Dominant Mutations of *Drosophila* MAP Kinase Kinase and Their Activities in *Drosophila* and Yeast MAP Kinase Cascades

Young-Mi Lim,^{*,†,1} Leo Tsuda,^{*,1,2} Yoshihiro H. Inoue,^{*,1} Kenji Irie,[†] Takashi Adachi-Yamada,^{*} Mami Hata,[†] Yoshimi Nishi,[†] Kunihiro Matsumoto[†] and Yasuyoshi Nishida^{*}

^{*}Laboratory of Developmental Biology and [†]Laboratory of Cell Regulation, Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan and [‡]Laboratory of Experimental Radiology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan

Manuscript received December 2, 1996
Accepted for publication February 4, 1997

ABSTRACT

Eight alleles of *Dsor1* encoding a *Drosophila* homologue of mitogen-activated protein (MAP) kinase kinase were obtained as dominant suppressors of the MAP kinase kinase kinase *D-raf*. These *Dsor1* alleles themselves showed no obvious phenotypic consequences nor any effect on the viability of the flies, although they were highly sensitive to upstream signals and strongly interacted with gain-of-function mutations of upstream factors. They suppressed mutations for receptor tyrosine kinases (RTKs); *torso* (*tor*), *sevenless* (*sev*) and to a lesser extent *Drosophila* EGF receptor (*DER*). Furthermore, the *Dsor1* alleles showed no significant interaction with gain-of-function mutations of *DER*. The observed difference in activity of the *Dsor1* alleles among the RTK pathways suggests *Dsor1* is one of the components of the pathway that regulates signal specificity. Expression of *Dsor1* in budding yeast demonstrated that *Dsor1* can activate yeast MAP kinase homologues if a proper activator of *Dsor1* is coexpressed. Nucleotide sequencing of the *Dsor1* mutant genes revealed that most of the mutations are associated with amino acid changes at highly conserved residues in the kinase domain. The results suggest that they function as suppressors due to increased reactivity to upstream factors.

A cascade of protein kinases composed of mitogen-activated protein kinase (MAPK), MAPK kinase (MAPKK or MEK) and MAPKK kinase (MAPKKK or MEKK) plays pivotal roles in the transduction of extracellular signals controlling cellular proliferation and differentiation (NEIMAN 1993; NISHIDA and GOTOH 1993). The chain of phosphorylation reactions is initiated by Raf-1, a MAPKKK, that is activated by a yet unproved mechanism after being recruited to the plasma membrane through binding of its amino terminal CR1 region to GTP-Ras (VOJTEK *et al.* 1993; CHUANG *et al.* 1994; FABIAN *et al.* 1994). Raf-1 then activates MAPKK by phosphorylating two adjacent serine/threonine residues in the activation lip located between the kinase subdomain VII and VIII (YAN and TEMPLETON 1994; ZHENG and GUAN 1994a). MAPKK is a dual specific kinase and in turn phosphorylates both threonine and tyrosine residues in the threonine-glutamate-tyrosine tripeptide motif in the activation lip of MAPK (CREWS and ERIKSON 1992; KOSAKO *et al.* 1992; ROSSOMANDO *et al.* 1992).

The cascade is highly conserved during evolution,

and the budding yeast, *Saccharomyces cerevisiae*, contains at least three distinct MAPK cascades controlling mating pheromone response, cell wall integrity, and response to osmotic stress (Figure 4; DAVIS 1994; MAEDA *et al.* 1995). The MAPKKK components of the yeast cascades comprise a family distinct from the Raf family. MEKK, a homologue of yeast MAPKKKs, has been identified in mammals and has been shown to activate MAPKK/MEK *in vitro* (LANGE-CARTER *et al.* 1993). Their MAPK components can be classified into subfamilies according to the tripeptide motif at the phosphorylation site in the activation lip; threonine-glutamate-tyrosine in MPK1, FUS3 and KSS1 and threonine-glycine-tyrosine in HOG1 (DAVIS 1994). Three subfamilies of MAPKs have been identified in mammals; MAPK/ERK, JNK/SAPK and p38/MPK2 subfamilies, whose tripeptide motives are threonine-glutamate-tyrosine, threonine-asparagine-tyrosine and threonine-glycine-tyrosine, respectively (NISHIDA and GOTOH 1993; DAVIS 1994).

Drosophila contains a single *raf* gene, *D-raf*, that is at least involved in the regulation of cellular proliferation, differentiation of muscle or peripheral nervous system in the thorax necessary for eclosion, and in transduction of diverse developmental signals from receptor tyrosine kinases (RTKs) encoded by *torso* (*tor*), *sevenless* (*sev*) and *Drosophila* EGF receptor (*DER*) (PERRIMON *et al.* 1985; NISHIDA *et al.* 1988; AMBROSIO *et al.* 1989; DICKSON *et al.* 1992; HATA *et al.* 1994; BRAND and PERRIMON

Corresponding author: Yasuyoshi Nishida, Laboratory of Developmental Biology, Division of Biological Science, Nagoya University Graduate School of Science, Furo-cho, Chikusa-ku, Nagoya 464-01 Japan. E-mail: nishida@bio.nagoya-u.ac.jp

¹ The first three authors made equal contributions to this work.

² Present address: Department of Biological Chemistry, MacDonald Medical Research Laboratories, Howard Hughes Medical Institute, University of California, Los Angeles, CA 90095-1662.

1994). It is not known how specific developmental responses are induced by the single shared pathway in response to the different RTKs. Further characterization of the pathway components is necessary to understand how this signal specificity is achieved.

In the course of studies of genes encoding factors acting downstream of D-raf, dominant suppressors of *D-raf* were screened and 18 mutants were obtained. Analysis on one of them, *Su1*, has led to the identification of *Dsor1* (*Dsor* is for *Downstream suppressor of raf*), which encodes the Drosophila homologue of MAPKK (TSUDA *et al.* 1993). The dominant allele, *Dsor1^{Su1}*, was found to interact not only with *D-raf* but also with mutations for factors acting further upstream of D-raf in the Tor pathway. Genetic interactions of the dominant allele and the loss-of-function phenotypes of *Dsor1* revealed the roles of *Dsor1* in cell proliferation and in Tor-mediated signaling (TSUDA *et al.* 1993). *Dsor1* is also referred to as *D-mek* and temperature-sensitive mutants revealed the involvement of *Dsor1/D-mek* in the Sev and DER pathways as well (HSU and PERRIMON 1994). We have identified seven additional *Dsor1* alleles among the remaining suppressors of *D-raf* that showed changes in signal strength and specificity. Their molecular characterization and an analysis of their activities in Drosophila and budding yeast were undertaken in an attempt to address the question of signal specificity in development.

MATERIALS AND METHODS

Fly stocks and culture: Fly cultures and crosses were performed according to standard procedures at 25° unless otherwise noted. *sev¹²*, *spd Sos¹²* was provided by U. BANERJEE, *Ras1^{2F}/TM3* by M. A. SIMON, *Elp¹/CyO*, *Elp^{B1}/CyO*, and *phyl/CyO*, *Ras1^{V12}* by G. M. RUBIN, *Sev¹¹* and *D-raf^{tor19}* by E. HAFEN, *cn top¹/Cy*, *top²/Cy* by T. SCHÜPBACH, and *cn flb^{2W74} bw sp/CyO* by B-Z. SHILO. For description of the genetic markers and balancers, see LINDSLEY and ZIMM (1992).

Molecular procedures: DNAs were extracted from hemizygous/homozygous mutant flies as described previously (NISHIDA *et al.* 1988). Using the mutant fly DNAs as templates, the mutant *Dsor1* genes were cloned as sets of six overlapping fragments by PCR with the following sense and anti-sense primer sets: GTTGCACACCGCACCGTCTG (sense primer 1N; the nucleotide residues 1–20 as described in TSUDA *et al.* 1993) and CGGTGTTATTGGAATGGGTGC (anti-sense primer 1R; nucleotide residues 588–566); CCGCCACAGTGCGCCGAC (sense primer 2N; nucleotide residues 473–491) and CGAACTGACTTTGTCTATCCC (anti-sense primer 2R; nucleotide residues 901–881); TCCTTTCGTGTGACGCACAGG (sense primer 3N; nucleotide residues 791–812) and TCCGTGTCGCCCATGTCCAGACC (anti-sense primer 3R; nucleotide residues 1240–1218); GCTGGGAAGCCCAAGACGAGC (sense primer 4N; nucleotide residues 1169–1190) and CTTGATCTCGCCGCTGCTATTGACG (anti-sense primer 4R; nucleotide residues 1718–1694); TCCACGTGACGTGAAGCCGAGC (sense primer 5N; nucleotide residues 1662–1685) and CTGTTTCTTCAGGCAGATGTCC (anti-sense primer 5R; nucleotide residues 2284–2263); and CCAAGCTAGAGCACAAGATCTTCTCC (sense primer 6N; nucleotide residues 2219–2242) and CGCTTATGTATT-

CAACCTTTTGG (anti-sense primer 6R; nucleotide residues 2538–2517). These primers were flanked by a *Bam*HI recognition sequence at their 5' ends and PCR-amplified fragments were cloned into the *Bam*HI site of pBluescript (Stratagene) after *Bam*HI digestion. Ampli Taq DNA Polymerase PCR Kit (Takara Co. Ltd, Japan) was used for the PCR reactions. The nucleotide sequences of these cloned PCR fragments were determined by the chain termination method using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (ABI).

The nucleotide alterations found in *Dsor1^{Su4}* and *Dsor1^{Su5}* were introduced into the normal *Dsor1* cDNA by site-directed mutagenesis with appropriate mutagenic primers using the Transformer Site-directed Mutagenesis Kit (Clontech) according to the procedure recommended by the manufacturer. The normal and mutant *Dsor1* cDNAs were cloned into the yeast high copy number expression vector, pNV11S (YAMAGUCHI *et al.* 1995).

Yeast strains and culture: The *S. cerevisiae* strains used were SY1491 (*MATa ura3 his3 leu2 trp1 FUS1::HIS3 pep4Δ::ura3 can1 ste4Δ::LEU2*), SY1493 (*MATa ura3 his3 leu2 trp1 FUS1::HIS3 pep4Δ::ura3 can1 ste7Δ::URA3*), SY1984 (*MATa ura3 his3 leu2 trp1 FUS1::HIS3 pep4Δ::ura3 can1 ste11Δ::ura3*), DL251 (*MATa/MATa ura3/ura3 leu2/leu2 his4/his4 trp1/trp1 can1/can1 bck1Δ::URA3/bck1Δ::URA3*), 3233-1B (*MATa ura3 leu2 his3 trp1 mkk1Δ::LEU2 mkk2Δ::HIS3*), KAN-4C11U (*MATa its3 leu2 trp1 bck1Δ::LEU2 ste11Δ::URA3*) (IRIE *et al.* 1993), and TM334 (*MATa ura3 leu2 trp1 his3 lys2 pbs2Δ::HIS3*) (MAEDA *et al.* 1995). Yeast cultures were grown in YEP (1% Bacto yeast extract/2% Bacto Peptone) supplemented with 2% glucose. SD medium [0.7% yeast nitrogen base without amino acids (Difco)/2% glucose], supplemented with the appropriate nutrients, was employed for the selection of cells with plasmids. Yeast cells were transformed by the lithium acetate method. General genetic manipulations were carried out as described (GUTHRIE and FINK 1991).

Other procedures: Transmission electron microscopy of ultra-thin sectioned adult compound eyes embedded in Epon was performed according to standard procedures. Cuticular preparations of the embryos were made as described (WIESCHAUS and NÜSSLEIN-VOLHARD 1986) and observed with dark-field optics.

RESULTS AND DISCUSSION

***Dsor1* mutations are highly sensitive to upstream signals:** *D-raf^{C110}* is a hypomorphic allele of *D-raf* and its hemi- or homozygotes die during or soon after eclosion, showing a rough eye phenotype due mainly to loss of the inner R7 and some outer photoreceptor cells (Figure 1, B and G). Among the 18 mutant lines obtained in a screen for suppressors of *D-raf^{C110}* (TSUDA *et al.* 1993), eight mapped to the *Dsor1* locus. All fully rescued the lethality and the external eye morphology of *D-raf^{C110}* mutant flies (Figure 1C). However, detailed analysis of the rescued eye by electron microscopy revealed incomplete suppression of the eye defect by some alleles (Figure 1H; Table 1, column 2). Single copies of these alleles had a full effect in females homozygous for *D-raf^{C110}* (data not shown), indicating their complete dominance over the normal allele.

As the *Dsor1* mutations were induced on the X chromosome as *D-raf^{C110}*, the alleles were separated from *D-raf^{C110}* by meiotic recombination to examine their

FIGURE 1.—Scanning (A–E and M–O) and transmission (F–L) electron microscopy of adult compound eyes. (A and F) Canton-S (normal) male. (B and G) *y D-raf^{C110} sn/Y*. The rough eye phenotype in the *D-raf^{C110}* hemizygous male (B) is mainly due to loss of inner and some outer photoreceptor cells in each ommatidium (G). (C and H) *y D-raf^{C110} sn Dsor1^{Su5}/Y*. The defects of *D-raf^{C110}* were suppressed by the *Dsor1* alleles but suppression by weak alleles such as *Dsor1^{Su5}* was incomplete in some ommatidia (arrows). (D) *+/Y; sev-Raf^{torY9}/CyO*. (E) *sn Dsor1^{Su4}/Y; sev-Raf^{torY9}/CyO*. The rough eye phenotype generated by constitutively activated *D-raf* (D) is greatly enhanced by the *Dsor1* mutations (E). (I) *sn Dsor1^{Su1}/Y*. Extra R7-like photoreceptor cells are occasionally formed (arrowheads). (J) *sn Dsor1^{Su1}/Y; spd Sos^{Jc2}/+*. Each ommatidium contains extra R7-like cells and some outer photoreceptor cells are also occasionally affected (arrow). (K) *sev^{E4}/Y*. The inner R7 cell is missing in all the ommatidia. (L) *sn Dsor1^{Su1} sev^{E4}/Y*. The R7 cell is formed in a significant fractions of ommatidia (arrowheads). (M) *+/+; Elp^{B1}/+* female. (N) *sn Dsor1^{Su7}/+; Elp^{B1}/+* female. (O) *Elp^{B1}/Sos^{Jc2}* female.

independent phenotypes. Flies hemi- or homozygous for the *Dsor1* alleles showed no visible phenotype and no significant effect on viability. However, observations of ultra-thin sections of the mutant eyes revealed that extra R7-like photoreceptor cells are formed in a small fraction of ommatidia with an average of 1.00–1.20 R7 cells per ommatidium seen (Figure 1I; Table 1, column 3). This suggested some constitutive activity of the mutant *Dsor1* protein and/or increased sensitivity to upstream signals so that the kinase is significantly activated even with basal level activities of upstream factors. This prompted us to study their sensitivity to gain-of-function mutations of upstream factors.

Sos^{Jc2} is a weak gain-of-function mutation of *Son of sevenless* (*Sos*), encoding a guanine nucleotide exchanger of Ras1, and has been demonstrated to par-

tially suppress an allele of *sevenless* (*sev*) (ROGGE *et al.* 1991). *Sos^{Jc2}* itself never produces extra R7 cells even when flies are homozygous for it making it a reliable indicator of genetic interaction (Table 1, column 4). In contrast, two to five R7-like photoreceptor cells were formed in most of the ommatidia in the flies hemizygous for a *Dsor1* allele while heterozygous for *Sos^{Jc2}* (Figure 1J; Table 1, column 4). Outer photoreceptor cells are also affected to lesser extents, and ommatidia with five or seven outer photoreceptor cells were occasionally observed instead of the normal six cells (Figure 1J). Thus, the *Dsor1* mutant proteins seem to be highly sensitive to upstream stimuli, and a slight increase in signals by the *Sos* mutation seems sufficient to ectopically activate the signaling pathway for R7 cell fate determination and to interfere with

TABLE 1

Suppressing activities of the dominant *Dsor1* alleles on the loss-of-function mutations on *D-raf*, *tor* or *sev* and their dominant effects on the eye development

Allele	1 Amino acid alteration	2 Percentage of normal ommatidia in <i>D-raf</i> ^{C110} /Y	3 Mean number of R7-like cells in +/+	4 Mean number of R7-like cells in <i>Sos</i> ^{JC2} /+	5 Percentage of ommatidia with R7 in <i>sev</i> ^{E4} /Y	6 Percentage of <i>tor</i> ^{PM} embryos with A8 segment
+		3.8 (369)	1.00 ± 0.00 (378)	1.00 ± 0.00 (186)	0.0 (264)	0.3 (392)
<i>Dsor1</i> ^{Su1}	Gly ¹⁹² to Ser	100.0 (243)	1.09 ± 0.29 (101)	2.62 ± 0.83 (571)	28.7 (164)	80.3 (585)
<i>Dsor1</i> ^{Su4}	Gly ¹⁴⁴ to Asp	100.0 (362)	1.11 ± 0.33 (495)	3.01 ± 0.72 (89)	8.9 (360)	99.7 (328)
<i>Dsor1</i> ^{Su5}	Ser ²¹⁰ to Asn	89.1 (274)	ND	ND	ND	10.6 (266)
<i>Dsor1</i> ^{Su6}	Thr ⁵² to Met, Met ¹⁶² to Ala	100.0 (261)	1.02 ± 0.15 (232)	ND	19.2 (395)	44.4 (389)
<i>Dsor1</i> ^{Su7}	Pro ²⁷⁸ to Ala	96.3 (109)	1.20 ± 0.47 (170)	2.76 ± 1.07 (110)	21.6 (389)	54.0 (378)
<i>Dsor1</i> ^{Su12}	Met ¹⁵⁹ to Leu	99.0 (305)	1.01 ± 0.09 (255)	ND	3.1 (323)	27.7 (649)
<i>Dsor1</i> ^{Su18}	Lys ²⁰⁸ to Asn	92.4 (210)	1.00 ± 0.00 (240)	1.54 ± 0.71 (174)	1.0 (288)	17.3 (226)
<i>Dsor1</i> ^{Su24}	Glu ⁶² to Lys	100.0 (302)	1.11 ± 0.33 (276)	3.02 ± 1.05 (97)	21.1 (341)	18.0 (455)

The numbers in parentheses indicate numbers of ommatidia (columns 2 to 5) or embryos (column 6) counted. ND, not done.

the development of some of the outer photoreceptor cells.

The *Dsor1* alleles also interacted strongly with the *Sev*^{S11}, *Ras1*^{V12} and *Raf*^{torY9} transgenes that express constitutively active forms of Sev, Ras1 and D-raf, respectively, in the eye disc under the control of the *sev* enhancer (BASLER *et al.* 1991; FORTINI *et al.* 1992; DICKSON *et al.* 1996). Extreme rough eye phenotypes that were not observed with the transgenes alone were generated (Figure 1, D and E).

From the above described experiments, the eight *Dsor1* alleles can be classified into three groups according to their differential activities: *Dsor1*^{Su1}, *Dsor1*^{Su4}, *Dsor1*^{Su7} and *Dsor1*^{Su24} are strong, *Dsor1*^{Su5} and *Dsor1*^{Su18} are weak, and *Dsor1*^{Su6} and *Dsor1*^{Su12} are intermediate.

Different activities of the *Dsor1* alleles in different RTK pathways: We have previously shown that *Dsor1* acts downstream of D-raf in the Tor signaling pathway and that *Dsor1*^{Su1} significantly rescues the posterior but not the anterior defects of the embryos that are devoid of maternal *tor* activity when it was introduced into the maternal genome (TSUDA *et al.* 1993). The remaining *Dsor1* alleles also showed obvious and different activities in suppressing the posterior defects (Figure 2, A–D; Table 1, column 6). A weak *Dsor1* allele, *Su(raf)34B*, obtained in a genetic screening for dominant suppressors of *D-raf*^{C110} similar to ours failed to suppress *tor* (LU *et al.* 1994). *Dsor1* is essential for the normal expression of *tailless* (*tl*), a target gene of the Tor pathway (PIGNONI *et al.* 1990; TSUDA *et al.* 1993), at both ends of the embryo, but the anterior defect was not affected by any of the mutations. This may suggest another pathway or mechanism parallel to *Dsor1* in regulating the anterior expression pattern of *tl*.

Dsor1 has also been shown to function in the Sev and DER pathways using temperature-sensitive mutants of *Dsor1*, *D-mek*^{ts} (HSU and PERRIMON 1994). We have

analyzed the activities of our *Dsor1* alleles in these pathways. They significantly suppressed *sev*, which causes loss of the inner R7 photoreceptor cell in all ommatidia (Figure 1K), showing R7 cell formation in up to 30% of the ommatidia in flies hemizygous for both *sev* and one of the *Dsor1* alleles (Figure 1L; Table 1, column 5).

DER is multi-functional and is required for the determination of the dorso-ventral polarity of follicle cells in the egg chamber, embryonic development, wing vein formation, eye development and cell proliferation (PRICE *et al.* 1989; SCHEJTER and SHILO 1989; CLIFFORD and SCHÜPBACH 1994). It has been shown that the DER pathway is highly sensitive to reduced levels of *Dsor1* and most of the eggs laid by *D-mek*^{ts} at a permissive temperature (18°) show a weak ventralized eggshell phenotype (HSU and PERRIMON 1994). On the other hand, we could not see any suppression by our *Dsor1* alleles of this defect in the dorso-ventral polarity of the eggshell in *torpedo*¹ (*top*¹) or *top*^{CJ} maternal effect mutations of DER (data not shown).

The gain-of-function mutations of *DER*, *Elp*¹ and *Elp*^{B1}, are highly sensitive to the activity of downstream factors, and the gain-of-function mutations of *Sos* and *rolled* (*rl*), which encodes a Drosophila homologue of MAPK, enhanced *Elp* to produce a strong rough eye phenotype (Figure 1O; ROGGE *et al.* 1991; BRUNNER *et al.* 1994). On the contrary, all the *Dsor1* alleles showed no significant interaction with *Elp*^{B1} (Figure 1N). Examination of ultra-thin sections of the compound eyes of *Dsor1*^{Su1}/*Dsor1*^{Su1}; *Elp*^{B1}/+ flies showed no difference in the photoreceptor cell development from those of *Elp*^{B1}/+ flies in which some of the outer photoreceptor cells are occasionally missing (data not shown). Therefore, we have no evidence for excess activation of the *Dsor1* alleles by *Elp*^{B1}.

On the other hand, significant activity of *Dsor1* alleles in the DER pathway was observed during embryonic

FIGURE 2.—The cuticular patterns of embryos defective for maternal *tor* or zygotic *flb* activity and suppression of the defects by the dominant *Dsor1* mutations. (A) An embryo laid by a Canton-S (normal) female. In embryos laid by *tor*^{PM} females the structures posterior to the seventh abdominal segment (A7, indicated with arrowheads) and the head regions are missing (B). Embryos laid by *sn Dsor1*^{Su1}/*Binsc*; *tor*^{PM} (C) and *sn Dsor1*^{Su4}/*Binsc*; *tor*^{PM} (D) females. The posterior defect was variably suppressed by the dominant *Dsor1* mutations but the anterior defect was not affected by any of them. *flb*^{2W74} (E) and *sn Dsor1*^{Su1}; *flb*^{2W74} (F) embryos laid and allowed to develop at 25°. *flb*^{2W74} (G) and *sn Dsor1*^{Su1}; *flb*^{2W74} (H) embryos laid and allowed to develop at 28°. Anterior is to the top. The vitelline membrane was removed from the embryos shown in A–D.

development. A temperature-sensitive allele of *DER*, *faint little ball*^{2W74} (*flb*^{2W74}), affects the development of embryonic cuticle and central nervous system, in addition to head involution and germband retraction at 25° (Figure 2E). About 90% of the homozygous embryos failed to undergo head involution. In contrast to this, only about half of the mutant embryos failed when both parents were homo- or hemizygous for one of the two strong alleles, *Dsor1*^{Su1} or *Dsor1*^{Su4} (other alleles are not yet tested) (Figure 2F). *flb*^{2W74} is nearly null functional at 28° and affects embryonic development severely, exhibiting the faint little ball phenotype with poor cuticular formation (Figure 2G). Maternal expression of the *Dsor1* alleles allowed formation of some ventral denticle belts in several per cent of embryos (Figure 2H). The observed activity of the *Dsor1* alleles in embryonic development could be due to the large amount of maternal *Dsor1* mRNA in the ooplasm (TSUDA *et al.* 1993).

The *Dsor1* alleles showed strong activity in the Tor and Sev pathways while their activity in the DER pathway was less obvious and was seen only in the embryo in

which a large amount of mutant *Dsor1* products were provided by its maternal expression. The results indicate that DER requires a higher level of *Dsor1* activity than Sev and Tor. Furthermore, no significant interaction was observed between *Elp* and the dominant *Dsor1* alleles, and this is in contrast to the strong interaction of *Elp* with gain-of-function mutations of *Sos* and *rl*, whose products act upstream and downstream of *Dsor1*, respectively. The results suggest that the DER pathway activates *Dsor1* less efficiently than the Sev and Tor pathways or that activation of *RI* by *Dsor1* is less efficient in the DER pathway than in the others. The differential activity of *Dsor1* may be explained by the involvement of a factor or factors that reduce(s) required levels of *Dsor1* activity in the Sev and Tor signaling pathways but not in the DER pathway. One candidate for such a factor is STE5, which tethers multiple protein kinases in the MAPK cascade required for mating in budding yeast (CHOI *et al.* 1994). MAPKK/MEK-enhancing factor (MEF) purified from rabbit skeletal muscle is another candidate. In the presence of MEF, molar equiva-

FIGURE 3.—Complementation of yeast *mkk1Δ mkk2Δ* and *ste11Δ* mutations by *Dsor1*. (A) Yeast strain 3233-1B (*mkk1Δ mkk2Δ*) was transformed with pNV11S (control) or the same vector harboring *Dsor1*, *Dsor1^{Su1}*, *Dsor1^{Su4}*, *Dsor1^{Su5}* or *MKK1*. The transformants were streaked on YPD medium and allowed to grow at 35°. (B) Yeast strain SY1984 was transformed with pNV11S (control) or the same vector harboring *Dsor1*, *Dsor1^{Su1}*, *Dsor1^{Su1, Su5}* or *STE7*. The transformants were streaked on YEP-glucose plates seeded with a lawn of yeast strain 15Dau. Mating was allowed to proceed overnight before transfer onto synthetic glucose minimal medium plates, supplemented with the appropriate requirements, for monitoring formation of diploids. Two independent clones were tested.

lents of MEK to MAPK are sufficient to fully phosphorylate MAPK *in vitro*, while a 40:1 molar excess ratio of MEK to MAPK is required in its absence (SCOTT *et al.* 1995). These observations suggest a mechanism that provides diversity and specificity to signals of RTK pathways that are mediated by the common MAPK cascade, and *Dsor1*/MAPKK would be one of the points for such differential regulation.

Activities of *Dsor1* alleles in budding yeast: Dominant activity of the *Dsor1* alleles was analyzed in yeast. The budding yeast, *Saccharomyces cerevisiae*, contains at least three MAPK pathways controlling mating pheromone response, cell wall integrity, and response to osmotic stress (Figure 4; DAVIS 1994; MAEDA *et al.* 1995). *Dsor1* cDNA was *in vitro* mutagenized according to the nucleotide alterations found in two strong alleles, *Dsor1^{Su1}* and *Dsor1^{Su4}*, and in a weak allele, *Dsor1^{Su5}* (see below), and their constitutive activity was assayed by their ability to complement deletions of genes of the three yeast pathways (*ste7Δ*, *mkk1Δ mkk2Δ* and *pbs2Δ*) (Figure 3 and Table 2). The normal *Dsor1* allele or *Dsor1^{Su4}* and *Dsor1^{Su5}* showed no complementing activity while *Dsor1^{Su1}* complemented the mating defect of *ste7Δ* and the temperature-sensitive cell-lysis defect in the *mkk1Δ mkk2Δ* double mutant. Sensitivity to osmotic pressure in *pbs2Δ* was not rescued by any of them.

Dsor1^{Su1} suppressed a deletion of the *MKK1/MKK2* activator *BCK1* (*bck1Δ*), however, it failed to complement a deletion of the *STE7* activator *STE11* (*ste11Δ*) or both *BCK1* and *STE11* (*bck1Δ ste11Δ*) (Table 3). This suggests a strict dependency of *Dsor1^{Su1}* on *STE11*, indicating a lack of constitutive activity in yeast and the necessity of phosphorylation for its activity. *Dsor1^{Su1}* also weakly suppressed a yeast strain bearing a deletion of the *STE11* activator *STE4* (*ste4Δ*) that encodes the β

TABLE 2
Complementation of yeast MAPKK mutations by *Dsor1*

Gene	<i>ste7Δ</i> ^a	<i>mkk1Δ mkk2Δ</i> ^b	<i>pbs2Δ</i> ^c
Vector	—	—	—
<i>Dsor1</i>	—	—	—
<i>Dsor1^{Su1}</i>	+	+	—
<i>Dsor1^{Su4}</i>	ND	—	ND
<i>Dsor1^{Su5}</i>	—	—	ND
<i>STE7</i>	++	ND	ND
<i>MKK1</i>	ND	++	ND
<i>PBS2</i>	ND	ND	++

ND, not done.

^a Assayed for mating ability.

^b Assayed for growth on YPD medium at 35°.

^c Assayed for growth under an osmotic pressure.

TABLE 3
Suppression of yeast mutations by *Dsor1*

Gene	<i>ste4</i> Δ^a	<i>ste4</i> Δ^b	<i>ste11</i> Δ^a	<i>ste11</i> Δ^b	<i>bck1</i> Δ^c	<i>bck1</i> Δ <i>ste11</i> Δ^c
Vector	—	—	—	—	—	—
<i>Dsor1</i>	—	+	—	—	—	—
<i>Dsor1</i> ^{Su1}	—	++	—	—	+	—
<i>Dsor1</i> ^{Su4}	—	+	ND	ND	ND	ND
<i>Dsor1</i> ^{Su5}	—	—	ND	ND	ND	ND

ND, not done.

^a Assayed for mating ability.

^b Assayed for growth dependent on the *FUS1-HIS3* fusion construct.

^c Assayed for growth on YPD medium at 35°.

subunit of the mating factor receptor-coupled G protein (WHITEWAY *et al.* 1989), and the suppression was also detected with a more sensitive assay system using a *FUS1-HIS3* fusion construct (STEVENSON *et al.* 1992; IRIE *et al.* 1994) (Table 3).

The results suggest that *Dsor1*^{Su1} is activated by basal level activity of *STE11* (Figure 4). The activated *Dsor1* now acts as an activator of FUS3, KSS1 and MPK1 but

not of HOG1. It should be noted that the target sequence in the activation lip of FUS3, KSS1 and MPK1 is threonine-glutamate-tyrosine, while the corresponding sequence of HOG1 is threonine-glycine-tyrosine. This suggests that *Dsor1* has strict substrate-specificity determined by the target sequence requiring the target tripeptide motif threonine-glutamate-tyrosine. *Dsor1*^{Su1} has also been demonstrated to suppress the vulvaless

FIGURE 4.—MAPK signal transduction pathways in *S. cerevisiae* and *Drosophila* and possible mechanisms for activation of *Dsor1* in yeast. Three of the yeast MAPK cascades each controlling mating pheromone response, cell wall integrity or response to osmotic stress (DAVIS 1994) and the *Drosophila* cascade are shown. The yeast MAPKKK components belong to a family distinct from the D-raf family, while MAPKK and MAPK components of both species are structurally closely related to each other. The yeast MAPK components can be classified according to their tripeptide motives at the putative phosphorylation site in the activation lip; threonine-glutamate-tyrosine (TEY) in FUS3, KSS1 and threonine-glycine-tyrosine (TGY) in HOG1. Rolled has a TEY motif. *Dsor1* activates yeast MAPKs belonging to the subfamily with the tripeptide motif of TEY only when its appropriate activator, human RAF-ΔN in this case, is provided. On the contrary, the product of *Dsor1*^{Su1} can also be activated by STE11 whose primary structure considerably differs from D-raf. The mutation may affect the specificity of *Dsor1* for recognition by its activators.

TABLE 4

Activation of *Dsor1* by coexpressed human *RAFΔN* in yeast

Gene	<i>ste11Δ</i> ^a	<i>ste11Δ</i> ^b
<i>RAFΔN</i>	—	—
<i>Dsor1</i> + <i>RAFΔN</i>	+	++
<i>Dsor1</i> ^{Su1} + <i>RAFΔN</i>	+	++

^a Assayed for mating ability.^b Assayed for growth dependent on the *FUS1-HIS3* fusion construct.

phenotype of *lin-45*, *let-23* and *lin-3* in *Caenorhabditis elegans*, which encodes the Raf homologue, the EGF receptor homologue and its ligand, respectively (KOGA and OHSHIMA 1995).

Coexpression of normal *Dsor1* or *Dsor1*^{Su1} with *RafΔN*, a constitutively activated human *Raf-1*, which has been demonstrated to complement *D-raf* (CASANOVA and STRUHL 1994), significantly suppressed the phenotype of *ste11Δ* (Table 4). Expression of *RafΔN* alone did not complement *ste11Δ*. Thus, *Dsor1* is functional in yeast if an appropriate activator is provided. This provides an excellent system for screening novel MAPKKK family members.

Molecular characterization of the *Dsor1* mutations: Molecular lesions associated with the eight dominant *Dsor1* mutations were analyzed by determining the nucleotide sequences of the mutant *Dsor1* genes. The analysis revealed that the mutant genes contain single or, in the case of *Dsor1*^{Su6}, two point mutations that substitute amino acid residues (Figure 5; Table 1, column 1). The alterations were found mostly at the highly conserved residues in the kinase subdomains IV to VIB (HANKS *et al.* 1988), which locate outside the activation lip.

Phosphorylation of two adjacent serine/threonine residues in the activation lip of human MEK1 and yeast STE7 is necessary and sufficient for their activity (YAN and TEMPLETON 1994; ZHENG and GUAN 1994a), and substitutions of these residues in human MEK1 with acidic residues resulted in constitutive activation (HUANG and ERIKSON 1994; YAN and TEMPLETON 1994). The sequence of the kinase subdomains VII and VIII that include the phosphorylation lip is highly conserved (Figure 5), indicating its functional importance. The most plausible mechanism for the increased sensitivity of the *Dsor1* alleles to upstream signals would be alterations of the conformation of the activation lip to become more accessible to activators or less accessible to inactivating phosphatases. Such cases have been reported for gain-of-function mutations of *STE7* and *MKK1* that are associated with serine-to-proline substitutions at analogous positions in their putative activation lips (YASHAR *et al.* 1995). It is remarkable that no amino acid changes were found in the kinase subdomains VII and VIII and isolation of truly constitutively

active alleles of *Dsor1* may be inefficient by the methods used here. However, a possibility remains that alterations distantly located from the putative phosphorylation lip can somehow affect the conformation or flexibility of the lip.

Dsor1 must associate with a number of factors, such as activators, phosphatases, substrates and putative enhancers as discussed above, for its function, and the sites where the amino acid changes were found in association with the *Dsor1* mutations may be involved in the interaction with these factors. It has been shown that a proline-rich (PR) sequence, which is absent from the yeast homologues and SEK1/MKK4, and is located between the kinase subdomains IX and X in mammalian MEK is necessary for binding to Raf-1 and B-Raf and, consequently, for their activation (CATLING *et al.* 1995). The corresponding amino acid sequence in *Dsor1* is shorter than the PR sequence of MEK and is not rich in proline (Figure 5). *RafΔN* can utilize *Dsor1* as a substrate (Table 2), and the sequence may be responsible for binding to *RafΔN*. The highly conserved proline residue near the base of the putative Raf-binding loop was altered to alanine in *Dsor1*^{Su7}, and this change may affect the affinity to its authentic activator, D-raf. The mammalian MEK is phosphorylated *in vitro* by MEKK, which belongs to the same family as yeast MAPKKK (LANGE-CARTER *et al.* 1993), and the MEK lacking the PR sequence is activated by a factor other than the Raf family (CATLING *et al.* 1995). The allele-selective interaction between *Dsor1*^{Su1} and *STE11* (Figure 3; Table 2) suggests that the residue 192 is within the site for interaction with *STE11*.

The subdomain VIB contains the catalytic loop and the aspartic acid at residue 206 would be the catalytic base that accepts the proton from the hydroxyl group of the substrate (KNIGHTON *et al.* 1991; HARDIE and HANKS 1995; JOHNSON *et al.* 1996). It should be noted that two weak alleles, *Dsor1*^{Su5} and *Dsor1*^{Su18}, contain amino acid changes in the putative catalytic loop; the serine at residue 210 to asparagine in *Dsor1*^{Su5} and the lysine at residue 208 to asparagine in *Dsor1*^{Su18}. These alterations should affect the catalytic activity, and, in fact, the activity of *Dsor1*^{Su1} in yeast is reduced considerably by introducing the substitution found in *Dsor1*^{Su5} in combination with that found in *Dsor1*^{Su1} (Figure 3B). As these weak alleles significantly interact with gain-of-function mutations of upstream factors in *Drosophila*, the amino acid substitutions may have dual effects; one on the catalytic activity and the other on the interaction with regulatory factors.

Mammalian MEK localizes exclusively in the cytoplasm (ZHENG and GUAN 1994b), and it has been reported recently that a short leucine-rich sequence in the amino-terminal region of *Xenopus* MAPKK acts as a nuclear export signal (NES) (FUKUDA *et al.* 1996). A sequence similar to NES is also found in the corresponding region of *Dsor1* (Figure 5, underline), and

FIGURE 5.—Molecular lesions associated with the dominant *Dsor1* mutations. The amino acid sequence of Dsor1 is aligned with those of MEK from mouse (CREWS *et al.* 1992), MKK1 (IRIE *et al.* 1993), MKK2 (IRIE *et al.* 1993), STE7 (TEAGUE *et al.* 1986) and PBS2 (BOGUSLAWSKI and POLAZZI 1987) from *S. cerevisiae*. Only the sequences in the kinase domain of the yeast homologues are shown. The amino acid residues identical to those in Dsor1 are shaded. The kinase subdomains I–XI (HANKS *et al.* 1988) are indicated with overlines, and the serine/threonine residues at the putative phosphorylation sites by their activators are indicated with asterisks. The putative nuclear export signal (NES) (FUKUDA *et al.* 1996) is underlined. Amino acid substitutions associated with the dominant *Dsor1* mutations are indicated over the Dsor1 sequence. The amino acids are indicated with one letter code.

An important aspect of signal transduction that is not understood is how specific responses are induced by the same phosphorylation cascade functioning under different RTKs. Precise regulation of signal strength, interaction with specific modulators and between parallel pathways are thought to be crucial. Taken together the results suggest that the eight *Dsor1* alleles have increased sensitivity to upstream signals rather than constitutive activity. The significance of the individual peptide changes in receiving upstream signals may become clear when the higher structure of the *Dsor1* protein and proteins interacting with it become known. The *Dsor1* alleles show increased activity to upstream signals without obvious effects on the viability or morphology of the fly. They may prove valuable for analyzing genetic interactions with mutations affecting signal transduction and for screening novel signaling factors in both *Drosophila* and yeast. We are currently trying to isolate P-insertional mutants whose phenotypes can be either suppressed or enhanced by the *Dsor1* mutations, and

We are grateful for the fly stocks to U. BANERJEE, M. A. SIMON, G. M. RUBIN, E. HAFEN, T. SCHÜPBACH and B-Z. SHILO, for critical reading of this manuscript to S. SUGIYAMA and for technical assistance to T. TSUBOI. This work was supported by grants from The Mitsubishi Foundation, The Kurata Foundation and The Ministry of Education, Culture and Science of Japan.

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Communicating editor: N. TAKAHATA