Inducible cell ablation in Drosophila by cold-sensitive ricin A chain

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Summary

We have developed a system for temperature-inducible killing of specific cells in the fruitfly Drosophila melanogaster. The system overcomes many of the limitations of existing cell ablation methods and is in principle applicable to any non-homeothermic eukaryote. Temperature-sensitive and cold-sensitive mutations in the ricin toxin A chain (RTA) of castor bean were generated in yeast. One cold-sensitive mutation, RAcs2, produced temperature-dependent ablation of eye cells in Drosophila when expressed under control of the eye-specific sev enhancer. At 29°C, cell death was observed within 7 hours in the developing eye and no obvious toxic effects were observed elsewhere; at 18°C, extremely low toxicity was observed. DNA sequencing of RAcs2 revealed a single amino acid substitution in the RTA active site cleft.

Key words: ablation, Drosophila, ricin, sevenless.

Introduction

Cell ablation is a powerful tool in eukaryotic developmental biology: ablation of cells can provide information about their origin, their fate or their function (Lohs-Schardin et al., 1979; Doe and Goodman, 1985; Behringer et al., 1988; Sulston, 1988). Physical ablation methods, such as a UV laser microbeam, have been widely used, but these are often limited by problems of small size and of inaccessibility of the cells to be ablated.

More recently, a toxigenic approach to cell ablation has been used; a gene encoding a toxic product is expressed from a cell-type-specific promoter to ablate cells in which the promoter is active (Behringer et al., 1988; Sulston, 1988). Physical ablation methods, such as a UV laser microbeam, have been widely used, but these are often limited by problems of small size and of inaccessibility of the cells to be ablated.

We chose RTA for temperature-inducible ablation because it has been used previously for cell ablation in mice (Landel et al., 1988), its mechanism of action is well understood (Endo and Tsurugi, 1988) and it is active in all eukaryotes tested, including yeast (Olsnes and Pihl, 1982; Frankel et al., 1989). RTA catalytically inactivates eukaryotic ribosomes by a specific depurination event in 28S rRNA, thus inhibiting protein synthesis (Endo and Tsurugi, 1988). Hence, expression of RTA will disrupt normal cell function faster than ablation methods that affect DNA replication (Borelli et al., 1988; Heyman et al., 1989). In this study, we used a truncated RTA gene that does not encode the secretory signal peptide (Lamb et al., 1985; Haling et al., 1985), or the ricin B chain which would cause any extracellular toxin to be internalized by adjacent cells (Olsnes et al., 1974); previous work suggested that the toxic effects of such an RTA product are, as expected, restricted to cells in which it is synthesized (Landel et al., 1988).

Materials and methods

Plasmid construction

An integrative derivative of the yeast galactose-inducible expression plasmid pEMBLyex4 (gift of John Murray, University of Cambridge), pJG2, was constructed which
lacked the Hpal-HindIII fragment carrying yeast 2μ circle sequences necessary for autonomous replication, from nucleotides 3426 to 4778, and carried a transcription termination site from the PGK gene on a fragment of 375 bp (Mellor et al., 1983) between the PstI and HindIII sites of the pEMBLex4 polyclinker. A 0.9kb Xhol-SalI fragment carrying the RTA gene (nucleotides -74 to 822, numbered from the 5' end of the proricon coding sequence; Lamb et al., 1985) was cloned into the SalI site of pJG2, generating pJG2RA.

The sev enhancer-dependent expression vector, P[ry+; sevPT], was constructed carrying the ry+ gene on a 7.2kb HindIII fragment (Rubin and Spradling, 1982), the sev enhancer on a 1.2kb Xhol fragment (nucleotides 6347-7564; Basler et al., 1989), a fragment carrying the TATA box and part of the 5' untranslated leader of hsp70 (hs43), from nucleotide -43 to the PstI site at nucleotide 83 (gift of M. Akam; originally from the group of V. Pirrotta), a 0.9kb fragment carrying the hsp70 polyadenylation site (Hiromi et al., 1985), and Bluescript + KS (Stratagene, La Jolla, CA). The RTA, RAts and RAcs genes were cloned as Xbal-SalI fragments into the unique Xbal and SalI sites between the TATA box and polyadenylation sites of P[ry+, sevPT], to give P[sev-RTA], P[sev-RAts] and P[sev-RAcs] respectively.

The DNA sequence of RAcs2 was determined using a Sequenase dideoxy sequencing kit (United States Biochemicals), using three primers that hybridized at regular intervals along the RTA gene. The template was the P[sev-RAcs2] plasmid preparation that was used to obtain the sev-RAcs2 transgenic fly lines.

Embryo injections
P element vectors were co-injected as described by Spradling (1986) into ry+embryos with helper P element pUCHs82-3 (Misra and Rio, 1990). Between 300 and 600 embryos were injected with each construct. Individuals injected with RAts and RAcs constructs, and their G1 progeny, were subsequently maintained at 28°C and 18°C respectively, to minimise RTA activity. We also tried to inject some injected embryos at 30°C but obtained extremely low survival rates, even when we injected P[ry+; sevPT], which did not carry any RTA gene. In most injection experiments, about 20% of injected embryos survived to adulthood, regardless of whether the construct carried an RTA gene. The control injections using P[ry+; sevPT] gave rise to two independent transformants; hence the presence of the RAcs2 gene (five independent transformants obtained with P[sev-RAcs2]) had no detectable effect on survival of G0 or G1 flies at 18°C.

Selection of RAts and RAcs mutations
Yeast strain J20 (Gould et al., 1991) carrying an integrated, single-copy, galactose-inducible RTA gene was mutagenized with EMS (ethylmethane sulphonate) to 16%-25% survival, washed three times in 0.1 M pH 7.4 sodium phosphate buffer, and plated on galactose medium at 30°C (for RAts selection) or 18°C (for RAcs selection). After galactose selection at 30°C, approximately 2000 survivors were recovered from 10^6 viable cells plated. After repeated checking, 15 of these were found not to grow at 18°C on galactose medium and to grow normally on YPD medium containing glucose as sole carbon source (Sherman, 1991) at both 18°C and 29°C, and hence carried a putative RAts mutation. The 15 putative RAts genes were cloned into Escherichia coli by plasmid rescue and used to retransform JRY188 yeast (a leu2 ura3 trp1 his4); 14 were found to confer the expected cold-sensitive growth phenotype, and hence were genuine RAts mutations. After selection of mutagenized cells on galactose medium at 18°C, 250 survivors were recovered from 10^7 viable cells plated. After checking for heat-sensitive growth on galactose and normal growth on YPD, 3 putative RAcs mutations were recovered and all 3 were shown by cloning and retransformation to be genuine RAcs mutations. Strain J20 was grown routinely on YPD medium. Galactose selection was performed on synthetic complete medium minus uracil (Sherman, 1991) containing 2% galactose as carbon source.

Histology and microscopy
For SEM, 5 to 10 whole flies of each genotype were fixed overnight at 4°C in 2.5% glutaraldehyde, dried in a graded acetone series, plated with gold and viewed in a Joel SEM. Eye sections were prepared as described by Saint et al. (1988); 3 to 5 flies were sectioned for each combination of genotype and culture temperature examined. Flies were reared routinely in an incubator maintained at the desired temperature. For temperature-shift experiments, white prepupae were placed on small Petri dish lids floating on the surface of a 25°C waterbath, brushed after the desired time interval onto the side of a food vial that had been pre-equilibrated to 18°C, and allowed to continue development at 18°C; 5 to 10 flies were examined by SEM for each set of temperature-shift conditions. Acridine orange staining was performed as described by Masucci et al. (1990); 5 to 10 individuals were examined for each set of experimental conditions.

Results
Selection of RTA mutants
To isolate temperature-sensitive and cold-sensitive mutations in RTA (RAts and RAcs respectively), we used a simple selection scheme based on lethality of RTA expressed in yeast (Fig. 1). A yeast integrating plasmid, pJG2RA (Fig. 2A), in which the RTA structural gene was fused to a galactose-inducible promoter, was used to transform haploid yeast strain JRY188. One transformant, J20, carried a single copy of pJG2RA integrated at the leu2 locus (Gould et al., 1986) into ry+ embryos with helper P element pUCHs82-3 (Misra and Rio, 1990). Between 300 and 600 embryos were injected with each construct. Individuals injected with RAts and RAcs constructs, and their G1 progeny, were subsequently maintained at 28°C and 18°C respectively, to minimise RTA activity. We also tried to inject some injected embryos at 30°C but obtained extremely low survival rates, even when we injected P[ry+; sevPT], which did not carry any RTA gene. In most injection experiments, about 20% of injected embryos survived to adulthood, regardless of whether the construct carried an RTA gene. The control injections using P[ry+; sevPT] gave rise to two independent transformants; hence the presence of the RAcs2 gene (five independent transformants obtained with P[sev-RAcs2]) had no detectable effect on survival of G0 or G1 flies at 18°C.

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expressed in any other tissue or at any other stage of development (Basler et al., 1989; Banerjee et al., 1987a, 1987b; Hafen et al., 1987). Hence, disruption of sev-expressing cells should be easily detectable and should not affect survival to adulthood.

A sev-RTA fusion and six sev-RAts fusions were injected into Drosophila embryos. No confirmed transformants were obtained with the sev-RTA fusion and five of the six sev-RAts fusions (although in one case, sev-RAts7, we obtained an infertile putative transformant). In all cases, we observed transient expression of the \( r^+ \) marker on the P element vector; this suggests that lack of transformants was not due to failure of the injections, but to residual toxicity of most RAts variants at 29°C and leaky expression from the sev-hsp70 enhancer/promoter fusion. A fertile transformant line was obtained for only one of the RAts variants, RAts1; however, its eyes were normal at both 18°C and 29°C, suggesting that RAts1 is non-toxic or unstable in flies at both temperatures, or is not expressed in this line.

**Temperature-dependent eye defects in sev-RAcs2 flies**

One of the three sev-RAcs fusions, sev-RAcs2, was injected into embryos and five independent transformant lines were obtained. An approximately similar number of transformant lines was obtained when a control construct lacking any RTA gene, but which was otherwise identical, was injected into a similar number of embryos. As expected, sev-RAcs2 flies had almost normal eyes when reared at 18°C (the RAcs restrictive temperature) and severely disrupted eyes when reared at 29°C (the RAcs permissive temperature). To visualize the disruption more precisely, we performed scanning electron microscopy (SEM) on eyes of all the lines, and in addition examined eye sections of one transformant line (Fig. 3). Four of the five lines showed almost identical defects that are on the whole consistent with specific ablation of sev-expressing cells, as discussed below. The fifth line in addition showed defects that are probably due to position-dependent ectopic expression of RAcs in cells that do not normally express sev. Except where otherwise indicated, the following descriptions are based on the four lines that showed almost identical defects.

SEM analysis of sev-RAcs2 flies reared at 18°C showed very occasional eye defects; less than 1% of ommatidia had either misplaced bristles or a lens defect (Fig. 3B). Sections of several eyes showed the R7 photoreceptor missing in about 1-2% of ommatidia and the R8 photoreceptor missing in almost 50% of ommatidia. Therefore, sev-RAcs2 flies were as viable and fertile as wild-type flies at all temperatures tested, with no obvious defects outside their eyes. This suggests that there is normally no expression of RAcs2 outside the normal sites of sev expression.

**Inducible cell ablation in Drosophila**

Transforming Drosophila with RAts mutant genes

To determine whether any of the RAts or RAcs mutants also showed temperature-dependent activity in Drosophila, they were fused to the sev (sevenless) enhancer on a P element vector (Fig. 2B). This enhancer is active transiently in subsets of cells in the developing eye (Basler et al., 1989; Bowtell et al., 1989) and subsequently in adult heads, but is not known to be

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**Fig. 2.** Plasmids for expression of RTA, RAcs and RAts in yeast and flies. Drawings are not to scale; only restriction sites relevant to construction are shown. Full restriction maps and construction details are available on request. Abbreviations for restriction sites: E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SalI; St, SacI; X, XbaI. (A) Yeast integrating plasmid pJG2RA, shown integrated after linearisation at the unique EcoRI site in the LEU2 gene. RTA is expressed from the galactose-inducible GAL10-CYC hybrid promoter of pEMBL18. The RTA fragment carries a stop codon at codon 268, and translation is putatively initiated at AUG codon –24 (numbered from N terminus of proricin), resulting in an RTA product that lacks the first 11 amino acids of the secretory signal sequence and all amino acids from proricin residue 268 onwards, including the B chain (Lamb et al., 1985; Halling et al., 1985). The polyadenylation/transcription termination site of the PGK gene (T) is downstream of RTA. The plasmid also carries a URA3 selectable marker and pEMBL18 plasmid sequences. (B) P element vector P[sev-T] is downstream of RTA. The vector also carries a URA3 selectable marker and pEMBL18 plasmid sequences. (B) P element vector P[sev-RTA]. Mutant and wild-type RTA genes are transcribed from the galactose-inducible GAL10-CYC hybrid promoter of pEMBL18. The RTA fragment is downstream of RTA. The vector also carries the \( r^+ \) marker, and Bluescript plasmid sequences.

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1991). The parental strain, JRY188, grows on medium containing either glucose or galactose as sole carbon source, but J20 fails to grow on galactose medium due to induction of RTA. Hence, any mutant J20 cell that survives on galactose medium probably carries an RTA mutation; RTA-resistant mutations are extremely rare (Frankel et al., 1989; Gould et al., 1991), and cells that fail to induce RTA because of a mutation that inactivates a GAL regulatory gene will not grow on galactose as sole carbon source. J20 cells were therefore mutagenized and plated on galactose medium at either 29°C or 18°C. Surviving cells were checked for temperature-dependent galactose resistance, and putative cold-sensitive and temperature-sensitive RTA genes were cloned in Escherichia coli and used to retransform JRY188 yeast (Fig. 1). After retransformation, we recovered 14 RAts and 3 RAcs variants which conferred temperature-dependent galactose resistance.

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Fig. 3. Effects of RAc2 on Drosophila eyes. SEM preparations of a wild-type eye (A), an eye of a heterozygous sev-RAc2 fly raised at 18°C (B), and an eye of a heterozygous sev-RAc2 fly raised at 29°C (C). At 18°C, mild perturbations of bristle pattern are occasionally seen in most sev-RAc2 eyes; a few eyes also have occasional lens defects (not shown). In three of the four lines with the phenotype illustrated, SEM showed no consistent differences between heterozygous and homozygous flies at either 18°C or 29°C. In the fourth case, homozygotes had a phenotype as illustrated at 29°C and heterozygotes had a slightly less severe phenotype (data not shown). Sections of typical eyes of sev-RAc2 flies raised at 18°C (D), 29°C, apical region (E) and 29°C, basal region (F); scale bar is 10µm. One ommatidium can be seen in the 18°C eye which lacks R7 (arrowhead). SEM preparations of eyes of sev-RAc2 flies that were raised at 18°C and transferred at the white prepupal stage to 29°C for 4 hours (G) or 10 hours (H). Homozygotes and heterozygotes gave similar results. (I) A higher magnification micrograph of an eye like that in C, showing typical lens and bristle defects. Most lenses have some kind of defect, ranging from large craters to smaller depressions.

defects. Eye sections showed between 4 and 8 photoreceptors per ommatidium apically, and between 4 and 15 photoreceptors per ommatidium basally; the pigment cells surrounding each ommatidium appeared greatly reduced in size and were barely detectable in basal sections (Fig. 3E,F).
Fig. 4. Cell death in the eye-antennal disc of sev-RAc2 larvae. Anterior is to the left; scale bar is 100 µm. Dead or dying cells do not exclude acridine orange and hence fluoresce brightly. (A) Eye-antennal disc of a sev-RAc2 larva reared at 18°C. No consistent differences were found between these discs and the discs of wild-type flies reared at 29°C (data not shown). There is some cell death in the antennal area and a few cells which appear to be underneath the disc epithelium, as reported previously (Masucci et al., 1990). (B) Eye-antennal disc of a sev-RAc2 larva reared at 29°C. An irregular band of cell death appears in the disc epithelium about 50 µm posterior to the morphogenetic furrow (arrowhead). The degree of cell death observed with this method varies from about the extent illustrated to about half this number of cells, but always begins at a constant distance behind the morphogenetic furrow. The stained cells just anterior to the furrow, which are not in the epithelial layer, and the cells in the more anterior antennal portion of the discs, are also found in wild-type discs (Masucci et al., 1990).
The fifth sev-RAc2s2 line, which had a phenotype at variance from the other four lines, had the standard sev-RAc2s2 phenotype on the eye surface when heterozygous. Homozygotes, however, had greatly reduced viability at 29°C. Surviving homozygotes had the standard sev-RAc2s2 phenotype over most of the eye surface, but in addition had a patch in the ventral posterior eye quadrant which had a much lower density of ommatidia, and these ommatidia were harder to distinguish from surrounding cuticle (data not shown). It is hence likely that there is position-dependent ectopic expression of RAc2s2 superimposed on the normal sev expression pattern in this line.

**Estimating killing time**

We took two approaches to estimate the time taken for RAc2s2 expression to disrupt cell function. First, we used acridine orange staining as a criterion to detect dead cells (Masucci et al., 1990) in third larval instar eye-antennal discs. We detected almost no cell death in the epithelium of wild-type discs at 29°C and of sev-RAc2s2 discs at 18°C, and extensive cell death in 29°C sev-RAc2s2 discs (Fig. 4). Eye development proceeds across the disc in a posterior-to-anterior direction at an average rate of 1 ommatidial column every 70 minutes at 25°C (Basler and Hafen, 1989), and we observe that development in general proceeds at approximately similar rates at both 25°C and 29°C. As sev is expressed initially in the moving morphogenetic furrow (Banerjee et al., 1987b; Tomlinson et al., 1987) and cell death is seen from about 6 columns behind the furrow, we estimate that cell death begins within about 7 hours of initial RTA expression. We also performed timed temperature-shift experiments with sev-RAc2s2 white prepupae (Fig. 3G,H). In these temperature-shift experiments, we observed defects spread over a broad area of the adult eye - this is expected because of the broad band of sev expression that is found in the prepupal eye disc at any one time point. Periods of as short as 4 hours at 29°C caused lens defects similar to, but less widespread than, those of sev-RAc2s2 flies reared continuously at 29°C. Hence, we estimate that de novo RAc2s2 expression in sev-expressing cells can cause cell death within 7 hours; a temperature shift of as short as 4 hours can severely disrupt cell function.

**Sequence of the RAc2s2 mutant**

DNA sequencing of RAc2s2 revealed a single amino acid substitution, Gly-212 changed to Arg. This residue is in the active site cleft of RTA (Frankel et al., 1989; Montfort et al., 1987) and Glu-212 and Trp-212 substitutions have been shown to inactivate ricin toxicity in yeast (Frankel et al., 1989). We are not aware that the Glu-212 or Trp-212 substitutions have been tested for cold- or temperature-sensitivity.

**Discussion**

The properties of the RAc2s2 mutant that we have used should make it an extremely useful tool in *Drosophila* and in other non-homeothermic eukaryotes. We observe very low toxic effects of RAc2s2 at its restrictive temperature in the cells where it is expressed, and we had no difficulty in obtaining sev-RAc2s2 transgenic lines at this temperature. Hence, it should be straightforward to obtain transformants carrying fusions of RAc2s2 to other enhancers and promoters, in spite of the aberrant gene expression that usually occurs after injection of DNA into embryos.

At the RAc2s2 permissive temperature, we detect severe cell damage and cell death within a few hours in the developing eye. As sev-RAc2s2 flies reared at 29°C, the RAc2s2 permissive temperature, are apparently as viable and fertile as wild-type flies reared at the same temperature and have no obvious defects outside their eyes, there is no detectable toxicity due to basal levels of expression in other cells and tissues.

**Defects in sev-expressing cells**

The eye defects observed at 29°C are consistent with specific disruption of a variable subset of sev-expressing cells by the sev-RAc2s2 fusion. First, the sev enhancer is not active in the earliest photoreceptor cells to form, R8, R2 and R5, and is active only weakly, if at all, in R1 and R6; it is active in R3, R4, R7, the two 'mystery cells' and the four cone cells (Basler et al., 1989; Bowtell et al., 1989). One might therefore expect a normal number of ommatidia, with at least 5 photoreceptors each - R1, R2, R5, R6 and R8 (though only 4 may sometimes be visible because R8 does not extend throughout the length of each ommatidium). The presence of more than 5 photoreceptors in some ommatidia might be due to some sev-expressing cells not expressing sev-RAc2s2 for long enough to be killed, to uncommitted cells inappropriately assuming a photoreceptor fate because of disrupted cell-cell interactions, or to ommatidial fusions caused by the reduction in pigment cells surrounding each ommatidium. Second, the lens region of most ommatidia shows some defect, in many cases very severe; this is consistent with the strong prolonged expression of sev in cone cells (Tomlinson et al., 1987). Third, lateral inhibition may be important in determination of eye bristles (Cagan and Ready, 1989); we speculate that cells that inhibit bristle formation are either missing or defective. We cannot easily explain the pigment cell defect, as these cells are not known to express sev; however, they are recruited to ommatidia subsequent to the cone cells, and killing of the cone cells may disrupt cues that the pigment cells require for normal development. Non-autonomy of the ablation is unlikely, given that we used an RTA that does not encode a secretory signal sequence or a B chain and given the presence of apparently normal photoreceptor cells - one would expect photoreceptors to be affected as much as pigment cells if the toxin action was non-autonomous.

The fact that the actual number of photoreceptors and the extent of lens ablation varies between ommatidia is probably due to the transient dynamic expression pattern of sev and the stochastic nature of many of the complex cell-cell interactions of the developing eye.
disc. Similar variability is seen in *in vitro* flies, which carry a fusion of the *rough* gene to the *sev* enhancer (Basler et al. 1990; Kimmel et al. 1990). We anticipate that expression of RAc2 from more stably expressed regulatory elements in differentiated cells will give more consistent defects.

**Uses for RAc2**

The speed of cell killing that we observe should make the system extremely useful for ablation of stably differentiated cell types (e.g. in the developed larval or adult nervous systems). However, it may be of more limited use in situations where there is a relatively rapid succession of cell-cell interactions and cell states (e.g. early embryogenesis, and eye disc development).

A major advantage of the RAc2 system is its conditionality. Firstly, the fact that toxicity of RAc2 is so low at its restrictive temperature should make it straightforward to obtain stable transgenic lines when it is expressed under control of most promoters or enhancers; the aberrant expression that occurs after embryo injection (Kunes and Steller, 1991, and personal communications cited therein) will have little or no toxic effect. Secondly, the temperature dependence of RAc2 toxicity makes it possible to use particular promoters or enhancers to drive RAc2 expression at its permissive temperature in cells that one wishes to ablate later in development, regardless of whether those promoters or enhancers are expressed earlier in development. One should be able to avoid unwanted cell killing in, say, embryos, larvala and pupae by rearing them at the RAc2 restrictive temperature, and then ablate given cells in adults by shifting them to the RAc2 permissive temperature.

The applicability of RAc2 should be further widened by expressing it in a “binary weapon system”, under control of a regulatory element, UAS<sub>Gal4</sub>, whose activity is dependent on the presence of the yeast transcriptional activator GAL4. In this case, RAc2 will only be expressed when the strain carrying it is crossed to an appropriate GAL4-expressing strain (Fischer et al., 1988; Ornitz et al., 1991). The use of “enhancer-trapping” (O’Kane and Gehring, 1987) to express a P-GAL4 fusion in a wide variety of cell types (Brand and Perrimon, 1991) should hence allow the ablation of virtually any cell type of the fly, and avoid the need to generate new transgenic fly lines every time one wishes to express RAc2 in a given cell type.

We have successfully used a cold-sensitive RTA isolated in yeast for conditional cell ablation in another non-homeoerotic eukaryote, *Drosophila*. We propose the name RAMBO (Ricin A-Mediated Bioinducible Obliteration) for this conditional toxicogen approach, which should be applicable in other non-homeoerotic eukaryotes for which transgenic methods are available.

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