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Review

STAT and SMAD signaling in cancer

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Summary. STAT and SMAD often exert opposite biological effects on diverse cellular functions. Recent studies have shown that STAT can interface with SMAD at molecular level and that some novel molecules, such as SOCS (also called CIS) and APRO6 (also called TOB), modulate this signaling. A cofactor p300/CBP might act as a bridging molecule to mediate the interface. Thus, STAT and SMAD signaling pathways may crosstalk each other with interweaved regulatory mechanisms. Interestingly, the importance of all the proteins' function has been shown by the increasing evidence of their involvement in cancer. These recent progresses have been made in attributing novel exciting functions. Accordingly, we would like to review the latest advances of those pathways on a cross-section in cancer signaling.

Key words: STAT, SMAD, SOCS/CIS, JAK, APRO/TOB

Introduction

STAT (signal transducers and activators of transcription) and SMAD proteins are essential components of the intracellular signaling pathways. However, they have rather opposite effects on diverse cellular functions. Members of both family proteins can directly transduce a signal from the plasma membrane to the gene. It has been implied that STAT-signaling pathways can interface with SMAD signaling pathways significantly increasing combinative signaling possibilities. For example, interferon (IFN)-gamma through acting JAK1 and STAT1 induces the expression of SMAD7, which prevents the interaction of SMAD3 with the TGF-beta receptor (Ulloa et al., 1999). In cooperative signaling between Leukemia inhibitory factor (LIF: an interleukin -6 family member) and Bone morphogenetic protein-2 (BMP-2: a TGF-beta superfamily member), an indirect interaction between STAT3 and SMAD1 with the transcriptional co-activator CBP/p300 acting as a bridging molecule has been found (Nakashima et al., 1999). These findings clearly indicate the crosstalk between the STAT and SMAD signaling pathways. Strikingly, the importance of these proteins' function is also shown by their activity involved in carcinogenesis. Accordingly, the link between STAT and SMAD signaling pathways should be of great interest to cancer biologists. In the present paper, we present a review of the latest advances in our understanding of the function from a viewpoint of cancer signaling. In addition, we discuss recently characterized molecules, SOCS/CIS and APRO6/Tob, which negatively modulate STAT and SMAD, respectively.

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STAT structure and signaling

Seven members of mammalian STAT (1, 2, 3, 4, 5a, 5b, and 6) have been cloned (Fig. 1a). While about 200 amino acids of the central domain contribute to DNA binding, Src-homology 2 (SH2) domain mediates dimer formation through the interaction between SH2 and a specific phosphorylated tyrosine residue adjacent to SH2 domain. Amino terminal domain of STAT also promotes its binding to DNA, probably, by inducing oligomerization of the nuclear STAT dimers bound to target sequences. A carboxyl terminal portion acts as a transcriptional transactivation and phosphorylation of seine residues within this domain; in certain cases, it is required for full activation of the STAT function. Cytokines bind their cognate cell surface receptors that are associated with subsets of JAK kinases (JAKs; JAK1, JAK2, JAK3, TYK2) to exert their biological effect. Cytokine-induced receptor dimerization causes the activation of JAKs, tyrosine phosphorylation of the cytoplasmic domains, and subsequent recruitment of various signaling proteins to the receptor complex (Ihle, 1995). The tyrosine-phosphorylated STATs form homo- or hetero-dimers and translocate into the nucleus, where they bind to their specific target sequences and regulate the expression of the cytokine-induced genes (Ihle,

1996; Darnell, 1997). Each cytokine selectively activates its own sets of STATs, although they are rather various due to stimulated cells. Thus, STATs family proteins are a kind of transcription factor as well as cytoplasmic mediators.

Three suppressive regulatory mechanisms for the above signaling have been documented (Kile et al., 2001). The first group of inhibitors is a family of SH2-containing protein tyrosine phosphatase (SHP1/2). Mice with a mutation in the SHP2-binding motif in gp130 showed prolonged activation of STAT3, suggesting that SHP2 negatively regulates STAT3 (Ohtani et al., 2000). The second is a protein inhibitor of activated STAT (PIAS) family proteins binding to dimerized STATs, therefore diminishing their DNA-binding activity (Chung et al., 1997; Liu et al., 1998). The third is a recently identified suppressor of cytokine signaling (SOCS/CIS) proteins (Krebs and Hilton, 2000; Yasukawa et al., 2000). To date, eight members of the SOCS family (CIS1, SOCS1, 2, 3, 4, 5, 6, 7) have been isolated and they contain a central SH2 domain and a C-terminal SOCS box (Fig. 1). A member of this family, CIS1, which was first cloned, inhibits the STAT5 phosphorylation by binding to the STAT5-docking sites, the autophosphorylation sites of tyrosine residues within the cytoplasmic region of cytokine receptor (R) such as interleukin-3 (IL-3) R, erythropoietin (Epo) R (Yoshimura et al., 1995). The second member of the SOCS family, SOCS1 (also called JAB or SSI-1) is induced by a variety of cytokines and growth factors. As SOCS1 binds to the kinase domain of JAKs, it suppresses phosphorylation and activation of STATs (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). Biochemical characterization has revealed that the interaction between SOCS1 and JAK2 consists of two independent bindings. One is the amino terminal kinase inhibitory region (KIR) binding to the catalytic groove of JH1 and the other is the SH2 domain binding to the phosphorylated tyrosine residue (Y1007) in the activation loop of JAK2 (Yasukawa et al., 1999). While SOCS2 and SOCS3 are also cytokine-induced STAT inhibitors, the SOCS family seems to regulate a wide range of signal transduction molecules such as insulin-like growth factor 1 receptor (IGF-1R) (Dey et al., 1998) or c-kit (De Sepulveda et al., 1999). However, function of other SOCS members has not yet been well understood. Knockout mice studies have confirmed the role of the SOCS family as a negative feedback regulator of cytokine-signaling in living animals. For example, SOCS1 proved to be a vital inhibitor for IFN-gamma signaling (Krebs et al., 2000; Yasukawa et al., 2000).

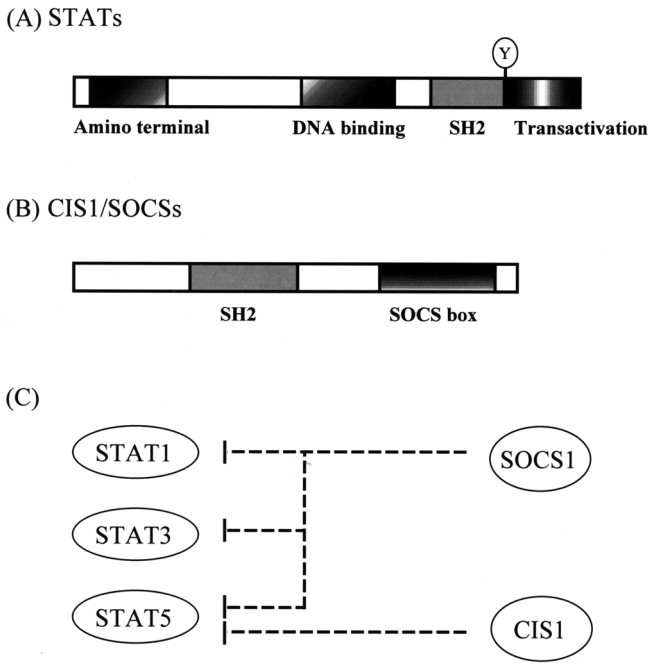

Fig. 1. A and B. Simplified diagrammatic representation of typical STAT and SOCS/CIS proteins. Functional domains are depicted. Y represents tyrosines whose phosphorylation is essential for the dimerization of STATs. **C.** Schematic representation of possible indirect inhibition of STATs by the SOCS family in cancer cells is shown.

Involvement of STAT in cancer development

A growing number of reports have shown that activated STAT molecules are functioning in cancer cells (Bowman et al., 2000). For example, constitutive activation of STAT1, STAT3, and STAT5 was observed in some human malignancies including leukemias, multiple myelomas, head and neck cancers, and breast carcinomas (Migone et al., 1995; Grandis et al., 1998; Catlett-Falcone et al., 1999; Garcia et al., 2001). In most of the cases, STAT activation was accompanied by constitutive phosphorylation of JAK1, JAK2, and/or JAK3. Aberrant activation of JAK is sometimes linked to chromosomal abnormality. TEL-JAK2 fusion proteins resulted from t(9;12)(p24;p13) chromosomal translocations and were observed in T-cell acute lymphoblastic leukemia (ALL), pre-B-cell ALL, and atypical chronic myelogenous leukemia (CML) (Schwaller et al., 1998). TEL is the ETS-variant gene 6 (ETV6), a transcription factor family that contains the pointed domain (PNT) mediating oligomerization of proteins. In TEL-JAK2, the JAK2 kinase domain is constitutively activated by PNT-induced oligomerization. These results suggest that JAK-STAT signaling may be involved in the oncogenic transformation of diverse cells.

It has been often reported that introduction of certain oncogenes into a variety of cells causes STAT activation. Yu et al. (1995) delineated that JAK1, JAK2 and STAT3 were constitutively activated in fibroblasts transformed by v-Src. Two groups clearly indicated that dominant negative (dN) STAT3 suppressed fibroblast transformation by v-Src, suggesting that activation of STAT3 is a critical step in v-Src-transformation (Bromberg et al., 1998; Turkson et al., 1998).

Furthermore, Bromberg et al. (1999) demonstrated that the constitutively active form of STAT3 could lead to cellular transformation by itself. In NIH 3T3 cells transformed with v-Src, aberrant activation of JAK1 and JAK2 was observed (Campbell et al., 1997; Iwamoto et al., 2000). Consistent with these observations, Zhang et al. (2000) recently showed that a cooperation of v-Src and JAK1 is required for full activation of STAT3 in NIH3T3 cells. On the other hand, forced expression of v-Abl in pre-B lymphocytes caused constitutive activation of STAT1 and STAT5. In this case, the activation of JAK1 through the direct interaction between JAK1 and v-Abl was essential for STAT activation (Danial and Rothman, 2000). However, JAK activation would be dispensable for activation of STAT1 and STAT5 in BCR-Abl-transformed hematopoietic cells (Carlesso et al., 1996; Ilaria et al., 1996). Thus, involvement of JAKs would not be necessarily required for the activation of STATs in transformation with some oncoproteins. Further studies using dNSTAT5 demonstrated that STAT5 is a key molecule in BCR-Abl-transformation (de Groot et al., 1999; Nieborowska-Skorska et al., 1999). More recently, Schwaller et al. (2000) showed that mice transplanted with bone marrow cells, which were infected with retroviruses containing constitutive active STAT5a gene, developed a fatal myeloproliferative disease.

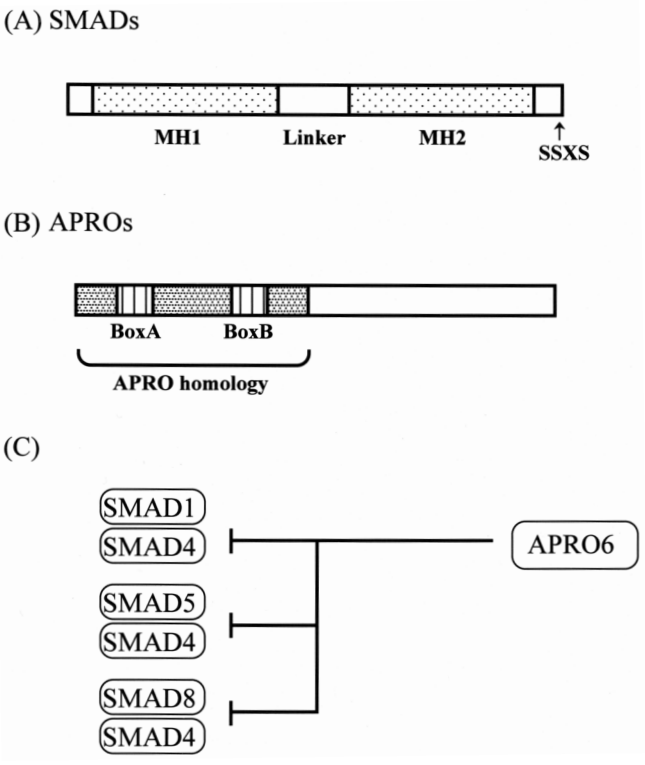

Fig. 2A and B. Simplified diagrammatic representation of typical SMAD and APRO proteins. Functional domains are depicted. Only the MH2 domain is conserved in the I-SMADs (SMAD6, SMAD7). SSXS at the carboxyl terminus indicates phosphorylation sites by receptor. **C.** Schematic representation of a possible direct inhibition of SMADs by APRO6 is shown.

SMAD structure and function

The family of SMAD molecules is a pivotal intracellular effector of transforming growth factor beta (TGF-beta) family signaling. To date, eight members of this family have been identified. SMADs are molecules of relative molecular weight 42-60 kDa with two homology regions at the amino- and the carboxyl-terminals termed Mad homology domain, MH1 and MH2 respectively, connected with a proline-rich linker sequence (Fig. 2). Experiments with truncation mutants indicated that both MH1 and MH2 domains of SMADs were necessary for the activity (Imai et al., 2001; Yuan and Varga, 2001). The MH1 domain may be involved in direct DNA binding in addition to its role as a repressor of the MH2 domain, which is suggested to be responsible for protein-protein interaction including homo- or hetero- oligomer formation of SMADs. Ligand-induced activation of TGF-beta family receptors with intrinsic serine/threonine kinase activity trigger phosphorylation of receptor-regulated SMADs (R-SMADs). The R-SMADs are anchored to the cell membrane by interaction with membrane-bound proteins, including SMAD anchor for receptor activation (SARA) (Tsukazaki et al., 1998). Whereas SMAD2 and SMAD3 (these are subclassified to AR-SMADs) are phosphorylated by TGF-beta and activin type I receptors, SMAD1, SMAD5 and SMAD8 (subclassified to BR-SMADs) act downstream of BMP type I receptors (Heldin et al., 1997). In the carboxyl terminal region, R-SMADs have a characteristic S-S-X-S motif, the two most carboxyl-terminal serine residues of which are phosphorylated by activated receptors. After the phosphorylation, R-SMADs form hetero-oligomeric complexes with common-partner SMAD (Co-SMAD), e.g. SMAD4. The oligomeric SMAD complexes then translocate efficiently into the nucleus, where they regulate the transcription of target genes by direct binding to DNA, in cooperation with other transcription co-factors, co-activators and co-repressors. Although transcriptional responses can result from direct SMAD binding to DNA, a more commonly functional interaction of SMADs with other factors is thus required. In many TGF-beta-regulated genes, SMAD-binding sequences are located adjacent to AP-1 recognition sites. Thus, some of SMAD family proteins are also a kind of transcription factor as well as cytoplasmic mediators. A third class of SMADs, inhibitory SMADs (I-SMADs, also called Anti-SMADs), acts in an opposite manner to R-SMADs and Co-SMADs. For example, SMAD7, a structurally and functionally divergent member of the SMAD family, forms a stable association with the activated TGF-beta receptor complex, thereby preventing phosphorylation of SMAD3 and blocking downstream TGF-beta signaling. Thus, SMADs fall into

three classes based on sequence similarity and function (Massague et al., 2000). Since expression of I-SMADs is induced by the TGF-beta superfamily proteins, SMADs constitute an autoinhibitory signaling pathway. In addition, SMAD activity seems to be intricately regulated by other signaling pathways such as the MAP kinase pathway. Recently, it has been reported that APRO6 (also called Tob: an antiproliferative gene product) is involved in the SMAD pathway in osteoblastgenesis, as described later. Furthermore, analyses by gene targeting revealed critical roles of SMADs in early embryogenesis, angiogenesis, and immune functions in vivo.

Involvement of SMAD in cancer development

TGF-beta has a multifunctional role in tumorigenesis. An idea supporting a tumor suppressive role for the type-II receptor of TGF-beta came from the analysis of an inherited form of colon cancer (Markowitz et al., 1995). It has been documented that TGF-beta is overexpressed in both benign papillomas and malignant squamous cell carcinomas (Go et al., 1999). In addition, it is suggested that activin receptor and SMAD pathways are activated and can regulate the activin response in breast cancer cells (Cocolakis et al., 2001). Moreover, loss of function of certain SMADs also seems to be involved in tumorigenesis, e.g., pancreatic and colorectal cancers. Certain SMADs are somatically mutated at high frequency in the particular cancers. At least two different SMADs, SMAD2 and SMAD4 (also called DPC4), have been implicated in the cancer and appear to have tumor-suppressor functions. In particular, loss of function of SMAD4 is most strongly associated with malignancy. Also, germline mutations in SMAD4 have been associated with juvenile polyposis syndrome, although loss of the SMAD4 allele was infrequently found (Howe et al., 1998). The MH2 domain is often the target for point mutations and frameshift mutations that lead to premature stops. These mutations may disrupt the structure of the protein then perturb the ability of SMAD4. Furthermore, the works from several different groups have shown that loss of SMAD4 expression is identified in various TGF-beta-resistant cancer cells, and that transfection of SMAD4 rescues the responsiveness to TGF-beta in these cells (Schutte et al., 1996; de Caestecker et al., 1997; Zhang et al., 1997). In these cases, loss of SMAD signaling in tumor cells may contribute to a change the TGF-beta responsiveness. SMAD3 has not been found mutated in human cancers. However, loss of SMAD3 function in mouse leads to metastic colon carcinomas (Zhu et al., 1998). During skin carcinogenesis, loss of SMAD1 through SMAD5 and overexpression of SMAD7 may contribute to tumor progression (He et al., 2001). These data clearly indicate that SMAD signaling is involved in some suppressive steps against cancer development. For one of the critical steps, loss of c-Myc repression mediated by Smad complexes seems a target of oncogenic signals in breast cancer (Chen et al., 2000).

Cell-growth regulation by SOCS or APRO

Accumulating evidence has shown that JAK inhibitors can inhibit cell growth and induce apoptosis of tumor cells in vitro and in vivo (Meydan et al., 1996; Nielsen et al., 1997), suggesting an idea that SOCS molecules might function as a tumor suppressor. Is SOCS1 a real tumor suppressor? To date, several groups have approached this issue. Kamizono et al. (2001) showed that introduction of SOCS1 into factor-independent Ba/F3 cell lines expressing TEL-JAK2 induces their apoptosis by inhibiting the JAK2 activity. Frantsve et al. (2001) further indicated that this inhibitory effect of SOCS1 also occurred on the cells in living mice. Interestingly, this inhibition was dependent on the SOCS box-mediated proteasomal degradation of JAK2 rather than on JAK2 kinase inhibition. Previously, it was demonstrated that SOCS proteins can associate with a complex containing elongins B and C (elonginBC) through their SOCS box, which was also conserved in proteins containing Ras, WD-repeat, ankyrin repeat and SPRY domain families (Kamura et al., 1988). The elongin BC complex binds to E3 ubiquitin ligase, cullin-2. Kamizono et al. demonstrated that SOCS1-induced degradation of JAK2 was cullin-2-dependent. Thus, SOCS1 seems to bind tyrosine-phosphorylated JAKs and suppresses the kinase activity and degrades JAK2 by recruiting ubiquitin ligase complex. Whereas these experimental data suggest that SOCS1 might work as a tumor suppressor in vivo, there has been no evidence suggesting physiological involvement of SOCS1 for suppressing tumor formation. However, Yoshikawa et al. (2001) recently reported that the CpG island within the promoter region of SOCS1 gene was frequently aberrant-methylated in human primary hepatocellular carcinomas (HCCs), in which JAK2 and STAT3 were constitutively activated. Furthermore, introduction of the SOCS1 gene into these cells suppressed their proliferation as well as anchorage independent cell growth. These data suggested that the silencing of SOCS1 might be involved in tumor progression in HCCs by eventual activation of JAK/STAT signaling. Tauchi et al. (2001) have recently reported that CIS1 suppresses the BCR-Abl-mediated transformation via the STAT5 pathway. Collectively, these results suggest a possibility that the SOCS protein family might physiologically inhibit tumor proliferation (Fig. 1c). However, we found that SOCS1 does not inhibit v-Src-induced JAK/STAT activation in NIH3T3 cells (Iwamoto et al., 2000). Zhang et al. (2000) demonstrated a hypothetical model where JAK1 is initially phosphorylated by v-Src, following subsequent phosphorylation of membrane receptors by JAK1 to recruit STAT3, where v-Src directly phosophorylates STAT3. On the other hand, the auto-phosphorylation of JAKs is believed to happen due to the receptor-oligomerization by cytokine stimulation or by fusing

with TEL. We presume that SOCS1 might preferentially suppress auto-phosphorylation of JAKs rather than their transphosphorylation by v-Src. Together, the SOCS protein family would provide valuable clues to regulate aberrant proliferation of certain malignant cells.

I-SMADs are known as general inhibitors for the SMAD pathway. Recently, a new member of inhibitors, APRO6/Tob, has emerged (Yoshida et al., 2000). The APRO6 was primarily detected as a molecule associating to ErbB2 receptor tyrosine kinase (Matsuda et al., 1996). Now, six distinct proteins of the APRO family have been identified in human cells (Matsuda et al., 2001). Several experiments have shown that the forced expression of these genes (APRO1, APRO2, APRO3, APRO4, APRO5, and APRO6) was significantly antiproliferative for the cells (Bradbury et al., 1991; Fletcher et al., 1991; Rouault et al., 1992; Guehenneux et al., 1997; Ikematsu et al., 1999). The schematic structure of the APRO is shown in Figure 2. An APRO homology domain in the amino-terminus can be divided into two short, relatively more conserved elements, box A and box B, separated by a spacer sequence of less conserved amino acids. Both APRO5 and APRO6, but not the others, contain a predicted nuclear localization signal in this region. As the APRO gene family often contains some copies of the ATTTA motif, which is known as a common determinant of the RNA stability in mammalian cells, the half life of APRO molecules seems short in vivo. Recently, APRO6 has been shown to act as a negative regulator of SMAD signaling in osteoblasts (Yoshida et al., 2000). The authors showed that APRO6 associated with receptor-regulated SMAD1, SMAD4, SMAD5 and SMAD8, and colocalized with these SMADs in the nuclear bodies after bone morphogenetic protein (BMP) stimulation. MH2 plus linker region of SMADs is responsible for the interaction with APRO6. Furthermore, APRO6 represses ligand-induced SMAD-dependent transcriptional activation. The data clearly suggest that APRO6 is a novel inhibitor of BMP/SMAD signaling. This notion implies an attractive idea that the other members of the APRO family are also involved in SMAD signaling. This is an urgent issue to be clarified.

Perspective of STAT and SMAD pathways in cancer biology

How do STAT molecules promote cancer? Bowman et al. (2001) indicated that STAT3-mediated Myc expression is a key step for v-Src-induced transformation. However, the signaling pathway regulated by STATs is becoming diverse. For example, we recently demonstrated that one of the Ras effectors, RalGDS, is induced by IL-6 in a STAT3-dependent manner and that cooperation of Ras and JAK/STAT3 is necessary for full activation of Ral (Senga et al., 2001). Interestingly, it was reported that Ral enhanced the transforming activity of H-Ras and Raf, suggesting a possible involvement of JAK/STAT3/RalGDS/Ral in several different steps of oncogenesis (Urano et al., 1996). Another crosstalk between JAK/STAT and Ras signaling was previously documented by Turkson et al. (1999). They showed that serine phosphorylation of STAT3, which is essential for Src-transformation, is dependent on the Ras activation. Furthermore, it was recently delineated that STAT3 and c-jun cooperatively suppress the expression of Fas in melanoma cells (Ivanov et al., 2001). Thus, intertwined synergy between JAK/STAT and other signaling pathways should contribute the development of a cancer.

As for the SMAD pathway, although SMAD proteins are important elements in the TGF-beta superfamily receptor signaling, it is not the sole pathway activated by the receptor complexes. In other words, the TGF-beta superfamily activates different signaling pathways in addition to the SMADs. For example, TGF-beta itself can activate TGF-activated kinase (Yamaguchi et al., 1995), a member of the MAPKKK family of kinases, which then activates the stress-activated kinase p38 and the transcription factor ATF2, a member of the basic-ZIP family of DNA binding proteins. In vitro studies suggested that the transcription factor ATF2 could interact with the MH1 domains of two activin-responsive SMAD3 and SMAD4 (Hanafusa et al., 1999). In addition, TGF-beta correlates with SMAD4-independent inhibition of MAP kinase (ERK) activation (Giehl et al., 2000). Furthermore, both TGF-beta and the Mullerian inhibiting substance (MIS) were also shown to mediate some of their biological effects through an NFkB-mediated pathway in addition to the SMAD activation (Sovak et al., 1999; Segev et al., 2000). TGF-beta also induces growth inhibition by upregulation of the cyclin-dependent kinase-inhibitor, p15, in certain epithelial cells (Hannon and Beach, 1994). Thus, TGF-beta receptors intricately propagate signals downstream through direct interaction with cytoplasmic SMADs, and possibly other proteins as well. Recent studies have revealed that the involvement of SMADs in cancer is also intricate. Several transcriptional factors such as AMLs involved in cancer have been shown to interact with SMADs (Hanai et al., 1999). The binding arises from the MH2 domain of SMADs and the complexes may function in the TGF-beta pathway. However, the physiological significance remains to be better clarified.

Interestingly, recent studies have revealed that the complicated STAT and SMAD pathways are eventually connected. It was suggested that the ciliary neurotrophic factor and TGF-beta synergistically induce pathway-specific transcription through stimulating a novel combination of SMAD and STAT protein (Pitts et al., 2001). Although CNTF and TGF-beta stimulate different signaling pathways to activate gene expression, it is likely that crosstalk between these pathways exists. Moreover, these transcription factors seem to be able to form transcriptional complexes which are more active when activated as a STAT-SMAD supercomplex than when activated each alone. Recently, evidence for the nuclear integration of TGF-beta and IFN-gamma

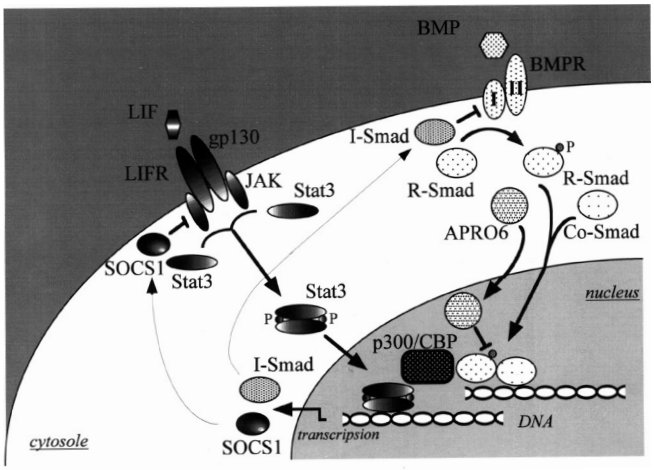

Fig. 3. Schematic representation of the STAT and SMAD signaling pathways. The signaling components involve a family of molecules in some cases. The signaling system is indicated along with the sites of activation of various positive and negative regulators. Various other molecules also participate in the described pathways including MAPK, SARA, Maf, FKBP12, Myc and so on, although these are not shown because of a terseness-figure and space-limitation.

signaling by sharing cofactor p300/CBP has been presented (Ghosh et al., 2001). STAT1 employs p300/CBP to bring about the effects on transcription (Horvai et al., 1997). Interaction between STAT1 and p300/CBP was observed in a ligand-independent fashion, indicating that both unphosphorylated monomeric, and phosphorylated dimeric STAT1 can interact with p300/CBP. Furthermore, it has already been shown that SMAD proteins interact directly with the p300/CBP (Feng et al., 1998; Janknecht et al., 1998). Likewise, p300/CBP is implicated in the synergistic interaction between LIF and BMP2 for induction of the GFAP in fetal neuroepithelial cells through bridging STAT3 and SMAD1 (Ulloa et al., 1999). The bottom line is that p300/CBP might act as a bridging molecule to mediate the synergy. However, a different form of crosstalk between STAT and SMAD pathways has been shown (Nakashima et al., 1999). The authors suggested that IFN-gamma induced the expression of SMAD7 through JAK/STAT pathway. As SMAD7 is a repressor of TGF-beta signaling, these data probably explain the opposing effects between STAT and SMAD pathways.

Taken together, STAT and SMAD signaling pathways crosstalk each other with interweaved regulatory mechanisms (Fig. 3). By modulating those pathways, SOCS and APRO play an important role in cell growth regulation. Indeed, further investigation of these combinatorial actions may bring us novel findings that are useful for understanding a molecular basis of cancer development.

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