Signal transducer and activator of transcription 3 regulation by novel binding partners

Tadashi Matsuda, Ryuta Muromoto, Yuichi Sekine, Sumihito Togi, Yuichi Kitai, Shigeyuki Kon, Kenji Oritani

Tadashi Matsuda, Ryuta Muromoto, Yuichi Sekine, Sumihito Togi, Yuichi Kitai, Shigeyuki Kon, Kenji Oritani, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Hokkaido 060-0812, Japan

Kenji Oritani, Department of Hematology and Oncology, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan

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Correspondence to: Tadashi Matsuda, PhD, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan. tmatsuda@pharm.hokudai.ac.jp
Telephone: +81-11-7063243
Fax: +81-11-7064990

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Abstract
Signal transducers and activators of transcription (STATs) mediate essential signals for various biological processes, including immune responses, hematopoiesis, and neurogenesis. STAT3, for example, is involved in the pathogenesis of various human diseases, including cancers, autoimmune and inflammatory disorders. STAT3 activation is therefore tightly regulated at multiple levels to prevent these pathological conditions. A number of proteins have been reported to associate with STAT3 and regulate its activity. These STAT3-interacting proteins function to modulate STAT3-mediated signaling at various steps and mediate the crosstalk of STAT3 with other cellular signaling pathways. This article reviews the roles of novel STAT3 binding partners such as DAXX, zipper-interacting protein kinase, Krüppel-associated box-associated protein 1, Y14, PDZ and LIM domain 2 and signal transducing adaptor protein-2, in the regulation of STAT3-mediated signaling.

Key words: Janus kinase/signal transducer and activator of transcription; Signal transduction; Signal transducer and activator of transcription 3; DAXX; Zipper-interacting protein kinase; Krüppel-associated box-associated protein 1; Y14; PDZ and LIM domain 2; Signal transducing adaptor protein-2; Nuclear factor-κB

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Core tip: Signal transducer and activator of transcription 3 (STAT3) has been proposed its physiological and pathological significance in malignant and inflammatory diseases; therefore, the targeting of the STAT3 pathways is likely to be suitable for clinical application. In this review, we introduced novel regulatory molecules of STAT3 binding partners, such as DAXX, zipper-interacting protein kinase, Krüppel-associated box-associated protein 1, Y14, PDZ and LIM domain 2 and signal transducing adaptor protein-2. These proteins positively or negatively regulate critical steps of STAT3-mediated signals via individually unique mechanism. We hope that the information described here will help to develop a new strategy to clinically control the STAT3 activities.
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INTRODUCTION

Cytokines selectively activate Janus kinases (JAKs), which in turn activate one or more signal transducers and activators of transcription (STATs) via their tyrosine phosphorylation[1-3]. STATs have cytoplasmic signaling regions, such as a Src-homology 2 (SH2) domain and tyrosine phosphorylation sites. Upon cytokine stimulation, STATs are phosphorylated and dimerize via their SH2 domains and then move into the nucleus[4]. STAT3 is a central member of the STAT protein family, and is activated by various cytokine signals, such as interleukin 6 (IL-6)[5,6,7], which plays a role in immune regulation, hematopoiesis, inflammation and oncogenesis[6,7] (Figure 1). The majority of IL-6 functions are in turn mediated by STAT3[5,9]. Of importance, abnormal expression of STAT3 has been reported in several cancer cells as well as autoimmune diseases, suggesting the involvement of STATs in a wide range of diseases[5,10-13]. Because of important physical roles, STAT3 activity is strictly regulated by multiple molecular mechanisms. For example, the protein inhibitor of activated STAT suppresses transcriptional activities of STAT3 from DNA binding in the nucleus[14]. Suppressor of cytokine signaling (SOCS), which is induced by STAT3, participates in the negative feedback of STAT3 activities[15]. Cytoplasmic tyrosine phosphatases, such as SH2-containing phosphatase 1 (SHP1), SHP2 and protein-tyrosine phosphatase 1B, function to stop STAT activities[14,15]. Nuclear tyrosine phosphatases, such as TC45, also dephosphorylate nuclear STAT3, resulting in their translocation from the nucleus to the cytoplasm[14,17].

We have identified some STAT3-interacting molecules, including DAXX[18,19], zippering-interacting protein kinase (ZIPPK)[19,21], Krüppel-associated box-associated protein 1 (KAP1)[21], Y14[22,23], PDZ and LIM domain 2 (PDLIM2)[23] and signal transducing adaptor protein-2 (STAP-2)[26-28]. Here, we describe functions of each of these molecules in the STAT3-mediated signaling pathway. DAXX negatively regulates STAT3-mediated transactivation and cell proliferation through the IL-6 signal transducer gp130[18,19]. ZIPPK positively regulates STAT3 transactivation through STAT3 Ser727 phosphorylation[20,21]. KAP1 negatively regulates STAT3 Ser727 phosphorylation and transactivation by interacting with HDAC3 within the nucleus[22]. Y14 is a novel type of STAT3 binding partner and influences IL-6-induced STAT3 transactivation through altering its tyrosine-phosphorylation state[23,24]. PDLIM2 acts as a nuclear E3 ligase for STAT3 and terminates STAT3-mediated signaling[25]. STAP-2 is a novel adaptor protein, composed of pleckstrin homology (PH) and SH2-like domains, and a STAT3-binding (YXXQ) motif[26-28]. Taken together, STAT3 activity is positively and negatively regulated at multiple steps.

NOVEL STAT3 BINDING PARTNERS

The STAT3 activities are strictly regulated, and recent reports have suggested several novel STAT3 regulators, whose characters are summarized in Table 1[25,26,29-33]. A nuclear STAT3 repressor, Death domain-associated protein (DAXX)

DAXX, which mainly located in the nucleus, has an ability to modulate transcription as well as cell death[34]. DAXX interacts with a number of transcription factors, including ETS1[35], PAX5[36], Glucocorticoid receptor[37], RelA[38], RelB[39], TCF4[40], SMAD4[41], C/EBPβ[42] and AIRE[43], and regulates their transcriptional activities. Because DAXX is also known to bind to histone deacetylases[44], DNA methyltransferases and their associated proteins[45-46], and the chromatin-modifying α-thalassemia syndrome protein[47,48], DAXX is likely to regulate cellular processes by regulating the transcription of specific genes via epigenetic modification. We found that DAXX regulates STAT3-transcriptional activity and that IFN-induced DAXX functionally links to IL-6/LIF/STAT3-mediated signaling[48]. Pretreatment of HeLa and Hep3B cells with IFN caused a decrease of IL-6-induced STAT3 transcriptional activities. Importantly, DAXX directly interacts with STAT3 in the nucleus, leading to the decreased STAT3-transcriptional activities. Indeed, knockdown of DAXX significantly enhanced STAT3 activation and gene expression after IL-6-stimulation.

The IL-6 family cytokines recognize gp130 membrane protein as a signal-transducing receptor component[49,50]. Dimerization of gp130 activates JAK family proteins (JAK1, JAK2 and TYK2), which then phosphorylate and activate STAT3. In lymphocytes, STAT3 is involved in IL-6- and/or IL-27-dependent cell growth[49,50]. In addition, STAT3 is also required for pro-B cell survival as well as efficient B lymphocyte production[49]. When progenitor B lymphocytes were treated with IFN-β, DAXX expression and nuclear localization were enhanced in parallel to Bcl-2 down-regulation[52]. Therefore, DAXX has an important function to control STAT3 activity and Bcl-2 expression during cytokine
stimulation.

A STAT3 Ser727 kinase, ZIPK
Tyrosine and/or serine residues of STATs are phosphorylated in response to ligand stimulation\[^{53,54}\]. In the case of STAT3, a single serine phosphorylation (serine residue at the position of amino acid 727; Ser727) in the transcriptional activation domain is needed for its maximal transcriptional activity. A mutant form of S727A of STAT3, in which serine 727 was replaced by alanine, was estimated to have approximately 50% of transcriptional activity when compared with wild type \[^{54}\]. To analyze the meaning of Ser727 phosphorylation in vivo, SA mutant mice whose STAT3 Ser727 was substituted to alanine, were produced \[^{55}\]. Embryonic fibroblasts from SA/SA homozygous mice displayed approximately 50% of the transcriptional cellular responses when compared with wild-type mice; therefore, Ser727 phosphorylation is important for maximal transcriptional activities of STAT3 even in vivo. Serine phosphorylation increases STAT3 activity via the association with some cofactors, such as p300 \[^{56}\]. Several kinases were implicated in serine phosphorylation of STAT3, and interactions between STAT signaling and serine kinase signaling pathways have been proposed \[^{53}\].

With a yeast two-hybrid screen using the C-terminal region of STAT3 as bait, we identified ZIPK as a new STAT3-binding protein \[^{20}\]. ZIPK selectively bound to STAT3, but not other STAT proteins, in mammalian cells. Furthermore, the kinase domain of ZIPK interacted with the DNA binding and C-terminal domains of STAT3 although ZIPK kinase activities were not essential for their binding. Of importance, ZIPK phosphorylates STAT3 Ser727 in the nucleus, and functionally enhances STAT3-mediated transcription after IL-6- or LIF-stimulation. siRNA-mediated knock down of endogenous ZIPK expression also proposed participation of ZIPK in STAT3-mediated transcriptional activation and target gene expression after LIF-stimulation. ZIPK, a serine/threonine-specific protein kinase, binds to ATF4, which belongs to the activating transcription factor/cyclic AMP-responsive element binding protein family \[^{57}\]. ZIPK aggregates via its leucine zipper domain to become an active enzyme form. Over-expression of wild type ZIPK, but not the kinase-inactive mutant ZIPK K42A, induces apoptosis in NIH 3T3 cells, indicating that ZIPK stimulates the apoptotic process via its catalytic activity \[^{57}\]. The kinase domain of ZIPK shows high sequence homology to that of death-associated protein kinase (DAPK), and these proteins establish a family with DAPK2/DRP-1, DRAK1 and DRAK2, all of which are related to apoptosis \[^{58-60}\]. In collaboration with DAXX and Par-4, ZIPK induces apoptosis by way of nuclear PML oncogenic domains (PODs) \[^{61}\]. We previously reported that activated STAT3 enhanced ZIPK activity after IL-6- or LIF-stimulation\[^{20,21}\]. In this regard, IL-6/LIF/STAT3 signaling is likely to mediate apoptotic activity via inducing the translocation of ZIPK into PODs, together
with PML and DAXX. Conversely, ZIPK induces STAT3 Ser727 phosphorylation, and enhances STAT3-mediated transcription. However, ZIPK K42A expression decreased STAT3 Ser727 phosphorylation in early but not late phase of IL-6-stimulation, suggesting that other kinases may be involved in the late phase of STAT3 Ser727 activation after IL-6-stimulation. Phosphorylation of Ser727 can increase STAT3 activity via associations with some co-activators, such as p300. Of importance, ZIPK also interacts with p300 and forms a complex with STAT3. Therefore, the binding of STAT3 to ZIPK in the nucleus may contribute to the stabilization of coactivator-transcription factor complexes.

A nuclear STAT3 binder, KAP1
KAP1, also known as transcriptional intermediary factor 1β and Tripartite motif-containing 28 (TRIM28), is a co-repressor of Krüppel-associated box-domain-containing zinc finger proteins. KAP1 has an ability to coordinate various components involving in gene silencing; therefore, it can control the histone deacetylase (HDAC) complex and a histone methyltransferase. In other words, KAP1 inhibits the transcription of its target genes via orchestrating functions of the co-repressor complexes.

We isolated KAP1 as a STAT3-interacting protein using a yeast two-hybrid screening of a mouse embryo cDNA library. Co-immunoprecipitation experiments confirmed that KAP1 binds to STAT3 in Hep3B cells. Endogenous KAP1 was present within the nucleus even in the absence of stimulation. After IL-6 stimulation, STAT3 was predominantly found in the nucleus, where it overlapped with KAP1, demonstrating that activated STAT3 translocates into the nucleus and interacts with KAP1. In Hep3B cells, KAP1 knockdown by specific siRNA significantly enhanced STAT3 activation as well as mRNA expression of SOCS3 and C/EBPδ in response to IL-6. Thus, KAP1 negatively regulates STAT3-mediated transcriptional activation and gene expression after IL-6-stimulation. Importantly, phosphorylation of STAT3 Ser727, but not STAT3 Tyr705, increased in parallel to reduction of KAP1 expression. Coincident with these data, reduction of KAP1 expression showed enhanced nuclear accumulation of STAT3 phosphorylated at Ser727. This may be in part related to the association with some cofactors, such as p300. Therefore, KAP1 is likely to recruit protein phosphatases to dephosphorylate STAT3 Ser727 in the nucleus. Alternatively, the direct interaction of KAP1 with HDACs may also be another mechanism for KAP1-mediated transcriptional repression because STAT3 has an ability to associate with HDAC3.

Therefore, KAP1 has a potential to suppress transcrip- tional activities of STAT3 in multiple ways.

A novel type of STAT3 binder, Y14
We identified Y14 as a novel associating protein with STAT3. Y14, an RNA-binding protein, forms an exon-junction complex (EJC) with MAGOH. This complex selectively recognizes spliced forms of mRNAs immediately upstream of exon-exon junctions, and the binding is kept even after nuclear export. In general, mRNAs produced by splicing are translated more efficiently than those from similar intronless precursors. The EJC is in part involved in this translational enhancement because both Y14 and MAGOH recognize spliced form of mRNAs in the cytoplasm until mRNAs are translated. Human Y14 is known to shuttle mRNAs to interact with MAGOH. However, only limited information is available regarding

Table 1: Novel signal transducer and activator of transcription 3-interacting proteins and their knockout mice phenotypes

| Protein | STAT3-binding site | Binding site in STAT3 | Function | Reported KO mice phenotype | Ref. |
|---------|--------------------|-----------------------|----------|-----------------------------|------|
| DAXX    | N-terminal (1-240)  | DNA-BD (320-493)      | STAT3 suppression | Extensive apoptosis and embryonic lethality | [29] |
| ZIPK    | Kinase domain (1-275) | DNA-BD (320-493) and SH2-TAD (494-750) | STAT3 activation | Not yet reported | |
| KAPI    | Not determined     | Coiled-coil (138-319) and DNA-BD (320-493) | STAT3 suppression | Severe hypoproliferative anemia (hematopoietic-restricted deletion of KAPI) | [30] |
| Y14     | Not determined     | DNA-BD (320-493) and SH2-TAD (494-750) | STAT3 activation | Enhanced Th17-cell dependent responses | [25] |
| PDLIM2  | LIM domain         | N-terminal (1-137), DNA-BD (320-493) and SH2-TAD (494-750) | STAT3 suppression | Not yet reported | |
| STAP-2  | SH2 domain YXXQ motif | Coiled-coil (138-319), DNA-BD (320-493) and SH2-TAD (494-750) | STAT3 activation | Reduction in LPS-induced acute phase response | [26] |
an mRNA shuttling protein involved in the regulation of transcription factors, such as STAT3.

We found that endogenous Y14 directly binds to STAT3 in Hep3B cells and affects STAT3 transactivation activity at several steps of IL-6-mediated signaling, including the tyrosine-phosphorylation, the nuclear accumulation and the DNA-binding of STAT3\(^{[23,24]}\). Furthermore, MAGOH inhibits complex formation between STAT3 and Y14, and MAGOH knockdown by specific siRNA enhances IL-6-induced gene expression.

Therefore, Y14 positively regulates IL-6-induced STAT3 activation, and MAGOH interferes with this effect by displacing Y14 from STAT3.

**PDLIM2, a nuclear E3 ligase for STAT3**

STAT3 activation is tightly regulated at multiple levels, including the ubiquitin/proteasome-dependent degradation of STAT3\(^{[75,76]}\). We found that a nuclear ubiquitin E3 ligase, PDLIM2 (also known as SLIM or mystique) binds to and degrades STAT3. PDLIM2 is a nuclear protein, composed of PDZ (postsynaptic density 65-discs large-zonula occludens 1) and LIM (abnormal cell lineage 11-isket 1-mechanosensory abnormal 3) domains\(^{[77,78]}\). PDLIM2 promoted to polyubiquitinate and degrade STAT3 in a proteasome-dependent manner by binding to and degrades STAT3, caused insufficient STAT3 degradation, leading to nuclear accumulation of STAT3 and enhanced STAT3-mediated gene expression.

The LIM domain of PDLIM2 is needed for the recognition of STAT3. Ubiquitination reactions require three types of enzymes: An ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) an ubiquitin ligase (E3). RING-type E3 ligases provide the polyubiquitin chain from E2 to their substrate by binding to E2 via their RING-finger domain, as well as by interacting with substrate proteins via the other domain\(^{[79]}\). The LIM domain forms a zinc finger structure and PHD domains. Proteins containing these domains generally possess ubiquitin E3 ligase activity and polyubiquitinate their target proteins\(^{[80]}\). Thus, the LIM domain of PDLIM2 is thought to be enough to bind to both E2 and its substrate STAT3. This possibility was consistent with the finding that the LIM domain of PDLIM2 could individually polyubiquitinate STAT3 in vitro. Interestingly, PDLIM2 binds to both phosphorylated and unphosphorylated STAT3, but PDLIM2 was shown to bind to phosphorylated but not unphosphorylated STAT4.

Therefore, PDLIM2 may regulate STAT3 activation via a different mechanism from that used on STAT4.

**STAP-2 as a novel adaptor protein for STAT3**

STAP-2, which we isolated as a c-fms-interacting protein, is composed of an N-terminal PH domain and an SH2-like domain\(^{[26]}\). A proline-rich region as well as a STAT3-binding YXXQ motif are also present in its C-terminal region\(^{[26]}\). STAP-2 is a murine homologue of an adaptor molecule BKS, which is a substrate of BRK tyrosine kinase\(^{[81]}\). Upon stimulation with epidermal growth factor, STAP-2 is tyrosine-phosphorylated and moves to the plasma membrane in STAP-2-overexpressing fibroblasts. IL-6 strongly induced STAP-2 mRNA in cultured hepatocytes; in addition, lipopolysaccharide-injection also induced STAP-2 mRNA in mice liver. In STAP-2-deficient hepatocytes, mRNA expression of acute-phase proteins and the tyrosine-phosphorylation of STAT3 are specifically impaired at the late phase of IL-6 stimulation. Thus, STAP-2 regulates IL-6/STAT3-mediated acute-phase protein responses during systemic inflammation. Furthermore, transient overexpression of STAP-2 mutant constructs revealed that STAP-2 enhances STAT3 activation through the YXXQ motif\(^{[85]}\). STAP-2 tyrosine-250 (Tyr250), a major tyrosine phosphorylation site by v-Src, JAK2 and LIF is also required for the enhancement of STAT3 activity\(^{[87]}\). Indeed, the Y250F mutant, in which Tyr250 is substituted with phenylalanine, does not enhance STAT3 transcriptional activity.

As announced first, STAP-2 is a substrate of BRK\(^{[81]}\). BRK, also known as PTK6, is a non-receptor tyrosine kinase, composed of an SH3 domain, an SH2 domain and a tyrosine kinase catalytic domain, lacking an N-terminal myristoylation site for membrane targeting\(^{[82]}\). BRK is expressed by several malignant cells, such as metastatic melanomas and colon and prostate tumors as well as breast cancers\(^{[83-87]}\). In mammary gland, a large proportion of breast cancer cells express BRK, while normal mammary cells do not\(^{[88]}\). Notably, growth of breast cancer cells was impaired by siRNA-mediated down-regulation of BRK expression\(^{[89]}\). Our manipulation of STAP-2 expression indicates that STAP-2 plays an essential role in STAT3 activation by BRK. Indeed, STAP-2 bound to both BRK and STAT3, and STAP-2 knockdown by specific siRNA greatly decreased STAT3 activation induced by BRK in a breast cancer line T47D. Notably, an artificial STAP-2-BRK fusion protein had robust kinase activity and strongly induced activation and tyrosine phosphorylation of STAT3\(^{[88]}\).

Therefore, STAP-2 is involved in BRK-mediated STAT3 activation and tumor cell growth.

**Possible clinical utility of targeting STAT3-related molecules in future**

Of note, most of these STAT3 binding proteins also directly interact with nuclear factor-κB (NF-κB) (p65/RelA) or NF-κB signaling molecules\(^{[82,83,90-92]}\). NF-κB, as well as STAT3, is a central signaling hub in inflammation and oncogenesis. NF-κB is also a transcription factor, which regulates gene expression of antiapoptosis as well as proinflammatory cytokines and chemokines\(^{[83,93,94,96]}\). Like STAT3, constitutively active NF-κB is found in many types of cancers\(^{[93,94]}\). Both STAT3 and NF-κB are also involved in the expression of target genes relating to tumor cell growth, migration and invasion\(^{[93,94,96]}\). Furthermore, target genes regulated by positive or negative crosstalk between STAT3 and NF-κB are gradually increasing\(^{[96,97]}\).
In normal immune cells, activated STAT3 promotes serine-phosphorylation and subsequent proteasome-mediated degradation of 1xβ, resulting in the activation of 1xβ kinase[80]. In cardiomyocytes as well as non-small cell lung cancer cells, the activation of NF-κB up-regulates STAT3 expression[89,100]. Importantly, STAT3 is known to directly bind to the transactivation domain of NF-κB through its DNA-binding domain[101,102]. Furthermore, it has been shown that, besides nuclear translocation after cytokine-stimulation, STAT3 continuously shuttles between the cytoplasm and the nucleus, independently of its tyrosine phosphorylation. Unphosphorylated STAT3 can interact with transcription factors, such as NF-κB, bind to DNA and drive gene expression in a distinct manner from phosphorylated STAT3[103]. Thus, direct interactions between STAT3 and NF-κB can regulate gene expression in several forms of NF-κB-dependent transcription. Therefore, STAT3-regulating molecules as well as STAT3 are likely to be key players during oncogenesis or inflammation, proposing that STAT3 could be a suitable target for malignant and/or inflammatory diseases. Although many manuscripts have showed that STAT3 has physiological and/or pathological significance, clinical meanings of the interactions with STAT3 and its binding partners should be clarified in future. STAT3 binding proteins described here are likely to have a potential to regulate STAT3 activity under some malignant or inflammatory circumstance; therefore, further experiments, including the establishment of low molecular compounds to inhibit their interaction with STAT3 could help for us to gather information about their clinical utility as well as physiological and/or pathological significance. Because STAT3 deficient mice are embryonic lethal[104], the targeting of STAT3 binding proteins may have fewer adverse effects than that of STAT 3 itself.

CONCLUSION

In this review, we summarized the functions of newly identified STAT3-interacting proteins. DAXX negatively regulates STAT3-mediated transactivation and cell proliferation through an IL-6 signal transducer, gp130[18,19]. ZIPK positively regulates STAT3 transactivation through STAT3 Ser727 phosphorylation[10,21], KAP1 negatively regulates STAT3 Ser727 phosphorylation and transactivation by interacting with HDAC3 inside the nucleus[22]. Y14 regulates STAT3 transactivation via influencing tyrosine-phosphorylation after IL-6-stimulation[23,24]. PDLIM2 acts as a nuclear E3 ligase for STAT3 and terminates STAT3-signals[25]. STAP-2, a new adaptor protein, recognizes STAT3 through its YXXQ motif and stimulates STAT3 transactivation[26]. Although constitutive STAT3 activation is frequently observed in malignancies, few mutations in the STAT3 gene have yet been described. Therefore, it is very informative to clarify the mechanism how STAT3 is activated in malignant cells. Although direct proof is lacking, STAT3-associated proteins described here may be involved in this malignant process.

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