The EMBO Journal vol.15 no.15 pp.3943–3950, 1996

Phosphorylation of *Drosophila* Jun by the MAP kinase Rolled regulates photoreceptor differentiation

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Drosophila **Jun (D-Jun) is a nuclear component of the receptor tyrosine kinase/Ras signal transduction pathway which triggers photoreceptor differentiation during eye development. Here we show that D-Jun is a substrate for the ERK-related *Drosophila* MAP kinase Rolled, which has previously been shown to be a part of this pathway. A D-Jun mutant that carries alanines in place of the Rolled phosphorylation sites acts as a dominant suppressor of photoreceptor cell fate if expressed in the eye imaginal disc. In contrast, a mutant in which the phosphorylation sites are replaced by phosphate-mimetic Asp residues, as well as a VP16–D-Jun fusion protein, can promote photoreceptor differentiation. These data implicate Jun phosphorylation in the choice between neuronal and non-neuronal fate during *Drosophila* eye development.**

Keywords: *Drosophila* development/Jun/MAP kinase/signal transduction/transcription factor

Introduction

Jun is a signal-responsive bZIP transcription factor of the AP-1 family. Studies in mammalian systems have shown that its activity can be regulated by a broad range of signals, implicating the protein in the regulation of a wide spectrum of biological phenomena as diverse as cell growth, differentiation and apoptosis (Angel and Karin, 1991; Bohmann *et al.*, 1994; Ham *et al.*, 1995). Several mechanisms, both transcriptional and post-translational, have been reported to mediate the induction of Jun activity by extracellular stimuli. Among the post-translational mechanisms which directly modulate Jun function at the protein level, phosphorylation and dephosphorylation reactions have prominent roles. A group of N-terminally located MAP kinase (MAPK) target sites function as primary recipients for intramolecularly transduced signals. Phosphorylation of these sites stimulates the transcriptional activity of Jun (Binetruy *et al.*, 1991; Smeal *et al.*, 1991). Based on tissue culture and biochemical studies, the extracellular-signal-regulated protein kinase (ERK)-type MAPKs pp42/44 and pp54 have been proposed as the enzymes which mediate signal-dependent Jun phosphorylation and activation (Pulverer *et al.*, 1991). This role for the ERKs is consistent with their position downstream of Ras in growth factor-activated signaling pathways. More recently, another class of MAPKs with substrate specificity for Jun has been identified. These enzymes, called stress-activated protein kinases (SAPKs) or Jun N-terminal kinases (JNKs), are responsive to a number of signals that are often associated with cell stress, such as radiation, exposure to protein synthesis inhibitors or TNFα (Hibi *et al.*, 1993; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994). SAPK activation is mediated by a signaling cascade that is distinct from that leading to ERK activation (Minden *et al.*, 1994, 1995; Sánchez *et al.*, 1994; Yan *et al.*, 1994; Coso *et al.*, 1995). *In vitro* SAPKs are more efficient kinases of Jun than the ERKs, which has caused a controversy about which enzyme 'truly' phosphorylates and activates Jun *in vivo*.

To gain insight into the signaling cascades and phosphorylation events targeting Jun in the organism, we turned to *Drosophila*, where the question can be addressed with both genetic and biochemical methods. The developing *Drosophila* eye is a particularly suitable system to monitor signaling events, including Jun regulation. Each ommatidium of the adult compound eye contains one R7 photoreceptor cell. Differentiation of this specialized UV-sensitive photoreceptor is triggered in the eye imaginal disc, during late larval to early pupal stages of development, by ligand-induced activation of the Sevenless receptor tyrosine kinase (RTK). It has been established that the Sevenless signal is relayed to the nucleus of the R7 precursor by a Ras-dependent pathway which results in activation of the *Drosophila* ERK-type MAPK Rolled (Biggs *et al.*, 1994; Brunner *et al.*, 1994b). We have previously reported that differentiation of R7 and also of the other photoreceptor cells requires the *Drosophila* transcription factor D-Jun (Bohmann *et al.*, 1994). Sevenless-dependent activation of D-Jun appears to occur at the post-translational level, as D-Jun expression in the developing eye is not affected by changes in Sevenless activity (Bohmann *et al.*, 1994). Here we show that D-Jun is directly phosphorylated by Rolled and present evidence that this phosphorylation is part of the switch that triggers neuronal differentiation of photoreceptors.

Results

Rolled, a Drosophila ERK, phosphorylates D-Jun

To investigate the potential regulation of D-Jun by protein phosphorylation, we searched for kinase(s) specific for D-Jun. Crude extracts derived from Schneider cells or from young wild-type *Drosophila* pupae, harvested at the time of photoreceptor differentiation, were incubated with a glutathione *S*-transferase (GST)–D-Jun fusion protein that was immobilized on glutathione–Sepharose beads. After extensive washing which removed more than 95% of the input protein (data not shown), an activity that could specifically phosphorylate D-Jun was retained on

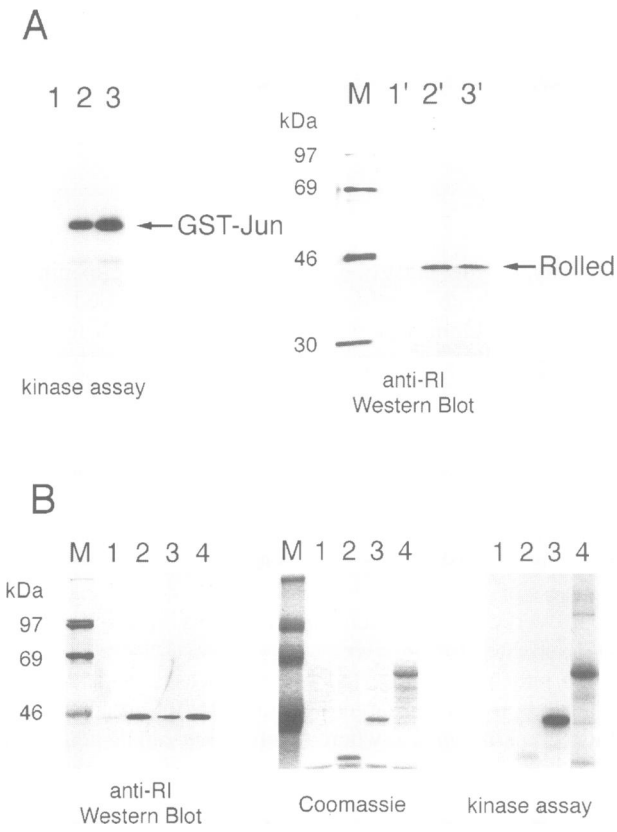

Fig. 1. Rolled binds to D-Jun within the 81 N-terminal amino acids and co-purifies with a Jun-specific kinase activity. (**A**) D-Jun-specific kinase activity was affinity purified from Schneider 2 cell extracts (lane 2) or from early pupal extracts (lane 3) on Sepharose bound GST–D-Jun. Lane 1 shows a control in which unfused GST was used for affinity purification. Kinase activity was monitored by adding [γ-^{32}P]ATP to allow phosphorylation of GST–D-Jun by purified kinase(s) (in lane 1 GST–D-Jun was added after the washing step to provide the same concentration of substrate as in the other reactions). Radioactive GST–D-Jun (indicated by an arrow) was visualized by SDS–PAGE and autoradiography (left panel). An aliquot of the same column eluates was also assayed by Western blotting with an anti-Rolled antibody (lanes 1′–3′). A cross-reactive band with the apparent molecular mass of Rolled is marked with an arrow. Molecular masses (in kDa) of protein standards are indicated on the left side of the panel. (**B**) The same experiment as in (A) was performed with the following GST fusion proteins: lanes 1, unfused GST; lanes 2, GST–D-Jun 1–81; lanes 3, GST–D-Jun 1–134; lanes 4, GST–D-Jun 1–289 (full-length). Pupal extracts were used as starting material for the 'pull-down' assays. The different panels show different analyses of the eluate fractions, from left to right: an immunoblot developed with anti-Rolled antiserum to visualize the purified kinase; a Coomassie stained SDS gel showing the different GST–D-Jun derivatives; a D-Jun kinase assay (autoradiography of the Coomassie stained gel).

the beads, as monitored by phosphorylation of the GST–D-Jun protein after addition of [γ-^{32}P]ATP (Figure 1A, lanes 2 and 3). In a control experiment with unfused GST protein no kinase activity was recovered (Figure 1A, lane 1). Previous genetic experiments had indicated that the *Drosophila* MAPK Rolled acts upstream of D-Jun in the pathway that determines photoreceptor fate (Biggs *et al.*, 1994; Bohmann *et al.*, 1994; Brunner *et al.*, 1994b). To examine whether this epistatic relationship between Rolled and D-Jun reflects a direct interaction between the two proteins, we probed the eluate from the GST–D-Jun resin with a Rolled antiserum. The immunoblot in Figure 1A documents that Rolled is indeed present in the eluate fraction and thus specifically co-migrates with the D-Jun kinase activity. Two further GST fusion proteins containing the 81 and the 134 N-terminal amino acids of D-Jun, respectively, were also tested for their ability to sequester Rolled from a crude extract. As shown in the immunoblot in Figure 1B (left panel) both proteins can pull down Rolled with an efficiency comparable with full-length D-Jun fused to GST. This finding delimits the part of D-Jun that is required for Rolled binding to the N-terminal 81 amino acids. Interestingly, however, this 81 amino acid D-Jun fragment, while competent to bind Rolled, can no longer function as a kinase substrate (Figure 1B right panel). This result is reminiscent of the properties of mammalian Jun and SAPK, where the kinase binding and phosphoacceptor sites also map to physically separable peptide sequences (Hibi *et al.*, 1993).

To confirm that Rolled directly phosphorylates D-Jun and to find out whether additional D-Jun-specific kinases might be present in pupal extracts, we performed in-gel kinase assays. Crude extracts were prepared from young pupae and loaded onto a polyacrylamide gel that had been polymerized in the presence of bacterially expressed D-Jun protein. Soaking of the gel with [γ-^{32}P]ATP after a renaturation step permits the phosphorylation of the gel-bound substrate by specific kinases, should they be present among the electrophoretically separated pupal proteins. The one- and two-dimensional gels displayed in Figure 2 show that only one such activity, with the apparent molecular mass of Rolled, can be detected.

Taken together, the biochemical and immunological data presented above identify Rolled as a D-Jun kinase in *Drosophila*. To also gain genetic evidence for this function of Rolled, we performed in-gel kinase assays with larval extracts from various different genotypes. As previously reported, gain-of-function alleles of components of the Sevenless/Ras pathway, such as activated alleles of *ras*1 (*ras*^{Val12}) or *rolled* (*rl*^{Sem}), expressed during eye development, cause the appearance of ectopic photoreceptors in the adult eye (Figure 3A). A corresponding increase in D-Jun-specific kinase activity can be measured in extracts from the mutant pupae (Figure 3B). In heterozygous flies carrying one *rolled* null allele (*rl*^{10a}), the phenotypic effects of *ras* gain-of-function alleles are suppressed (Biggs *et al.*, 1994; Brunner *et al.*, 1994b; Figure 3A). Consistently, the *ras*^{Val12}-dependent stimulation of D-Jun kinase activity is reduced in extracts from larvae that lack one copy of the *rolled* gene (Figure 3B). Thus, whenever Rolled activity is stimulated genetically, increased phosphorylation of D-Jun can be measured, further supporting the identity of D-Jun as a substrate for Rolled. Significantly, in all tested genotypes the strength of the ectopic photoreceptor phenotype correlates well with the measured D-Jun kinase activity, suggesting an important role of D-Jun phosphorylation in the determination of R cell fate (see below).

D-Jun is phosphorylated on three conserved MAPK sites

Next, we sought to determine on which sites D-Jun is phosphorylated by Rolled. As discussed above, the N-terminal 134 amino acids of D-Jun can be bound and phosphorylated by Rolled *in vitro*. GST–D-Jun 1–134 phosphorylated in this manner with ^{32}P and the affinity-

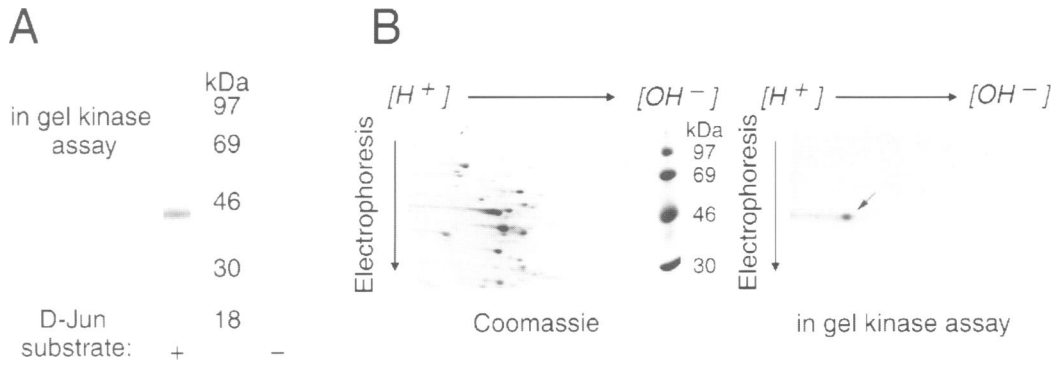

Fig. 2. A single 45 kDa D-Jun kinase activity is detectable in *Drosophila* extracts using one- and two-dimensional in-gel kinase assays. (**A**) An extract from wild-type *Drosophila* white prepupae was subjected to in-gel kinase assays with (+) or without (−) bacterially expressed D-Jun as a substrate. Molecular masses of protein standards are indicated. (**B**) Two identical aliquots of pupal extract were separated by two-dimensional gel electrophoresis and Coomassie stained (left) or subjected to an in-gel kinase assay using D-Jun as a substrate (right). For this experiment extracts from ras^{Val12} pupae were used to obtain a better signal. The arrows on the top of the panels show the direction of migration of the proteins in the non-equilibrium pH gradient electrophoresis of the first dimension. The direction of the second dimension is indicated by arrows on the left side of the panels. The arrow in the right panel points to the single spot with observed Jun kinase activity. The molecular masses of protein standards are indicated on the right side of the left panel.

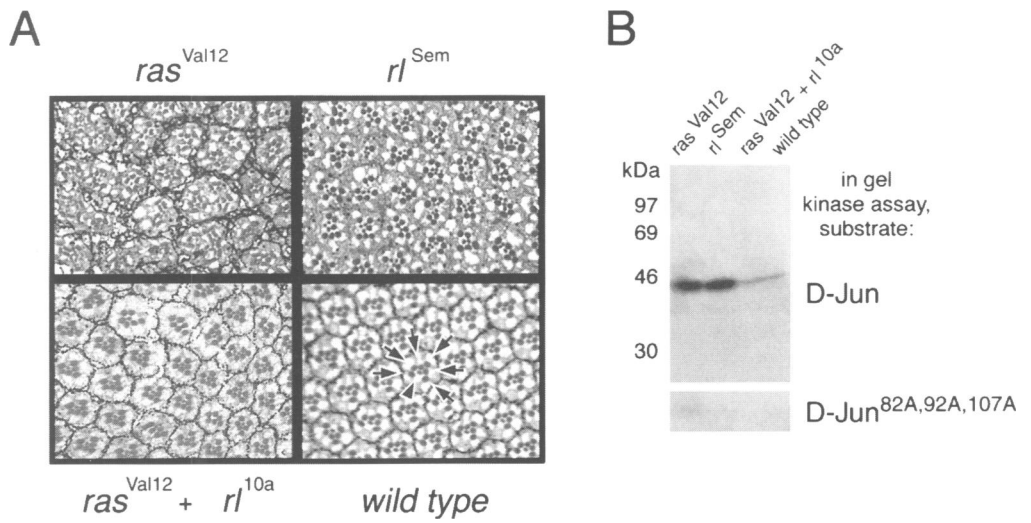

Fig. 3. Flies in which Rolled is genetically activated display ectopic photoreceptor differentiation and increased Jun-specific kinase activity. (**A**) Tangential eye sections of flies with the indicated genotypes are shown. In the bottom right panel, showing a section through a wild-type eye, the positions of the six outer photoreceptor cells of a typical ommatidium are indicated with black arrows, the R7 cell is marked with an arrowhead. Both the gain-of-function alleles of *rolled* (rl^{Sem}) and of *ras* (ras^{Val12}) give rise to additional photoreceptors as compared with the wild-type (wt). Reduction of the *rolled* gene dose by introducing the null allele rl^{10a} suppresses the phenotypic effects of ras^{Val12} (ras^{Val12} + rl^{10a}). (**B**) Extracts from *Drosophila* white prepupae of the same genotypes analyzed in (A) were subjected to in-gel kinase assays using wild-type D-Jun or a point mutant in the indicated phosphoacceptor sites as substrates. A Jun kinase with the apparent molecular mass of Rolled is detected. Note that the kinase activity is higher in extracts from prepupae that carry gain-of-function alleles of *ras* and *rolled* (ras^{Val12} and rl^{Sem}) and repressed if one copy of the *rolled* gene is removed due to the rl^{10a} allele. A parallel in-gel kinase assay performed with the D-Jun point mutant is shown at the bottom of the figure, indicating that the Jun kinase activity is abolished by mutating the indicated phosphoacceptor sites to Ala.

purified Rolled preparation was further analyzed by tryptic digestion and two-dimensional peptide separation. Figure 4A shows that three tryptic phosphopeptides can be discerned by this analysis. Consistent with this result, three potential MAPK target sites, Ser82, Thr92 and Thr107, can be identified in the protein sequence of D-Jun 1–134. These amino acids correspond in their sequence context and relative location to established phosphorylation sites in human c-Jun (Papavassiliou *et al.*, 1995; Figure 4A). To investigate whether these residues might serve as target sites for Rolled, we engineered a mutant of GST–D-Jun 1–134 in which all three of them were converted to Ala. Figure 4B (left panel) shows that this 'triple Ala' protein is not an efficient phosphorylation substrate: none of the previously identified phosphopeptides could be detected after kinase assay and tryptic mapping. To confirm the identity of Ser82, Thr92 and Thr107 as phosphorylation sites, we tested 'double Ala' mutants in which combinations of two of the three putative target sites were mutated to Ala, i.e. only one presumptive phosphorylation site was preserved. Phosphopeptide maps generated from these three mutant proteins each showed only one of the three spots of the wild-type pattern (Figure 4B, right three panels). This result is consistent with our assignment of phosphorylation sites and, furthermore, reveals the identity of the spots in the two-dimensional tryptic analysis (Figure 4D).

After establishing that Ser82, Thr92 and Thr107 serve as substrates for Rolled *in vitro*, it was important to determine whether they are also phosphorylated *in vivo*.

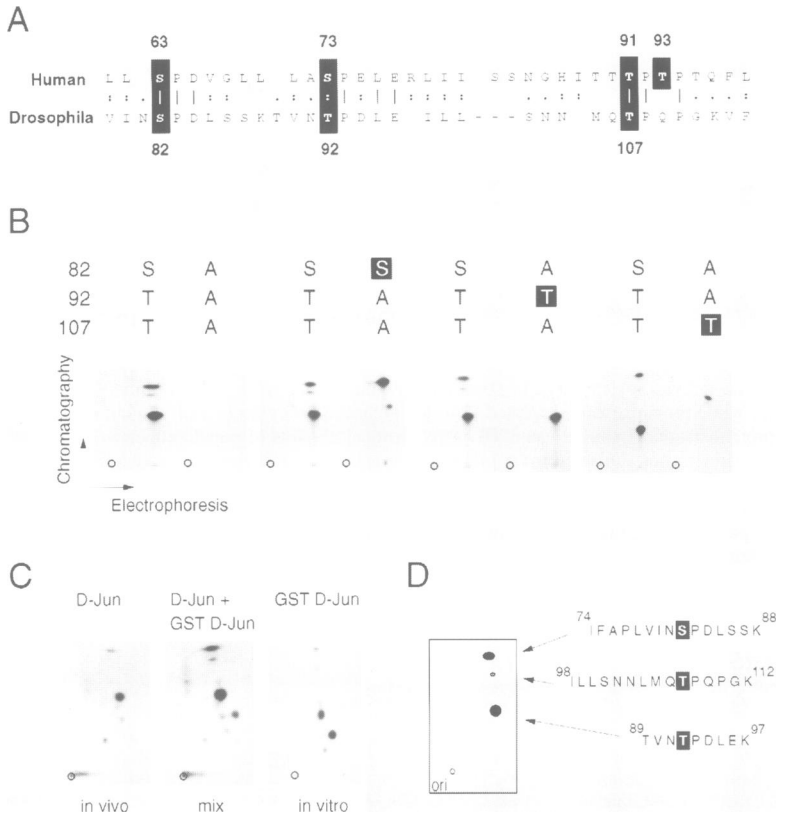

Fig. 4. Three phosphorylation sites are conserved between human and *Drosophila* Jun. (**A**) Comparison of the human and *Drosophila* Jun sequences around the N-terminal phosphorylation sites (marked by boxes). (**B**) Tryptic phosphopeptide maps of D-Jun phosphorylated by Rolled *in vitro*. N-Terminal wild-type GST–D-Jun (GST–D-Jun 1–134) or a set of mutants in which the amino acids indicated on the top of each panel have been converted to Ala were used to affinity purify the Jun-binding kinase activity. After *in vitro* kinase reaction (see legend to Figure 1) the ^{32}P-labeled proteins were purified by SDS–gel electrophoresis and analyzed by tryptic phosphopeptide mapping and autoradiography. Tryptic digests of the wild-type GST–D-Jun 1–134 were loaded along with one mutant (right) on the same TLC plate for direct comparison. Electrophoresis was followed by chromatography as indicated. The origin of the peptide separation is marked by a circle. (**C**) Comparison of D-Jun phosphorylation *in vitro* and *in vivo*. D-Jun was immunoprecipitated from Schneider 2 cells metabolically labeled with [^{32}P]orthophosphate and analyzed by tryptic phosphopeptide mapping as described in (B). The phosphopeptide patterns of *in vivo* labeled D-Jun (left panel), *in vitro* labeled GST–D-Jun 1–289 (right panel) and a mix of both (middle panel) are shown. The spots at the bottom of the panels originate from phosphorylations C-terminal to amino acid 134 of D-Jun (data not shown). (**D**) Schematic representation of the three N-terminal tryptic phosphopeptides. The peptide sequences and their coordinates are depicted on the right of the panel. The putative phosphoacceptor sites are highlighted. The arrows indicate the position of the corresponding peptides relative to the origin (ori) after two-dimensional fractionation.

Phosphopeptide maps from D-Jun immunoprecipitated from Schneider cells after metabolic labeling with ^{32}P were generated and compared with the maps obtained after *in vitro* phosphorylation of full-length D-Jun (Figure 4C). This experiment shows that the phosphopeptides containing Ser82 and Thr92 are also found in the *in vivo* labeled material. The peptide containing Thr107, which was the most weakly labeled *in vitro*, was only barely discernible in the *in vivo* phosphopeptide map. Taken together, these data indicate that the *in vitro* characterized phosphorylation sites are also utilized in the cell and are thus potentially relevant for the regulation of D-Jun activity *in vivo*.

D-Jun phosphorylation and photoreceptor differentiation

To investigate whether phosphorylation of D-Jun on the thus identified amino acids would play a role in photoreceptor differentiation, we expressed D-Jun^{82A,92A,107A} in transgenic flies under the control of the eye-specific *sevenless* enhancer. Microscopic inspection of the eye phenotype of flies carrying this transgene indicated that D-Jun^{82A,92A,107A} acted like a dominant negative form of D-Jun: it suppressed the differentiation of photoreceptor cells (Figure 5, left panels). This effect was most clearly visible in a sensitized background where ectopic R7 cells are induced by gain-of-function mutations in the Sevenless/Ras pathway, for example by the activated *raf* allele *raf*^{tor4021} (Figure 5, middle panels) or the *rolled* allele *Sevenmaker* (Figure 5, right panels). Flies expressing both Raf^{tor4021} or Rl^{Sem} and D-Jun^{82A,92A,107A} show significantly reduced numbers of R7 cells in comparison with flies carrying the gain-of-function *raf* or *rolled* alleles alone. Suppression of R cell differentiation is not detected in transgenic flies that express wild-type D-Jun from otherwise identical constructs to that used here for D-Jun^{82A,92A,107A} (see Figure 6 and Bohmann *et al.*, 1994). Thus, abrogating the ability of D-Jun to serve as a Rolled substrate interferes with photoreceptor differentiation.

To complement the loss-of-function effect observed with the dominant negative Ala substitution mutants of D-Jun, we attempted to mimic the phosphorylated form of D-Jun by replacing the three Rolled phosphorylation sites with negatively charged Asp residues. When the

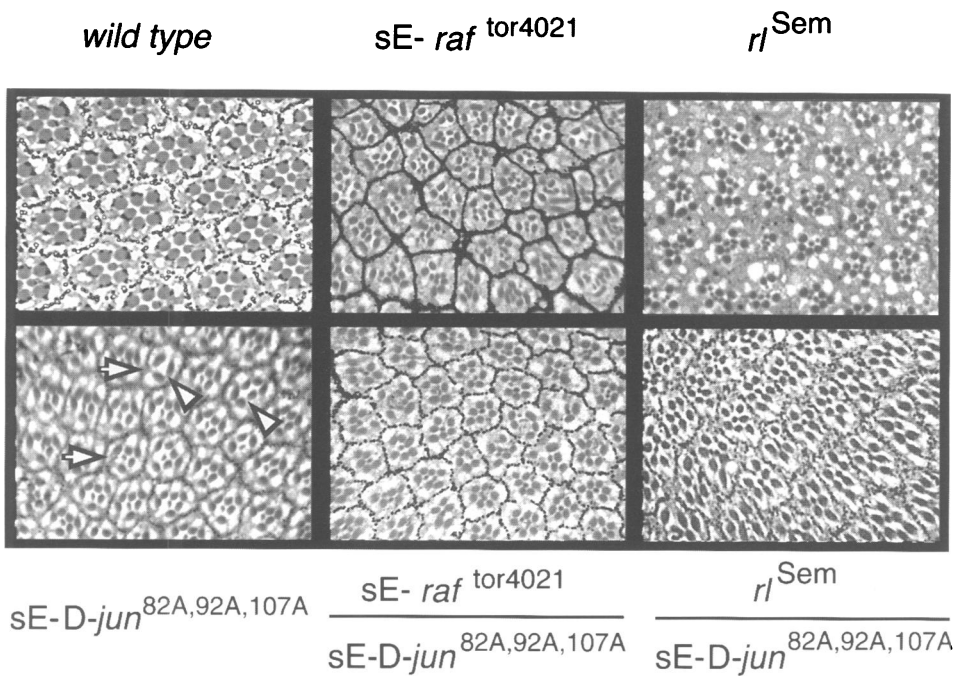

Fig. 5. Mutation of the D-Jun phosphorylation sites to Ala interferes with photoreceptor differentiation. The effect of D-Jun^{82A,92A,107A} expression on eye morphology was analyzed in different genetic backgrounds. Tangential sections through eyes of adult flies with the indicated genotypes are shown. In flies expressing D-Jun^{82A,92A,107A} (lower left panel) ommatidia lacking outer photoreceptors (empty arrows) or R7 cells (empty arrowheads) are found. Note that gain-of-function alleles of *raf* (top middle) and of *rl* (top right) cause the differentiation of additional R7 photoreceptor cells. The appearance of these ectopic R7 cells is suppressed in flies expressing D-Jun^{82A,92A,107A} from the *sevenless* enhancer (bottom panels).

resulting protein, D-Jun^{82D,92D,107D}, was expressed in the eye imaginal disc under the *sevenless* enhancer, no phenotypic alterations could be detected (Figure 6A). Presumably, the triple Asp replacement does not generate a strong enough activation of D-Jun to alter the stable wild-type pattern of photoreceptor cell differentiation. Thus we used a sensitized background in which more subtle changes in neurogenic signaling can be detected. Flies that carry the hypomorphic *raf* allele *raf*^{HM7}, in which the expression of Raf protein is strongly reduced, provide such a genotype. The eyes of *raf*^{HM7} flies display defects in photoreceptor cell recruitment, with ~70% of the ommatidia lacking one or more photoreceptor cells (Figure 6A and B). Expression of D-Jun^{82D,92D,107D} in the *raf*^{HM7} background partially rescues this phenotype, giving rise to the differentiation of otherwise missing photoreceptors. In the D-Jun^{82D,92D,107D}/*raf*^{HM7} flies 60–70% of the ommatidia have the wild-type complement of photoreceptor cells (Figure 6A and B). Wild-type D-Jun does not rescue the *raf* phenotype to the same extent. This implies that D-Jun phosphorylation by Rolled, as mimicked by the triple Asp mutation, does promote photoreceptor differentiation.

Together with previous genetic data (Bohmann *et al.*, 1994; Treier *et al.*, 1995), these results strongly suggest that the induction of neuronal fate triggered by Sevenless activation requires the phosphorylation of D-Jun by the Rolled kinase. Based on experiments in mammalian systems (Binetruy *et al.*, 1991; Pulverer *et al.*, 1991; Smeal *et al.*, 1991; Papavassiliou *et al.*, 1995), it is quite conceivable that this involves activation of the D-Jun transcription activation potential by MAPK phosphorylation. If this was the case, one might predict that photoreceptor differentiation could be artificially triggered by attaching a strong constitutive transcription activation domain to D-Jun. We tested this hypothesis by constructing a fusion protein in which the N-terminal region of D-Jun, including the Rolled substrate sites, was replaced by the transactivation domain of the herpesvirus VP16 protein. A similar fusion has previously been shown to possess strong AP-1 activity (Baichwal *et al.*, 1992). As shown in Figure 6C and D, expression of VP16–D-Jun under the *sevenless* enhancer in the eye imaginal disc causes the differentiation of supernumerary photoreceptors, predominantly R7 cells, in the adult eye, a phenotype that is reminiscent of gain-of-function mutations in the Sevenless/Ras/Raf/Rolled pathway. These results indicate that activation of the D-Jun transactivation properties, either naturally by MAPK phosphorylation or artificially by fusion to a heterologous transcription activation domain, is a key step in the determination of photoreceptor fate during *Drosophila* eye development.

Discussion

The phosphorylation of Jun by MAPK-type enzymes is a paradigm for signal-dependent transcription factor activation (Hill and Treisman, 1995). Here we report how this process is employed to make a developmental decision, namely the choice between a non-neuronal and a neuronal photoreceptor cell fate during *Drosophila* eye development. The dominant negative properties of D-Jun^{82A,92A,107A}, as well as the gain-of-function effects of D-Jun^{82D,92D,107D} and VP16–D-Jun, suggest that Sevenless-induced D-Jun phosphorylation by Rolled results in D-Jun activation and is necessary for proper R7 cell differentiation. It is not clear based on these data whether this event

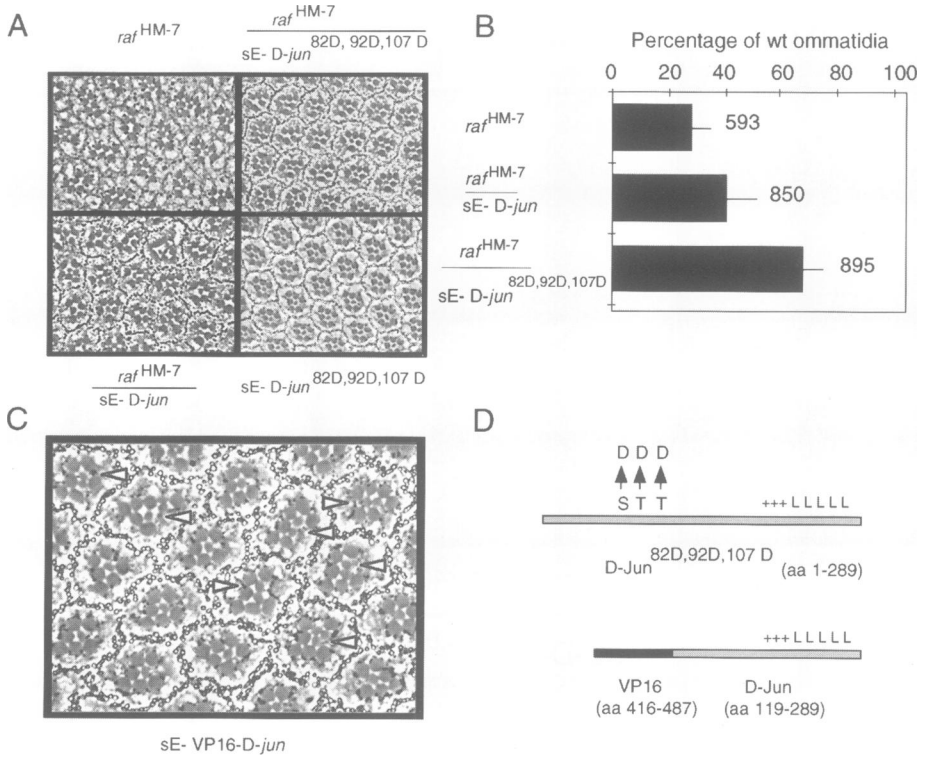

Fig. 6. Gain-of-function D-Jun mutants that mimic N-terminal phosphorylation or carry a VP16 transactivation domain promote photoreceptor differentiation. (**A**) The D-Jun^{82D,92D,107D} mutant, in which the identified phosphoacceptor sites were replaced by phosphate-mimetic Asp residues, was expressed from the *sevenless* enhancer and the effects on eye morphology were analyzed in different genetic backgrounds. Tangential sections through adult flies with the indicated genotypes are shown. The phenotype caused by a hypomorphic *raf* allele, *raf*$^{HM-7}$ (top left panel), showing a dramatic reduction in the number of R7 and outer photoreceptors, is rescued by D-*jun*^{82D,92D,107D} (top right panel), but not by the wild-type D-*jun* (lower left panel). D-*jun*^{82,92,107D}, however, is not strong enough to promote extra R7 photoreceptor differentiation in a wild-type background (lower right panel). (**B**) Statistical analysis of the D-*jun*^{82D,92D,107D} rescue of the *raf*$^{HM-7}$ phenotype. The histogram indicates the percentage of wild-type ommatidia observed in the specified genotypes. The numbers beside the error bar refer to the total number of ommatidia examined. (**C**) The effects of VP16–D-Jun on eye development. A tangential section of an adult fly expressing the *VP16*–D-*jun* cDNA from the *sevenless* enhancer in a wild-type background is shown. The arrowheads indicate ommatidia with extra R7 photoreceptors. (**D**) D-Jun mutant proteins which promote photoreceptor differentiation. D-Jun protein is depicted by an empty box. The position of the basic and leucine zipper (+++LLLLL) domains which are required for DNA binding and dimerization are shown. In D-Jun^{82D,92D,107D} the serine (Ser82) and the threonine (Thr92 and Thr107) residues were converted to Asp. In the VP16–D-Jun protein, the 118 N-terminal residues, including the phosphorylation sites, were replaced by the transactivation domain (black box) of VP16 (residues 416–487).

is also sufficient, i.e. whether Jun phosphorylation by itself is enough to trigger photoreceptor differentiation or whether other Rolled targets are required in addition. One such component might be the Ets protein Pointed, which has recently been identified as a nuclear target of the Sevenless/Ras/Rolled cascade and a positive regulator of R cell differentiation (Brunner *et al.*, 1994a; O'Neill *et al.*, 1994). Consistent with the idea of several cooperating nuclear effectors of Sevenless, our studies (Treier *et al.*, 1995) indicate that phosphorylated Jun synergistically interacts with Pointed in gene activation and eye development.

It is worth noting that Rolled is more related to ERK-type than to SAPK-type MAPKs, demonstrating that members of the former class of enzymes can interact with Jun and contribute to target gene activation. It appears that the role of D-Jun in *Drosphila* eye development and photoreceptor differentiation is governed by Rolled rather than by *Drosophila* SAPK. This conclusion is supported by studies on a recently identified *Drosophila* homolog of SAPK. Somatic recombination studies using mutant alleles indicate that, in contrast to Rolled, *Drosophila* SAPK is not required for photoreceptor differentiation (Ernst Hafen, personal communication). Jun functions in many different biological signaling systems, as for example in control of the $G_0 \rightarrow G_1$ transition, the cellular stress response or developmental processes such as photoreceptor differentiation in the *Drosophila* facet eye. The question of what determines the specificity of the particular biological readout after Jun activation in these different situations remains a largely unresolved issue. It is tempting to speculate, however, that the identity of the kinase that phosphorylates Jun, be it an ERK as in cell differentiation or a SAPK as in stress response, might influence the nature of the response.

As mentioned in the Introduction, D-Jun activity is tightly controlled both transcriptionally and post-translationally. In the developing *Drosophila* eye these two regulatory levels appear to play distinguishable roles. The D-Jun expression pattern in the eye imaginal disc is temporally and spatially well defined by a so far uncharacterized mechanism that is independent of the Sevenless pathway (Bohmann *et al.*, 1994). D-Jun is specifically expressed in cells of the R cell equivalence group, i.e. all those cells which are competent to adopt the neuronal photoreceptor fate (in the wild-type situation not all cells of this equivalence group actually do that). The determination of photoreceptor fate is triggered by activa-

tion of the Ras/MAPK pathway, in the case of the R7 cell by Sevenless. This results in D-Jun phosphorylation on positive stimulatory sites (Treier *et al.* 1995; this work) and presumably in the activation of D-Jun target genes that are involved in the execution of the R cell differentiation program. Thus, D-Jun expression correlates with the competence of imaginal disc cells to undergo neuronal differentiation, while signal-dependent D-Jun phosphorylation appears to be at least part of the molecular trigger which initiates this process. Taking advantage of the genetic and biochemical possibilities of the *Drosophila* system, it should be possible to identify further components that cooperate with D-Jun in the developmental decision-making processes discussed here, as well as relevant downstream target genes that are turned on to execute the neuronal differentiation process.

Materials and methods

Plasmid constructions

To generate mutant forms of D-Jun the codons for Ser82, Thr92 and Thr107 were replaced in various combinations by PCR-mediated mutagenesis (Landt *et al.*, 1990) with Ala or Asp codons.

Bacterial expression vectors. The GST–D-Jun fusion protein used for the 'pull-down' experiments is encoded by the plasmid pFP115, which contains the GST gene fused to a D-Jun–His_6 sequence in a modified pGEX-2T vector (pFP98). GST–D-Jun 1–134 and GST–D-Jun 1–81 were derived from pFP115 by removing the appropriate 3′-coding sequence of D-*jun*. These constructs also carry a C-terminal histidine tag. The D-Jun and D-$Jun^{82A,92A,107A}$ proteins used for in-gel kinase assays were C-terminally histidine tagged and expressed in bacteria from a pAR3040-derived plasmid (Bohmann *et al.*, 1994).

P-Element transformation vectors. A fragment encoding the activation domain of VP16 protein (amino acids 416–487; Peverali *et al.*, 1994) was used to replace codons 2–118 of D-*jun* by PCR-mediated mutagenesis to generate the *VP16*–D-*jun* fusion gene. For the generation of transgenic flies, the D-$jun^{82A,92A,107A}$, D-$jun^{82D,92D,107D}$ and *VP16*–D-*jun* cDNAs were cloned into the pKB267 vector (Basler *et al.*, 1991), which contains a tandem duplication of the sevenless enhancer (sE) element and an hsp70 promoter. The sE–D-*jun* construct used as a control has been described previously (Bohmann *et al.*, 1994).

Transgenic and mutant fly strains

Germline transformation was performed by standard procedures (Spradling and Rubin, 1982) using w^{1118} as host strain and pUCHSpΔ2-3 (Mullins *et al.*, 1990) as helper plasmid. Four independent lines were analyzed for the sE–D-$jun^{82A,92A,107A}$ construct. Three of them had a mild effect on eye development in a wild-type background (see Results). These lines were tested for suppression of sE–$raf^{tor4021}$ and rl^{Sem}. Three transgenic lines carrying the sE–D-$jun^{82D,92D,107D}$ expression vector and one previously characterized sE–D-*jun* line were tested for genetic interaction with the hypomorphic *raf* allele, raf^{HM-7} (Melnick *et al.*, 1993). Three independent lines carrying the sE–*VP16*–D-*jun* construct were examined for the occurrence of extra photoreceptors. The following fly strains were used for genetic interactions, phenotypic and biochemical analyses: *sev-*ras^{Val12} (Fortini *et al.*, 1992), carrying P[ry^+, *sev-*ras^{Val12}] insertions either on the Cy0 or TM3 balancers; sE_$raf^{tor4021}$ (Dickson *et al.*, 1992), carrying an insertion of P[w^+, sE–$raf^{tor4021}$] on the third chromosome in a sev^- background; rl^{Sem} (Brunner *et al.*, 1994b), a gain-of-function allele of *rl*; rl^{10a} (Hilliker, 1976), carrying a deletion on the second chromosome which deletes the *rl* gene.

Bacterial expression of wild-type and mutant D-Jun proteins

GST–D-Jun, GST–D-Jun 1–134 and GST–D-Jun 1–81 were expressed in *Escherichia coli* XL1-Blue. Overnight LB cultures containing 100 μg/ml ampicillin and 10 μg/ml tetracycline were diluted 10-fold and 1 h later induced with 1.5 mM isopropyl-thio-galactopyranoside (IPTG) for 2 h at 30°C. The bacteria were harvested and lysed for 30 min on ice in phosphate-buffered saline (PBS) containing 100 μg/ml lysozyme, 0.05% Triton-X100, in the presence of the following protease inhibitors: 3 μg/ml leupeptin, 5 μg/ml aprotinin, 3 μg/ml pepstatin, 1 μg/ml E64 and 1 mM phenylmethylsulfonyl fluoride. After sonication and centrifugation at 25 000 *g* the supernatant fraction was mixed with Ni^{2+}-NTA–agarose beads. Bound proteins were eluted in PBS with 200 mM imidazole and subsequently dialyzed against PBS. Recombinant D-Jun proteins were then further purified by chromatography on glutathione–Sepharose beads and used for the 'pull-down' experiments. The pAR3040-derived expression vectors were transformed into *E.coli* BL21(DE3) and the expressed proteins were purified under denaturing conditions on Ni^{2+}–NTA–agarose columns as described (Bohmann *et al.*, 1994).

In vivo labeling of Schneider cells, immunoprecipitation and two-dimensional phosphopeptide analysis

Schneider 2 cells (6×10^6 per 6 cm plate) were grown for 2 h in Grace's phosphate-free medium in the presence of 0.7 mCi/ml [^{32}P]orthophosphate. Cells were lysed in Ripa buffer and D-Jun was immunoprecipitated with an affinity purified anti-D-Jun antibody (Bohmann *et al.*, 1994; Papavassiliou, 1994). Immunoprecipitated material was loaded onto a 12% SDS–polyacrylamide gel. After gel purification ^{32}P-labeled D-Jun was subjected to two-dimensional tryptic phosphopeptide mapping as previously described (Papavassiliou *et al.*, 1992).

Western blot analysis and solid phase kinase assay

Whole-cell extracts were prepared from Schneider 2 cells or early pupae and mixed with 10 μg GST–D-Jun or unfused GST bound to glutathione–Sepharose beads. Extract preparation, washing and elution of the affinity resin were performed as described (Hibi *et al.*, 1993). Western blotting was carried out with polyclonal anti-Rolled antisera (Biggs and Zipursky, 1992), using a chemoluminescence detection method. The solid phase kinase reaction has been described (Papavassiliou *et al.*, 1995).

Two-dimensional electrophoresis (NEPHGE-SDS)

Proteins from sE–ras^{Val12} prepupae were separated by two-dimensional gel electrophoresis (O'Farell *et al.*, 1977). Briefly, extracts were prepared by lysing prepupae in 9.5 M urea, 2% Nonidet P-40 (NP-40), 5% β-mercaptoethanol containing 0.4% ampholines (Pharmacia) pH range 3.5–10 and 1.6% ampholines pH range 5–7. The first dimension non-equilibrium pH gradient electrophoresis (NEPHGE) gels were poured in glass tubes (100×1.5 mm inner diameter) with a gel mixture containing 9.2 M urea, 2% NP-40, 4% acrylamide/bisacrylamide (28.4:1.6), 2% ampholines pH range 3.5–10 and 66.7 μl 10% ammonium persulfate per 10 ml gel mixture. Extract samples were loaded and overlaid with sample overlay solution: 8 M urea, 5% NP-40 and 1% ampholines (0.2% pH range 3.5–10 and 0.8% pH range 5–7). The gels were run at 300 V for 2 h. Following electrophoresis, the gels were removed from the glass tubes, equilibrated briefly in Laemmli sample buffer and placed horizontally onto a 1.5 mm thick SDS–polyacrylamide gel for separation in the second dimension. Gels were polymerized in the presence of 100 μg/ml D-Jun protein for the two-dimensional in-gel kinase assay (see below).

In-gel kinase assay

Protein extracts of white prepupae of different genotypes were prepared in sample buffer as described (Marais *et al.*, 1993). In-gel kinase assays were performed as described (Hibi *et al.*, 1993) with the following modifications. The extract proteins were separated on 10% SDS–polyacrylamide gels or on two-dimensional gels (see above), which were polymerized in the presence of 100 μg/ml D-Jun or D-$Jun^{82A,92A107A}$. After electrophoresis, the gel was soaked twice for 45 min in 200 ml 20% 2-propanol, 50 mM HEPES–NaOH, pH 7.5, and twice for 30 min in 200 ml buffer A (50 mM HEPES–NaOH, pH 7.5, 5 mM β-mercaptoethanol). The gel was then incubated twice for 45 min in 200 ml 6 M urea in buffer A at room temperature, followed by serial 20 min incubations with 200 ml buffer A containing 0.05% Tween 20 and 3, 1.5 or 0.75 M urea respectively at 4°C. After washing five times for 20 min with 200 ml buffer A containing 0.05% Tween 20, the gel was preincubated for 20 min at 30°C in 30 ml kinase buffer (20 mM HEPES–NaOH, pH 7.5, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate, 0.1 mM Na_3VO_4, 2 mM dithiothreitol, 5 mM $MgCl_2$ and 2 mM $MnCl_2$) and then incubated for 1 h in 20 ml kinase buffer containing 50 μM ATP, including 5 μCi/ml [γ-^{32}P]ATP. Finally, the gel was washed in 5% trichloroacetic acid, pH 5.7, 1% sodium pyrophosphate and 2 mM ATP overnight at room temperature, followed by drying and autoradiography.

Histology

Retinae of adult flies were fixed, embedded, sectioned and stained as described (Tomlinson and Ready, 1987).

Acknowledgements

We thank Ernst Hafen, Barry Dickson, Konrad Basler, William Biggs, Lawrence Zipurski, Mark Fortini and Gerry Rubin for generous gifts of reagents and fly strains. The members of the Bohmann and Mlodzik laboratories, especially Lutz Kockel, are acknowledged for helpful discussions and technical advice. Sandra Scianimanico and Ann-Marie Voie generated all transgenic fly lines used in this study. We are grateful to Steve Cohen and Julia Zeitlinger for comments on the manuscript. This work was supported by an HFSP fellowship to F.A.P. and an HFSP grant to D.B.

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Received on September 13, 1995; revised on April 22, 1996