Transcriptional Regulation of the Drosophila raf Proto-oncogene by Drosophila STAT during Development and in Immune Response*

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The Drosophila raf (D-raf) gene promoter contains a recognition consensus sequence for Drosophila STAT (D-STAT). By band mobility shift assay, we detected a factor binding to the D-STAT-recognition sequence in extracts of cultured Drosophila cells treated with vanadate peroxide. UV-cross-linking analyses suggested the size of the binding factor to be almost same as that of D-STAT. Furthermore, the binding activity was increased in cells cotransfected with HOP and D-STAT expression plasmids. These results strongly suggest that D-STAT binds to the D-STAT recognition sequence in the D-raf gene promoter. Transient luciferase expression assay using Schneider 2 cells indicated that the D-raf gene promoter is activated by D-STAT through the D-STAT-binding site. Furthermore, analyses with transgenic flies carrying Draf-lacZ fusion genes with and without mutations in the D-STAT-binding site pointed to an important role in D-raf gene promoter activity throughout development. We also found that the D-STAT-binding site is required for injury-induced activation of the D-raf gene promoter. Here we propose that D-STAT can participate in regulation of the mitogen-activated protein kinase cascade through D-raf gene activation.

The mitogen-activated protein kinase (MAPK)1 cascade, activated in response to a variety of ligands, is highly conserved among eukaryotic organisms including yeast, Drosophila, and mammals (1–3). Raf, a constituent of the MAPK cascade, belonging to a family of serine/threonine protein kinases, acts as an important mediator of signals between upstream tyrosine kinases and downstream serine/threonine kinases in regulation of cell proliferation, differentiation, and development (4, 5). D-raf, a Drosophila homolog of the human c-raf-1 gene, has been cloned, and mutants defective for this gene have been identified (6). Through analysis of the D-raf mutant phenotypes, it was found that D-raf functions in regulation of cell proliferation, as does mammalian c-raf-1, and in the determination of cell fates at embryonic termini (6–8). D-raf is expressed throughout development in a wide range of tissues with high levels in tissues containing rapidly proliferating cells (7, 8). Although multiple roles for D-raf in the regulation of cellular proliferation and differentiation have been demonstrated, little is known about the mechanisms controlling D-raf gene expression. In previous studies, we showed regulation by the DNA replication-related element (DRE/DRE-binding factor) (DREF) regulatory system (9), which appears to be of general importance for DNA replication- (10, 11), cell cycle- (12), and proliferation-related (9) genes in Drosophila. Furthermore, the D-raf gene is probably another target of the Zerknüll homeomain protein-like DNA replication-related genes, such as DNA polymerase α and proliferating cell nuclear antigen (9, 13, 14).

In addition to DRE, the D-raf gene promoter contains a Drosophila signal transducer and activator of transcription (D-STAT) recognition consensus sequence, 5′-TTCNNNGAA (15). The transcription factor STAT is known to be activated by the Janus kinase (JAK) in response to a variety of cytokines, growth factors, and interleukins in mammals (16, 17). In Drosophila, a single JAK encoded by the gene hopscotch (hop) and STAT encoded by the gene marello, also known as STAT92E or D-STAT, have been characterized (18–20). Both have high homology with their mammalian counterparts (18–20). Regarding D-STAT as an activator of transcription, only the pair rule gene even-skipped (eve) has been identified as a target (20).

In this study, we examined the role of D-STAT in regulation of the D-raf gene promoter. The obtained results indicate that the D-raf gene is a target of D-STAT activated by HOP and suggest the possibility of participation in regulation of the MAPK cascade.

EXPERIMENTAL PROCEDURES

Oligonucleotides—The sequences of double-stranded oligonucleotides containing the D-STAT recognition sequence in the D-raf gene (Draf/STATwt) and its two base-substituted derivatives (Draf/STATmut1 and Draf/STATmut2) were described previously (15). The oligonucleotide ODBS (optimum D-STAT-binding site) determined by sequence selection was prepared as described previously (20). The oligonucleotides eveS1 and eveS2 containing D-STAT-binding sites in the even-skipped (eve) gene stripe 3 promoter region were described as earlier (21). The following double-stranded oligonucleotides contained a 6-base pair linker sequence recognizable by BglII and BamHI and were chemically synthesized. The 2×ODBSwt, 2×Draf/STATwt, and 2×Draf-
D-STAT Is a Transcriptional Regulator of the D-raf Gene

STATmut1 oligonucleotides used for luciferase reporter constructs were as follows: 2×ODBSwt, 5′-gatcGGTTTTTTTGCAGAAAATGCTGGAGAATTTTTCCGGGAATTGTGGCa, 3′-gCCTAAAAAGGGGCTTACAGGCTAAAGGGCGGCTTTACACCCGGAAGGTTACTACAGGAAAT/CTGGGAAAATGCCCGCTTTTGGTTAATTCGCGGAAAGTAAATAAA TTGTTATAGC; PR-DrafACTATAACAATTTAT.

To amplify a sequence containing full amino acids of D-STAT (amino acid 1–761), a set of PCR primers were synthesized: 5′-spec oligonucleotide (5′-GGGCCGGATCCGCGAGCATGAGCTTGTGGAAGCGC) and 3′-spec oligonucleotide (5′-GACAAGCTGTGACGCGTCGCC). The UV-cross-linking assay, single-stranded oligonucleotides containing DrafSTAT sites were chemically synthesized, the oligonucleotides as follows: UV-Draftstat, 5′-TAAAAAATTCGGCGAAAGATTAAATATTCCGGCGAAGATTAGa, 3′-ATTATTAGCGGCTTTAGTTAATTAGTCGCGGAAAGTAAg, 5′-gATTTTAAGCTCGCGGAAAGTAAATAAAATTCGCGGAAAGTAAg, and 3′-gCCTAAAAAGGGCCTTTACCAGCC-TTTTTTCCCGGAAATGGTCGGA-

FIG. 1. Structure of the 5′-upstream region of the D-raf gene and base-substituted mutants in D-STAT recognition sequence. The transcription initiation site is indicated by the arrowhead and numbered as +1. Relative locations of each site are indicated by numbers. The open box represents the DRE sequence, and the closed box represents the D-STAT recognition site in the D-raf gene promoter. The nucleotide sequences of the DrafSTAT site and its base-substituted mutants are shown in boxes below with lowercase letters for substituted nucleotides.

formed as described earlier (32). The molecular weights of the protein bands were estimated by comparing their mobilities with those of prestained marker proteins (Bio-Rad): myosin (200,000), β-galactosidase (116,500), phosphorylase b (106,000), bovine serum albumin (80,000), ovalbumin (49,500), carbonic anhydrase (32,500), soybean trypsin inhibitor (27,500), and lysozyme (18,500).

Establishment of Transgenic Flies and Quantitative Measurement of β-Galactosidase Activity in Extracts—To establish transgenic flies carrying p5′-663Draf-DrafSTATmut1-lacZ, P element-mediated germ line transformation was carried out as described earlier (33, 34). Quantitative measurements of β-galactosidase activity in larval extracts were carried out as described previously (34). In this study, the transgenic flies carrying p5′-663Draf-Drafmut2-lacZ (previously called p5′-1103Draf-lacZ) (35) were used as a positive control. To correct for endogenous β-galactosidase activity, extracts from the wild-type strain (white) were included in each experiment, and this background reading was subtracted from readings obtained with each transformant line.

Treatment of Embryos with Vanadate/Peroxide Mixture, Injury Experiments of Larvae, and X-Gal Staining of Fat Bodies—Embryos carrying one copy of p5′-663Draf-lacZ or p5′-663Draf-SHATmut1-lacZ fusion genes were collected 5 h after mating of the transgenic lines with host strain w, dechorionated, and treated with octane. They were then exposed to 1 mM sodium orthovanadate and 2 mM hydrogen peroxide for 30 min at 25°C, washed in phosphate-buffered saline buffer, and further incubated for 3 h at 25°C. Quantitative measurements of β-galactosidase activity were carried out as described above. Injury experiments were performed by pricking third instar larvae 72 ± 4 h after egg laying, with a fine needle (34). Histochemical analyses of lacZ expression were conducted as described earlier (34–36).

RESULTS

Location of a Potential D-STAT Recognition Sequence in the D-raf Gene Promoter—Since the transcription initiation site of the D-raf gene was uncertain, we determined the site by primer extension analysis.2 The mapped transcription initiation site is 215 base pairs upstream from the reported putative cap site (9), predicted from the consensus signal sequence for transcription initiation of Drosophila mRNA (37). In the region between –82 and –74 with respect to the newly determined transcription initiation site, we found the nucleotide sequence 5′-TTCCGGG-AA (15) that perfectly matches the D-STAT recognition consensus sequence, 5′-TTTCCNNNGGA (20) (Fig. 1). Since the sequence is located close to the transcription initiation site, we considered that D-STAT may be involved in regulation of the D-raf gene promoter. We designated this putative D-STAT

2 E.-J. Kwon, E.-J. Oh, Y.-S. Kim, F. Hirose, K. Ohno, Y. Nishida, A. Matsukage, M. Yamaguchi, and M.-A. Yoo, manuscript in preparation.

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recognition sequence the Draf-STAT site. As noted previously (9), DRE is located in the region between −155 and −142 (Fig. 1).

Detection of Factors Binding to the ODBS and Draf-STAT Site in Vanadate/Peroxide-treated Cells—It has been noted that activated D-STAT protein in Drosophila cells treated with vanadate/peroxide mixture can bind to oligonucleotide ODBS with high affinity (20). It is well known that vanadate/peroxide inhibits tyrosine phosphatases and maintains D-STAT in an active phosphorylated form (20, 38). In a band mobility shift assay using extracts of Kc cells treated with vanadate/peroxide, the binding activity to oligonucleotide ODBS proved greater in vanadate/peroxide-treated Kc cell extracts than in vanadate-treated cell extracts (Fig. 2A). The DNA-protein complex was diminished effectively by adding unlabeled oligonucleotides ODBS, eveS1, and eveS2 as competitors (Fig. 2B). Effective competition was also observed with the addition of the DrafSTATwt oligonucleotide, while oligonucleotide DrafSTATmut1 (Fig. 1) did not compete for binding (Fig. 2B). From the extent of competition, it appears that the Draf-STAT and eve gene D-STAT-binding sites have comparable affinity to the vanadate/peroxide inducible factor, probably a D-STAT protein. Essentially similar results were obtained with Schneider 2 cell extracts (data not shown).

To determine if the shifted band represents a ODBS-D-STAT complex, anti-D-STAT antibody was added to the binding reaction with vanadate/peroxide-treated Kc cell nuclear extracts. As shown in Fig. 2C, the addition of the anti-D-STAT antibody supershifted the DNA-protein complex, and this supershifted complex formation was effectively diminished by adding unlabeled oligonucleotides ODBS and DrafSTATwt as competitors.

Since the vanadate/peroxide-inducible factor binding to ODBS was competitively blocked by the DrafSTATwt oligonucleotide, a band mobility shift assay was carried out again using the latter as a probe. As observed with ODBS, binding activity was enhanced in cells treated with increasing concentrations of vanadate/peroxide (Fig. 3A). The DNA-protein complex disappeared on adding unlabeled DrafSTATwt oligonucleotide as a competitor but not the mutant oligonucleotide carrying a base-substitution in the D-STAT core binding sequence, DrafSTATmut1, even when added in excess (Fig. 3B). The oligonucleotide DrafSTATmut2 (Fig. 1) carrying a base-substitution in a noncritical sequence for binding of D-STAT competed effectively (Fig. 3B). These results taken together indicate that the vanadate/peroxide-activated D-STAT protein has affinity for ODBS, eveS1, eveS2, and Draf-STAT sites.

Size Determination for the Polypeptide(s) Binding to the Draf-STAT Site by UV-cross-linking Analysis—To determine the size of the polypeptide(s) binding to the Draf-STAT site, a UV-cross-linking assay was carried out using the UV Draf-STAT oligonucleotide as a probe and extracts of vanadate/peroxide-treated cells. As shown in Fig. 4, polypeptides at around 88 kDa were specifically cross-linked with the probe. A lesser amount of radiolabeled polypeptides was observed on adding unlabeled DrafSTATwt oligonucleotides as competitors. However, the DrafSTATmut1 oligonucleotides did not compete. Essentially the same results were obtained with different cell lines, Kc (Fig. 4A) and Schneider 2 (Fig. 4B). Since the molecular weight of D-STAT protein is reported to be 87,500 and the D-STAT protein migrates to a position corresponding to 88 kDa, it is again very likely that the protein bound to the probe is the D-STAT protein.

Increase in Binding to the D-STAT Recognition Sequence in D-STAT and HOP Transiently Expressed Cells—To confirm that the factor binding to the D-STAT recognition sequence is activated by HOP, we performed a band mobility shift assay using D-STAT and HOP transient expression cell extracts. The DNA-protein complex formed with oligonucleotide ODBS in-
creased effectively upon D-STAT and HOP coexpression (Fig. 5A, lane 4) but was diminished when unlabeled oligonucleotides, ODBS, eveS1, eveS2, or DrafasTATwt were added as competitors. Little competition was observed when the DrafasTATmut1 oligonucleotide was added (Fig. 5B). From these results, it is likely that D-STAT activated by HOP has high affinity for the DrafasTAT site and D-STAT-recognition sites in the eve gene and ODBS.

**D-STAT and HOP Enhance Drafas Gene Promoter Activity—**

Since D-STAT very likely binds to the DrafasTAT site, we conducted the following experiments to test whether D-STAT activated by HOP can regulate activity of the Drafas gene promoter. Expression of the HOP protein had little effect on the Drafas gene promoter activity (Fig. 6A). However, expression of D-STAT protein gave an enhanced transcriptional signal possibly because of endogenous HOP expression (Fig. 6B). When D-STAT and HOP protein were expressed simultaneously, the p5'-663Drafwt-luc produced the strongest transcriptional signal (Fig. 6A). These results indicate that Drafas gene promoter activity is dependent on the amounts and ratio of D-STAT to HOP.

For further confirmation of the DrafasTAT sequence as a target of the activated D-STAT, three luciferase reporter constructs were prepared in which transcription of the luciferase genes was driven by the Drosophila metallothionein gene basal promoter carrying two copies of the ODBS wild type sequences (2×ODBS), the Drafas wild type (2×DrafasTATwt), or its mutant type (2×DrafasTATmut1). The HOP and D-STAT expression alone induced transcriptional activation of 2×DrafasTATwt-TATA-luc by 2-fold (Fig. 6B). When both HOP and D-STAT were expressed in Schneider 2 cells, the promoter activity of 2×DrafasTATwt-TATA-luc increased much more effectively (17-fold) (Fig. 6B). In contrast, little activation by HOP and D-STAT expression was observed with 2×DrafasTATmut1-TATA-luc (Fig. 6B). Thus, the DrafasTAT sequence functions as a target of the D-STAT protein, which appears to be activated by HOP protein.

**Role of the DrafasTAT Site in Transcriptional Activation of the Drafas Gene during Development—**

To confirm a role of the DrafasTAT site for Drafas gene promoter activity in vivo, transgenic Drosophila were used. Previously, we established three independent transgenic fly lines carrying a wild type Drafas gene promoter-lacZ fusion gene (p5'-663Drafwt-lacZ) (35). In the present study, four independent transformant lines carrying the p5'-663DrafwtTATmut1-lacZ fusion gene that has a mutation in the D-STAT core binding sequence were established. As shown in Fig. 7, mutation in the DrafasTAT site resulted in extensive reduction of lacZ expression throughout development.

**The DrafasTAT Site Is Required for Vanadate/Peroxide-inducible Transcriptional Activation of the Drafas Gene in Living Flies—**

To confirm whether expression of the Drafas gene is regulated by the activated D-STAT in living flies, the dechorionated and permeabilized embryos were incubated in vanadate/peroxide mixture. Transcriptional activation of the p5'-663Drafwt-lacZ transgene by vanadate/peroxide treatment is shown in Fig. 8. The level of β-galactosidase activity of the vanadate/peroxide-treated embryos bearing one copy of the p5'-663Drafwt-lacZ fusion gene was 2.2-fold higher than that of untreated embryos. In embryos bearing one copy of p5'-663DrafTATmut1-lacZ fusion gene, the expression of lacZ was little induced by vanadate/peroxide treatment. Thus, it was confirmed in living embryos that the DrafasTAT site is required for vanadate/peroxide-inducible activation of the Drafas gene promoter.

**Role of the DrafasTAT Site in Activation of the Drafas Gene Promoter in the Immune Response—**

The hemocytes play an important role in the events that lead to the activation of the immune response. Drosophila l(2)mbn hematocyte cell line is derived from larval hemocytes of the mutant lethal (2) malignant blood neoplasm (l(2)mbn) (39), in which the dipterinc and cecropin genes are rapidly induced by the addition of LPS (40, 41). Mammalian STAT has been implicated in a variety of immunity-related signaling pathways, including the response to proinflammatory cytokines (42).

Previously, we reported that the Drafas gene promoter was activated by injury or bacterial challenge (34). To investigate whether the DrafasTAT site is involved in this activation, the band mobility shift assay was carried out with nuclear extracts of the l(2)mbn cells treated with LPS and the DrafasTATwt oligonucleotides as a probe. Specificity of binding was confirmed in competition with DrafasTATwt and its base-substituted derivative DrafasTATmut1. A 2-fold increase of the DNA-protein complex was detected in l(2)mbn nuclear extracts 1 h after LPS treatment (Fig. 9), indicating that D-STAT is activated in response to treatment with LPS.

To further examine the role of the DrafasTAT site in the immune response in vivo, larvae carrying one copy of the p5'-663Drafwt-lacZ fusion gene or p5'-663DrafTATmut1-lacZ fusion gene were injected with fine needles, and after 3 h, corresponding to the time of maximum induction of the Drafas gene in larvae (34), quantitative analysis of β-galactosidase activity in total crude extracts of the larvae was carried out. After injury, the level of β-galactosidase activity in larvae carrying one copy of the p5'-663Drafwt-lacZ fusion gene was induced 1.7-fold, but that in larvae carrying the p5'-663DrafTATmut1-lacZ gene did not change as shown in Fig. 10A. Since activation of the Drafas gene promoter after injury was most prominent in the larval fat body, known to be involved in innate immunity, serving as the functional homologue of the vertebrate liver (34), expression of the Drafas-lacZ fusion gene in this tissue was examined by histochemical staining. lacZ staining signals in larval fat bodies carrying the p5'-663Drafwt-lacZ after injury were dramatically increased, whereas lacZ expression was not induced in p5'-663DrafTATmut1-lacZ transgenic larval fat bodies (Fig. 10B).
D-STAT Is a Transcriptional Regulator of the D-raf Gene

DISCUSSION

The D-raf gene promoter contains a D-STAT recognition sequence in the region between −82 and −74 (Fig. 1). In this study, we identified a factor binding to the Draf-STAT site as having almost the same molecular weight as D-STAT and showed that the Draf-STAT site plays an important role in activation of the D-raf gene promoter by HOP/D-STAT. We also observed that the Draf-STAT site is required for D-raf gene promoter activation during development and in response to injury. These results lead us to suggest that the D-raf gene is a target of activated D-STAT protein through which the latter can participate in regulation of the MAPK cascade.

In the embryos homozygous for null alleles of D-raf, the embryogenesis proceeds normally depending on the maternal D-raf activity. However, the proliferation in imaginal discs and other proliferating tissues is severely affected during larval stages, indicating that the zygotic D-raf activity is essential after hatching (6, 7). It is remarkable that expression of D-raf during larval stages, especially in early stages, is largely dependent on the transcriptional activation through the D-STAT-binding site (Fig. 7). It has been reported that mutants defective in hop or D-STAT showed the similar proliferation defects (19, 43). These observations suggest that the HOP/D-STAT signaling pathway contributes to the D-raf gene activation at significant stages of development.

Recently, the mosquito (Anopheles gambiae) STAT gene was cloned (44). It is noteworthy that bacterial challenge results in nuclear translocation of A. gambiae STAT protein in mosquito fat bodies and induction of DNA binding activity that recognizes an A. gambiae STAT target site (44). Furthermore, in vitro treatment with vanadate/peroxide enhances translocation of A. gambiae STAT into the nucleus in midgut epithelial cells (44). These observations provide evidence of direct participation of the STAT pathway in the immune response in insects and are in agreement with our conclusion that the Draf-STAT site is required for D-raf gene promoter activation after injury, which is supported by the results from the band mobility shift assays with the LPS-treated l(2)mbn cells (Fig. 9) and the injury experiment using transgenic flies (Fig. 10). In addition, it is well known that some STAT family members in mammals participate in the immune response (45) so that a function in immunity appears to have been conserved during evolution in mammals and insects.

In mammals, several lines of evidence pointing to cross-talk
between JAK/STAT, the mammalian homologues of Drosophila HOP/D-STAT, and MAPK pathways have been reported. First, MAPKs activate some STAT family members, STAT1a, STAT3, and STAT4, by direct phosphorylation on the serine residue of STAT proteins, participating in the mechanism by which interferon stimulated ligand-induced early response genes (46, 47). MAPKs also directly phosphorylate the interferon-α/b receptor and stimulate the JAK/STAT pathway (48). On the other hand, it was recently shown that epidermal growth factor receptor is phosphorylated by Jak2 in signaling by growth hormone, thereby providing docking sites for Grb2 and activating MAPKs and their target gene expression, independently of the intrinsic tyrosine kinase activity of epidermal growth factor receptor (49). These reports suggest that the two signaling pathways are activated by mutual stimulation. Direct activation of the raf gene by STAT has not been reported. However, the human A-raf-1 gene promoter contains putative STAT binding sites (31). Although further analyses are necessary to generalize our findings to include mammals, the present study,
for the first time, suggests a novel mechanism by which STAT can participate in regulation of the MAPK cascade throughraf
gene activation.

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