Getting the message across, STAT! Design principles of a molecular signaling circuit

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The STAT transcription factors, usually referred to as "latent cytoplasmic proteins," have experienced a fundamental reevaluation of their dynamic properties. This review focuses on recent studies that have identified continuous transport factor–independent nucleocytoplasmic cycling of STAT1, STAT3, and STAT5 as a basic principle of cytokine signaling. In addition, molecular mechanisms that modulate flux rates or cause retention were recognized, and together these findings have provided novel insight into the rules of cellular signal processing.

The JAK/STAT pathway

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways, first identified in the interferon systems, are responsive to a wide range of cytokines and growth factors (Levy and Darnell, 2002). The STAT proteins receive cytokine signals at intracellular receptor chains in the cytoplasm and carry them into the nucleus, where they then act as transcription factors (Levy and Darnell, 2002). Thus, these proteins need to cross the nuclear envelope to functionally link the cell membrane with the promoters of cytokine-responsive genes. Movement of STATs in either compartment is diffusion-controlled and not directed along permanent structures (Lillemeier et al., 2001). Yet, passage through the nuclear gateways, named nuclear pore complexes (NPCs), provides a formidable diffusion barrier to proteins the size of STATs (>85 kD for the monomer; Fig. 1), as only ions and small molecules not exceeding 40 kD can freely enter the cell nucleus (Suntharalingam and Wente, 2003). The analysis of their nucleocytoplasmic translocation has to consider that the STAT proteins exist in two different states in terms of signaling: before the stimulation of cells with cytokines the STAT molecule exists in a nontyrosine phosphorylated state, the oligomerization status of which is still debated (Sehgal, 2000). Stimulation with cytokines increases the activity of receptor-associated JAK kinases and leads to the formation of tyrosine-phosphorylated STATs, which instantly assemble into homo- or heterodimers via canonical phosphotyrosine-SH2 domain interactions (Shuai et al., 1994; Greenlund et al., 1995). Tyrosine phosphorylation is often described as STAT activation since only the dimer is a high affinity DNA-binding protein required for the induction of cytokine-responsive genes (Shuai et al., 1994). This is achieved by directly targeting cognate recognition elements named gamma activated sites (GAS) (Horvath et al., 1995).

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Abbreviations used in this paper: JAK, Janus kinase; STAT, signal transducer and activator of transcription; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; Nup, nucleoporin.

Modes of nuclear translocation

Carrier-dependent as well as carrier-independent modes of translocation are known to exist for the passage of proteins through the nuclear pore (Komeili and O'Shea, 2001). The NPC is a multi-protein structure that creates an aqueous channel spanning the double membrane of the nuclear envelope (Suntharalingam and Wente, 2003). This structure with an estimated molecular mass of 125 MDa in mammalian cells is composed of several copies of ~30 different proteins collectively called nucleoporins (Nups), many of which contain multiple phenylalanine-glycine (FG) repeats that are interspersed with polar residues of varying number (Suntharalingam and Wente, 2003). The key tenets of the NPC translocation models are based on the translocating molecules having weak binding affinities to a number of nucleoporins (Suntharalingam and Wente, 2003). Biochemically carrier-free and carrier-dependent nucleocytoplasmic translocation are related. Docking to the NPC is diffusion controlled for the two pathways, and in both cases the actual translocation process appears to occur independent of metabolic energy via identical interactions with channel components (Kose et al., 1997; Schwoebel et al., 1998). Proteins that contain regions that enable them to directly engage in productive interactions with the nucleoporins are capable of carrier-independent nucleocytoplasmic transport, whereas the remaining proteins (also called cargo proteins) need to associate with transport factors, which act as chaperones during passage through the NPC. The vast majority of transport factors belongs to the karyopherin superfamily of proteins, which mediate either import or export from the nucleus (Wozniak et al., 1998). Recognition of cargo proteins by the transport factors requires the presence of loosely conserved stretches of amino acids on the cargo surface, termed nuclear localization signals (NLSs) or nuclear export signals (NESs), respectively (Wozniak et al., 1998). The stability of cargo/

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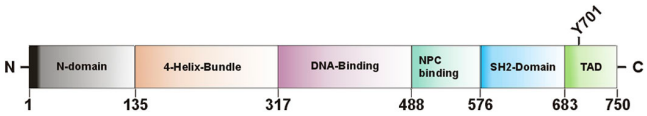

Figure 1. **Domain structure of STAT1.** The residue count is given below the diagram. Dimerization can occur via SH2 domain interactions after phosphorylation of Tyr701. TAD, transcription activation domain.

karyopherin complexes is determined by the small GTPase Ran, as RanGTP disrupts importin/cargo complexes but stabilizes the formation of exportin/cargo complexes (Komeili and O'Shea, 2001). Thus, the RanGDP/RanGTP gradient that forms through the asymmetric distribution of nucleotide exchange factors across the nuclear envelope discriminates cytosol from nucleoplasm and hence confers directionality to the transport process (Komeili and O'Shea, 2001). In the following, the author will describe how the STATs make use of both of these translocation mechanisms.

Nucleocytoplasmic cycling of STATs in resting cells

The recognition that cytoplasmic and nuclear pools of STAT proteins exchange rapidly and quantitatively even before their activation is in stark contrast to conventional textbook knowledge, where unphosphorylated STATs usually are described as exclusively cytoplasmic proteins. It has become clear, though, that only the unphosphorylated STAT molecule is endowed with both nuclear import and export capability (Fig. 2 A, blue arrows; Marg et al., 2004). Although unphosphorylated STATs were reported in the cell nucleus before stimulation (Chatterjee-Kishore et al., 2000; Meyer et al., 2002b), the highly dynamic behavior of STATs in resting cells was uncovered only recently. The intracellular precipitation of STAT1 after the microinjection of specific antibodies in conjunction with the microinjection of recombinant STATs was used to assess the flux rates in living cells. These experiments were complemented by permeabilized cell assays and together these approaches lead to the discovery that unphosphorylated STAT1, STAT3, and STAT5 are constantly shuttling between the cytosol and the nucleus via a mechanism that does not require metabolic energy or transport factors (Meyer et al., 2002a; Marg et al., 2004).

As STAT1 was also demonstrated to bind to nucleoporins, the STATs can be added to a still short but growing list of transcriptional regulators that directly interact with the NPC (Marg et al., 2004; Xu and Massagué, 2004). The carrier-free translocation of STATs is saturable and appears to entail interactions with pore proteins that are shared among the STAT proteins and with importin-β (Marg et al., 2004). Despite this, a common structural denominator for binding to the nuclear pore complex is not discernable (Xu and Massagué, 2004). In vitro alkylation with N-ethyl-maleimide of a single cysteine precluded nucleocytoplasmic translocation of STAT1. The modified residue is situated in a hydrophobic surface groove in the α-helical linker domain. Hence, we tentatively designate the functionally poorly characterized linker domain as a NPC binding domain. However, further regions of the STAT molecule may also participate (Marg et al., 2004).

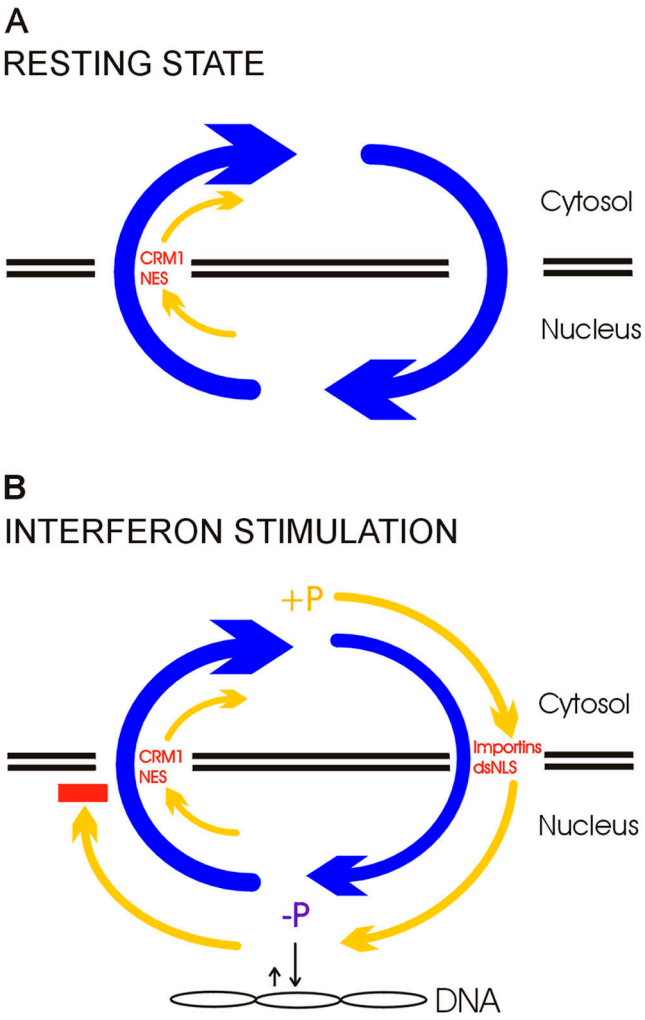

Figure 2. **Schematic representation of STAT1 nucleocytoplasmic cycling in resting cells (A) and during interferon stimulation (B).** Carrier-independent cycling is depicted with blue arrows, carrier-dependent translocation with yellow arrows. Imperatives for carrier-dependent transport are indicated in red; the red bar represents the export block of phosphorylated STAT dimers. Phosphorylation and dimerization are indicated (+P). The reverse reaction, which is under control of the DNA off-rate, is also indicated (−P). Arrow widths are proportionate to their corresponding flux rates.

Despite their constant nucleocytoplasmic cycling, immunofluorescence microscopy images show a predominantly cytoplasmic localization of most STATs in resting cells. For other shuttling signal transducers the deviation from a pancellular distribution was attributed to their binding to anchoring factors (Xu and Massagué, 2004). For the STATs, an alternative mechanism appears to operate. In addition to carrier-free transportation, transferable leucine-rich export signals have been identified in varying numbers and location in mammalian STAT1, STAT3, STAT5 (Begitt et al., 2000; Bhattacharya and Schindler, 2003; McBride et al., 2000; Zeng et al., 2002), slime mold STATa, and STATc (Ginger et al., 2000; Fukuzawa et al., 2003). Such signals, which constitute the prototypical binding surface for the export receptor CRM1 (Komeili and O'Shea, 2001), were initially believed to function only during the termination of cytokine-induced nuclear accumulation, but it has now been demonstrated that the CRM1-dependent export oper-

ates constitutively (Andrews et al., 2002; Zeng et al., 2002; Marg et al., 2004). Moreover, the inactivation of Ran-dependent transport or the specific inactivation of CRM1 both turned the predominantly cytoplasmic localization of STAT1 into a pancellular distribution (Marg et al., 2004). This indicated that carrier-independent and carrier-dependent transport cooperatively determine the subcellular distribution. Even so, the blocking of CRM1 by small molecule inhibitors did not measurably reduce the flux rate of endogenous STAT1 as determined by antibody microinjection, indicating that carrier-dependent transport plays only a minor role in the overall flux rate (Fig. 2 A, yellow arrow; Marg et al., 2004). Whether cell type-specific and STAT-specific distribution differences are due to modulations of actual translocation events, or whether additional mechanisms such as retention are required, remains an open question. Interestingly, nuclear export of *Dictyostelium* STATa, which contains consensus CRM-1 binding sites, was found to be enhanced after glycogen synthase kinase-3 mediated serine phosphorylation (Ginger et al., 2000), which demonstrated a role for post-translational modifications in transport modulation. As of now, constitutively acting nuclear import signals that mediate carrier-dependent nuclear import have not been reported for a STAT protein, but STATs contain conditional NLSs that operate during cytokine stimulation of cells.

Nucleocytoplasmic cycling of STATs in cytokine-stimulated cells

Cytokine stimulation of cells increases the activity of receptor-associated JAK kinases leading to tyrosine phosphorylation and dimerization of STATs, the two requirements for the concomitant nuclear translocation of transcriptionally active STATs that were recognized from early on (Schindler et al., 1992; Shuai et al., 1994). Fluorescence microscopy of stimulated living or fixed cells revealed the characteristic nuclear accumulation that is associated with tyrosine phosphorylation of STATs (Schindler et al., 1992). Depending on the cell type and stimulus intensity, nuclear accumulation is discernable as early as 10 min after the addition of cytokines and it can last for several hours, before the STATs gradually return to their resting distribution. However, cytokine treatment induces the phosphorylation of no more than ~30% of the STAT1 molecules in Bud-8 fibroblasts or HeLa cervix carcinoma cells (Haspel et al., 1996; unpublished data). Functional data indicate that this number also represents the active dimers. This was inferred from the coexpression of wild-type STAT1 and a mutant with defective tyrosine phosphorylation (Tyr701Phe). Although interferon stimulation induced nuclear accumulation of the wild-type molecules, a translocation of the mutant was not discernable (unpublished data). In the case of STAT1/STAT2 heterodimers, however, singly tyrosine-phosphorylated dimers were detected in vitro, but whether or not these molecules participated in cytokine-induced nuclear import was not resolved (Gupta et al., 1996). Thus, in cytokine-stimulated cells at any given time point at sizeable portion of the STAT molecules continue their carrier-free shuttling unchanged (Fig. 2 B, blue arrows). This explains why subcellular fractionation experiments could not confirm the apparent quantitative nuclear translocation of STATs seen by immunofluorescence microscopy (Haspel et al., 1996; Meyer et al., 2002a).

The absence of easily discernable importin binding sites in the STAT molecule has sparked the search for cytokine-triggered events other than tyrosine phosphorylation that are involved in the nuclear import of dimeric STATs, such as complexation with ligands or the formation of endocytotic vesicles (Johnson et al., 1998). Those events may occur, but STAT1 dimers do not require cytokine stimulation for their nuclear translocation and accumulation. This was clearly demonstrated by microinjection of recombinant phosphorylated STAT1 (Meyer et al., 2003).

The autonomous carrier-free nuclear import of STAT1 is incapacitated by dimerization (Marg et al., 2004; Fig. 2 B, yellow arrows), whereas the standard model of STAT functioning stated that dimerization induces nuclear import. Thus, what distinguishes STAT dimers is not at all their ability of nuclear import but the switch to carrier-dependent translocation. Long before the discovery of a STAT NLS, it was concluded from precipitation experiments with STAT1 that dimerization is conducive to the association with specific importin transport factors (Sekimoto et al., 1997). Similar to other NLS-containing proteins, dimeric STAT1 is transported into the nucleus in a Ran-dependent manner by an importin-β and importin-α containing complex, the stoichiometry of which is unknown (Sekimoto et al., 1996, 1997; Fagerlund et al., 2002). Among the six different human importin-α proteins, only importin-α5 (hSRP1, NPI-1) appears to recognize the STAT1 homodimer and the STAT1/STAT2 heterodimer (Sekimoto et al., 1997; Fagerlund et al., 2002). The association with karyopherins has thus far not been studied for other STAT proteins. Notably, the region of importin-α5 required for the binding of conventional NLSs is distinct from that needed for STAT binding (Sekimoto et al., 1997; Melén et al., 2003).

In the recent past, a conserved NLS has been identified in the DNA-binding domain of STAT1, STAT2, and STAT3 (Melén et al., 2001; McBride et al., 2002; Meyer et al., 2002a; Ma et al., 2003). For STAT1 this was a rather serendipitous discovery, because isolated peptides containing the NLS confer nuclear export, but not nuclear import activity on a heterologous protein (McBride et al., 2000; Meyer et al., 2002a). Thus, this nontransferable signal of an unusual amino acid sequence is functional and necessary only in the STAT dimer and was thus termed dimer-specific NLS (dsNLS; Meyer et al., 2002a). Nevertheless, mutation of this signal in one monomer appears sufficient to preclude association of the dimer with importin-α and hence interferes with cytokine-induced gene transcription (Fagerlund et al., 2002; Meyer et al., 2002a), whereas the contribution of this signal to nuclear export of STAT1 has not been demonstrated unambiguously (McBride et al., 2000). The dsNLS is not sufficient for nuclear import of STAT dimers. Removal of the aminoterminal domain, or mutations in the coiled-coil domain both preclude nuclear import after cytokine stimulation (Strehlow and Schindler, 1998; Ma et al., 2003).

It has become evident that the switch to carrier-dependent nuclear import is not the cause of nuclear accumulation of STATs, as carrier-dependent and -independent nuclear import appear to proceed at similar or identical rates (Meyer et al.,

2003; Marg et al., 2004). Hence, the crucial event associated with tyrosine phosphorylation of STATs is the induction of a STAT conformation capable of retention in the nucleus. This conclusion is based on two observations made for STAT1 (Meyer et al., 2003). First, the inhibition of tyrosine phosphatases precluded nuclear export both of endogenous and microinjected recombinant phosphorylated protein. Second, the engineering of STAT1 DNA-binding mutants demonstrated that the duration of nuclear accumulation correlated with the avidity of DNA binding. From this, it was further deduced and then demonstrated that dephosphorylation is blocked when STAT1 is bound to DNA (Meyer et al., 2003). These findings complemented earlier studies that demonstrated a role for nuclear phosphatases and tyrosine dephosphorylation in the nuclear buildup of STATs (Shuai and Liu, 2003).

So what role does DNA binding play in nuclear retention of STATs? An intuitively appealing model of STAT1 nuclear accumulation stated that an NES was masked by DNA binding (McBride et al., 2000). According to this model, which makes no explicit distinction between phosphorylated and nonphosphorylated STATs in terms of export, loss of DNA binding is inevitably associated with the loss of nuclear retention. However, this assumption was disproved by experimental evidence, since a STAT1 mutant without any DNA-binding capabilities could nevertheless accumulate normally if dimer break-up resulting from dephosphorylation was prevented (Meyer et al., 2003, 2004). As a corollary of these system characteristics, nuclear export and DNA-binding reactions do not compete for STATs. Although in principle DNA binding is thus dispensable for nuclear retention of STATs, it nevertheless provides protection from the otherwise exceedingly high dephosphorylation activity in the nucleus. The key parameter to consider is the STAT/DNA dissociation off-rate, which can differ significantly between various STAT binding sites (Vinkemeier et al., 1996). It was found that the rate of tyrosine dephosphorylation was directly proportional to the DNA off-rate (Meyer et al., 2003). These experiments revealed a critical role for nonspecific DNA binding in the nuclear retention of wild-type STAT1. Hence, studies of the kinetics of nonspecific DNA binding are needed also to better understand how STATs locate their target sites on chromatin (von Hippel, 2004).

Importantly, this setup predicts that the activity of the transcription factor is extended at sites with a low DNA off-rate, in other words at GAS-containing target promoters. This expectation was confirmed in vivo for STAT3 binding to a natural promoter (Lerner et al., 2003). Nevertheless, rapid dephosphorylation results in the close coupling of receptor activity to the transcriptional activity in the nucleus. It was found for STAT3 that the half-life of the dimer was only 15 min even on a target promoter (Lerner et al., 2003). Accordingly, rapid shuttling occurs also during nuclear accumulation (Meyer et al., 2003), when efficient export rates are particularly important to allow for successive rounds of rephosphorylation in order to maintain a steady level of transcriptionally active molecules in the nucleus (Andrews et al., 2002; Meyer et al., 2003; Swameye et al., 2003).

Conclusions

Recent insight into the dynamic redistribution of STAT transcription factors confirmed that their name, which was coined in the Darnell lab, was well chosen. Already before stimulation with cytokines these proteins engage in rapid nucleocytoplasmic cycling via a simple, karyopherin-independent mechanism. Concurrent nuclear export via a classical exportin-dependent pathway was determined for STAT1 to achieve the predominantly cytoplasmic steady-state distribution that is characteristic for many different cells. Upon exposure to cytokines, a considerable portion of the STATs are tyrosine phosphorylated and dimerize via SH2 domain interactions. This change in structure has important functional implications. It allows for high affinity DNA binding, and at the same time a nonconventional, but nevertheless karyopherin-dependent nuclear localization signal is exposed. Macroscopically, these events become apparent as a transient nuclear accumulation, which does not, however, result from the switch to carrier-dependent import. Rather, dimerization not only precludes further carrier-free nucleocytoplasmic cycling of STATs, but their nuclear export in general. Although the intranuclear mobility of STAT dimers remains at the diffusion limit (Meyer et al., 2003, 2004), they are nonetheless trapped in this compartment until their tyrosine dephosphorylation. Importantly, the dephosphorylation rate was determined to be directly proportional to the DNA off-rate, which implicated nonspecific DNA binding into STAT nucleocytoplasmic cycling. Accordingly, at target promoters, the transcriptionally active factor is preserved best. Despite that, the dephosphorylation reaction proceeds with half-times of less than 15–30 min, making nuclear accumulation a dynamic process that is sustained by continous shuttling and kinase activity.

Future research needs to focus on the molecular mechanisms that give rise to flux modulations, either physiologically or in pathological situations such as microbial infections. This is critical in order to determine the functional implications of nucleocytoplasmic cycling for cytokine signaling and beyond.

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