Transcription co-activator SAYP mediates the action of STAT activator

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ABSTRACT

Jak/STAT is an important signaling pathway mediating multiple events in development. We describe participation of metazoan co-activator SAYP/PHF10 in this pathway downstream of STAT. The latter, via its activation domain, interacts with the conserved core of SAYP. STAT is associated with the SAYP-containing co-activator complex BTFly and recruits BTFly onto genes. SAYP is necessary for stimulating STAT-driven transcription of numerous genes. Mutation of SAYP leads to maldevelopments similar to those observed in STAT mutants. Thus, SAYP is a novel co-activator mediating the action of STAT.

INTRODUCTION

Signaling pathways in metazoans orchestrate complex developmental events. This process usually requires an elaborate transcription machinery controlling the expression of multiple genes. One of important pathways having a role in all metazoans is the Jak/Stat pathway. It has multiple functions, being responsible, in particular, for germ-cell function, morphogenesis and patterning, as well as for cell differentiation and proliferation (1). The final effector of the pathway is the family of STAT transcription activators (2). From a researcher’s standpoint, Jak/Stat in Drosophila has an advantage of being simple: it consists of unique receptor dome, Janus kinase hop, and transcription factor STAT92E (below, referred to as STAT).

The recruitment of STAT onto chromatin occurs in cooperation with other factors (3). An important role in mediating the function of STAT in transcription activation is played by its C-terminal portion carrying the activation domain (3,4). Reliably identified co-activators for the STAT family are histone-modifying acetyltransfases CBP/p300 (5,6), the GCN5-containing complex (7) and chromatin-remodeling factor Brahma (8–11). In addition, some novel components of the pathway have been revealed among transcription factors (12,13). In particular, transcription factors Brahma, TFIID and SAYP have proved to be positive regulators of the pathway. SAYP was previously described as a transcription co-activator-mediating gene activation via a novel mechanism, by coupling chromatin remodeler Brahma and transcription initiation factor TFIID into one co-activator complex BTFly (14). SAYP is a conserved factor in metazoans. Its vertebrate homologue, named PHF10, shares with SAYP a conserved core consisting of the SAY domain an two PHD fingers (15).

Here, we describe the participation of SAYP in mediating STAT-driven transcription activation. Mutation in the gene encoding SAYP manifests itself similarly to those in the Jak/Stat pathway. Both SAYP and STAT co-occupy multiple loci in the genome. We have demonstrated the association of STAT with the SAYP-containing complex and revealed the domains mediating this interaction. The presence of SAYP is important for activation of STAT-dependent genes. As shown by ChIP analysis, SAYP is recruited onto STAT-dependent genes together with Brahma and TFIID.

MATERIALS AND METHODS

Experiments with S2 cell culture

Schneider cell line 2 (S2) of Drosophila were maintained at 25°C in Schneider’s insect medium (Sigma) containing 10% FBS (HyClone). Conditions optimal for activation

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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of STAT were determined experimentally. Pervanadate solution (PV) was prepared from sodium vanadate and hydrogen peroxide and then treated with catalase. Cells were treated with 100 µM PV for 2 h (for measuring mRNA level) or 30 min (for ChIP).

DNA fragments encoding SAYP with 3×FLAG epitope and STAT (form F, 761 amino acids) with HA epitope were cloned into pAc5.1/V5-HisB vector (Invitrogen). The cell line stably expressing tagged SAYP was established as described (14).

Antibodies and western blot analysis

Antibodies used in this study were described previously (14,16). Antibodies against STAT (261–456 amino acids fragment of form F) were raised in rabbits and affinity purified. These and other antibodies raised in our laboratory were used in a 1:500 dilution for western and in an amount of 5 µg for immunoprecipitation. Antibodies against fasciclin III, (obtained by C. Goodman) and beta-tubulin (obtained by M. Klymkowsky) were from the Developmental Studies Hybridoma Bank.

Genes expression analysis by reverse transcription–PCR

The following STAT-dependent genes were chosen for analysis: SOCS3/6E (17); dm [(Drosophila homologue of vertebrate STAT-dependent c-myc (18)]; buffy and debcl [Drosophila homologues of vertebrate STAT-dependent bcl-2 (19)]; slbo (20); eve (21); dpp (22); apontic (23); and DIAP1 (24).

For measuring gene expression, RNA was extracted with Trizol (Ambion) from four pairs of ovaries or 3×10⁶ S2 cells and treated with DNase I. Reverse transcription (RT) was performed from random hexanucleotide primers and measured by qPCR. The sequences of the primers are given in the Supplementary Data. As a reference, we used the levels of actin and histone H1 mRNAs, which were stable upon PV treatment.

ChIP and Quantitative (q) PCR Analysis

The protocol for ChIP with S2 cells was described previously (25). As a negative control, measurements at rDNA and ChIP with nonspecific antibodies were used in each experiment, the signal in the latter case being at least 10 times weaker than in the former. The sequences of the primers are given in the Supplementary Data.

Immunostaining

Ovaries were stained as described (16). Polytene chromosomes were stained with rat anti-SAYP, rabbit anti-STAT, and the corresponding secondary antibodies (Molecular Probes) following the procedure described previously (16), fixation with 4% FA was performed for 2 min. Salivary glands were treated with 500 µM PV for 1 h.

Gel filtration of nuclear extract and immunoprecipitation

Preparation of the nuclear extract from Drosophila embryos, gel filtration, and immunoprecipitation were performed as described (14).

Drosophila genetic crosses

Cultivation of flies and genetic crosses were described previously (26). Females y+w+/e(y)3u1/FM4 and males carrying Stat92E mutation were selected for crossing. The mutation was caused by P-element insertion in the line 11681 (ry306 P(ry;+;7.2 = PZ)Stat92E68344/TM3, ry171 SbI Ser). All genetic crosses were carried out at 25°C and repeated no less than three times. At least 50 flies of each viable genotype were screened for each strain.

RESULTS

Phenotypic manifestations of SAYP mutation

To check the possibility of SAYP participation in signaling pathways, we thoroughly analyzed the phenotype of flies with the e(y)3u1 mutation in the gene encoding SAYP (16). The main molecular manifestation of this hypomorphic mutation was a lower level of e(y)3 transcripts. Initially, e(y)3u1 was reported to suppress the expression of yellow2 allele in bristles (27,28). All flies homozygous for e(y)3u1 had lower viability, homozygous females were sterile, hemizygous males had a characteristic bent-leg phenotype and early embryos showed defects in cell cycle progression (14–16). In addition, the presence of ectopic longitudinal veins was revealed in the posterior wing blade (Figure 1A). Such a phenotype was observed in all flies carrying the e(y)3u1 allele in either homo- or hemizygous state. It is noteworthy in this context that STAT also regulates wing venation, with the hypomorphic mutation Stat92E06346 resulting in the formation of similar ectopic longitudinal veins (29).

SAYP is abundant in various cells of the growing ovary, and females homozygous for e(y)3u1 are sterile (16). We checked the possible source of female sterility by inspecting the structure of ovaries in mutant flies. The ovary of an adult female consists of ovarioles, each representing an assembly line of developing ovarian follicles (egg chambers). Each follicle is covered by a monolayer of follicular cells and contains a pair of specific cells located at its anterior and posterior poles, named polar cells; adjacent follicles are connected by a column of stalk cells (30). Until Stage 2, epithelial follicular cells in the egg chambers express high levels of Fasciclin III (Fas3) (31), which marks immature proliferating follicular cells in the germarium. Progressive down-regulation of Fas3 takes place in the vitellarium after Stage 2, with the level of its expression in the stalk and polar cells remaining high.

Different degrees of disturbance were revealed in e(y)3u1 mutants. A mild mutant phenotype was characterized by maldevelopment of follicular cells: the number of Fas3-positive cells was increased, and they were detected in follicles of the vitellarium until stage 4–5 (Figure 1B). Moreover, they appeared to retain their proliferative potential, as follows from the
fact that egg chambers with several layers of follicular cells and aggregates of these cells were detected (Figure 1C and D). In extreme cases, we observed the formation of fused follicles (cysts) at different stages of development, with epithelial cells within the cysts often showing disorganized, invasive growth (Figure 1D).

Jak/Stat plays an important role in follicle cells differentiation, and a similar phenotype of fused egg chambers
has been reported for flies with hypomorphic mutations of hop (Janus kinase in Drosophila) and STAT92E (32,33).

Finally, we checked the effect of combining of mutation in e(y)3 and Stat92E. We observed that amorph Stat92E1758,1760 in a heterozygous state enhances the phenotypic expression of the e(y)3 mutation, which is manifested in almost complete suppression of yellow2 in bristles and increased frequency of flies with the bent femur phenotype (Figure 1E). Stat92E is expressed at the stage of pupae and in larval imaginal discs (34,35) and, therefore, may have a role in the development of this phenotype.

Thus, we have found that mutations in the genes encoding SAYP and components of the Jak/Stat pathway manifest themselves similarly. However, the described phenotypes are not unique for this pathway, being also manifested in cases of mutations in other cascades (36–38). Moreover, the cross-talk of pathways in development (39) does not allow any unequivocal conclusion concerning the participation of SAYP in the Jak/Stat pathway. That is why we further checked the STAT–SAYP interaction at the molecular level.

**STAT interacts with SAYP**

To confirm the interaction of SAYP with STAT biochemically, antibodies against STAT were raised in rabbits, affinity purified, and their specificity was checked on both endogenous and recombinant tagged STAT (Figure 2A).

Gel filtration of the nuclear extract from Drosophila embryos showed that the elution profiles of SAYP and STAT partially overlapped. SAYP migrated in fractions 16 and 17 (Figure 2B) as a component of high-molecular-weight protein complex BTFly including also Brahma and TFIID (14). A significant proportion of STAT was also found in these fractions. To confirm STAT–SAYP association in fractions 16–17, a co-immunoprecipitation experiment was performed. The results showed that anti-SAYP antibodies co-precipitated STAT, and vice versa (Figure 2C). Moreover, antibodies against STAT were capable of co-precipitating the components of Brahma and TFIID (Figure 2D), although to a lesser extent than SAYP.

To verify the SAYP–STAT interaction, both proteins with tags were co-expressed in cell culture. HA-tagged STAT was able to co-precipitate Flag-tagged SAYP, and vice versa (Figure 2E). It is noteworthy that the strength of this interaction was lower than that of SAYP–BAP170 and SAYP–TAF5 interactions in the stable BTFly complex (14), as could be expected for a transient activator–co-activator interaction.

To further study this interaction, we mapped the interacting domains within the proteins. Flag-Gal4-tagged separate domains of SAYP (described in ref. 14) and the HA-tagged C-terminal portion of STAT carrying the activation domain (AD) of STAT (40) were co-expressed in S2 cells (Figure 2F). As a result, a fairly strong interaction of STAT AD with the conserved SAY-PHD fragment of SAYP was revealed (Figure 2G).

We have previously demonstrated that the SAY domain interacts with TFIID and Brahma (14). However, the STAT AD has shown no comparable interaction with the subunits of endogenous TFIID and Brahma. Therefore, these factors are unlikely to mediate the observed STAT AD–SAY-PHD interaction. A relatively strong association of the two overexpressed proteins indicates that they probably interact with each other directly, although we cannot exclude that some other protein mediates their interaction in the lysate.

Thus, a portion of STAT is associated with the SAYP-containing protein complex, and STAT, via its activation domain, can interact with the conserved core of SAYP.

**SAYP and STAT jointly control numerous genes**

We assessed STAT–SAYP co-localization in the genome using preparations of polytene chromosomes from salivary glands. Both hop and STAT are expressed in this organ in larvae (35), indicating that the corresponding pathway is active. Co-immunostaining of chromosomes with antibodies against STAT and SAYP revealed a significant co-localization of these factors, although not complete (Supplementary Figure S1A). To further activate STAT in the salivary glands, they were treated with pervanadate (PV); in this case, the factors showed an even higher degree of co-localization (Figure 3, Supplementary Figure S1B). These results showed that STAT was present in multiple loci of euchromatin, which were often co-occupied by SAYP.

To directly check the importance of SAYP for the activity of STAT-dependent genes in vivo, we used the ovaries of adult flies, in which the Jak/Stat pathway is active in many cells (32). Several genes were chosen which are known to be STAT-dependent (see ‘Materials and Methods’ section). Measurements of the level of gene expression showed that the reduced content of SAYP in hypomorphic e(y)3 mutants led to a drop in the expression of the STAT-dependent genes (Figure 3B). Importantly, the level of hop and STAT expression in these mutants was not reduced, indicating that SAYP acted downstream of STAT in the pathway.

We conclude that STAT and SAYP occur together on multiple sites genome-wide, with the presence of SAYP being important for the activity of STAT-dependent genes in the organism.

**SAYP is recruited onto STAT-driven genes**

To further investigate activation of STAT-dependent genes, we used Schneider (S2) cell line expressing the components of the Jak/Stat pathway (41). Western blot analysis confirmed that the level of the STAT protein (relative to the levels of tubulin and TBP) in these cells was markedly higher than in embryos (Figure 2A), while the embryo in Drosophila is the stage characterized by the highest STAT activity (35). Therefore, S2 cells are an appropriate model for studying the functioning of the pathway of interest. Treatment of S2 cells with PV, which causes the accumulation of phosphorylated STAT in them (42,43) is used to study short-term gene activation.
Using this approach, we found that STAT-driven transcription took place in the untreated cell line and that PV treatment moderately stimulated STAT-dependent gene expression (see below).

The PV treatment for 2 hours resulted in induction of STAT-dependent genes, with their expression in S2 cells increasing several fold (Figure 4A) but the levels of STAT, SAYP, and BTFly components remaining unchanged (Supplementary Figure S2). To study the role of SAYP in this process, we changed its content in cells by either its RNAi knockdown or 5-fold overexpression (Supplementary Figure S3). The relative level of induction of STAT-dependent genes by PV proved to be the highest in cells overexpressing SAYP and the lowest in SAYP knockdown cells (Figure 4, Supplementary Figure S4). It should be noted that the observed changes in gene

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**Figure 2.** STAT is associated with SAYP-containing complex. (A) Western blot analysis of total proteins from embryos or S2 cells with antibodies against STAT. To confirm their specificity, lysate of S2 cells expressing HA-tagged STAT was stained with anti-HA antibodies. Staining for tubulin and TBP is shown as a loading control. (B) Fractions after gel filtration of DNase I-treated nuclear extracts from embryos on Superose 6 were analyzed for the presence of SAYP and STAT proteins. STAT was detected not only as a free protein (fractions 16–17) but also in association with high-MW complexes, together with SAYP (fractions 16–17). (C) STAT and SAYP co-immunoprecipitated with each other from gel filtration fractions 16–17 of the extracts from embryos. IP with pre-immune IgG was used as a negative control. Equal portions of the input (In) and precipitated (IP) material were tested. (D) Antibodies against STAT co-immunoprecipitated MOR (component of Brahma) and TAF1 (component of TFIIID) from gel filtration fractions 16–17. (E) Recombinant Flag-SAYP and HA-STAT co-immunoprecipitated with each other. Both proteins were co-expressed in cells, then IP with antibodies against either FLAG or HA was performed. In control IP, only one recombinant protein was expressed. Equal portions of the input (In) and precipitated (IP) material were tested. (F) Scheme of SAYP and STAT proteins. Boundaries of the tested regions and the total protein length (number of amino acids) are indicated. (G) Recombinant Flag-Gal4-fusions of SAYP fragments and the HA-tagged activation domain of STAT were tested for co-immunoprecipitation (the left column). Both proteins were co-expressed in cells, then IP with antibodies against HA was performed. In control IP, only Flag-tagged recombinant protein was expressed. Recombinant Flag-SP fragment co-immunoprecipitated HA-AD of STAT and HA-STAT (the right column). Equal amounts of the input (In) and precipitated (IP) material were tested.
activation were not due to changes in the expression of hop and STAT in the cells (Supplementary Figure S3). Thus, SAYP has a positive effect on short-term induction of STAT-dependent genes in S2 cells.

We then directly checked whether SAYP is recruited onto STAT-dependent genes upon their activation by ChIP. After the PV treatment, an increase was observed in the contents of not only STAT but also of SAYP, TFIID and Brahma components and PolII on the promoters of several genes studied (Figure 4B).

To confirm the positive role of SAYP in this process, the recruitment of the above factors was measured upon SAYP knockdown. The results showed that the recruitment of STAT was not impaired, while the contents of TFIID, Brahma and especially PolII on the promoters dropped significantly. As shown in our previous study (14), SAYP knockdown did not affect the total content of TFIID and PolII but reduced the content of Brahma in the cells. Testing of the control promoters of housekeeping genes hsp70 (14) and actin (Supplementary Figure S5) upon SAYP knockdown showed that the recruitment of TFIID and PolII was not affected, while the level of Brahma was reduced to a lesser extent than on STAT-dependent genes. The increase of STAT signal after SAYP knockdown may be explained by higher accessibility of the STAT protein to antibodies on the promoter without co-activators.

The presence of SAYP and BTFly components on STAT-dependent genes was also checked by ChIP in early embryos (0–6 h), in which STAT is expressed at

**Figure 3.** SAYP controls multiple STAT-dependent genes. (A) Immunostaining of polytene chromosomes with antibodies against STAT and SAYP shows significant co-localization of these factors. (B) The levels of expression of different genes in the ovaries of homozygous e(y)3BR1 mutants relative to those in wt flies, with the ratio for actin mRNA taken as 1. CG11400 is a SAYP-dependent gene (14) shown as a control.

**Figure 4.** SAYP is important for STAT-driven response in S2 cells. (A) The relative induction of different genes expression (the ratio of levels of expression after and before 2-h PV treatment) in normal S2 cells and cells with SAYP overexpression and SAYP knockdown. (B) The levels of different factors on promoters of genes before and after PV treatment in normal cells and cells with SAYP knockdown. The results of ChIP are shown as the percentage of input, gray staining shows the levels of the factors on rDNA.
a high level (35). Since STAT was recently shown to be important for activation of numerous genes in the early Drosophila embryo (44), we checked several known targets for this protein. The contents of STAT and SAYP were found to be elevated relative to those on the promoters of housekeeping genes (Figure 5A).

Thus, SAYP is recruited onto STAT-dependent genes and has a role in inducing their expression. Its recruitment is also important for subsequent engagement of other co-activators and PolII.

DISCUSSION

We have analyzed the role of co-activator SAYP in the Jak/Stat pathway in Drosophila and found that SAYP interacts with STAT and mediates its activation potential (Figure 5B). SAYP operates as a component of large protein complex BTFly, which also contains Brahma and TFIID, and mediates subsequent recruitment of PolII onto the promoter (14). The results of polytenic chromosome staining and measurement of multiple STAT-dependent genes testify to genome-wide cooperation of STAT and SAYP in gene activation.

The activation domain of STAT interacts with the SAY-PHD fragment of SAYP within the BTFly, and the STAT–BTFly association is not mediated by Brahma or TFIID. It is noteworthy that the interacting fragment of SAYP belongs to its conserved core, which is also found in vertebrate homologues of SAYP (16). Therefore, the above interaction may also take place in other species.

This finding broadens the known spectrum of transcription factors mediating the effect of STAT on gene expression. Such a diversity of cooperating factors appears to provide a basis for the specificity and strength of Jak/Stat-driven response in different cell types. In particular, BTFly may serve for rapid induction of transcription, as proposed previously (15). Indeed, we have observed the positive effect of SAYP content on short-term induction after STAT activation. SAYP is also important for PolII stalling (N.E.V., personal communication), which may provide for the precision of responses of target genes in the signaling pathway (44,45). One more point of interest is the putative role of SAYP in coordinating the crosstalk between Jak/Stat and other signaling pathways, which follows from the fact that SAYP is also involved in the ecdysone cascade (46) and probably in some other cascades (15).

Mutation of both STAT and SAYP leads to formation of excess numbers of ovarian follicular cells and ectopic wing veins. As shown in our previous study (15), SAYP is abundant in cells with high proliferative potential, and its mutation results in overproliferation of polar cells in embryos. Therefore, SAYP may participate in regulation of proliferation of certain cell types. Interestingly, PHF10—a vertebrate homologue of SAYP—is required for proliferation of stem/progenitor cells (47) and fibroblasts (48). On the other hand, it has been shown that STAT also has a role in proliferation and growth control (49). Therefore, it appears that SAYP and STAT are jointly involved in regulation of proliferation and differentiation of certain cell types during metazoan development.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–5 and Supplementary Materials.

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