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Abstract

The phyllopod (phyl) gene regulates the fates of a subset of cells in the developing Drosophila eye; in the absence of phyl function, the R1, R6, and R7 photoreceptors are transformed into additional cone cells, whereas ectopic phyl expression in the cone cell precursors transforms these cells into additional R7 cells. Within this group of cells, phyl expression thus mimics activation of the Raf pathway in its ability to induce photoreceptor rather than cone cell development. Furthermore, the transformation of cone cells into R7 cells in response to Raf activation is both accompanied by and dependent upon ectopic phyl expression. phyl thus represents a possible target gene of the Raf pathway during eye development, controlling the fates of a novel subset of photoreceptors.
Control of Drosophila Photoreceptor Cell Fates
by Phyllopod, a Novel Nuclear Protein
Acting Downstream of the Raf Kinase

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Summary

The phyllopod (phyl) gene regulates the fates of a subset of cells in the developing Drosophila eye; in the absence of phyl function, the R1, R6, and R7 photoreceptors are transformed into additional cone cells, whereas ectopic phyl expression in the cone cell precursors transforms these cells into additional R7 cells. Within this group of cells, phyl expression thus mimics activation of the Raf pathway in its ability to induce photoreceptor rather than cone cell development. Furthermore, the transformation of cone cells into R7 cells in response to Raf activation is both accompanied by and dependent upon ectopic phyl expression. phyl thus represents a possible target gene of the Raf pathway during eye development, controlling the fates of a novel subset of photoreceptors.

Introduction

The R7 photoreceptor of the Drosophila compound eye has received considerable attention in recent years as a system in which genetic techniques can be applied to the analysis of cell-cell interactions mediated by receptor tyrosine kinases. Such studies have identified a number of genes that are required in order that the presumptive R7 cell adopts a neuronal fate in response to an instructive signal from the neighboring R8 cell. These genes encode components of a highly conserved, multifunctional cytoplasmic signal transduction cascade (Drk, Son of sevenless, Ras1, Raf, and rolled/mitogen-activated protein kinase [r/MAPK]), an apparently dedicated ligand-receptor pair that activates this cascade (bride of sevenless [boss] and sevenless [sev]), and a set of nuclear factors that are required to interpret the signal (pointed, yan, D-Jun, and seven in absentia [sina]); reviewed by Zipursky and Rubin, 1994; Dickson et al., 1992; Biggs et al., 1994; Brunner et al., 1994a, 1994b; O'Neil et al., 1994). No target genes that are transcriptionally regulated as an immediate response to the activation of this pathway have yet been identified.

The R7 cell is the last of eight photoreceptors added to the developing ommatidial cluster. Assembly of each ommatidium begins approximately 20 hr earlier in a depression, the morphogenetic furrow, which sweeps from posterior to anterior across the eye-imaginal disc. Behind the furrow, 5-cell preclusters consisting of photoreceptors R2–R5 and R8 are first formed, after which all remaining undetermined cells undergo a final division. R1 and R6 then join the cluster, a few hours ahead of R7, which is followed in turn by the nonneuronal cone cells (reviewed by Wolff and Ready, 1993). Of those genes required for R7 development, the entire cytoplasmic signaling cascade (Drk, Son of sevenless, Ras1, Raf, and rl) appears to be additionally required for the determination of all other photoreceptors (Simon et al., 1991; Dickson et al., 1992; Brunner et al., 1994b). Since R1–R6 and R8 are unaffected by mutations in boss and sev, it is presumed that this pathway is activated by another receptor tyrosine kinase in these cells, the best candidate for which is the Drosophila EGF receptor homolog (Xu and Rubin, 1993). The possible requirements for the nuclear factors in other photoreceptors have yet to be clarified in detail, but it is evident that sina function, like that of boss and sev, is uniquely required for R7 determination (Carthew and Rubin, 1990).

The four cone cell precursors display a similar developmental potential to the R7 precursor, and all five cells are often referred to collectively as the R7 equivalence group (Greenwald and Rubin, 1992). If, by virtue of a mutation in either the boss, sev, or sina genes, the R7 precursor fails to respond to its inductive cue, it will instead develop as a cone cell (Tomlinson and Ready, 1986; Carthew and Rubin, 1990; Cagan and Zipursky, 1992). Conversely, the cone cell precursors can be triggered to initiate development as R7 cells by ectopic activation of the pathway at any of several different points (Basler et al., 1991; Van Vactor et al., 1991; Dickson et al., 1992; Fortini et al., 1992; Gaul et al., 1992; Lai and Rubin, 1992; Brunner et al., 1994b).

One such means of transforming cone cells into additional R7 cells is via ectopic activation of the Raf serine/threonine kinase (Dickson et al., 1992). With the aim of identifying genes acting downstream from Raf, we performed a genetic screen for mutations that would dominantly suppress this transformation. One of the loci we identified in this screen, rl, has been shown to encode a MAP kinase homolog (Biggs et al., 1994). Here, we report the characterization of a second locus, which we call phyllopod (phyl). In several respects, phyl is unique among the genes thus far known to be involved in R7 determination: first, it is not normally expressed in the equipotent, but uninduced, cone cell receptors; second, increased phyl expression is observed upon ectopic activation of the Raf pathway in the cone cell precursors; third, expression of the wild-type gene in the cone cell precursors is sufficient to induce their differentiation as R7 cells; and finally, phyl shows a novel photoreceptor subgroup specificity, it being required for the specification not only of R7 but also of R1 and R6. phyl is also unique at the molecular level, encoding a novel type of nuclear protein.
**Results**

**phyl: A Rate-Limiting Factor in the Response of Ommatidial Cells to Raf Activation**

The expression of a constitutively activated Raf kinase under the control of sev regulatory sequences (raf™™) results in the recruitment of the cone cell precursors as additional R7-like cells (Dickson et al., 1992). The presence of these additional photoreceptors disrupts the ordered ommatidial array, giving the eye a rough external appearance (Figures 1B and 1E). We recovered three ethyl methanesulfonate–induced (EMS-induced) and three X-ray-induced phyl alleles on the basis of their ability to dominantly suppress the raf™™ rough eye phenotype (Figure 1C), correlating with a near complete elimination of the additional R7 cells (Figure 1F). Thus, disruption of one copy of the phyl gene, presumably resulting in a 50% reduction in phyl protein levels, impairs the ability of the cone cell precursors to develop as additional photoreceptors in response to Raf activation.

To determine whether phyl gene dosage can also be rate limiting within the precursors of the endogenous photoreceptors, we tested phyl for genetic interactions with the loss-of-function raf allele raf™™. This temperature-sensitive hypomorphic allele is associated with lower levels of raf transcription, not an alteration of the Raf protein (Melnick et al., 1993), and results in a reduction in the numbers of photoreceptors of both the R7 and R1–R6 classes (Dickson et al., 1992). The raf™™ phenotype is dominantly enhanced by phyl mutations, leading to a further reduction in the numbers of both R7 and R1–R6 cells (from 0.50 _±_ 0.03 R7 cells per ommatidium and 5.62 _±_ 0.07 R1–R6 cells per ommatidium to 0.09 _±_ 0.02 and 4.99 _±_ 0.07, respectively). Similar results were obtained for the R7 cell when signaling was impaired by a hypomorphic sev mutation, sev™™ (a reduction from 0.32 _±_ 0.03 to 0.18 _±_ 0.02 R7 cells per ommatidium). phyl gene dosage is therefore a limiting factor in the response of the precursors of R7, and at least some other photoreceptors, to reduced signaling via the endogenous Raf kinase.

**R1, R6, and R7 Precursors Adopt a Cone Cell Fate in the Absence of phyl Function**

All six phyl alleles are lethal in homozygotes or in heteroallelic combinations, with the exception of the X-ray-induced allele phyl™, which is semiviable in combination with all other phyl alleles. phyl™/phyl™ animals have a survival rate comparable to wild type until late pupal stages, but either fail to eclose or die shortly thereafter. Since the dominant suppression of raf™™ is observed with the phyl™ allele is indistinguishable from a deficiency for the locus, we assume that the phyl™ allele is essentially amorphic for phyl function in R7 development and that the superior vigor of this allele is due to residual activity in other, vital processes. The availability of this allele allowed us to examine the recessive phyl/phenotype in phyl™/phyl™ mutant flies. Unless otherwise stated, the following phenotypic analysis has been performed with individuals of this genotype.

Ommatidia of phyl/™/phyl™ flies contain only five photoreceptors: an R8 cell plus four cells of the R1–R6 class (Figure 2A). This ommatidial arrangement was observed in the vast majority of cases, though rare ommatidia with five R1–R6 cells were also observed. To ensure that this phenotype is typical for the loss of phyl function during eye development, and not specific just to this particular allelic combination, we also examined clones of homozygous mutant cells induced by mitotic recombination in individuals heterozygous for either of the two EMS-induced alleles phyl™ or phyl™. By both genetic and molecular criteria (see below), the phyl™ allele represents a complete loss of phyl gene function, while the slightly weaker suppression of phyl™ suggests that it may retain some activity. Nevertheless, the phenotypes of both phyl™ and phyl™ mutant clones are indistinguishable from that of phyl™/phyl™ mutants (compare Figures 2B and 2A).

Analysis of third instar eye-imaginal discs revealed that the loss of photoreceptors in phyl mutants could be attributed to a transformation of the R1, R6, and R7 cells into cone cells. We used the expression of elav and cut as markers to distinguish the photoreceptor and cone cell fates, respectively (Robinow and White, 1991; Blochlinger et al., 1993). In phyl mutants, nuclei occupying the normal positions of the R1, R6, and R7 nuclei, which in wild-type discs are positive for elav expression (Figure 2C), instead express cut as the cone cell marker (Figure 2D). As in

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Figure 1. Suppression of raf™™ by phyl

Scanning electron micrographs (A–C) and tangential sections (D–F) of compound eyes of wild-type (A and D), raf™™/+ (B and E), and raf™™/phyl™ (C and F) flies. The eye of wild-type flies is a regular array of about 750 ommatidia (A), each consisting of 8 photoreceptor cells (R1–R8) and 12 accessory cells, including 4 cone cells (which secrete part of the ommatidial lens). Each photoreceptor contains a phototransductive organelle called the rhabdomere, which stains strongly in sectioned material (D). The rhabdomeres of R1–R6 have a larger diameter than those of R7 and R8 and are arranged in a trapezoidal configuration. The smaller R7 and R8 rhabdomeres lie in the center of this trapezoid, with the R7 rhabdomere located apically and that of R8 basally. The sections shown in (D–F) are from the apical part of the eye; all small diameter rhabdomeres therefore belong to R7 cells. In raf™™ transformants, the recruitment of excess R7 cells in each ommatidium (E) leads to a disruption in the ordered ommatidial array (B). Removing just one functional copy of the phyl gene prevents the formation of most of the additional R7 cells (F), reverting the eye back to an almost wild-type appearance (C). Scale bars indicate 100 μm in (C) and 10 μm in (F).
Figure 2. The phyL Mutant Phenotype

(A) A tangential section through the eye of a phyL/phyL mutant. Each ommatidium contains four (or in rare cases five) large rhabdomeres of the R1–R6 class of photoreceptors and no small rhabdomeres of the R7 class. The R8 rhabdomere is always seen in sections deeper than the one shown here and often extends apically into the space left vacant by the missing R7 rhabdomere. The few small rhabdomeres seen in this apical section are such extended R8 rhabdomeres, not R7 rhabdomeres.

(B) A homozygous phyL clone (unpigmented tissue) generated by FLP-induced mitotic recombination in a heterozygous mutant background. Pigmented tissue in the upper right corner is genotypically either heterozygous or wild-type and contains normally constructed ommatidia. Ommatidia lying entirely within the clone are identical to those in (A), with the exception that ommatidia with five large rhabdomeres are not observed. phyL appears to be an amorphic allele, whereas phyF might not completely eliminate phyL function in eye development.

(C and D) Confocal images of single ommatidial clusters from wild-type and phyF/phyL late third instar larval discs, respectively, stained for elav (green) and cut (red). These clusters are approximately 12 rows behind the morphogenetic furrow. In the wild-type cluster (C), all photoreceptor precursors express elav, and some of the surrounding cone cell precursors are beginning to express cut. In the phyF/phyF cluster (D), the nuclei of R1, R6, and R7 express the cone cell marker, cut. The numbers 1–7 indicate the nuclei of R1–R7; R8, R2, and R5 are below the plane of focus in (C).

Later stages of eye development, 40–45 hr after pupation, are shown for wild type and phyF/phyF in (E–H, M) and (I–L, N), respectively.

(E and I) Pupal discs stained for cut. The images in (F)-(H) and (J)-(L) show pupal discs stained for α-spectrin (revealing cell membranes, green) and elav (red). b, bristle groups. Confocal images at the level of the cone cell nuclei are shown in (F) and (J); confocal images at the level of the photoreceptor nuclei are shown in (G) and (K); and confocal images at the base of the disc are shown in (H) and (L).

Ommatidia in phyF mutant pupal discs typically contain six and occasionally five cone cells (three primary pigment cells are indicated by arrowheads in K). The arrangement of secondary and tertiary pigment cells is also considerably disrupted (L).

(M and N) Anti-β-galactosidase strongly stains only the R7 cell in wild-type flies carrying the H214-1acZ enhancer trap insertion (M), but it stains two or three cells per ommatidium in a phyF/phyF background (N).

(O and P) Scanning electron micrographs of the thorax of wild-type (O) and phyF/phyF (P) flies.

(Q) A higher magnification micrograph of a bristle structure observed on a phyF/phyF thorax, with multiple shafts and sockets. Duplication of shafts occurs frequently, but duplication of sockets occurs only rarely.

Scale bars indicate 10 μm in (B) and (O), 5 μm in (D), (L), and (N) and 100 μm in (P).
sev mutants, the cone cell derived from the R7 precursor supplants the endogenous equatorial cone cell; those derived from R1 and R6 do not supplant endogenous cone cells. There is thus a loss of three photoreceptors and a net gain of two cone cells (Figures 2I–2K), resulting in an ommatidial composition similar, but not identical, to that observed in some phyllopod crustacea, such as Leptodora (Nilsson et al., 1983). Examination of later stages of eye development (Figures 2I–2L) also revealed frequent disruptions in the pattern of pigment and bristle cells.

The transformation of R7 to an equatorial cone cell is identical to that observed in boss, sev, and sina mutants. The additional transformation of R1 and R6 to ectopic cone cells represents a novel cell fate change and was therefore examined in further detail. We found that the precursors of these cells do express a seven up–lacZ enhancer trap marker (Mlodzik et al., 1980b), but they do not express BarH1/H2 (Higashijima et al., 1992), which is normally expressed about 6–8 hr later and coincides with the onset of neuronal differentiation (data not shown). We also examined expression of the the H214–lacZ marker in pupal discs, in which strong staining is normally restricted to the R7 cell in wild-type flies or to the cone cell it is transformed into in sev mutants (Figure 2M) (Mlodzik et al., 1992). In phy+ mutants, up to three cells per ommatidium express H214–lacZ (Figure 2N), presumably the three cone cells derived from the R1, R6, and R7 precursors. The transformation of R1 and R6 into cone cells is thus accompanied by the expression of a marker typical for neither cell type, but indicative of cells transiently possessing the developmental potential of the R7 cell. The R1 and R6 precursors, failing to respond to the inductive cues specifying these cell fates, may initially face the same developmental options as the R7 precursor, but ultimately they select a cone cell fate along with the endogenous R7 precursor.

phy+/phy− files also display a variety of phenotypes unrelated to eye development. The most striking of these are their extremely short life span, lack of coordination, and a severe reduction in the number of sensory bristles coupled with frequent bristle duplications (Figures 2P and 2Q).

**phy+ is Required Only in R1, R6, and R7**

To determine in which cells phy+ function is required, we generated clones of homozygous phy+ mutant cells by mitotic recombination in heterozygous, and therefore phenotypically wild-type, animals. Due to the lack of clonal relationships among ommatidial cells, both wild-type and mutant cells can mix at the border of such clones to form mosaic ommatidia. In those that are phenotypically wild-type, it is possible to identify each photoreceptor and infer its genotype from the accompanying white marker. One can thus determine which cells must be phy+ in order to construct a normal ommatidium. At the borders of 22 phy+ clones, 115 mosaic (but morphologically normal) ommatidia were scored. In all cases, the R1, R6, and R7 cells were white+, and hence phy+. In contrast, any of the other photoreceptors could be genotypically phy− and still contribute to the development of a normal ommatidium (Table 1). Furthermore, 12 ommatidia were observed in which only R1, R6, and R7 were phy+ and all other photoreceptors were phy−, indicating that phy function is required exclusively in these three cells.

**phy+ is Epistatic to rllsm and yan**

As well as dominantly suppressing the rllsm phenotype, phy+ also strongly suppresses the identical phenotype caused by constitutive activation of Ras1 (Fortini et al., 1992; data not shown). Indeed, Chang et al. (1995 [this issue of Celi]) have independently isolated phy+ mutations on the basis of this interaction. Mutations in the rllMAPK similarly suppress both the activated Raf and Ras1 phenotypes (Biggs et al., 1994). Identical interactions with both Ras1 and Raf are reassuring but not surprising, since Ras1 is believed to act at least in part via the activation of Raf (Dickson et al., 1992; Egan and Weinberg, 1993).

Two mutant conditions have been identified that produce a similar multiple R7 phenotype and affect components believed to act downstream of both Ras1 and Raf: a gain-of-function rl mutation (rlsm; Brunner et al., 1994b) and loss-of-function mutations in the yan gene (Lai and Rubin, 1992); yan encodes an Ets domain DNA-binding protein that may be a target of rllMAPK (Lai and Rubin, 1992; Brunner et al., 1994a; O'Neill et al., 1994). Since both rlsm and yan are only weakly suppressed by removing just one copy of the phy+ gene, we introduced these mutations into a background in which both copies of the phy+ gene were mutant. An se− rll− construct that normally mimics the rlsm phenotype (Brunner et al., 1994b) (Figure 3A) is unable to induce R7 development in a phy+/phy− mutant background (Figure 3B). Similarly, no R7 cells are formed within a yan−, phy+ double mutant clone generated in a yan−, phy+/yan−, phy+ background (Figure 3C). The formation of additional R7 cells in both rlsm and yan mutants therefore requires phy+ function.

**phy+ Encodes a Novel Nuclear Protein**

phy+ was mapped to the cytological divisions 51A1–B4. A chromosomal walk of 280 kb spanning this region was established (Figure 4A), and a 15 kb fragment was identi-
phyllopod Controls Photoreceptor Cell Fa'es

A

Y

NPB ZSZ E PZHESPPPXX ZZZB E GBVGKE BZ P VZHE(~

YI %17 I I I%1%#~d II V J %IcP'~ II I %111~

p~13 breakpoint

C

MSTNQQQQAN PS~VAApAA SSEYLKRTCL ICGCHTNQTI NIYEPRSGPN 50

IVQLIQAKFK FQPLNEDKFL CFSCNNWLIN 9~SLQAVNSN EAESQSQSP$ i00

HMGNSVLQQE RTKLRPVAHV RPTVRVQPQL QPQVPINPTP APIVYSKRRA 150

SRRSASVSRM SRVLRQCCVE SLRRSPKKRN QQSVFVCLRP QGQKRSNAIC 200

KVECVAPRRK PVERLVKDVA ATATPTPVLN TQSTPTYQRF PQPSVDGKVV 250

~FRRLGTTL SREEPAAYSA ESNPACSKLP QIHSPLKEAP RWTRDLDDDE 300

ILLEFDTAIS EVLPTARYQV THEENKENQQ AQEMELELEE EEEEEEEVDG 350

RAELE~gQEA EAPL~PQSHH KQGNSHQNSH QASIQLAGLR LPMGLSISLV 400

D

Figure 3. The r/° and yan Phenotypes Are Suppressed by phyl

Tangential sections of adult eyes of sE-rI°NI+ (A) and phyl/; phyl°; sE-

r/°NI+ flies (B). Shown in (C) is a section through a yan°, phyl° homozy-

gous clone generated in a yan°, phyl°/; yan°, phyl° background. Clones

were generated by crossing w, hsFLP; yan°, FRT42D, P[w+]47A/CyO

females to w; yan°, FRT42D, phylVCyO males and shifting the progeny

to 37°C for 1 hr during the first instar larval stage. Pigmented areas

to the left and in the upper right corner are regions of

yan°, phyl°/; yan°, phyl° tissue, and they display the

yan phenotype of multiple R7 cells. Unpigmented tissue in the center

and lower right corner is homozygous

yan°, phyl° and shows a

phyl mutant

phenotype. All sections are from the apical region of the eye; the few

small rhabdomeres seen in (B) and within the clone in (C) are R8

rhabdomeres, which frequently extend apically in phyl mutants. The

scale bars indicate 10 μm.

Figure 4. Structure of the phyl Gene and Nuclear Localization of Phyl

Protein

(A) Map of cloned genomic DNA spanning 280 kb from the cytological

interval 51A1–B6. The insertion sites of P elements used to initiate

the walk and map the phyl locus are shown, as are the breakpoints

of Df(2R)trix and Df(2R)L48, both of which uncover phyl. Imprecise

excision of the P element P1201 generated an additional deficiency,

Df(2R)AP1201, which was used to further localize phyl proximal to

this insertion. The approximate distal breakpoint of Df(2R)LAP1201

is inferred from genetic rather than molecular data. Fine-scale mapping

of the phyl allele with respect to P2329 and P1.15 located this lesion

near position –64 kb. Coordinates are in kilobases, with 0 as the inser-

tion site of the P2329 element; centromere proximal is to the left. N

marks NotI restriction sites.

(B) A 15 kb phyl genomic rescue fragment. This fragment completely

rescues phyl°/; phyl° and phyl°/; phyl° mutants (three independent

transgenic lines were tested in each case). The phyl transcription

unit is shown, with stippling indicating coding sequences. The EMS-induced

allele phyl° was found to be due to a translocation between 51A1–A4

and the proximal part of the X chromosome, the breakpoint lying within

the indicated HindIII-EcoRI fragment. Restriction sites: B, BamHI; E,

EcoRI; G, BglII; H, HindIII; K, Kpnl; N, NotI; P, PstI; S, SalI; V, EcoRV;

X, XbaI; and Z, Xhol.

(C) Predicted amino acid sequence of the phyl protein. In phyl°, the

CAG codon for Q281 is replaced by a TAG stop codon; in phyl°,

the deletion of a single C in the codon for P190 results in a frame shift

and the termination of translation after the addition of 59 novel residues.

(D) COS cells transfected with a phyl-myc cDNA were fixed and

stained with MAb9E10 (Evan et al., 1985), which recognizes the c-myc

epitope attached to the C-terminus of the phyl protein. Transfected

cells showed intense nuclear staining. A fluorescent photomicrograph

of such a cell is shown.
minus (pI = 3.7). Within the N-terminal domain are two CxxC motifs that might contribute to a divalent metal-binding domain. To verify that this cDNA was derived from a transcript of the phyl gene, we amplified and sequenced genomic DNA from the parental strain and the two EMS-induced alleles, phyl<sup>i</sup> and phyl<sup>f</sup>. Point mutations leading to premature termination of translation were identified for both alleles.

The predicted sequence of the phyl protein contains a potential bipartite nuclear localization signal at residues 193–194 and 208–210 (Robbins et al., 1991). To determine whether phyl is indeed a nuclear protein, we prepared a construct in which sequences encoding a c-myc epitope tag had been inserted at the 3' end of the phyl<sup>i</sup> transcript to consist of three cells (Figure 5C). Since these clusters might correspond to the proneural clusters which cells known to correspond to the cone cell precursors are strongly stained with a sev probe (F and G) (Tomlinson et al., 1987). In contrast, in ra<sup>f</sup> sev<sup>−</sup> eye discs (D and E), phyl expression does extend into the posterior part of the disc, including clusters of cells showing distinct perinuclear staining (closed arrowhead in E). Scale bars indicate 10 μm.

The distribution of phyl mRNA in late third instar eye discs

Phyl transcripts are expressed in a highly dynamic pattern in the eye-imaginal disc (Figure 5A). Within the morphogenetic furrow and coinciding with the boundary of the Rhomyo-plex expression (data not shown), phyl mRNA can be detected in regularly spaced clusters of about 12 cells (Figure 5B). This pattern is similar to the expression pattern of scabrous (Mlodzik et al., 1990a), suggesting that these clusters might correspond to the proneural clusters from which the R6 cell is specified. We have not observed any requirement for phyl function for R8 development.

Posterior to the furrow, phyl mRNA is mostly confined to a thin stripe approximately spanning the region between ommatidial columns 4–8. Within this stripe, phyl mRNA accumulates in clusters that at high magnification appear to consist of three cells (Figure 5C). Since phyl is required in a cell-autonomous fashion in R1, R6, and R7 and the mutant phenotype first becomes apparent at the corresponding stages in their development, we presume that it is the precursors of these three cells that make up the posterior clusters of phyl-expressing cells. No phyl mRNA could be detected beyond these clusters, indicating that the cone cell precursors do not express detectable levels of phyl mRNA.

We were somewhat surprised to find such differences in the level of phyl expression in the R7 and cone cell precursors, since these cells appear to have equivalent developmental potential and express similar levels of other genes implicated in R7 induction (e.g., sev; Figures 5F and 5G). Furthermore, we had observed a genetic requirement for phyl function in the cone cell precursors in ra<sup>f</sup> sev<sup>−</sup> and yan flies. This raised the possibility that phyl is expressed at higher levels in the cone cell precursors under these conditions. We compared the phyl expression pattern in wild-type, ra<sup>f</sup> sev<sup>−</sup>, and yan eye discs by treating wild-type and mutant discs together in a single reaction and distinguishing them either by the expression of a decapentaplegic–lacZ marker or the method of dissection. Significantly higher levels of phyl mRNA could be detected in ra<sup>f</sup> sev<sup>−</sup> and yan discs than in wild-type discs (compare Figures 5C and 5E). In yan discs, strong phyl expression was observed throughout the disc (data not shown), whereas ectopic phyl expression in ra<sup>f</sup> sev<sup>−</sup> discs was limited to a subset of cells in the posterior part of the disc (Figures 5D and 5E). The position of these cells suggests that they are the cone cell precursors, the only cells in this part of the disc in which Raf is ectopically activated.

These results were highly reproducible: ra<sup>f</sup> sev<sup>−</sup> yan, and wild-type discs were compared in four separate experiments, and in each case all 5–10 mutant discs showed increased phyl expression compared with a similar number of wild-type discs.

Ectopic expression of phyl redirects cone cell precursors to the R7 fate

To induce directly high levels of phyl expression in the cone cell precursors, we generated transgenic flies in which cells known to correspond to the cone cell precursors are strongly stained with a sev probe (F and G) (Tomlinson et al., 1987). In contrast, in ra<sup>f</sup> sev<sup>−</sup> eye discs (D and E), phyl expression can also be detected in much smaller groups of cells in the first two ommatidial columns behind the morphogenetic furrow, but then it essentially disappears until the posterior clusters, roughly corresponding to columns 4–8 (open arrowhead in C). Note that no phyl staining is observed in the posterior part of the disc, in
which the *phyt* cDNA was expressed under the transcriptional control of one or two copies of the *sev* enhancer and the *hsp70* promoter (*sev-*phyl and *2sev-*phyl, respectively). We found that ectopic expression of the wild-type *phyt* gene in the cone cell precursors is sufficient to induce their differentiation as additional R7 cells. Of 13 *sev-*phyl transformants, 2 had a rough eye phenotype, with one copy of the *sev-*phyl construct, and all but 1 of the remaining 11 lines had rough eyes when the two copies were present. Similarly, of 14 *2sev-*phyl transformant lines, 12 had rough eyes when just one copy of the fusion construct was present. Sections through the apical regions of the eyes of *2sev-*phyl flies revealed the presence of multiple small rhabdomeres characteristic of R7 cells (Figure 6A). Many of the additional photoreceptors express the R7-specific rhodopsin Rh4, which is normally exclusively expressed by a random subset of R7 cells (Feiler et al., 1992) (Figures 6C and 6D). The recruitment of additional R7 cells in *2sev-*phyl transformants is independent of *sev* activity (data not shown), but does require *sina* function (Figure 6B).

Eye development in *2sev-*phyl transformants was examined by staining larval eye discs with antisera against Elav (Figure 6E). The nuclei of ommatidial precursor cells rise apically through the disc epithelium as cells are added to the developing clusters. Elav is normally expressed by photoreceptor nuclei shortly after their arrival at the apical side of the disc, after which they begin to sink back to their final location. The cone cell nuclei similarly rise within the disc, but remain apical and do not express Elav. If the cone cell precursors are transformed to an R7-like fate by the ectopic activation of sev, Ras1, or Raf, they do not express Elav until their nuclei have reached their apical positions. In contrast, in *2sev-*phyl transformants, additional elav-positive nuclei are initially seen deeper within the disc, indicating that they begin their neuronal differentiation considerably earlier. In this respect, the *2sev-*phyl phenotype more closely resembles that of *yan* mutants (Lai and Rubin, 1992).

The earlier appearance of the additional elav-expressing cells precludes a definite determination of their origin. The excess of elav-expressing cells is, however, accompanied by a deficit in the number of cone cells, as revealed by cobalt sulfide staining of *2sev-*phyl pupal eye discs (Figure 6F). We therefore conclude that, as in *raf/ten*, it is the cone cell precursors that give rise to the additional photoreceptors in *2sev-*phyl transformants. It is interesting to note that the extra photoreceptors resulting from ectopic activation of sev or Raf or from the loss of *yan* function are not formed at the expense of the cone cells. In fact, the eyes of these flies not only have additional photoreceptors, but often they also have excess rather than fewer cone cells. We do not know the reason for this difference, but speculate that, in these cases, additional cone cells are recruited by the extra photoreceptors and that this secondary induction might not occur in *2sev-*phyl transformants. *2sev-*phyl ommatidia also frequently lack one of the primary pigment cells, most likely as a consequence of the reduction in cone cell number (Figure 6F).
Discussion

**phyF: A Target of the Raf Pathway?**

Within the R7 equivalence group, cells respond to wild-type **phyF** expression in a manner that mimics their response to the activation, via sev, of the Raf pathway: activation of Raf and the presence of **phyF** both lead to differentiation as an R7 cell, whereas basal levels of Raf activity and the absence of **phyF** both result in selection of the alternative cone cell fate. These two events may be independent, with Raf and **phyF** acting in parallel pathways leading to R7 development. Alternatively, they may be linked in a common pathway, with **phyF** expression being a direct consequence of Raf activation. Although the former possibility cannot be definitively excluded, our data strongly support the latter. In particular, **phyF** is neither expressed nor required in the cone cell precursors for their normal development, yet their transformation into R7 cells in response to Raf activation does require **phyF**. This observation is not readily explained by the first model. Moreover, ectopic activation of Raf in the cone cell precursors produces a marked increase in the levels of **phyF** expression. Although it is not possible to positively identify the cells in which **phyF** is ectopically expressed, their position in the eye disc suggests that they are the cone cell precursors. This interpretation is clearly consistent with the fact that Raf is ectopically activated only in these cells, that they require **phyF** to select an R7 fate, and that **phyF** expression itself is sufficient to induce this transformation.

We therefore propose that **phyF** is transcriptionally regulated in response to the activation, via sev, of the Raf pathway. Our genetic data suggest that **phyF** expression may be an immediate consequence of activation of this pathway, and, within the R7 equivalence group, may control the choice between the alternative fates of R7 and cone cell development.

**phyF** and the Specification of Photoreceptor Subtype

An intriguing aspect of the **phyF** phenotype is that, along with R7, the R1 and R6 photoreceptors are also transformed into cone cells, apparently passing through an intermediate stage in which they acquire the developmental potential of R7. None of the other photoreceptors are affected. In contrast, the Raf pathway is involved in the recruitment of all photoreceptors. A major challenge is to understand how this generic signal is integrated with context-specific information to produce different responses in different cells, in this case the choice of a particular photoreceptor subtype. Specifically, why do the cells of the R7 equivalence group have the potential to differentiate only as either R7 or cone cells, whereas the precursors of the other photoreceptors select from a different set of fates in response to the same signal? **phyF** may provide some useful insights into this problem, appearing to define an intermediate stage in the process of subtype specification. Furthermore, it allows this question to be recast in a more concrete form: why, if the Raf pathway is activated in all photoreceptor precursors, do only some of them express and require **phyF**?

**Experimental Procedures**

**Genetics**

The genetic screen for dominant modifiers of the raf^wz phenotype will be described in detail elsewhere (B. D., A. v. d. S., M. D., and E. H., unpublished data). In brief, w^1185 males were mutagenized either with EMS or X-rays and were mated with females either heterozygous for a P element insertion carrying the raf^wz fusion under the transcriptional control of a single sev enhancer and the hsp70 promoter or homozygous for an insertion with the same fusion construct under the control of a duplicated sev enhancer and the sev promoter. The raf^wz fusion differs from the previously described raf^32 and raf^wz^24 fusions (Dickson et al., 1992) in that the torso domain contains the weakly activating Y9 mutation (Sprenger and Nüsslein-Volhard, 1992). This modification provided an intermediate level of activation suitable for detecting subtle genetic interactions. We screened ~200,000 F1 progeny of the EMS-mutagenized males, from which the alleles **phyF**^d4 were recovered, and ~50,000 progeny of the X-irradiated males, providing the alleles **phyF**^d4.

The dominant suppression phenotype associated with the **phyF** chromosome was mapped to chromosome 2, position 73.0 ± 0.7, using the markers ai, b, c and sp. Failure to complement the deficiencies Df(2R)irix and Df(2R)ta48, both of which dominantly suppress the raf^wz phenotype, localized **phyF** to the cytological interval 51A1–B4. **phyF** was further localized to the interval 51A1–A5 using a deficiency generated by the imprecise excision of the P element P1201. This deficiency was recovered by mating P1201/Df(2R)irix; D2–3, Sb^+ males to raf^wz, P1.15/Cyo females and by screening the Cy^+; Sb^− progeny for suppression of the raf^wz phenotype. One suppressor chromosome, P^5D101, was recovered from 1500 Cy^+; Sb^− flies screened and failed to complement the lethality of both **phyF** and P1.5. Additionally it failed to complement a lethal P element insertion, P2329, at 51A1–A2, as well as the original P1201 chromosome, but does complement Asex at 51A4–A5. P^5D101 thus appears to be deficient for an interval extending proximally from 51A1–A5 to at least 51A1–A2.

Fine-scale mapping of **phyF** within the 51A1–B4 region was performed using the lethal P element insertions P2329 and P1.15, located at either end of this interval and uncovered by Df(2R)irix. Virgins carrying **phyF** over either of these insertions were mated to Df(2R)irix/Cyo males, and the offspring were scored for Cy^+ (recombinant) progeny. Between P2329 and **phyF**, 17 recombination events were detected in 29,738 flies screened, and between **phyF** and P1.15, 36 recombination events were detected in 31,272 flies screened.

**phyF** alleles were maintained as stocks balanced over either a CyO, raf^wz chromosome or an Sb^+–TMB8 double balancer, which allowed the Tb marker to be used to distinguish mutant larvae and pupae. Clonal analysis was performed using the FLP–FRT system (Xu and Rubin, 1993). Flies carrying the raf^wz allele were raised at 18°C; all other crosses were performed at 25°C. sev^− flies are sev^2 null mutants partially rescued by a P element insertion carrying a sev cDNA construct in which the codons for Yid and Wid have both been replaced by alanine codons (B. D. and E. H., unpublished data).

**Histology and Immunohistochemistry**

Antibody stainings were performed essentially as described in Gaul et al. (1992); cobalt sulfide stainings were performed according to the protocol of Cagan and Ready (1989); and histological sections and scanning electron micrographs of adult eyes were performed as in Basler et al. (1991).

**Molecular Analysis**

The chromosomal walk through the 51A1–B6 region was established from a cosmid library provided by Dr. J. Tamkun. Starting points were phage clones from the trz2 region at 51B4–B6 (Dr. B. Baker), a cosmid from the Axx region at 51A4–A5 (Dr. D. Sinclair), and fragments obtained by plasmid rescue from several P element insertions within this region (Figure 4A). cDNA clones were isolated from a λgt10 third instar eye-antennal disc library (Dr. A. Cowman).

Several different fragments from a 1.8 kb cDNA were subcloned into M13 for DNA sequencing (Banger et al., 1977), which was completed for both strands. Three other cDNAs were partially sequenced. Corresponding genomic regions from the cosmids cosM3 were identified, and the sequences of both strands were determined. To determine the DNA alterations present in the **phyF** and **phyP**
alleles, genomic DNA from both exons was amplified in several independent polymerase chain reactions (PCRs) from phyF/h/ phyF+h, and +/+ flies, where the plus sign represents the parental wild-type chromosome. Fragments were amplified using leader-, intron-, and trailer-specific primers, subcloned into M13 and sequenced in one direction. When compared with the 3' exon sequences of three PCR products from the parental strain, single base pair changes were detected in 5/7 and 4/6 products from phyF and phyF heterozygotes, respectively. Three parental, six phyF, and six phyF PCR products had identical 5' exon sequences.

COS Cell Transfection

The phy/myc fusion construct was generated by introducing a BamHI site at the 3' end of the phy open reading frame via PCR mutagenesis and by then inserting an oligonucleotide encoding the c-myc epitope between this BamHI site and an SphI site in the phy trailer. These manipulations added codons for the amino acid sequence GSGTGTEQ-XKLEEDNL to the end of the phy open reading frame. This cDNA was then subcloned into the expression vector PM721 (Genetics Institute).

COS cells were transfected by the DEAE-dextran method (Klar et al., 1992), incubated for 48 hr, and then fixed and stained using MAβE10 (Evans et al., 1985), according to standard methods.

Germline Transformation

The 15 kb fragment used for genomic rescue was a NotI fragment from cosmid cos J1, extending from an endogenous NotI site to the 3' exon. The 3' genomic sequences extend to a PstI site 259 bp beyond the end of the cDNA. This cDNA-genomic fusion was then subcloned between this BamHI site and an SphI site in the cosmid. This fragment was subcloned into the transformation vector pCaSpeR4 (Pirotta, 1991).

In Situ Hybridizations

RNA in situ hybridizations to whole-mount imaginal discs were performed as described by Cubas et al. (1991). phy probes were prepared from a PCR-amplified fragment from position 218–1365; the sev probe was prepared from a full-length sev cDNA. To compare phy RNA levels in different strains, eye-antennal discs were treated together in a single section.

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References


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