stat3-Y705F-YFP, as indicated. One day post-transfection, the cells were stimulated with erythropoietin for 40 min. (**B**) Confocal image of HEK-293 cells co-transfected with Stat3–CFP and Eg 15 min after stimulation with erythropoietin. (**C**) HepG2 cells expressing Stat3–CFP and stimulated with IL-6 for 20 min. Note the formation of caveolae-like structures close to the plasma membrane in both cell types after cytokine stimulation.

cells we reproducibly observed an accumulation of the fluorescent protein in vesicles (results not shown). This effect was independent of the time after transfection, or the DNA concentration used and also occurred in HepG2 cells. Although the reason for this phenomenon is not known, we assume that it is probably due to a non-physiologically high expression level of fusion protein in these few cells.

As shown in Figure 3(A), stimulation through the Eg receptor hybrid yielded nuclear translocation of Stat3–CFP in HEK-293 cells. To quantify this process, the integral of the whole-cell fluorescence was divided by that of the nuclear area as defined by the DsRed-Nuc fluorescence in the red channel. According to this calculation the amount of nuclear Stat3–CFP rose from 25 % to 40 %, in untreated, and from 60 % to 80 % in cells treated with erythropoietin for 40 min. At earlier time points, a transient accumulation of Stat3–CFP in punctual structures near the plasma membrane became evident in both HEK-293 and HepG2 cells (Figures 3B and 3C). Comparable results were obtained with Stat3–YFP (results not shown). This likely reflects the transient association of Stat3 with gp130 after ligand binding and suggests that IL-6 receptor signalling complexes accumulate in caveolae or similar plasma membrane structures. In fact, caveolae have recently been described as being important for IL-6 signalling in multiple myeloma cells [24], and Stat3 has been reported to be associated with lipid rafts [25,26].

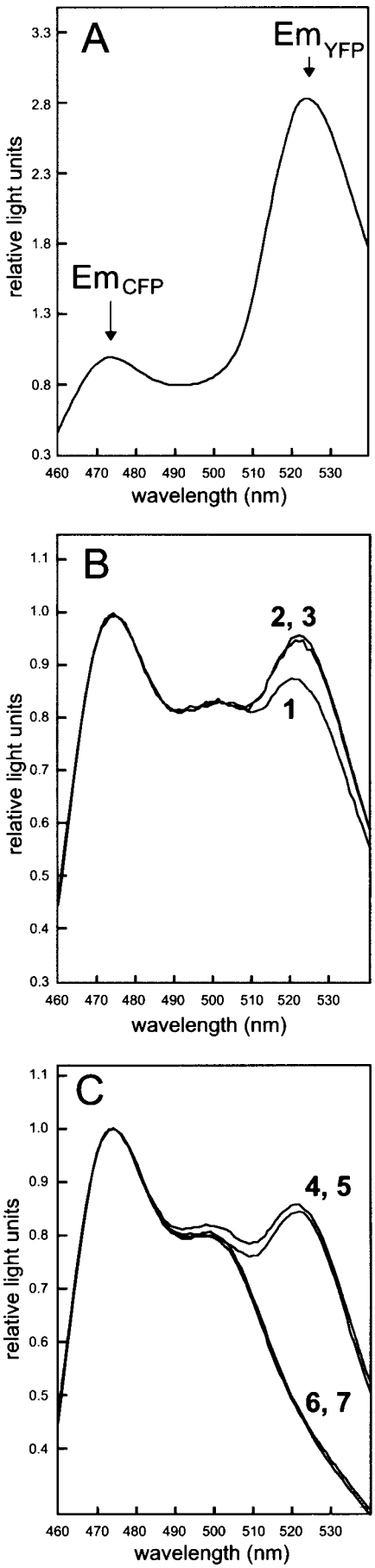
The distribution of the mutated fusion proteins Stat3-R609Q-CFP and Stat3-Y705F-YFP between cytoplasm and nucleus in non-stimulated cells was comparable with the wild-type protein. Furthermore, Stat3-Y705F-YFP, but not Stat3-R609Q-CFP, also accumulated with caveolae-like structures near the plasma membrane, after cytokine treatment, as did the wild-type fusions. Neither mutant, however, underwent cytokine-induced nuclear translocation (Figure 3A).

Stat3–CFP and Stat3–YFP dimerize prior to activation

In order to study the dimerization of Stat3 in living cells, FRET signals of co-expressed Stat3–CFP and Stat3–YFP were examined. FRET was first measured by fluorescence spectrometry in HEK-293 cells. As a positive control for FRET between CFP and YFP, a CFP–YFP fusion protein was constructed that was expected to permanently produce intense FRET. In fact, HEK-293 cells expressing this protein showed high FRET intensity, as evident from a strong emission peak at 525 nm upon excitation with 433 nm (Figure 4A). Co-expression of Stat3–CFP and Stat3–YFP yielded a much smaller but significant FRET signal even in non-stimulated cells (Figure 4B). This signal increased upon stimulation of the cells with erythropoietin through the co-expressed Eg hybrid receptor (Figure 4B) and decreased again after 60 min (results not shown). To verify that the FRET signal observed reflects a true interaction between Stat3 molecules and is not due to non-specific interactions of the CFP and YFP moieties, we co-expressed Stat3–YFP with the mutated Stat3-R609Q-CFP containing a non-functional SH2 domain. In both stimulated and non-stimulated HEK-293 cells the emission spectra measured with this mutant exhibited no FRET peak at 525 nm (Figure 4C) and were indistinguishable from the one obtained with Stat3–CFP alone. We conclude from these findings that the FRET signal observed upon co-expression of Stat3–CFP and Stat3–YFP does reflect specific dimerization of Stat3.

According to these data, therefore, Stat3 dimer formation occurs in living cells even in the absence of cytokine stimulus. The higher FRET intensity observed after Stat3 activation can be interpreted either as an increased Stat3 dimer/monomer ratio or as a change of dimer conformation that results in a closer proximity of the fluorophore domains and hence a higher resonance transfer efficiency.

The three-dimensional structure of Stat3 [20] showed the involvement of the SH2 domain in dimerization of the tyrosine-phosphorylated factor. Interestingly our observation that the Stat3-R609Q mutant was entirely incapable of producing FRET signals



now demonstrates that the dimer formed by non-phosphorylated Stat3 also depends on an intact SH2 domain.

By immunoblotting we could not detect any tyrosine phosphorylation of Stat3 fusion proteins in non-stimulated HEK-293 cells (see above). However, the theoretical possibility remained that the FRET signal observed under these conditions relies on a level of tyrosine-phosphorylated Stat3 too low to be detected by the immunoblot analysis. Therefore we co-expressed Stat3–CFP with Stat3-Y705F-YFP, in which the tyrosine phosphorylation site had been mutated into phenyalanine. As shown in Figure 4(C), the FRET signal observed was as high as with wild-type Stat3–YFP in non-stimulated cells. In contrast to Stat3–YFP, however, the mutated protein did not give rise to the enhanced FRET signal after cytokine treatment. Hence the Stat3 dimerization observed in untreated HEK-293 cells did not involve Tyr-705 phosphorylation, whereas the cytokine-induced increase of the FRET signal depended strictly on this phosphorylation. As one of the fusion proteins (Stat3–CFP) is wild-type and phosphorylated under these conditions, these findings also imply that tyrosine phosphorylation of only one partner does not suffice to support the dimer stabilization or conformational change underlying the increased FRET signal.

Visualization of Stat3 dimerization by FRET using live-cell fluorescence microscopy

In order to visually monitor dimer formation in living cells, FRET measurements were next carried out using a fluorescence microscope system. First it was necessary to measure the bleedthrough emission into the FRET channel of either fusion protein, according to Gordon et al. [17]. For this purpose, Stat3–CFP and Stat3–YFP were expressed separately in HEK-293 cells and the relative bleedthrough ratios of excitation at 433 nm into the FRET channel was estimated. The values obtained were 0.5 and 0.02 for Stat3–CFP and Stat3–YFP, respectively. Exciting CFP in cells expressing Stat3–CFP showed no fluorescence signal with the YFP filter set, and vice versa, proving the correct imaging conditions and specifications (Figure 5). For the estimation of FRET in cells co-expressing Stat3–CFP and Stat3–YFP, $FRET^c$ signals were calculated using these bleedthrough values from the primary signals obtained in the microscope FRET channel (see Equation 1 in the Experimental section). In addition, a relative $FRET^c$ was calculated by dividing the $FRET^c$ signals by the Stat3–YFP fluorescence ($FRET^c$/YFP). The resulting intensity-modulated images [18] represent the ratio of FRET signal to Stat3 fusion protein.

Stat3–CFP and Stat3–YFP were co-expressed in HepG2 cells and the $FRET^c$ and $FRET^c$/YFP signals calculated before and after IL-6 treatment. Stat3–CFP (or Stat3-R609Q-CFP) and Stat3–YFP (or Stat3-Y705F-YFP) showed a complete co-localization throughout the cytoplasm and the nucleus with only negligible quantitative differences (Figure 6). Expression of CFP–YFP as a positive control resulted in a high level of $FRET^c$ all over the cell (Figure 6A). No FRET was detected with co-expressed Stat3-R609Q-CFP and Stat3–YFP, confirming the data obtained with

Figure 4 Spectrofluorimetric FRET analysis of Stat3 dimerization

HEK-293 cells were transfected with Eg and CFP–YFP (**A**), Stat3–CFP and Stat3–YFP (**B**, traces 1, 2 and 3), Stat3–CFP and Stat3-Y705F-YFP (C, trace 4 and 5), or Stat3-R609Q-CFP and Stat3–YFP (**C**, traces 6 and 7), Emission spectra were taken from unstimulated cells (traces 1, 4, and 6) or from cells 20 min (trace 2) and 40 min (traces 3, 5 and 7) after treatment with erythropoietin. In (**A**), the emission maxima of separately recorded CFP and YFP spectra (Em_{CFP} and Em_{YFP}, respectively) are indicated.

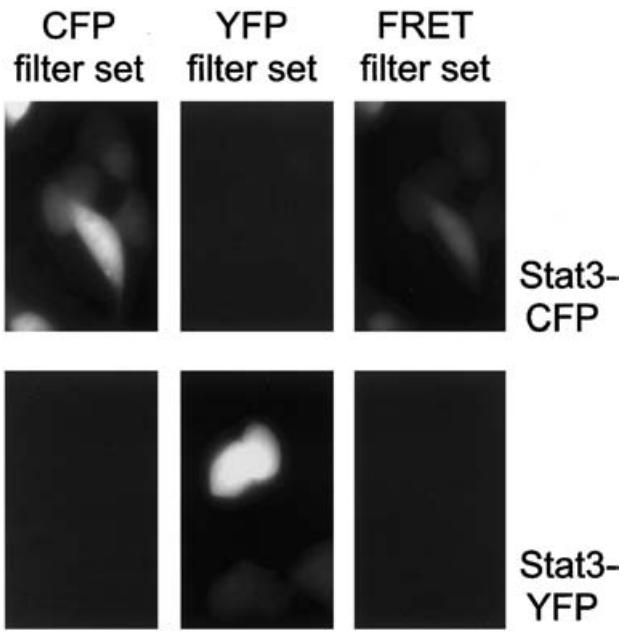


Figure 5 Imaging conditions for FRET analysis

Fluorescence microscopic pictures of HEK-293 cells transiently expressing either Stat3–CFP or Stat3–YFP were taken using CFP filter (left), YFP filter (middle), or FRET filter (right).

fluorescence spectrometry (Figure 6B). Cells expressing Stat3–CFP and Stat3-Y705F-YFP showed a basal FRET^c signal that did not change after stimulation with IL-6 (Figure 6C). The FRET^c/YFP signals were found evenly distributed within the cells indicating that dimer formation of non-phosphorylated Stat3 occurs throughout the cell.

Upon co-expression of Stat3–CFP and Stat3–YFP, a FRET^c signal in untreated cells was observed that was similar in intensity and distribution to the one obtained with the Y705F mutant (Figure 6D). After addition of IL-6 for 20 min and 40 min, the FRET intensity increased considerably. Quantitative analysis of the FRET^c signals revealed an approx. 2-fold increase in FRET intensity in response to cytokine stimulation (Figure 6E). After 60 min, the overall FRET^c signal decreased again (results not shown). As expected the highest FRET signals, and hence accumulation of Stat3 dimers, was found in the nuclear region.

When the Stat3 dimer/protein ratio was visualized in IL-6-treated cells, however, an entirely different distribution emerged. The relative FRET^c/YFP signals showed a gradient from high to lower intensities from the plasma membrane area to the cell interior. This is most obvious in Figure 7 showing microscopic pictures of Figure 6 at higher magnifications. Hence, in spite of the nuclear translocation of Stat3 the membrane-proximal area remained the location of highest relative FRET^c/YFP signals and the highest ratio of activated to total Stat3 protein. This distribution clearly demonstrates the steady state of Stat3 activation at the plasma membrane and inactivation in the nucleus.

Stat3 phosphorylation and DNA binding start to decrease after approximately 30 min of IL-6 stimulus and reach almost control levels after 1 h [6,7]. It is intriguing, therefore, that the high relative FRET signal at the plasma membrane persisted for up to 40 min while the relative FRET in the nuclear region had already begun to return to its pre-activation level. This finding suggests that upon down-regulation of Stat3 activation a drop of activation at the plasma membrane is preceded by increased nuclear dephosphorylation.

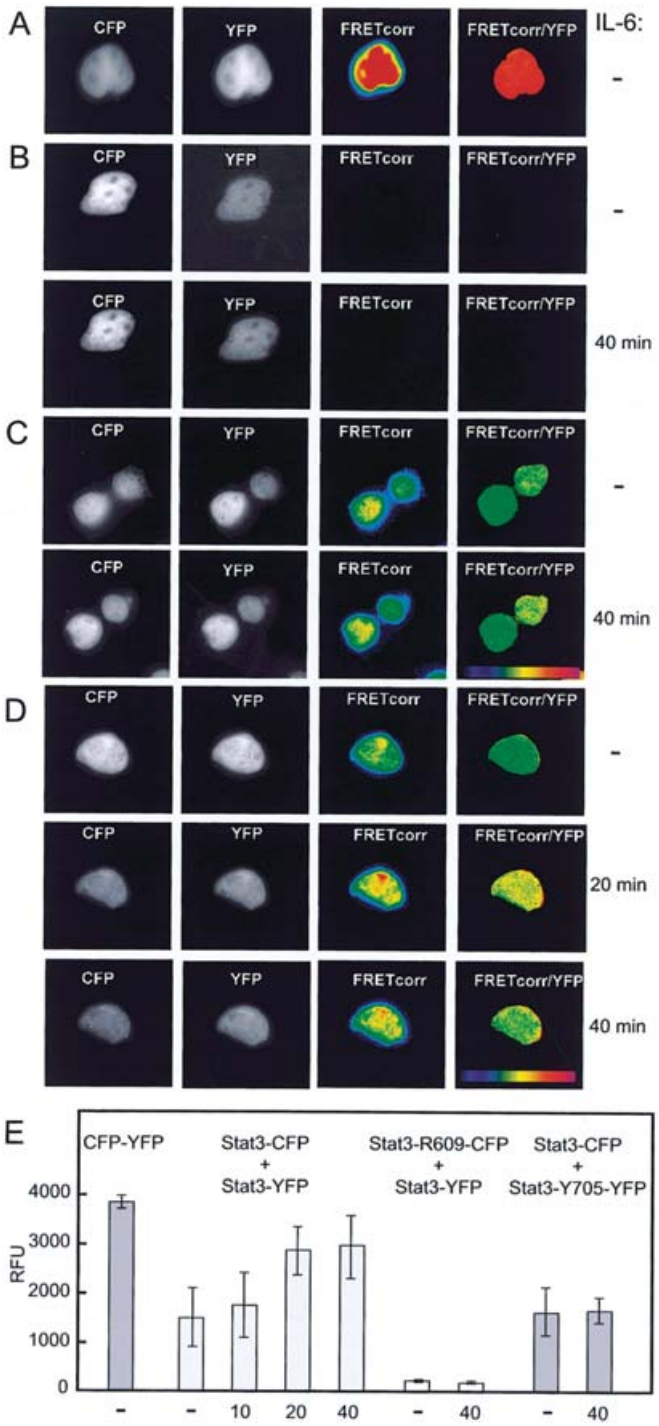


Figure 6 Microscopic FRET analysis in living HepG2 cells

FRET^c and corrected normalized FRET (FRET^{corr}/YFP) was calculated according to the Materials and methods section. HepG2 cells transiently expressing CFP–YFP (**A**), Stat3-R609Q-CFP and Stat3–YFP (**B**), Stat3–CFP and Stat3-Y705F-YFP (**C**), or Stat3–CFP and Stat3–YFP (**D**) were treated with IL-6 for the times indicated. FRET intensity is indicated by pseudocolours, with red and blue representing high and low intensities, respectively. (**E**) FRET intensities calculated for individual cells from the FRET^{corr}/YFP images. The values given are means ± S.D. of at least three individual experiments, each analysing 2–3 cells. RFU, relative light units.

DISCUSSION

Stat3 plays a key role in the signalling cascade in response to IL-6 and related cytokines. Its recruitment to cytokine receptors via its

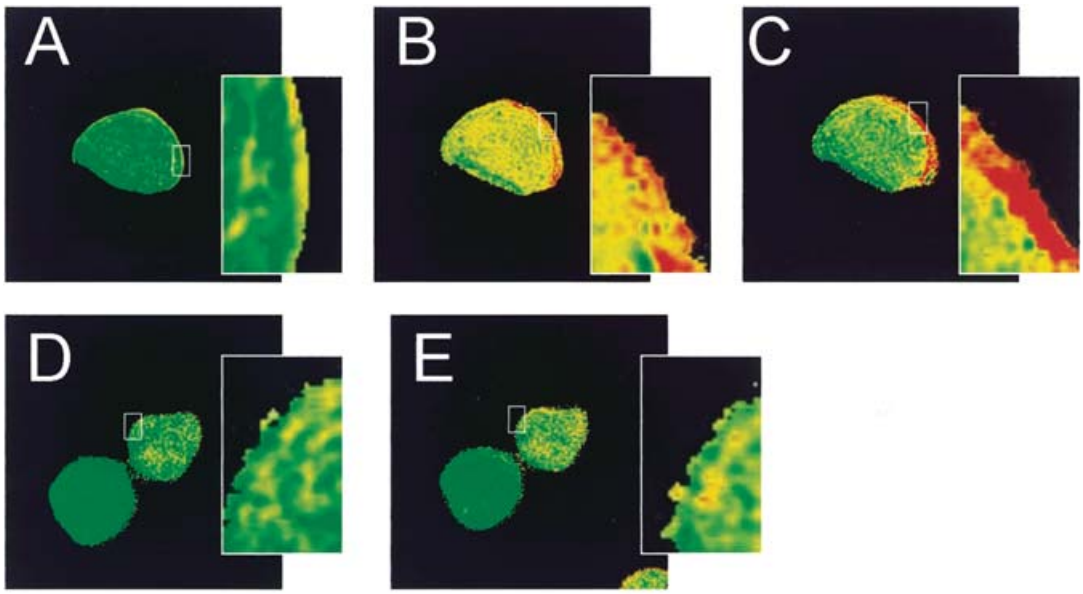


Figure 7 Spatiotemporal dynamics of Stat3 dimer formation

The corrected normalized FRET ($FRET^{corr}$/YFP) pictures from Figure 6(**C**) and 6(**D**) are shown at higher magnifications: HepG2 cells co-expressing either Stat3–CFP and Stat3–YFP (**A**–**C**) or Stat3–CFP and Stat3-Y705F-YFP (**D**,**E**) were treated with IL-6 for 20 min (**B**) or 40 min (**C**,**E**) or left untreated as a control (**A**,**D**).

SH2 domain, the phosphorylation at Tyr-705, and its subsequent dimerization and translocation to the nucleus are regarded as critical steps for the induction of target gene transcription.

Several laboratories, however, have reported pre-association of inactive cytoplasmic Stat3 molecules. Both Stat3–Stat3 and Stat3–Stat1 complexes can be co-immunoprecipitated from untreated cells [9,10]. In contrast with tyrosine-phosphorylated Stat3 dimers, these Stat3 complexes were found by Novak et al. [9] to be stable only in the presence of bivalent cations. The authors conclude from this observation that tyrosine-phosphorylated dimers differ in structural terms from the complexes formed by inactive Stat3. Haan et al. [10], however, could not reproduce this requirement for cations in their studies. Hence the issue remains as yet unresolved. While in both reports co-immunoprecipitation is taken as an evidence for the formation of Stat3 'dimers', the paper of Ndubuisi et al. [12] hints at another possible interpretation. By gel chromatography of cellular extracts, Ndubuisi et al. [12] demonstrated the existence of large multi-protein complexes that Stat3 is associated with ('statosomes'). Therefore, it appears possible that co-precipitation of Stat3 molecules is not a result of direct Stat3–Stat3 interaction (i.e. dimerization) but rather reflects the integration of multiple STAT molecules into these statosomes. Hence, on the basis of these studies, it is not possible to decide whether Stat3 molecules dimerize prior to their activation *in vivo* or not. Furthermore, all techniques used so far on this topic were invasive and might not reflect the true situation within living cells.

We therefore addressed this question by the FRET technique that allows visualization of protein–protein interactions within living cells. Both fluorescence spectrometric measurements and fluorescence microscopy showed considerable FRET signals between Stat3 molecules carrying the CFP and YFP fluorophores even in the absence of cytokine stimulus. Activating Stat3 increased this signal with a similar time-course as that observed for tyrosine phosphorylation. Since efficient resonance energy transfer between two fluorophores requires a distance of less than 100 Å, FRET signals are observed only if the proteins carrying the fluorophores are in immediate contact. Therefore, our data provide strong evidence that an equilibrium of monomeric and dimeric molecules exists for inactive Stat3.

A point mutation rendering the Stat3 SH2 domain incapable of binding its target peptides yields a Stat3 molecule that cannot associate with cytokine receptors and hence cannot be tyrosine-phosphorylated in response to cytokine treatment. As this mutation completely eliminated the generation of FRET signals, even in the absence of cytokine, dimer formation of non-phosphorylated Stat3 also requires a functional SH2 domain. Although for most SH2 domains high-affinity interaction with their target peptides requires its tyrosine phosphorylation, there are exceptions from this rule. The SH2 domain of SAP, the protein found mutated in X-linked lymphoproliferative syndrome XLP, binds efficiently to its target sequence in the signalling lymphocytic activation molecule ('SLAM') of T lymphocytes without tyrosine phosphorylation [27]. Our data suggest that the same might hold true for the SH2-domain interactions involved in Stat3 dimerization.

Activation of Stat3 by cytokine treatment considerably increased the FRET signal within the cells. It cannot be deduced from these experiments whether this observation reflects a higher number of dimers or a conformational change yielding a more efficient energy transfer. It is possible that dimers of non-phosphorylated Stat3 rely on an entirely different structural basis. In fact, for the cooperative binding of Stat3 to adjacent DNA binding sites, an N-terminal domain of Stat3 has been described as being essential [28]. Furthermore, DNA-binding of Stat3 requires tyrosine phosphorylation, at least under experimental conditions *in vitro* [2,15]. Therefore, one would expect some change of dimer conformation to account for the different DNA-binding capability. However, based on the notion that both the non-phosphorylated and phosphorylated dimers require intact SH2 domains, it appears unlikely that the Stat3 dimers adopt a completely different conformation upon phosphorylation. Whether or not

such a conformational change occurs, and whether it affects FRET efficiency, cannot be clarified without further structural information.

Gupta et al. [29] reported that on the basis of co-immunoprecipitation studies, Stat1 dimers are able to form when only one factor is phosphorylated. Our studies using the Stat3-Y705F mutant demonstrate that the basal dimer formation does not depend on Tyr-705. However, with one partner mutated, the FRET signal did not increase upon cytokine addition. Therefore, the active dimer forms only with two tyrosine-phosphorylated Stat3 molecules.

Several laboratories have provided evidence for the existence of nuclear Stat3 prior to stimulation. The extent of this effect remains controversial, i.e. ranging from marginal amounts [10] to up to one-third of Stat3 protein [30] in HepG2 cells. According to the latter report, this basal nuclear presence of Stat3 did not require tyrosine phosphorylation. Similarly, we found 25 % to 40 % of the Stat3 fusion proteins used in the present study to be located in the nucleus in both HepG2 and HEK cells prior to cytokine stimulation. The Stat3-Y705F and Stat3-R609Q mutants showed the same subcellular distribution as wild-type Stat3, suggesting that for nuclear import of non-activated Stat3 molecules neither tyrosine phosphorylation nor basal dimerization are required. In fact Bhattacharya and Schindler [31] recently reported the presence of three nuclear export signal elements in Stat3. When the nuclear export was blocked, Stat3 accumulated in the nucleus in the absence of tyrosine phosphorylation demonstrating a constitutive shuttle of Stat3 between cytoplasm and nucleus.

The results reported here, and by other laboratories, strongly suggest that in non-stimulated cells, the status of Stat3 underlies multiple equilibria of monomeric versus dimeric, free versus multiprotein complex-associated, and cytoplasmic versus nuclear forms. It appears most likely that these equilibria have substantial impact on the kinetics of Stat3 signalling. In addition, the possibility arises that the basal dimerization and/or nuclear occurrence of non-phosphorylated Stat3 are of immediate functional importance for yet unknown cellular responses. For Stat1, it has been demonstrated that the non-phosphorylated factor regulates the expression of genes involved in apoptosis control [32]. Recent reports have shown that for this effect, the DNA-binding domain of Stat1 is not required [33,34].

Our observation that Stat3 fusion proteins transiently associate with small punctual structures close to the plasma membrane upon cytokine-stimulation of the cells, supports the view that IL-6 signal transduction at the plasma membrane is organized into caveolae, as has recently been described for multiple myeloma cells [24]. These authors have shown that Stat3 activation by IL-6 is inhibited when caveolae are disrupted by β-cyclodextrin, which sequesters cholesterol from the membrane. Caveolae are specialized flask-shaped vesicular invaginations of the plasma membrane serving as a scaffold for various signalling molecules [35]. Intriguingly, we have recently observed that IL-6 induces genes encoding proteins associated with caveolae and lipid rafts, like caveolin-2, flotillin, and phospholipid scramblase 1 (K. Brocke-Heidrich and F. Horn, unpublished work). By analysing relative FRET intensities it became evident that the ratio of Stat3 dimer to Stat3 protein in IL-6-stimulated cells is highest close to the plasma membrane. This correlates well with the site of Stat3 tyrosine phosphorylation by receptor-associated janus kinases. In contrast, the basal monomer/dimer equilibrium of non-phosphorylated Stat3 was equal throughout the cell. While estimation of absolute FRET values provides information about the compartmentalization of protein–protein interactions, the latter observations illustrate that measurement of normalized FRET signals allow valuable insight into the spatiotemporal dynamics of signalling.

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