



“In vivo” toxicity of a truncated version of the *Drosophila* Rst-IrreC protein is dependent on the presence of a glutamine-rich region in its intracellular domain

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ABSTRACT

The *roughest-irregular chiasm C* (*rst-irreC*) gene of *Drosophila melanogaster* encodes a transmembrane glycoprotein containing five immunoglobulin-like domains in its extracellular portion and an intracytoplasmic tail rich in serine and threonine as well some conserved motifs suggesting signal transduction activity. In the compound eye, loss-of-function *rst-irreC* mutants lack the characteristic wave of programmed cell death happening in early pupa and which is essential for the elimination of the surplus interommatidial cells. Here we report an investigation on the role played by the Rst-irreC molecule in triggering programmed cell death. “In vivo” transient expression assays showed that deletion of the last 80 amino acids of the carboxyl terminus produces a form of the protein that is highly toxic to larvae. This toxicity is suppressed if an additional 47 amino acid long, glutamine-rich region (“opa-like domain”), is also removed from the protein. The results suggest the possibility that the opa-like domain and the carboxyl terminus act in concert to modulate *rst-irreC* function in apoptosis, and we discuss this implication in the context of the general mechanisms causing glutamine-rich neurodegenerative diseases in humans.

Key words: *Drosophila*, *rst-irreC*, glutamine-rich repeats, cell death.

INTRODUCTION

One of the key problems faced by multicellular organisms during development is the control of cell number. Unchecked proliferation may cause hyperplasia and interfere with growing of adjacent tissue while excess or inappropriate cell death can result in an insufficient number of cells to form a tissue, or in its degeneration. A delicate balance between cell

proliferation and the elimination of surplus or defective cells must therefore be maintained throughout morphogenesis so that the correct structural organization and function of a given organ or tissue can be achieved. Although a lot of attention has been devoted to the study of the molecular and cellular basis of the mechanisms controlling cell division, very little was known, until recently, about how programmed cell death is regulated during tissue growth and differentiation. In the last few years however, work carried out in several model organisms, especially the nematode *Caenorhabditis ele-*

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gans and the dipteran *Drosophila melanogaster*, as well as in vertebrate systems, resulted in the unraveling of a basic molecular mechanism by which programmed cell death is triggered during development. This apoptotic mechanism, highly conserved phylogenetically from worms to mammals, is dependent on the activation, inside the cell, of a cascade of cysteinyl aspartate-specific proteinases – caspases – in response to a variety of death signals, leading to characteristic morphological and molecular changes including cell shrinkage, membrane blebbing, chromatin condensation, and extensive DNA fragmentation, which culminate in the complete removal of the dying cell without lysis, its contents being engulfed by phagocytes or neighboring cells. (See Ashkenazi and Dixit 1998, Thornberry and Lazebnick 1998, Green and Reed 1998, Raff 1998 for recent reviews).

The final steps of compound eye development in *D. melanogaster* offer a particularly instructive example of the importance of programmed cell death in establishing the correct structural patterning of a functional structure. Ommatidial patterning in this insect occurs through a series of sequential decisions that are critically dependent on local cell interactions, and culminates, around 30% of pupal development, in a wave of programmed cell death by which the remaining unpatterned interommatidial cells are removed and the highly organized, crystalline-like structure of the wild type compound eye emerges. This last step is dependent on the correct function of at least two genes: *echinus* (*ec*) and *roughest-irregular chiasm C* (*rst-irreC*). Loss-of-function mutations in any of these loci lead to an absence of programmed cell death in the compound eye during pupal stages (Wolff and Ready 1991, 1993). *ec* has not yet been fully characterized at the molecular level but *rst-irreC* encodes a 764 amino acid long transmembrane glycoprotein displaying five immunoglobulin-like domains in its extracellular portion and a cytoplasmic tail rich in serine and threonine residues (Ramos et al. 1993). Several additional features can be found in the intracellular part of the molecule, including a putative consensus sequence recognized by PDZ domain

proteins (Ponting et al. 1997) at its carboxyl terminus as well as 24 amino acid long stretch composed predominantly of glutamines, (“opa-like” sequence: Wharton et al. 1985) between residues 637 and 660. This latter feature is of special interest, since expanded glutamine repeats were recently shown to be present in a number of important neurodegenerative disorders in humans including Huntington’s disease (Gusella et al. 1996), spinobulbar muscular atrophy, (La Spada et al. 1991) dentatorubralpallidolusian atrophy (Koide et al. 1994, Nagafuchi et al. 1994) and spinocerebelar ataxias such as the Machado-Joseph’s disease (Kawagushi et al. 1994). Besides its role in ommatidial patterning, wild type *rst-irreC* function is also required for embryonic myogenesis, (Strükelberg et al. 2001) as well as for axonal pathfinding in the optic lobe and bristle differentiation in the head (Boschert et al. 1990).

Molecular analysis of mutant alleles of *rst-irreC* showed that the integrity of the intracellular domain is critical for its role in ommatidial assembly: mutant *rst-irreC* flies producing a Rst-IrreC protein with a truncated intracellular domain, but having normal extracellular and transmembrane portions, show an eye “roughening” phenotype (caused by the lack of cell death and the presence of surplus cells, that disrupt the highly regular ommatidial structure) as severe as those observed in null alleles (Wolff and Ready 1991, Ramos et al. 1993).

As a first step to gain a better understanding of the functional role played by the different subdomains that comprise the intracytoplasmic portion of the Rst-IrreC protein we generated truncated versions of the *rst-irreC* cDNA, coding for polypeptides completely or partially lacking the intracellular region and examined the phenotypic effects of their transient expression after microinjection into wild type *Drosophila* embryos at preblastoderm stage. Here we report that injection of a construct encoding a protein lacking the last 80 amino acids of the carboxyl terminus of the wild type molecule leads to a dramatic decrease in post embryonic viability when compared both with controls injected only with the

vector and with a construct encoding the complete wild type protein. Interestingly, injection of a construct without the opa-like, glutamine-rich region shows post-embryonic survival levels comparable to the controls. Based on these findings we suggest a model in which the opa-like domain and the carboxyl terminus could act in concert to modulate an apoptosis-inducing function for Rst-IrreC.

MATERIALS AND METHODS

Drosophila STOCKS AND GENETIC MANIPULATIONS

Unless otherwise stated fly stocks were kept at 25°C in standard fly food. Grapejuice egg-collecting medium was prepared as described in Ashburner (1989). A complete description of the mutants mentioned in the text can be found in Boschert et al. (1990) and Lindsley and Zimm (1992).

NUCLEIC ACID MANIPULATIONS AND CONSTRUCTS

All DNA manipulations were performed using standard techniques (Sambrook et al. 1989). The pCa18Z construct was made in two steps: first the initial 1.19kb DNA fragment from *rst-irreC* cDNA-HB3 (Ramos et al. 1993), was subcloned in the correct orientation into the EcoRI site of P-element transformation vector pCaSpeR-hs (Thummel and Pirrota 1991) generating intermediate construct pCa18. This plasmid was then restriction digested with enzymes KpnI and BglII and ligated to a 1.18kb KpnI-BamHI fragment from cDNA-HB3. The resulting final construct encodes a 688 amino acid polypeptide composed of residues 1-684 from the wild type Rst-IrreC protein plus 5 residues, AAV, added to its carboxyl terminus.

Construct pCa18ZΔ1 was a derivative of pCa18Z in which 140bp were additionally removed from its insert by treating it with exonuclease III, followed by digestion with S1 nuclease (Machado 1997). The resulting construct encodes a 639 amino acid polypeptide composed of amino acids 1-637 from wild type Rst-IrreC protein plus two prolines added to its carboxyl terminus.

Plasmid pKB256-HB3 is a P-element transformation vector carrying an insert containing the complete open reading frame from *rst-irreC* cDNA HB3 (Schneider et al. 1995).

EMBRYO MICROINJECTION AND POST-INJECTION CARE

Microinjection of preblastoderm y^w^{1118} embryos was performed essentially as described in Spradling (1986) and Ramos (1988). Females were allowed to lay eggs for one hour on grapejuice agar plates. Eggs were dechorionated with a 50% solution of commercial bleach, and aligned manually on a strip of double stick tape (Scotch®) mounted on a microscope slide, briefly desiccated in dryerite®, covered with halocarbon oil and injected at their posterior pole with a capillary needle attached to a Leica micro-manipulator. Concentration of injected DNA was usually 0.5-0.8 mg/ml. After injection, the double stick tape strips containing the eggs were carefully removed from the slides and transferred to grapejuice plates. After incubation at 18°C for 36 hours, hatched larvae were individually picked, transferred to vials containing standard food and allowed to develop until adulthood at 25°C. The number of pupae and, afterwards, the eclosed adults were counted, and compared with the total number of larvae initially put into each vial, in order to establish the post embryonic viability. In order to prevent overcrowding, not more than 30 injected larvae were usually put into each vial.

RESULTS

INJECTION OF pCa18Z IN PREBLASTODERM EMBRYOS DRASTICALLY REDUCES THEIR VIABILITY

In designing a strategy for an ‘in vivo’ functional dissection of the Rst-irreC protein we initially divided the intracellular portion of the molecule in two major structural domains, based primarily on their hydrophobic properties, shown in Figure 1. Domain one spans a very hydrophilic region starting immediately after the transmembrane domain and comprising roughly residues 550 to 660. It can, in turn,

be further subdivided into a near transmembrane hydrophilic region (Domain Ia) and a glutamine-rich region (Domain Ib). The second domain includes the remaining 104 amino acids of the carboxyl part of the molecule and alternates small hydrophobic and hydrophilic regions.

In an attempt to correlate these structural domains with specific functions of the *rst-irreC* gene we generated several constructs, deleting progressively larger portions of the cytoplasmic region of the protein, while respecting the approximate boundaries of the above mentioned intracellular subdomains. Here we report the results of "in vivo" transient expression assays using two of the constructs, pCa18Z and pCa18Z Δ 1. Plasmid pCa18Z deletes most, but not all, of domain II (Fig. 1). Construct pCa18Z Δ 1 completely removes domain II plus the glutamine-rich region of domain Ib. Both constructs were put under the control of the heat shock protein 70 promoter (Lis et al. 1983) by cloning them into the *Drosophila* transformation vector pCaSpER-Hs (Thummel and Pirrota 1991). DNA was injected in embryos prior to the cellularization stage as described in Methods. As controls we used pCaSpER-Hs DNA, as well as pKB256-HB3, a construct containing the complete open reading frame of *rst-irreC* cDNA HB3 (Schneider et al. 1995). The percentages of injected embryos surviving up to adult stage were calculated for each construct and are shown in Figure 2. Individuals injected with pCa18Z showed a clear decrease in survival rate when compared with those injected with the vector alone, or with the construct carrying the complete *rst-irreC* open reading frame. Injection of pCa18Z Δ 1 on the other hand did not seem to affect survival beyond control levels, strongly suggesting that the 139bp sequence encoding a glutamine-rich region is responsible for the observed toxicity of pCa18Z construct.

PCA18Z AFFECTS ONLY POST EMBRYONIC VIABILITY

Having established that a truncated form of the Rst-irreC protein produced by plasmid pCa18Z induces

lethality when injected into early embryos we further investigated whether the actual death occurred during embryogenesis, at post embryonic stages or continuously throughout development. To this end embryos were injected with same plasmids as above and the percentage of hatched larvae determined for each construct. As shown in Figure 3, the viability of embryos injected with different constructs and with the vector pCaSpER are approximately the same. The low viability obtained with all plasmids is a consequence of the injection trauma rather than any non-specific toxic effect of the injected DNAs, since embryos injected only with injection buffer showed similarly low hatching levels (data not shown). Thus, only post embryonic viability seems to be affected by the injection of pCa18Z. This was confirmed by examining the percentage of hatched larva from the previous experiment that reached adulthood (Fig. 4). However, once only post embryonic survival rates were taken into account, a small, but consistent, lowering of viability could be observed in embryos injected both with KB256-HB3 and pCa18Z in comparison with those injected with the vector alone, suggesting that ectopic expression of the wild type IrreC-rst protein might have a slight toxic effect not associated to the glutamine-rich region.

DISCUSSION

Transient expression of genes microinjected in embryos before cellularization has been extensively documented and the fate of their DNA followed throughout embryonic and post embryonic stages (Steller and Pirrota 1984, 1985, Martin et al. 1986). Injected DNA can be found in the nuclei of most cells and tissues of the embryo, larvae and even adults. Developmental and tissue specificity of transcription tend to be at least partially retained, and are independent of actual integration in the genome. These characteristics make "in vivo" transient assays a convenient method to quickly assess viability, as well as the presence of dominant phenotypes, before attempting to generate germline transformed

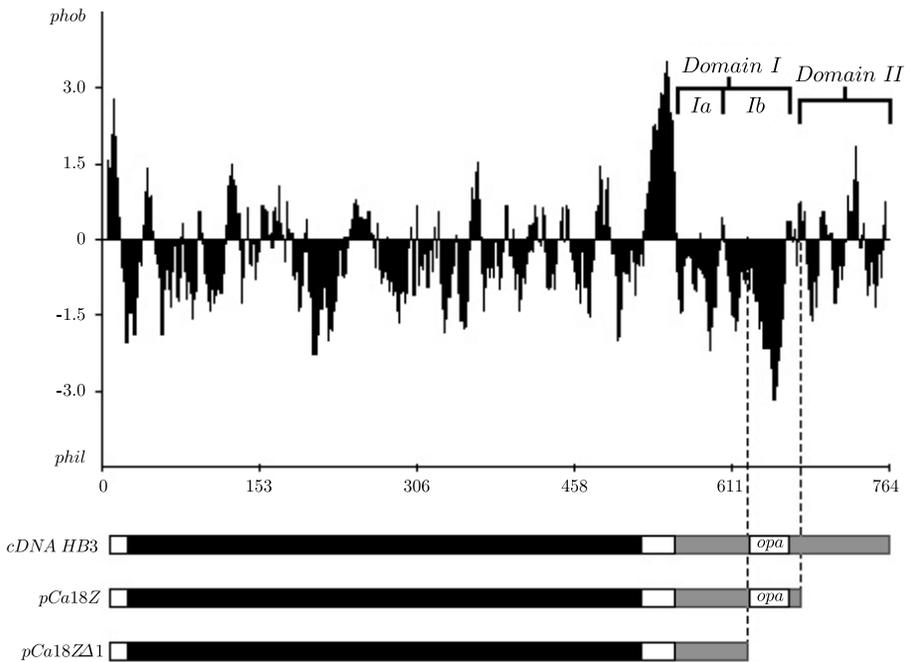


Fig. 1 – Structural organization of the complete Rst-IrreC protein and the truncated forms produced by the constructs tested in this work. A hydropathic profile of the Rst-IrreC protein is shown in the upper part of the figure. The intracellular subdomains and the opa region are indicated (see text for details). In the lower part, the boxes indicate how much of the wild type protein remains in the truncated products. White, signal peptide and transmembraned domains. Gray, intracellular domain: the approximate location of the opa-like sequence is shown. Black, extracellular domain.

individuals. Here we showed that transient expression of a truncated version of the *rst-irreC* cDNA encoding a protein lacking the last 80 amino acids present in the carboxyl terminus of the wild type molecule lead to a dramatic decrease in post embryonic viability compared to controls injected either with the vector alone or with a construct encoding the complete wild type protein. Removal of 140 additional nucleotides encoding a region unusually rich in glutamine restores the survival rates to levels comparable to the ones observed when the wildtype cDNA was injected.

Longer than normal glutamine tracts in the coding region of specific genes have been found consistently associated with a number of important human neurodegenerative disorders and are directly responsible for at least two of them, Huntington disease (Mangiarini et al. 1996) and Machado Joseph

Disease (Ikeda et al. 1996). The pathogenic effect of these expanded glutamine repeats is independent of the polypeptide in which it is present since the “in vitro” insertion of CAG repeats in non-disease protein can also induce neurodegeneration “in vivo” (Ordway et al. 1997). Also, the susceptibility of *Drosophila* to glutamine repeat disease has been demonstrated, very elegantly, by two studies: in one of them (Warrick et al. 1998) the expression, in the compound eye, of the carboxy-terminal fragment of ataxin-3 containing 27 glutamines (the wildtype number) plus the 43 downstream amino acids does not seem to cause any deleterious effect, while the expression of the same construct but with a 78 amino acid long expanded glutamine repeat present in patients with Machado-Joseph Disease shows late-onset progressive cell degeneration, with neurons being particularly sensitive. The cell

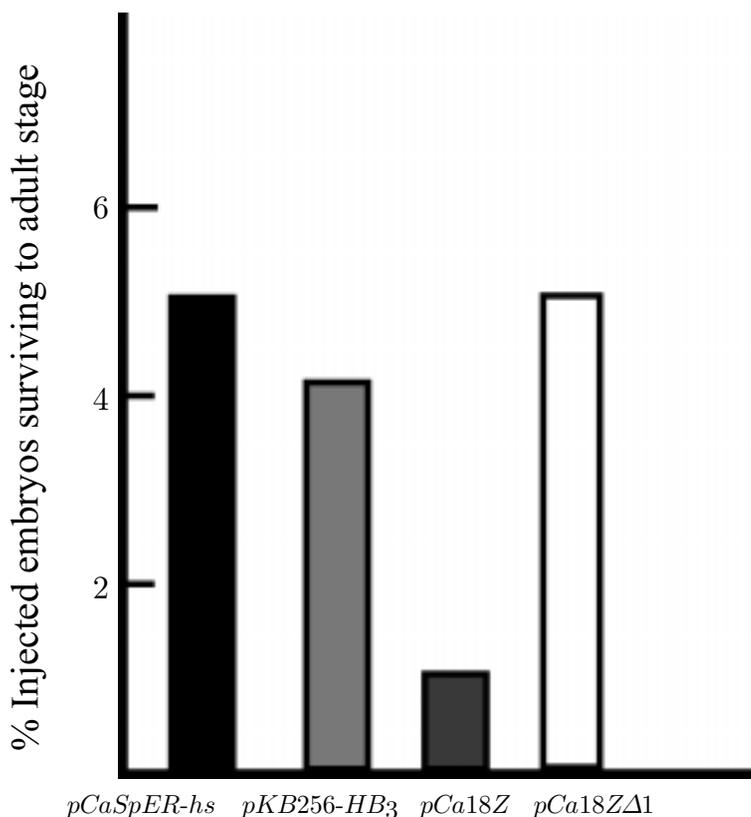


Fig. 2 – Percentage of embryos surviving to adulthood after having been injected, before cellularization, with plasmids carrying two different *rst-irreC* constructs (*pCa18Z*, *pCa18ZΔ1*) or with a control plasmid (*pCaSpER-hs*). A clear reduction in adult survivors is observed in the group of embryos injected with construct *pCa18Z*. Total number of injected individuals: *pCa18Z*, 2027; *pCa18ZΔ1*, 1019; *pCaSpER-hs*, 836; *pKB256-HB3* 356.

degeneration could be partially suppressed by the antiapoptotic protein p35. Similar results were observed with the expanded repeats present in Huntingtin (Jackson et al. 1998), although in this case the apoptotic nature of the cell degeneration remains unclear. Interestingly, expression of mutant ataxin 3 earlier in development, in presumptive mesoderm and muscle cells results primarily in larval lethality (Warrick et al. 1998). In our study it was the removal of sequences downstream to the glutamine-rich region, rather than its expansion, that caused the observed decrease in post embryonic viability. Besides, although the 47 amino acid sequence of

Rst-IrreC whose presence seems to be necessary to induce the toxic effect contains 13 glutamines clustered around a 23aa long stretch, the longest continuous glutamine repeat present is only 6 amino acids long, clearly much smaller than the minimal number of 35-40 residues reported to cause disease. This raises the question of whether the *pCa18Z* toxic effect has the same pathogenic causes of the aforementioned diseases, or it is a consequence of a completely different mechanism. A characteristic pathological feature, present in all glutamine repeat diseases reported so far is the aggregation, in the nucleus, of the expanded polyglutamine tracts present

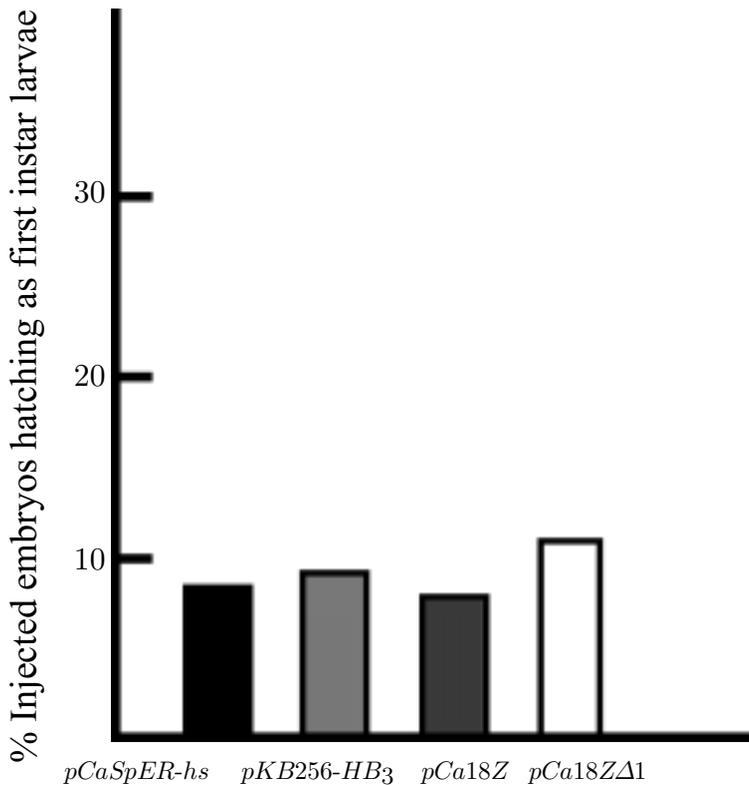


Fig. 3 – Percentage of larvae hatching from embryos injected before cellularization with plasmids carrying the same *rst-irreC* constructs and control as in figure 2. No obvious survival differences could be detected between the injected DNAs.

in the mutant proteins which became enclosed by a single amorphous intranuclear inclusion, although this seems to be a protection mechanism rather than a trigger for the apoptotic neuronal degeneration responsible for the onset of the disease (Klement et al. 1998, Saudou et al. 1998). Their presence in larvae of embryos injected with *pCa18Z* would strongly argue for a common pathogenic mechanism between glutamine repeat disease and the post embryonic lethality associated to *pCa18Z* described here.

Another important point raised by our results is their significance for *rst-irreC* wild type function. In principle, the decrease in viability caused by the *pCa18Z* construct could be simply a consequence of an accidental “gain of toxic function”, bearing no relationship whatsoever to Rst-irreC physiological role. On the other hand, the absence of the last

174 amino acids of the Rst-irreC carboxyl terminus, which includes the glutamine-rich domain, is sufficient to prevent apoptotic elimination of surplus interommatidial cells in the final steps of ommatidial development (Ramos et al. 1993), showing that these sequences are critical for *rst-irreC* function in the compound eye. The exact nature of the involvement of the Rst-irreC protein with programmed cell death in ommatidial pattern formation remains unclear and it was suggested (Reiter et al. 1996) that Rst-irreC could be primarily required for interommatidial cell sorting, this process being a pre requisite for the activation of the apoptotic program in the appropriate cells. Recent results, however, indicate that *rst-irreC* may trigger programmed cell death of surplus interommatidial cells independently of their reorganization (Miller and Cagan 1998). Our obser-

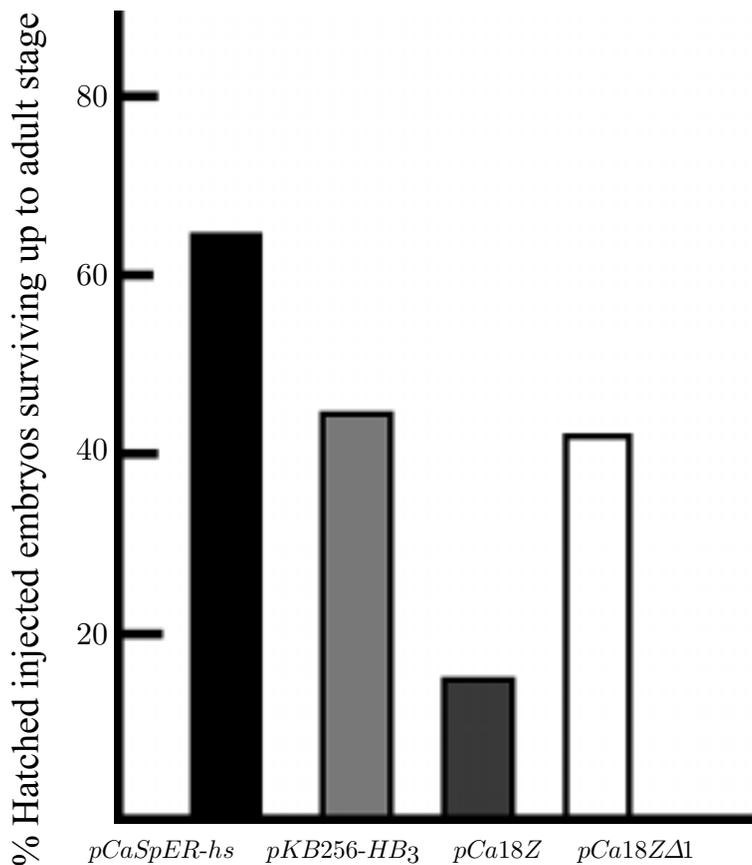


Fig. 4 – Percentage of adults eclosed from the surviving larvae obtained in the experiment shown in figure 3. The individuals injected with *pCa18Z* showed the lowest eclosion rate, but the other two *rst-irreC* constructs, *pCa18ZΔ1* and *pKB256-HB3* also seem to be able to decrease post embryonic viability, although not as severely as *pCa18Z*.

vations could be explained by a model in which the carboxyl terminus of the Rst-irreC molecule inhibits a “death inducing” activity of the glutamine-rich domain. Conformational changes resulting from the activation of the molecule (consequence of binding to an extracellular ligand, for example) could remove this inhibitory effect, resulting in a toxic effect leading to apoptosis. The sequence deleted in *pCa18Z* contain motifs such as putative tyrosine phosphorylation sites and the PDZ domain recognition sequence potentially capable of either bring about conformational changes or to stabilize those induced in the intracellular domain from the outside.

Additionally, some glutamine-rich domains seem to be able to directly activate transcription, (Courey and Tjian 1988, Courey et al. 1989) although in this case the question of how would the appropriate Rst-irreC sequences be translocated to the nucleus remains completely open.

Finally it is important to stress that, although compelling, the results reported in this study must be interpreted with caution, due to the intrinsic limitations of the transient assay methodology. For instance, the rate of protein translation may differ for the different constructs, or their RNA stability may be different. Both these circumstances could,

in principle, affect the survival rate of the injected individuals, leading to a false conclusion regarding the molecular basis of the observed toxicity. Further testing of these and the above mentioned hypothesis, together with a detailed investigation of the cellular basis of the toxic effect reported here will require the generation of germline transformed flies carrying pCa18Z and pCa18Z Δ 1, as well as other constructs specifically disrupting the carboxyl terminal motifs mentioned above. Such experiments are currently under way.

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RESUMO

O gene *roughest-irregular chiasm C (rst-irreC)* de *Drosophila melanogaster*, codifica uma glicoproteína transmembranar contendo cinco domínios semelhantes a imunoglobulina em sua porção extracelular e uma cauda intracitoplasmática rica em serina e treonina, assim como alguns sequências conservadas que sugerem atividade transdutora de sinais. No olho composto, mutantes *rst-irreC* de perda de função não apresentam uma característica "onda" de morte celular programada que ocorre no início do período pupal e que é essencial para a eliminação de células interomatiadas em excesso. Aqui descrevemos uma investigação sobre o papel desempenhado pela molécula Rst-IrreC no disparo da morte celular programada. Ensaio de expressão transiente "in vivo" demonstraram que a remoção dos 80 aminoácidos finais da porção carboxil terminal produz uma isoforma da proteína que é extremamente tóxica para as larvas. Esta toxicidade é suprimida se 47 aminoácidos adicionais, contendo uma região rica em

glutamina (região "opa-like"), forem também removidos da proteína. Os resultados sugerem a possibilidade de que o domínio "opa-like" e o terminal carboxila ajam em conjunto para modular a função de *rst-irreC* na apoptose, e esta implicação é discutida dentro do contexto dos mecanismos gerais causadores de doenças neurodegenerativas associados a proteínas ricas em glutamina em humanos.

Palavras-chave: *Drosophila*, *rst-irreC*, regiões ricas em glutamina, morte celular.

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