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Cytokine-induced nuclear translocation of signaling proteins and their analysis using the inducible translocation trap system

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Abstract

Binding of cytokines to their specific receptors induces activation of signal transduction pathways, many of which involve nuclear translocation of signaling proteins. In this review, an overview of cytokine-induced nuclear translocation of signaling proteins is provided. In addition, inducible translocation trap (ITT), a novel reporter-based system to detect nuclear translocation, and its application for identification of nuclear translocating proteins are elaborated. Finally, analysis of “nuclear translocatome”, the entire set of proteins that translocate into or out of the nucleus in response to extracellular stimuli, by ITT is discussed.

Keywords

Nuclear translocation; Inducible translocation trap; Signal transduction

1. Introduction

Extracellular signaling molecules bound to cell surface receptors can regulate nuclear events with consequences for cell proliferation, differentiation, and function. To regulate nuclear function, signals must be transduced across the nuclear envelope to propagate the signal from the cytoplasm to the nucleus. Therefore, many signaling responses induce the nuclear translocation of transcription factors, kinases and others [1,2]. Diverse mechanisms exist how these molecules are activated to move into the nucleus.

2. Mechanisms of cytokine-induced nuclear import and export of signaling proteins

2–1. Nuclear translocation of Stat proteins

Signal transducers and activators of transcriptions (Stats) are a family of latent cytoplasmic proteins that, when activated, link cytokine signals to changes in gene expression. Seven mammalian family members have been identified [3–5]. Upon binding of cytokines to their receptors, Jak kinases that are associated with the cytoplasmic tails of the receptors autophosphorylate tyrosine residues and phosphorylate residues on the cytoplasmic domains of the cytokine receptors [3–5]. The phosphorylated tyrosines on the receptors serve as docking sites for the Stat proteins via their Src-homology 2 domain. The binding of Stats to cytokine

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receptors brings them to close proximity with the Jak kinases which phosphorylate a single residue at their carboxyl-terminal domain. That event facilitates homo- or hetero-dimerization of the activated Stat proteins and their accumulation in the nucleus where they regulate gene expression [6-9].

Active transport across the nuclear membrane occurs, in general, through the nuclear pore complex (NPC). Proteins that need to be transported to the nucleus often contain a nuclear localization signal(s) (NLS) that is recognized by and binds to an importin α molecule [10-12]. Importin α has an importin β binding (IBB) domain and that interaction directs the complex to the cytoplasmic side of the nuclear pore, through which it translocates into the nucleus where Ran GTPase facilitates the dissociation of the protein-importins complex [13-15]. The opposite route (from nucleus to cytoplasm) requires, in general, leucine rich nuclear export signal(s) (NES) that is recognized by a protein called CRM1 [16,17]. CRM1 is an exportin that mediates the transport of cargo proteins from the nucleus to the cytoplasm through the NPC. Thus, large proteins need to have an NLS and an NES in order to travel to and from the nucleus.

Most characterized NLSs consist of either a classical motif with a single cluster of four or five basic residues or a bipartite motif having two clusters of basic residues separated by a spacer of about 10 amino acids (a.a.) in the primary sequence of the proteins [18-20]. The Stat proteins do not contain any classical or bipartite NLS motif in their primary sequence. There are, however, evidences that the activated dimers contain a structural NLS motif. An arginine/lysine-rich region within the DNA-binding domain (DB) of Stats has been shown to be required for interferon (IFN)-induced nuclear import of both Stat1 and Stat2 [21]. By creating a series of mutations in these elements, it was shown that a functional "structural" NLS requires that both monomers have their basic region intact. That group later showed that K410 and K413 in Stat1 are critical for interaction with importin α [22]. Another group showed that for the dimer to enter the nucleus, at least one monomer should have an intact residue (L407) in the NLS region [23]. Both groups showed that this particular arginine/lysine-rich region mediates interaction with importin α , particularly importin $\alpha 5$ [22,23]. In this regard, it has been previously suggested that the nuclear import of activated Stat1 is mediated by NLS interaction with importin $\alpha 5$ [24,25]. Sekimoto et al. showed that activated Stat1 associates with importin $\alpha 5$ and that its inhibition impairs nuclear translocation of Stat1 [24]. They also showed that dimerization is required for interaction with importin $\alpha 5$. Hawiger's group provided indirect evidence that activated Stat1 nuclear import depends on the importin/nuclear pore transport system [25]. Their paper showed that a peptide carrying the NLS of NF κ B p50 could competitively inhibit the nuclear import of Stat1 in Jurkat T cells in response to IFN stimulation [25]. That observation suggested that Stat1 and NF κ B depend on overlapping molecules necessary for nuclear translocation. Since different importin α s are used for Stat1 and NF κ B (Table 1), it is possible that importin β and other components of nuclear translocation machinery might be shared by these two transcription factors. It is important to note that different sequences have been identified in the DB of Stat5B as necessary for growth hormone-induced nuclear accumulation in some cell lines [26]. Beside the structural NLS identified in the DB of Stat1 [21,23,27], Stat3 had been shown to have an additional motif in its coiled-coil domain containing lysine, which is essential for its nuclear translocation upon activation [28]. While both the motif in the coiled-coil domain (R214/215) and the one in the DB (R414/417) of Stat3 are required for the full-length protein to enter the nucleus, only the one in the coiled-coil domain binds to importin α s, particularly importin $\alpha 5$ and 7 in response to epidermal growth factor (EGF) stimulation [29]. It is noteworthy that R414/417 in Stat3 corresponds to K410/413 in Stat1. Another small region in the coiled-coil domain (a.a.150–162) has been found by another group to be indispensable for constitutive as well as interleukin (IL)-6 induced nuclear translocation of Stat3 mediated by importin $\alpha 3$ [30]. Although that latter group claims that R214/215 had no effect on constitutive nuclear translocation of Stat3, they do not mention

whether or not its mutation affects nuclear import of activated Stat3 in their experimental system. It is possible that phosphorylated and constitutive nuclear import of Stat3 have different requirement in terms of NLS signals and importins α used. In *in vitro* experiments it has been shown that tyrosine-phosphorylated Stat3 can not only bind to importin $\alpha 5$ as previously reported but also importin $\alpha 1$ and importin $\alpha 3$ [31]. Table 1 shows a list of importins implicated in the nuclear translocation of various cytokine-dependent transcription factors. The N-terminus of Stats has also been shown to be required for cytokine-induced nuclear translocation [32,33].

As shown by the work of several groups, Stat proteins have leucine-rich NES that mediate nuclear export via the CRM1/NPC pathway. Functional NES was first identified in Stat1 [34-36]. It was suggested that the primary role of the nuclear export is to terminate cytokine-induced nuclear targeting of Stats. Studies by two other groups later showed that both Stat3 and Stat5B have functional NES [33,37]. Nuclear export, which occurs via exportin-dependent or -independent mechanisms, is an important process by which cytoplasmic accumulation of latent Stats occur [30,38,39].

In addition to cytokine-induced nuclear translocation, constitutive shuttling was detected for Stat1, Stat3, and Stat5 [30,33,38], leading to the observation that unphosphorylated Stats can enter the nucleus either through importin-dependent or -independent pathways [30,38]. Although non-phosphorylated Stat1 constitutively shuttles between the nucleus and the cytoplasm, nuclear import of the phosphorylated dimers does not use the same mechanism as the unphosphorylated monomer. It has been shown that unphosphorylated Stat1 does not use importins for its nuclear translocation but mediated by direct contact with NPC [38] while the phosphorylated dimers (homodimer or heterodimer with Stat2) nuclear entry is facilitated by importin $\alpha 5$ [22-24]. It was also reported that dimerization, which is mediated by tyrosine-phosphorylation of Stat1, is required for interaction with importins [24]. These reports indicate that nuclear import is facilitated by tyrosine-phosphorylation of Stat1. It has been shown that Stat3 also actively shuttles between the cytoplasmic and nuclear compartment. Several different importins, including importin $\alpha 1$, 3, 5, and 7 have been shown to mediate its nuclear translocation in various activated cells, and importin $\alpha 3$ has also been shown to mediate the nuclear import of the non-phosphorylated form [28-31]. In several studies, only phosphorylated dimers bind to importins to be transported to the nucleus [28,29]. In contrast, another group showed that phosphorylation is important not for nuclear import but for nuclear retention [30]. The current available data can be interpreted that the increase in the nuclear pool of Stats following cytokine stimulation is the result of changing rate of the two processes of nuclear import and export due to exposure of structural NLS by dimerization and hiding of NES by binding to DNA [40,41].

Molecules other than importins can also be required to direct some Stats to the nucleus. For example, it was suggested that Stat5A requires Rac1, a GTPase, and MgcRacGAP, a GTPase activating protein (GAP), for its nuclear translocation [42]. Both the GTPase and the GAP bind to Stat5A, a binding that is enhanced by IL-3 stimulation. The GAP protein and Stat5A enter the nucleus simultaneously upon IL-3 stimulation, and knocking-down of either the GTPase or the GAP inhibits the nuclear accumulation of phosphorylated Stat5A. The specific role of these molecules in the nuclear transport of Stat5A is not known. One possibility is that by forming a complex with Stats, they may provide an NLS for importin to bind to. In this regard, it has also been shown that other NLS containing molecules such as the cytoplasmic domain of ERBB4/HER4 for Stat5A can be required for the transport or functions of certain Stats in some cell lines [43].

2–2. Nuclear translocation of Smad proteins

Smad proteins are a family of inducible transcription factors that directly link signals from the transforming growth factor β (TGF β) receptors to gene expression regulation in the nucleus. They are grouped into three classes: (a) receptor-regulated Smads (Smad1, 2, 3, 5, and 8), (b) mediator Smad (Smad4), and (c) inhibitory Smads (Smad6 and 7). Like Stats, they are mainly in the cytoplasm in their inactivated forms and upon TGF β stimulation, phosphorylated R-Smads form a complex with Smad4 and accumulate in the nucleus to regulate transcription. Smads are directly phosphorylated on serine residues by the TGF β receptors, thus providing a direct link between cytokine binding and gene regulation [44–48].

Like the Stat proteins, Smads do not contain any classical NLS motif for their nuclear import. A conserved region of 5 basic a.a. in the N-terminal domain has been shown to be required for both signal-induced and constitutive nuclear translocation of Smad3 and Smad1. That region is conserved in the R-Smads and was hypothesized to mediate their nuclear translocation similarly [49,50]. Instead of binding to importin α which binds to importin β , it was found that Smad3 uses a more direct route by binding to importin β directly [51,52]. On the other hand, Smad4 uses an extended bipartite NLS that binds to importin α . This NLS is important not only for autonomous nuclear translocation of Smad4 but also its translocation in the presence of R-Smads [53]. The fact that R-Smads directly bind to importin β and Smad4 to importin α makes it an interesting issue to investigate whether importin β bound to R-Smad can still interact with IBB domain of importin α and whether such interaction contributes to stabilization of R-Smad/Smad4 complex during nuclear translocation. It was later found that Smad2 interacts directly with the NPC without the help of importins [54,55]. It is interesting to note that other studies have also suggested that the nuclear import of Smad3 and 4 are mediated by direct interaction with nucleoporin proteins without the use of any importin [56]. It is possible that different mechanisms of nuclear import are used in different context as is the case for certain Stat proteins.

Both R-Smads and Smad4 constantly shuttle between the cytoplasm and the nucleus in both resting and cytokine-stimulated cells [50,55,57–60]. Functional NES have been identified in several of the Smads including Smad1 and Smad4 and the nuclear export of these particular Smads have been shown to be CRM1-dependent [50,61,62]. However, Smad2 export is CRM1-independent and mediated by direct nucleoporins contact [55]. The cytoplasmic/nuclear ratio is higher for the latent form because those forms associate with microtubule in the cytoplasm as well as specific anchor proteins such as SARA and dissociate from them upon TGF β stimulation. Interaction of R-Smads to Smad4 also facilitates the retention of Smad4 in the nucleus [54,63,64]. In stimulated cells phosphorylated R-Smads/Smad4 complex enters the nucleus to regulate gene transcription, becomes dephosphorylated by a phosphatase [65], dissociates from each other, and exits the nucleus separately where R-Smads can be activated again. This cycle can be repeated for the duration of the stimulation [55,58–60].

2–3. Nuclear translocation of NF κ B

Rel/NF κ B family of proteins comprise a group of widely used transcription factors activated by numerous signal molecules including cytokines such as tumor necrosis factor (TNF)- α and IL-1 [66–68]. Unlike Stats and Smads that are in latent forms and activated by phosphorylation as a result of cytokine signaling, NF κ B are ready for transcription regulation except that they are retained in the cytoplasm by a group of proteins called I κ B. By binding to I κ B, their NLSs are masked and they are mostly prevented from entering the nucleus to affect gene transcription [69–71]. According to the literature, both I κ B α and I κ B β bind to and inhibit nuclear translocation of NF κ B dimers.

Upon stimulation, a cascade of events occurs resulting in the activation of kinases that phosphorylate the I κ Bs, leading to their ubiquitination and proteolysis. That frees the NF κ B transcription factors to enter the nucleus [66-68]. It has been shown that their actual entry is mediated by the importins/NPC complexes [72]. Unlike Stats and Smads, NF κ B proteins have recognizable NLS [25,69,73,74]. Both importin α 3 and α 4 have been shown to mediate their nuclear translocation [72]. It has been recently shown that *Plasmodium*, the malaria parasite, inhibits the nuclear translocation of NF κ B by producing a protein called the circumsporozoite protein (CS). CS translocates into the nucleus of the host cell in an importin α 3/ β 1-dependent manner, thus competitively inhibits binding of NF κ B to these importins [75].

The I κ B proteins also have a non-conventional NLS and a conventional NES that allow them to shuttle between the cytoplasm and the nucleus [76-83]. They can enter the nucleus and bind to NF κ B and facilitate their nuclear export [76,77]. This allows constitutive shuttling of the NF κ B proteins between the cytoplasm and the nucleus. As is the case for Stats, the nuclear import and export of the I κ B/NF κ B complex are very important for the cytoplasmic accumulation of the transcription factors in resting cells and for terminating a signal [76,77]. Several groups have shown its importance by mutating the leucine-rich motif of I κ B NES or by disrupting nuclear export with a CRM1-specific inhibitor. Basically, when the nuclear export of I κ B is inhibited, both I κ B and NF κ B accumulate in the nucleus [78,79].

There are also some evidences that proteins upstream of the I κ B such as the kinases that can phosphorylate them for destruction by ubiquitin ligase enter the nucleus [80]. That suggests that the “activation” or release of NF κ B may occur not only in the cytoplasm as was previously shown but also in the nucleus.

3. The inducible translocation trap (ITT) system

3-1. The ITT system

As described above, signal-induced nuclear translocation of signaling proteins plays critical roles in cytokine signaling. To analyze nuclear translocation of these proteins and identify novel signal-induced nuclear translocating proteins, we established the inducible translocation trap (ITT) system [84]. The method to detect nuclear translocation by extracellular stimuli is summarized in Fig. 2. The strategy is based on expression of a fusion protein, LGV, consisting of LexA DB, VP16 transactivation domain (TA), and the test protein encoded by cDNA subcloned downstream of VP16 TA (pLGV vector, Fig. 2A). GFP was inserted between LexA DB and VP16 TA as a marker of expression of the fusion protein. The fusion molecule is expressed in cells containing hCD2 reporter gene with multiple LexA-binding sites in its promoter (LexA-hCD2) (Fig. 2B). Following nuclear translocation of the fusion protein by ligand stimulation, the LexA DB targets the fusion protein to the LexA operator sites of the reporter gene. Subsequently, the VP16 TA activates the expression of hCD2 (Fig. 2C). Thus, nuclear translocation of the test protein is detected by the expression of hCD2.

To examine whether ligand-induced nuclear translocation can be detected with this system, we chose the IFN γ -Stat1 system, in which IFN γ induces nuclear translocation of Stat1 [4,5]. LexA-hCD2 reporter gene was transfected into murine IL-3-dependent Ba/F3 cells [85] to establish BL2 cell line. As shown in Fig. 2D, BL2 cells expressing LGV did not express hCD2 both in the absence and presence of IFN γ . Expression of hCD2 was not observed on BL2 cells expressing LGV- β -gal, which is a fusion protein of LGV and a cytoplasmic protein, β -galactosidase (β -gal), either in the absence or presence of IFN γ (Fig. 2E). In contrast, BL2 cells expressing LGV-NLS- β -gal, in which the NLS of SV40 T-antigen is fused between LGV and β -gal, expressed hCD2 both in the absence and presence of IFN γ (Fig. 2F). These results suggest that hCD2 expression faithfully reflects nuclear localization of the test proteins. When LGV-Stat1 was expressed in BL2 cells, low background expression of hCD2 was detected in

the absence of IFN γ (Fig. 2G). The background expression of hCD2 reflects amounts of Stat1 in the nucleus in the absence of ligand stimulation. In this regard, it has been shown that Stat1 shuttles between the cytoplasm and the nucleus even in the absence of ligand stimulation [38,39]. In contrast, significant upregulation of hCD2 was observed 7 hr after IFN γ stimulation. These results suggest that this system enables us to detect ligand-induced nuclear translocation of signaling molecules by expression of hCD2. When we examined kinetics of hCD2 induction by IFN γ in BL2 cells expressing LGV-Stat1, expression of hCD2 had a peak at 8 hr after IFN γ stimulation, then down-regulated to the basal level 72 hr after stimulation (Fig. 2H).

LGV-Stat1 was tyrosine-phosphorylated and translocated into the nucleus by IFN γ in BL2 cells expressing LGV-Stat1 [84]. Extra bands with smaller molecular weights (MW) were not detected in the nuclear extracts from IFN γ -treated cells with anti-LexA antibody (Ab) [84], excluding a possibility that hCD2 induction is caused by non-physiological processing such as a cleavage of the fusion protein. We also detected IFN γ -induced tyrosine-phosphorylation and nuclear translocation of LGV-Stat1 expressed in Stat1 $^{-/-}$ mouse embryonic fibroblasts [84]. These results showed that IFN γ -induced dimerization and nuclear translocation of LGV-Stat1 occur in the absence of endogenous Stat1, indicating that fusion with LGV does not severely affect dimerization and nuclear translocation of Stat1.

We also generated another system using GFP reporter gene. The modified system consists of pLG retroviral vector to express cDNA fused with LexA DB and Gal4 TA (Fig. 3A) and LexA-d1EGFP (destabilized enhanced GFP) reporter gene (Fig. 3B). GFP reporter enables us to dispense with staining of cells for flowcytometry. LexA-d1EGFP gene was transfected into Ba/F3 cells to establish BLG cell line. Stat1 fused with LexA DB-Gal4 TA (LG-Stat1) activates expression of GFP in BLG cells by IFN γ , and IFN γ -induced GFP expression in cells expressing LG-Stat1 reached its peak 4 hr after stimulation and down-regulated to the basal level within 24 hr after stimulation (data not shown). Human Smad3 cDNA [86] was inserted into pLG to express LG-Smad3. Expression of GFP was marginal in BLG cells expressing LG both in the absence and presence of TGF β 1 (Fig. 3D, left panel). In contrast, TGF β 1 stimulation induced expression of GFP in BLG cells expressing LG-Smad3 (Fig. 3D, right panel). Extra bands with smaller MW were not detected with anti-LexA Ab [84], excluding a possibility that GFP induction is caused by non-physiological processing such as a cleavage of the fusion protein. Thus, the ITT system works not only for the IFN γ -Stat1 system but also for the TGF β -Smad2/3 system, showing that the system is robust.

pLGV or pLG vectors express fusion proteins, in which LGV or LG locates to the N-terminal to the test protein. In some applications, for example, when nuclear translocation of cytoplasmic tail of membrane receptors is to be analyzed, LGV or LG should be placed to the C-terminal to the test protein. We have also developed a vector pLGV-N to express fusion proteins in which LGV is located to the C-terminal of the test proteins. By using this system, we have detected NGF-induced cleavage and nuclear translocation of the cytoplasmic domain of NGF receptor p75 (N. Zampieri, H.F., M. Chao, unpublished observation).

In addition to signal-induced nuclear translocation, signal-induced nuclear export can be analyzed using ITT. For example, we have detected growth factor-induced nuclear export of transcription factor Foxo3 by ITT (T. Kimura, N. Yamano, H.F., T. Nakano, unpublished observation).

3-2. Screening of cDNA library using the ITT system

The ITT system can be used for isolation of cDNAs that translocate into the nucleus by extracellular signaling. Screening of cDNA library using the ITT system is schematized in Fig. 4. A cDNA library constructed in the pLG vector is transfected into packaging cell lines to produce retrovirus particles. Then, an ITT reporter cell line containing LexA-d1EGFP is

infected with the supernatant of the packaging cells containing virus particles. Two days after infection, cells are stimulated with a ligand of interest, and GFP (+) cells are sorted by flowcytometry. Sorted cells are incubated for down-regulation of GFP and expansion of the sorted cells. Subsequently, GFP (–) cells are sorted. Several rounds of GFP (+) sorting after ligand stimulation and following GFP (–) sorting are performed. Finally, cells are subjected to single-cell sorting, and GFP expression is examined in the presence or absence of the ligand for each clone. Genomic DNAs extracted from these clones are used as templates for PCR amplification using viral vector primers. The amplified PCR fragments are subcloned into the pLG vector and sequenced. To verify that fusion proteins of LG and proteins or protein fragments encoded by the recovered cDNA inserts confer responsiveness for the ligand stimulation, the LG-fusion constructs are transduced into BLG cells and exposed to the ligand stimulation (re-transduction assay). We isolated Stat1 cDNA in a screening for proteins that translocate into the nucleus in response to IFN γ [84].

A few points should be taken into consideration in screening of cDNA library using the ITT system.

(i) Transduction efficiency: Recent technical advancement has enabled us to achieve high transduction efficiency (– 90%) in cultured cells. However, since high transduction efficiency increases the probability of integration of multiple cDNA inserts into the genome of a single cell, transduction efficiency should be 5 – 10%. Drug selection after transduction would be a good strategy to eliminate non-transduced cells.

(ii) Time after stimulation: Incubation time after ligand stimulation is determined by the expected kinetics of nuclear translocation of the proteins of interest. Signal transducers such as Stat proteins and Smad proteins quickly get into the nucleus after stimulation. For these proteins, GFP reporter expression reaches its peak 4 – 5 hr after stimulation. If translocation is expected to occur in the late phase of stimulation, sorting should be performed at later time points.

4. Applications of ITT for identification of nuclear translocating proteins

4.1. IL-3-induced nuclear translocation of pyruvate kinase

We applied the ITT system for screening of cDNA library to isolate cDNA encoding proteins that translocate into the nucleus in response to IL-3 [87]. We found that the M2 isoform of pyruvate kinase (M2-PK), a key enzyme in glycolysis, translocates into the nucleus by IL-3 but not by EGF stimulation. The C domain of M2-PK was sufficient for IL-3-induced nuclear translocation. IL-3-induced nuclear translocation of M2-PK was dependent on the activation of Jak2. Overexpression of M2-PK fused with an NLS enhanced cell proliferation in the absence of IL-3, suggesting that the nuclear pyruvate kinase plays an important role in cell proliferation. These data suggested a novel link between growth factor stimulation and the glycolytic pathway.

Recently, it was shown that somatostatin and its structural analogue induces nuclear translocation of M2-PK [88]. Nuclear translocation of M2-PK induced programmed cell death in Cos-7 cells. Thus, localization of M2-PK may be differentially regulated by various extracellular stimuli and affects important cell functions.

4.2. Nuclear translocation of 2-amino-3-ketobutyrate coenzyme A ligase (KBL) by cold and osmotic stress

Cells are continuously exposed to environmental stresses and respond to them to maintain cellular homeostasis. Failure to respond to these stresses may cause pathological states such as renal failure, complications of diabetes, and autoimmune diseases. Signal transduction

induced by osmotic and cold stresses is not fully understood. In addition, mechanisms of these stress responses are yet to be elucidated. We applied the ITT system for screening of cDNA library to isolate cDNA encoding proteins that translocate into the nucleus in response to cold and osmotic stress. We found that 2-amino-3-ketobutyrate coenzyme A ligase (KBL) [89], which is involved in the conversion of L-threonine to glycine [90,91], translocates into the nucleus in response to cold and osmotic stresses. Rapid nuclear translocation of KBL was confirmed by biochemical analysis and fluorescent microscopy. A large region of KBL was required for stress-induced nuclear translocation. The KBL reporter system will be a useful tool for the investigation of cold and osmotic stress responses.

4.3. Nuclear translocation of the CS protein of malaria sporozoites

The liver stages of malaria sporozoites develop in the hepatocyte cytoplasm inside a parasitophorous vacuole (PV). By using ITT, we showed that the CS protein, the major surface protein of sporozoites, translocates into the nucleus in malaria-infected hepatocytes [75]. CS traverses the PV membrane and enters the cytoplasm and nucleus of hepatocytes. The transport of CS into the host nucleus is mediated by importins $\alpha 3/\beta 1$ that binds to the NLS of CS localized in the conserved region II-plus. The NLSs of CS and NF κ B p50 share the same importins. The entry of NF κ B p50 into the nucleus is strongly inhibited in cell lines expressing CS, and in infected hepatocytes, thus explaining the notable absence of inflammatory cells surrounding exo-erythrocytic forms (EEFs). The presence of CS in the cytoplasm of hepatocytes enhances EEF growth both *in vitro* and *in vivo*. Applications of ITT so far are summarized in Table 2.

4.4. Application of the ITT system for drug screening

To examine whether this reporter system can be used for pharmacological assay, BLG cells expressing LG-Stat1 fusion protein was pre-treated with different concentrations of genistein, a general inhibitor of tyrosine kinases, for 1 hr. Cells were stimulated with IFN γ (10 ng/ml). GFP expression was analyzed 4 hr after IFN γ stimulation. We used normalized percentages of GFP (+) cells to generate a dose-response curve. Normalized GFP (+) cells (%) was calculated as follows:

$$\text{Normalized GFP (+) cells (\%)} = (\% \text{ of GFP (+) cells pretreated with genistein} - \% \text{ of GFP (+) cells in the absence of IFN}\gamma \text{ stimulation}) / (\% \text{ of GFP (+) cells in the absence of genistein} - \% \text{ of GFP (+) cells in the absence of IFN}\gamma \text{ stimulation}) \times 100$$

370 μ M (100 μ g/ml) of genistein completely inhibited IFN γ -induced GFP reporter expression in BLG cells expressing LG-Stat1 fusion protein (Fig. 5). This result is consistent with previous reports that 300 μ M of genistein completely inhibits IFN γ -induced tyrosine phosphorylation of cellular substrates and expression of Fc receptors in HL-60 cells [92]. These results suggest that the reporter system can be used for pharmacological assays.

5. Nuclear translocatome analysis by ITT

After the completion of the genome project, researchers are vigorously analyzing expression profile of transcribed genes (transcriptome analysis) and proteins (proteome analysis). After making spatio-temporal maps of gene expression from these analyses, one of next research goals will be to understand dynamics of gene products. Global understanding of translocation of the entire set of gene product ("translocatome" analysis) will be an emerging field of research. The ITT system will be useful in elucidating translocatome in various biological systems.

6. Concluding remarks

Cytokine signaling involves nuclear translocation of signaling proteins such as transcription factors. Pharmacological intervention of this process may be useful for regulation of cytokine-mediated cell function. The ITT system can be used for identification of novel nuclear translocating signaling molecules in cytokine signaling and other biological systems. This system also enables us to screen small compounds that affect nuclear translocation of signaling molecules.

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Abbreviations

ITT, inducible translocation trap
 Stat, signal transducer and activator of transcription
 NLS, nuclear localization signal
 IBB, importin β binding
 NES, nuclear export signal
 IFN, interferon
 NF κ B, nuclear factor κ B
 EGF, epidermal growth factor
 IL, interleukin
 GAP, GTPase activating protein
 TGF β , transforming growth factor β
 DB, DNA-binding domain
 TA, transactivation domain
 MW, molecular weight
 GFP, green fluorescent protein
 Ab, antibody
 M2-PK, M2 isoform of pyruvate kinase
 KBL, 2-amino-3-ketobutyrate coenzyme A ligase
 CS, circumsporozoite protein
 PV, parasitophorous vacuole
 EEF, exo-erythrocytic forms
 NGF, nerve growth factor.

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Figure 1.

(A) The Stat pathway. (B) The Smad pathway (C) The NF κ B pathway. In unstimulated cells (A - C, left panels), transcription factors are either in latent form [Stat (A) and Smad (B)] or bound to an inhibitor [NF- κ B bound to I κ B (C)]. They shuttle between the cytoplasm and the nucleus through the nucleoporin complex (NPC) but are for the most part unable to bind to promoters and affect gene expression. They are also actively transported back to the cytoplasm where they accumulate. Upon binding of cytokines to their receptors (A - C Right panels), latent Stats (A) become activated by tyrosine phosphorylation, homo- or heterodimerize and get transported to the nucleus by importins α/β and the NPC. The R-Smads (B) become serine phosphorylated, form dimers with Smad4 and translocate to the nucleus via the importins and

the NPC. The I κ Bs (C) are activated, ubiquitinated and lyzed and the free NF- κ Bs enter the nucleus where they activate transcription.

Figure 2.

pLGV/LexA-hCD2 reporter system. **(A)** pLGV expresses a fusion protein consisting of LexA DB, GFP, VP16 TA, and protein to be tested. LTR, long terminal repeat; Amp, ampicillin resistance gene; Ori, replication origin of pUC; ψ , packaging signal; MCS, multicloning site. **(B)** LexA-hCD2 reporter gene contains 8 $\times$ LexA binding elements, a minimal promoter from IFN β gene, and the full-length cDNA of hCD2. **(C)** Scheme of hCD2 expression by the fusion protein. When the LGV-fusion protein is in the cytoplasm, hCD2 is not expressed on the cell surface. If the fusion protein translocates into the nucleus by ligand stimulation, it binds to the LexA operators with LexA DB and activates hCD2 expression. **(D-G)** Inducible expression of hCD2 by IFN γ on BL2 cells expressing LGV-Stat1. Data for BL2 cells expressing LGV **(D)**, β -gal-fusion (LGV- β -gal, control as a cytoplasmic protein) **(E)**, NLS- β -gal-fusion (LGV-NLS- β -gal, control as a nuclear protein) **(F)**, and LGV-Stat1 **(G)** are shown. Dotted line: unstained control; thin line: mock-stimulation; thick line: IFN γ stimulation. **(H)** Time course of IFN γ -induced hCD2 expression in BL2 cells expressing LGV-Stat1. This figure is reproduced from [84].

Figure 3.

pLG/LexA-d1EGFP reporter system. **(A)** pLG expresses a fusion protein consisting of LexA DB, Gal4 TA, and protein to be tested. **(B)** LexA-d1EGFP reporter gene contains 8 $\times$ LexA binding elements, IFN β minimal promoter, and d1EGFP. **(C)** Inducible expression of GFP by TGF β 1 in BLG cells expressing LG-Smad3. BLG cells expressing LG (left panel) or LG-Smad3 (right panel) were mock-stimulated (thin line) or stimulated with TGF β 1 for 5 hr (thick line), and GFP expression was analyzed. This figure is reproduced from [84].

Figure 4.
Scheme of screening of cDNA library.

Figure 5.

Effects of genistein on GFP reporter gene expression in reporter cell line expressing LG-Stat1. BLG cells expressing LG-Stat1 fusion protein was pre-treated with different concentrations of genistein, a general inhibitor of tyrosine kinases, for 1 hr. Cells were stimulated with IFN γ (10 ng/ml). GFP expression was analyzed 4 hr after IFN γ stimulation.

Normalized GFP (+) cells (%) = (% of GFP (+) cells pretreated with genistein - % of GFP (+) cells in the absence of IFN γ stimulation) / (% of GFP (+) cells in the absence of genistein - % of GFP (+) cells in the absence of IFN γ stimulation) $\times$ 100 IC₅₀ = 27.1 μ M

Table 1
Signal transducers of cytokines and their karyopherin/chaperones

Cytokines	Transcription factors	Karyopherins and Chaperones (Ref.)
IL-2, IL-7, IL-9, IL-15, IL-21, Growth hormone	Stat5a, Stat5b, Stat3	Importin α , β , Rac1 (42), MgcRacGAP (42)
IL-3, IL-5, GM-CSF, Prolactin, Erythropoietin, Thrombopoietin	Stat5a, Stat5b	Importin β , Rac1 (42), MgcRacGAP (42)
IL-4, IL-13	Stat6	
IL-12, IL-23	Stat4	
IL-6, IL-11, OSM, CNTF, IL-10, LIF, CT-1, Leptin	Stat3	Importin α 1 (31), 3 (30,31), 5 (29), 7 (29)
IFN α , IFN β	Stat1, Stat2	Importin α 5 (22,23)
IFN γ	Stat1	Importin α 5 (22,23)
TGF- β	Smad2, 3, 4	Importin β 1 (49,52), α (53), Ran (52), CAN (54-56), Nup214 (54-56)
TNF- α , IL-1	NF- κ B	Importin α 3, 4 (72)

Table 2

List of proteins whose nuclear translocation was shown by ITT

Protein	Stimulation	Ref.
Stat1	IFN γ	84
Smad2, 3	TGF β	84
Pyruvate kinase M2 (M2-PK)	IL-3	87
2-amino-3-ketobutyrate coenzyme A ligase (KBL)	Cold and osmotic stress	89
Circumsporozoite protein (CS)	Malaria sporozoite infection into hepatocytes	75