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Molecular Basis for the Recognition of Phosphorylated STAT1 by Importin α5

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Interferon-γ stimulation triggers tyrosine phosphorylation of the transcription factor STAT1 at position 701, which is associated with switching from carrier-independent nucleocytoplasmic shuttling to carrier-mediated nuclear import. Unlike most substrates that carry a classical nuclear localization signal (NLS) and bind to importin α1, STAT1 possesses a nonclassical NLS recognized by the isoform importin α5. In the present study, we have analyzed the mechanisms by which importin α5 binds phosphorylated STAT1 (pSTAT1). We found that a homodimer of pSTAT1 is recognized by one equivalent of importin α5 with $K_d = 191 \pm 20$ nM. Whereas tyrosine phosphorylation at position 701 is essential to assemble a pSTAT1–importin α5 complex, the phosphate moiety is not a direct binding determinant for importin α5. In contrast to classical NLS substrates, pSTAT1 binding to importin α5 is not displaced by the N-terminal importin β binding domain and requires the importin α5 C-terminal acidic tail (505-EEDD-508). A local unfolding of importin α5 Armadillo (ARM) repeat 10 accompanies high-affinity binding to pSTAT1. This unfolding is mediated by a single conserved tyrosine at position 476 of importin α5, which is inserted between ARM repeat 10 helices H1–H2–H3, thereby preventing intramolecular helical stacking essential to stabilize the folding conformation of ARM 10. Introducing a glycine at this position, as in importin α1, disrupts high-affinity binding to pSTAT1, suggesting that pSTAT1 recognition is dependent on the intrinsic flexibility of ARM 10. Using the quantitative stoichiometry and binding data presented in this article, together with mutational information available in the literature, we propose that importin α5 binds between two STAT1 monomers, with two major binding determinants in the SH2 and DNA binding domains. *In vitro*, this model is supported by the observation that a 38-mer DNA oligonucleotide containing two tandem *cfos*M67 promoters can displace importin α5 from pSTAT1, suggesting a possible role for DNA in releasing activated STAT1 in the cell nucleus.

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Abbreviations used: NLS, nuclear localization signal; pSTAT1, phosphorylated STAT1; ARM, Armadillo; uSTAT1, unphosphorylated STAT1; dsDNA, double-stranded DNA; NPC, nuclear pore complex; cNLS, classical NLS; IBB, importin β binding; dsNLS, dimer-specific NLS; EBNA-1, Epstein–Barr virus nuclear antigen 1; ITC, isothermal titration calorimetry; pNLS, phosphorylated NLS; SPR, surface plasmon resonance; GST, glutathione *S*-transferase; PDB, Protein Data Bank; TCEP, tris(2-carboxyethyl)phosphine; EDTA, ethylenediaminetetraacetic acid.

Introduction

The JAK/STAT pathway, a key player in host immune response, transmits a cytokine signal from the extracellular space to the nucleus of the host cell to drive the transcription of genes responsible for host defense.[1] Products from seven different STAT genes are expressed in mammals, which share a fundamentally conserved topology consisting of three independent structural subunits.[1] The N-terminal helical domain, roughly 130 amino acids in length, promotes dimerization of unphosphorylated STATs and polymerization of phosphorylated dimers on DNA.[2,3] This is followed by a large STAT core of approximately 600 residues, containing a coiled-coil domain, a DNA binding domain, and an SH2 domain. Before cytokine stimulation of cells, STAT1 is a dimer that adopts AN anti-parallel conformation involving N-domain interactions.[4] Structural evidence indicates that such dimers are recruited to cytokine receptors for activation by phosphorylation of a C-terminal tyrosine residue.[5,6] Upon activation, an additional homodimer conformation emerges where the phosphotyrosine of one protomer reaches into the SH2 domain of the other.[7] SH2 phosphotyrosine-mediated dimers, also called parallel dimers, possess DNA-binding activity and evoke transcriptional responses to cytokines. While unphosphorylated STAT1 (uSTAT1) is a nucleocytoplasmic shuttling protein that efficiently enters the nucleus independent of carrier molecules, nuclear import of activated STATs requires importins. Moreover, activated STAT1 is barred from nuclear export. Although phosphorylated STAT1 (pSTAT1) is efficiently dephosphorylated in the nucleus by phosphatase TC45 ($t_{1/2} \sim 20$ min),[8] protracted nuclear export becomes apparent as nuclear accumulation of STAT1 occurs within minutes of cytokine stimulation.[9] These mechanisms ensure nuclear retention of the active transcription factor while linking the activity of cytokine receptors to their effector functions in the nucleus. Vaccinia virus and other members of the *Poxviridae* family of double-stranded DNA (dsDNA) viruses[10] encode the dual-specificity phosphatase VH1 that specifically dephoshorylates activated STAT1.[11,12] In contrast to TC45, VH1 is thought to dephosphorylate STAT1 in the cytoplasm, thus preventing the expression of antiviral genes.[12]

The carrier-dependent passage of proteins through the nuclear pore complex (NPC) is an active signal-mediated process that requires soluble transport factors of the importin β superfamily (also known as β-karyopherins) and the small GTPase Ran.[13–17] Import complexes form in the cytoplasm upon recognition of a nuclear localization signal (NLS) in import cargos by β-karyopherins. This interaction can be direct or mediated by transport adaptors such as importin α and snurportin.[18] There are six human importin α isoforms in humans that show a greater than 60% sequence similarity[19] and fall into three phylogenetically distinct groups: α1, α2, and α3.[19] All importin αs are made up of 10 stacked Armadillo (ARM) repeats,[20] each formed by three α-helices.[19] Different importin α isoforms have striking differences in substrate recognition, which enhance specificity for the nuclear import of diverse import cargos, but also share the ability to bind and import classical NLS (cNLS) substrates.[21] Despite the presence of six importin α isoforms, the majority of import cargos contain a classical SV40-like NLS that is recognized by importin α1.[13] Structural work has shown that the basic side chains of an NLS occupy a shallow groove within ARM repeats 1–4 of importin α, which is known as the major binding site, as well as a minor binding site between ARM repeats 4 and 8. Five points of contact between NLS and importin α (usually referred to as positions P1–P5) have been identified from the analysis of several importin α/NLS complexes.[22–27] Structural and mutational data have demonstrated the crucial role played by the NLS basic lysine at the P2 site, which contacts the Trp/Asn pair between importin α1 ARM repeats 3 and 4.[24,28,29] Interestingly, certain animal isoforms are very specific for nonclassical cargos. For instance, importin α5 (also known as NPI-1[30]) is involved in the nuclear import of dimeric pSTAT1[31] and influenza virus polymerase PB2.[32] The crystal structure of importin α5 was recently determined in complex with the C-terminal domain of influenza virus RNA polymerase PB2.[32] In this structure, the C-terminal folded core of PB2 (residues 686–741) makes extensive interactions with the C-terminus of importin α5, where ARM repeat 10 deviates from its canonical structure to wrap around the polymerase subunit.[32] In addition, an extended N-terminal moiety of PB2 (residues 749–757) extends inside the major NLS binding site (ARM repeats 1–4) to adopt a stretched conformation similar to that seen for the SV40-like NLS.[24] Overall, the recognition of PB2 by importin α5 is more extended and complex than that of cNLS peptides bound to importin α1 and requires the C-terminal domain of importin α5. Despite differences in cargo specificity and recognition, all importin α isoforms bind the receptor importin β via an N-terminal importin β binding (IBB) domain. Import complexes formed in the cytoplasm move through the NPC in a process that likely involves multiple rounds of interactions with phenylalanine–glycine nucleoporins (*FG*-nups). Ran is naturally compartmentalized in eukaryotic cells with a nuclear RanGTP pool, while cytoplasmic Ran is predominantly in RanGDP form.[13,15] RanGTP facilitates translocation through the NPC by releasing import complexes from high-affinity binding sites in nucleoporins and by disassembling import complexes in the cell nucleus.[13,15] GTP hydrolysis by the small GTPase Ran is thought to impose directionality of transport through the NPC.[33]