# Preassociation of nonactivated STAT3 molecules demonstrated in living cells using bioluminescence resonance energy transfer: a new model of STAT activation?

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**Abstract: Signal transducers and activators of transcription (STATs) are crucial molecules in cytokine signaling. In the conventional model of STAT activation, STAT molecules are recruited from a latent pool of cytoplasmic monomers to the activated cytokine receptor. After binding to the receptor, they get tyrosine-phosphorylated, dissociate from the receptor, and translocate to the nucleus as activation-induced dimers. Recently, several publications questioned this model of STAT activation and showed the existence of preassociated STAT molecules before activation. We were able to demonstrate the existence of these preassociated STAT3 molecules in living mammalian cells using bioluminescence resonance energy transfer. Our results support the new hypothesis that STAT molecules exist in the cytoplasm as dimers or multimers and point to an activation-induced change in STAT3 conformation. Therefore, we propose a new model of STAT activation and discuss a hypothetical structure of "cytoplasmic" STAT dimers as opposed to the known "activation-induced" dimer. *J. Leukoc. Biol.* 75: 792–797; 2004.**

***Key Words:*** *cytokine · signal transduction · dimerization signal transducer and activator of transcription (STAT) · energy transfer · BRET*

## INTRODUCTION

Cytokines are crucial molecules for orchestrating the host response against invading pathogens, and signal transducers and activators of transcription (STATs) play a central role in the signaling pathways activated by cytokine receptors. The model of STAT activation says that cytokine binding to their receptors leads to receptor aggregation and phosphorylation of specific tyrosine residues by Janus kinases (Jaks). Subsequently, STAT molecules bind to their phosphorylated receptor-docking sites with their Src homology (SH)2 domains and get tyrosine-phosphorylated by the Jaks. The STATs then dissociate from the receptor and dimerize by means of the SH2 domain, binding to the phosphorylated tyrosine residues of another STAT monomer. Afterwards, they translocate to the nucleus and induce the expression of target genes. This widely recognized model of STAT activation is based on the hypothesis that STAT molecules are recruited from a monomeric pool in the cytoplasm and dimerize only after activation-induced phosphorylation [1, 2]. However, some publications have recently suggested the existence of STAT dimers before activation, and Ndubuisi et al. [3] have even proposed the existence of a multimeric "statosome" in which STATs are associated not only with each other but also with additional chaperone proteins [4–6]. Nevertheless, the structure and function of the putative cytoplasmic dimers or multimers are largely unknown. It is very likely that they differ from the activation-induced nuclear dimers, which form via the SH2 domains and the phosphorylated tyrosine residues, both located close to the C-terminus. The structure of the STAT3 dimer binding to the DNA has been solved and shows a pliers-like structure with the C-termini on top of the DNA strand and the N-termini stretching apart at either side of the strand [7].

It has been described that not only the SH2 domain but also the N-terminal domain of the STATs are required for receptor binding [8, 9], and very recently Zhang et al. [10] proposed an intramolecular interaction between the C- and the N-terminal domains of STATs, which is necessary for receptor binding. These observations queried the conventional model of STAT activation and evoked the need for further investigations.

Bioluminescence resonance energy transfer (BRET) is a recently developed method to investigate protein–protein interactions in living mammalian cells. BRET takes advantage of a natural phenomenon, namely the Förster energy transfer between a light-emitting luciferase and an acceptor fluorophore. A bioluminescent luciferase [Renilla luciferase (Rluc)] is fused to one of the proteins of interest and a green fluorescent protein [enhanced yellow fluorescent protein (EYFP)] to the other. In the presence of its substrate, Rluc emits light ($\lambda_{max}$ 480 nm), which excites the acceptor fluorophore EYFP, if the two molecules are close enough for energy transfer to occur. It is important to note that those do not naturally

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associate with each other [11]. An interaction between the two fusion proteins should bring the luciferase and EYFP close enough for energy transfer to occur. By means of this method, protein interactions can be detected in living cells and be followed over a time-course or under varying conditions. BRET has been successfully used to investigate membrane receptor dimerization and has proven its suitability to detect existing protein interactions [12–14].

We used the BRET assay to investigate the association of STAT3 molecules before and after activation. For the first time, we detected an interaction of nonphosphorylated STAT molecules in living cells. Furthermore, our experiments may allow some conclusions about the structure of the cytoplasmic-preassociated STAT molecules, suggesting a reorganization of the STAT dimers after activation.

## MATERIALS AND METHODS

### Cell culture

COS-1 cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum, L-glutamine, and penicillin/streptomycin. One day before transfection, cells were plated at a density of $5 \times 10^5$ cells per 5 cm dish.

### Plasmid construction

The BRET expression vectors are based on the pcDNA3.1 vector containing the open reading frame for EYFP or for Rluc [14]. We inserted the complete coding sequences of STAT3 or STAT6 in a way that led to the expression of N-terminal or C-terminal fusion proteins with EYFP or Rluc. The inserts were generated by polymerase chain reaction amplification from IMAGE_3347434 clone (STAT3) or IMAGE_2958389 clone (STAT6; rzpd, Berlin, Germany) using mutagenic primers introducing the appropriate restriction sites.

### Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were performed using standard procedures. The phosphorylated form of the STAT3 fusion protein was detected with phospho-specific antibodies against STAT3–Tyr705 (Cell Signaling Technology, Beverly, MA).

### BRET assay

COS-1 cells were transfected with the Rluc–STAT3 protein alone or in combination with EYFP or the STAT3–EYFP fusion protein. Transfections were performed using the Superfect® reagent (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Total plasmid DNA (3 μg) was used for transfection, and the empty vector was used to equalize DNA amounts in each sample. After 48 h, cells were harvested, washed twice with phosphate-buffered saline, and used for BRET analysis. Flow cytometric analysis was performed to check for comparable EYFP expression of EYFP and the EYFP–STAT3 fusion protein.

Approximately 50,000 cells/well were distributed in a 96-well plate, the cell-permeable Rluc substrate coelenterazine (h form; Molecular Probes, Eugene, OR) was added to a final concentration of 5 μM, and readings were collected immediately following this addition. Repeated readings were taken for at least 5–10 min using a custom-designed BRET instrument (Berthold, Australia), which allows for sequential integration of the signals detected in the 440–500 and 510–590 nm windows. Human recombinant epidermal growth factor (EGF; Promega, Annandale, Australia) was added to a concentration of 100 ng/ml to activate STAT3, and readings were collected for another 10–20 min afterwards. Data were analyzed as a normalized BRET ratio, which is defined as the BRET ratio for the coexpression of the Rluc and EYFP constructs normalized against the BRET ratio for the Rluc expression construct alone.

To quantify the BRET signal generated, the ratio of the light emitted by the EYFP fusion protein (510–590 nm) over that emitted by the Rluc fusion protein (440–500 nm) was determined by using the following equation [12]: [(emission at 510–590 nm)–(emission at 440–500 nm)×*cf*]/(emission at 440–500 nm). *cf* corresponds to (emission at 510–590 nm/emission at 440–500 nm) for the Rluc construct expressed alone in the same experiment.

## RESULTS

To use the BRET assay for investigating homophilic interactions of STAT molecules, we made fusion proteins with Rluc or EYFP fused to the C-terminus of STAT3 (STAT3–Rluc and STAT3–EYFP). As the STAT3 molecule is relatively large and spans ~110 Å [7], and the critical Förster radius for energy transfer is ~50 Å [15], we also fused Rluc N-terminally to the STAT3 molecule (Rluc–STAT3). Thereby, we hoped to detect interactions independent of the orientation of the STAT3 molecules in the dimer/multimer.

### EGF rapidly phosphorylates the STAT3–Rluc fusion protein

We aimed at investigating the interaction between STAT3 molecules in the nonactivated state as well as after tyrosine phosphorylation. It has been reported previously that EGF is able to efficiently induce tyrosine phosphorylation of transfected STAT3 in COS-1 cells [9]. These cells also have reported low levels of endogenous STAT molecules, which is additionally convenient for our analysis, as high levels of endogenous STAT3 might lower the obtained energy transfer signal by forming dimers with fusion proteins, sequestering them in non-BRETting complexes. When we stimulated our transfected COS-1 cells with 100 ng/ml recombinant human EGF we were able to induce a strong, rapid, and lasting tyrosine phosphorylation of the STAT3–Rluc fusion protein, which we detected by Western blotting with phospho-specific antibodies against Tyr705 of STAT3 (**Fig. 1**).

### STAT3 molecules are preassociated in living COS-1 cells

For the BRET assay, COS-1 cells transfected with STAT3–Rluc and STAT3–EYFP or Rluc–STAT3 and STAT3–EYFP were incubated with the luciferase substrate coelenterazine, and light-emission acquisition was performed immediately. The energy transfer was quantified using the equation [12] described in Materials and Methods. Basically, the difference

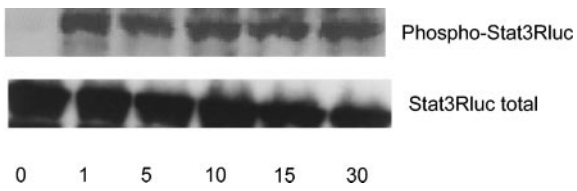


**Fig. 1.** COS-1 cells transfected with STAT3–Rluc were stimulated for the time periods indicated with 100 ng/ml human recombinant EGF. Cells were then lysed and subjected to Western blot analysis. Phosphorylated fusion protein was detected with a phospho-specific antibody for STAT3 and identified by its molecular weight.

between the BRET ratio for the Rluc construct alone and the BRET ratio for cells expressing the Rluc and the EYFP fusion protein was used as a measure for the occurring energy transfer and normalized to Rluc expression (normalized BRET ratio). Energy transfer was detected before activation with EGF between STAT3–Rluc and STAT3–EYFP (**Fig. 2A**) as well as Rluc–STAT3 and STAT3–EYFP (Fig. 2B). The detected signal was similar for both combinations and very stable and reproducible. A preactivation of these cells was ruled out by Western blotting (Fig. 1) with a phospho-specific antibody for STAT3. There was no phosphorylated STAT3 detectable in the cells before activation with EGF, which was also followed over a time-course of 30 min (data not shown). As a control, we overexpressed EYFP at a similar level to the fusion protein (controlled by flow cytometry analysis) together with either of the STAT3 luciferase constructs. There was no BRET signal detectable in this control, not even when EYFP was expressed at a much higher level. Therefore, the observed energy transfer between the STAT3 fusion proteins was not a result of a vast overexpression of EYFP in the cells. Furthermore as another control, no energy transfer occurred between STAT3–Rluc and a STAT6–EYFP construct, which points toward a specific interaction between the STAT3 fusion molecules.

### What happens to the BRET signal after activation?

Next, we wondered whether the signal obtained for the energy transfer between the STAT3 fusion proteins would change in any way after activation-induced phosphorylation by EGF treatment. As one of the advantages of the BRET technology, we were able to follow the same cells before and after activation. To this end, we used the same experimental procedure as described above. After the collection of 10 readings, we added EGF to a final concentration of 100 ng/ml and continued to collect data for another 15–20 min.

For the combination of the two C-terminal constructs (STAT3–Rluc and STAT3–EYFP) the signal remained unchanged after addition of EGF (**Fig. 3A**).

However, when we used the N-terminal luciferase construct (Rluc–STAT3) in combination with the C-terminal fusion protein STAT3–EYFP, energy transfer occurring between Rluc–STAT3 and STAT3–EYFP rather seemed to decrease after activation with EGF (Fig. 3B). This unexpected finding might be explained by a qualitative difference in the "cytoplasmic dimmer" and the activation-induced "nuclear dimmer" as discussed further below.

## DISCUSSION

With the BRET assay, we were able to detect the existence of associated STAT3 molecules before activation and tyrosine-phosphorylation in living mammalian cells. The BRET assay is based on the overexpression of fusion proteins, and therefore, higher than physiologic concentrations of STAT molecules are present in the cells and might influence their interaction behavior. Nevertheless, BRET as well as the related fluorescence RET (FRET) technique have been used for a variety of studies and have proven their suitability to detect real protein interactions [16]. We modestly overexpressed our fusion proteins and included the necessary controls so that our results are unlikely to be mere overexpression artifacts. Furthermore, Haan et al. [5] and Novak et al. [4] have shown the existence of preassociated STAT molecules by immunoprecipitation studies. We believe that our results are important in that they support these findings and uniquely demonstrate the occurrence of these STAT associates in living mammalian cells. Although we continue to refer to these associates as dimers, we do not exclude the existence of higher-order complexes, as has been suggested by Ndubuisi et al. [3], as the BRET assay cannot differentiate between dimers and higher-order multimers. The actual composition of these cytoplasmic associates should be the target of further investigations.

There are also at least two reports in the literature, which would suggest that STATs bind to the activated cytokine receptors in a dimeric rather than the monomeric form suggested

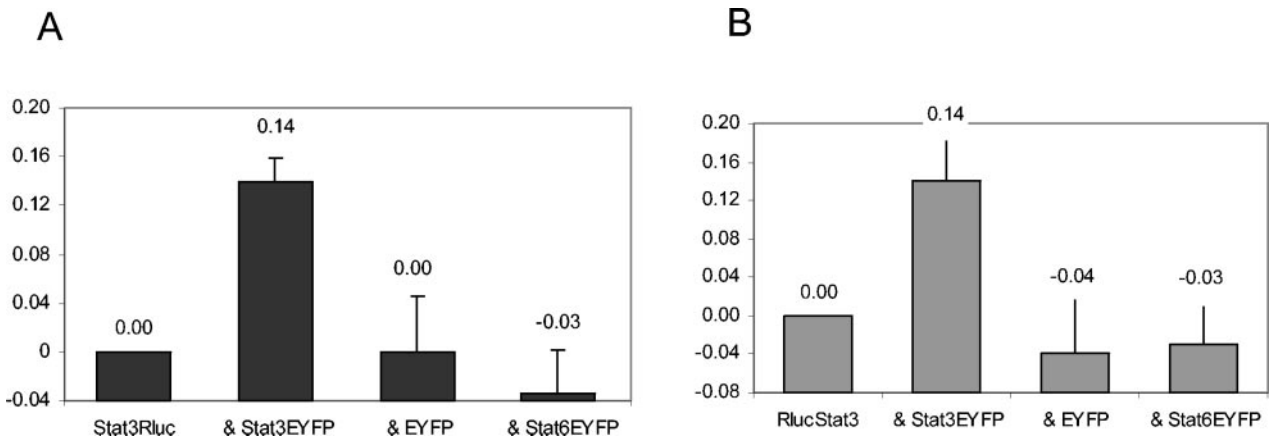


**Fig. 2.** (A) The C-terminal fusion protein STAT3–Rluc (0.5 μg) was transfected alone or in combination with the C-terminal fusion protein STAT3–EYFP (2 μg), EYFP (1 μg), or STAT6–EYFP (2 μg) into COS-1 cells. Cells were harvested 48 h post-transfection, and the BRET assay was performed at 37°C. Ten repetitive readings were collected, and an average of the normalized BRET ratio is depicted in this diagram. Shown is one representative experiment out of four. (B) The N-terminal fusion protein Rluc–STAT3 (1.5 μg) was transfected alone or with the above-mentioned constructs into COS-1 cells. The assay was performed as described above.

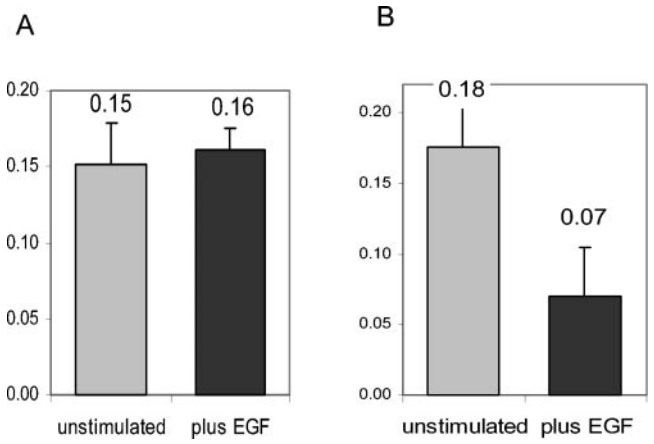


**Fig. 3.** (A) The C-terminal fusion protein STAT3–Rluc (0.5 μg) was transfected alone or in combination with the C-terminal fusion protein STAT3–EYFP (2 μg) into COS-1 cells. Cells were harvested 48 h post-transfection, EYFP expression was checked by flow cytometry, and the BRET assay was performed at 37°C. Ten repetitive readings were collected (approximately 10 min) before addition of EGF. Cells were then incubated for 5 min at 37°C to achieve tyrosine phosphorylation of STAT3, before 15 more readings were collected. The column diagram depicts the normalized BRET ratios before (gray column) and after addition of EGF (black column). (B) The N-terminal fusion protein Rluc–STAT3 (1.5 μg) was transfected alone or with the above-mentioned constructs into COS-1 cells. The assay was performed as described above.

by the current model of STAT activation. Behrmann et al. [17] demonstrated that a single STAT-binding site in a chimeric receptor complex is sufficient to activate STATs. In a very interesting study, the group of Sidney Pestka [18] used the FRET technique to demonstrate that interferon receptors are preassembled on the cell surface and that their intracellular domains move apart upon ligand binding. This might allow for the binding of a STAT dimer in the groove between the two receptor chains. We included this hypothesis into the new model of STAT activation that we suggest below.

We made the surprising observation that the BRET signal decreased after activation when Rluc was fused to the N-terminus of one, and EYFP was fused to the C-terminus of the other STAT3 molecule. This decrease was not observed when EYFP and Rluc were fused to the respective C-termini. We cannot exclude that this different behavior is simply caused by different properties of the luciferase constructs, such as a decreased stability of the Rluc–STAT3 protein. Furthermore, RET is not only dependent on the distance between the two fusion proteins but also on their proper orientation to each other. It is therefore thinkable that binding to the DNA or interaction with other proteins influences the orientation of the tag in the N-terminal but not the C-terminal fusion protein and reduces the ability of the former to elicit energy transfer.

Keeping these reservations in mind, we wondered whether our observation could also be explained by assuming qualitative differences between the cytoplasmic STAT3 associates (before activation) and the nuclear STAT3 dimer, which forms after activation.

From the crystal structure of the nuclear STAT3 dimer, it has been determined that the C-termini are very close together, the N-termini stretch apart, and each N-terminal domain alone spans ~80 Å [7]. Therefore, it would be plausible and in accordance with our results, that BRET occurs more efficiently when both tags are fused to the C-termini than when one is fused to the C-terminus and one to the N-terminus (**Fig. 4**).

On this assumption, the BRET experiments indicate a different orientation of the terminal domains in the cytoplasmic associates. The BRET signal decreased after activation, which points to a smaller distance between the C-terminus of one and the N-terminus of the other monomer in the cytoplasmic associates than in the nuclear dimer. In contrast, the C-termini should be close to each other also in the cytoplasmic associates, as no change in the BRET signal was detected after activation when the two C-terminal fusion proteins were used for the BRET assay. The finding of Novak et al. [4], who demonstrated that the formation of the nonactivated STAT3 associates was dependent on the C-terminal domain but independent of the SH2 domain, might support this.

A recent publication by Zhang et al. [10] showed an interaction between the N-terminal and the C-terminal domain of STAT3, which was necessary for receptor binding. The authors suggested that this is an intramolecular association between these two domains, which renders the SH2 domain accessible for binding to the receptor. Nevertheless, they cannot exclude that this binding property is also part of an intermolecular association between the STAT3 monomers, which would be favored by our results. Another possibility would be that the intramolecular interaction proposed by Zhang et al. [10] leads to a condensed structure of the STAT3 associates, which brings the C-termini and the N-termini into the close proximity necessary for efficient energy transfer.

Based on our data and the findings of Krause et al. [18], Zhang et al. [10], and Novak et al. [4], we suggest a new model of STAT activation, which comprises a latent cytoplasmic dimer and the known activation-induced nuclear dimer (**Fig. 5**). Activation-induced phosphorylation of the STATs renders the cytoplasmic dimer unstable and leads to a reorganization

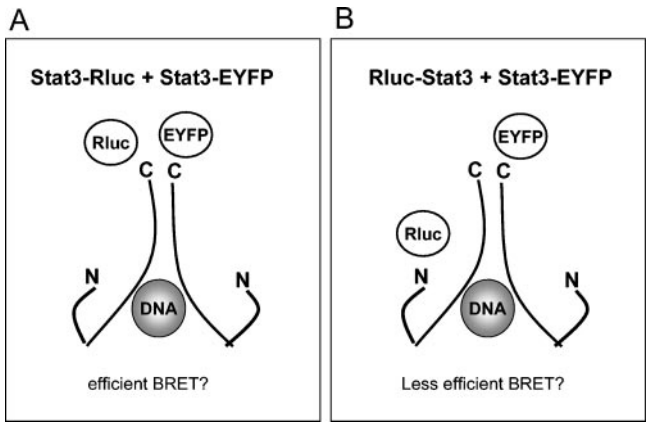


**Fig. 4.** Schematic drawing of the nuclear STAT3 dimer, indicating the positions of EYFP and Rluc, respectively. (A) EYFP and Rluc are fused to the C-termini (C) of the respective STAT3 monomers. The C-termini are close to each other, and therefore, BRET might occur very efficiently from Rluc to EYFP. (B) Rluc is fused to the N-terminus of one STAT3 monomer and EYFP to the C-terminus of the other STAT3 monomer. As the N-terminus (N) is very elongated in the nuclear dimer, Rluc and EYFP are relatively distant, and therefore, BRET might occur less efficiently than in A.

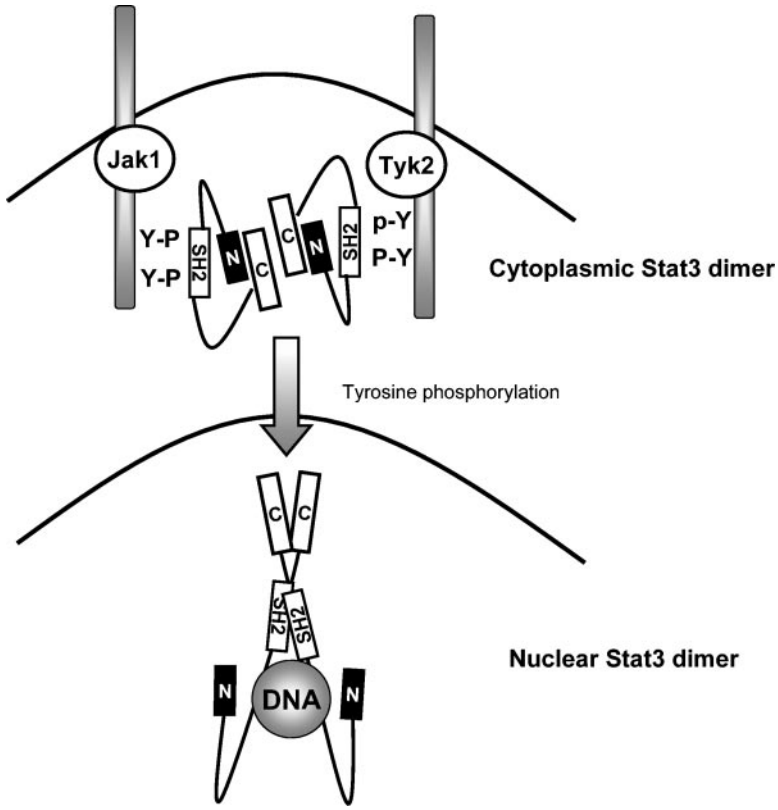


**Fig. 5.** A new model of STAT activation. This simplified schematic drawing shows the intramolecular interaction between the C- and the N-terminus (C and N, respectively) of STAT3, which was proposed by Zhang et al. [10] and suggests a model of receptor-binding of the hypothetical, cytoplasmic STAT dimer. In this dimer, the STAT monomers associate via their C-termini, and the intramolecular interaction between the C-terminus and the N-terminus of one STAT monomer renders the SH2 domain accessible for receptor-binding. After tyrosine phosphorylation, a conformational change occurs, which leads to the formation of the known activation-induced STAT dimers. Jak1, Janus kinase 1; Tyk2, tyrosine kinase 2; Y-P and P-Y, phosphorylated tyrosine residues; SH2, src-homology domain 2; C, C-terminus; N, N-terminus.

resulting in the formation of the known nuclear dimer. We are aware that this model is highly speculative, but we believe that it could provide a platform for discussion and further research. The conventional model of STAT activation did not provide a satisfying explanation for the dissociation of the STAT molecules from the receptor and for their nuclear transport. It is highly unlikely that a nuclear localization signal is revealed by a dimerization event. The new model might explicate this process more feasibly. Tyrosine phosphorylation of the STAT molecule has been shown to disturb the intramolecular interaction necessary for receptor binding and is therefore likely to provoke dissociation from the receptor. This structural change then probably leads to the formation of the known activation-induced STAT dimer, which is associated via the phosphorylated tyrosine residue and the SH2 domain of the second STAT molecule. It is rather conceivable that this structural change might display a nuclear localization signal, which can be recognized by importin molecules, and leads to the selective nuclear import of the activation-induced STAT dimers.

We hope that our alternate hypothesis (Fig. 5) leads to a reconsideration of STAT activation and reanimates research in this area.

Further investigation of the structure and function of the proposed cytoplasmic STAT dimers is necessary. An interesting, open question is whether the cytoplasmic STAT molecules actually form dimers or higher-order complexes and whether they only form homogeneous or also heterogeneous complexes with other STAT molecules or other signaling or chaperone proteins. Processes such as receptor-binding and dimerization as well as nuclear translocation of STAT molecules might be strongly connected to the structural change implicated by our model, and its investigation might lead to a better understanding of cytokine signaling.

## NOTE

While this paper was in proof, the group of K. Murphy published a study showing that association of nonphosphorylated STAT4 molecules also occurs and is even necessary for activation of STAT4. The N-terminal domain of STAT4 was crucial for this interaction to occur [19]. Also during the preparation of this paper, Kretzschmar et al. published very similar results to ours for murine Stat3 using fluorescence resonance energy transfer. Interestingly they found that the SH2 domain of Stat3 was essential for formation of the preassociated dimers [20].

## ACKNOWLEDGMENTS

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