Control of triglyceride storage by a WD40/TPR-domain protein

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Obesity is a metabolic disorder related to improper control of energy uptake and expenditure, which results in excessive accumulation of body fat. Initial insights into the genetic pathways that regulate energy metabolism have been provided by a discrete number of obesity-related genes that have been identified in mammals. Here, we report the identification of the *adipose* (*adp*) gene, the mutation of which causes obesity in *Drosophila*. Loss of *adp* activity promotes increased fat storage, which extends the lifespan of mutant flies under starvation conditions. By contrast, *adp* gain-of-function causes a specific reduction of the fat body in *Drosophila*. *adp* encodes an evolutionarily conserved WD40/tetratricopeptide-repeat-domain protein that is likely to represent an intermediate in a novel signalling pathway.

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INTRODUCTION

In the past decade, obesity has been recognized as an increasingly global healthcare problem that is associated with co-morbidities including diabetes mellitus and coronary heart disease (Kopelman, 2000). Recently, genetics has been applied successfully to the identification of factors that control this multi-factorial metabolic disorder (Barsh & Schwartz, 2002). Nevertheless, the known mendelian determinants of human obesity, and of obesity in model mammalian systems, such as mouse, represent only a small fraction of obesity-related genes (Barsh & Schwartz, 2002). Conservation of gene sequence and function throughout evolution has shown that most

human disease-associated genes are present in the genome of the fruitfly *Drosophila melanogaster*, thus supporting the use of this model organism in the molecular analysis of human disease processes (Kornberg & Krasnow, 2000; Rubin *et al.*, 2000). Therefore, we asked whether obesity can also be studied in invertebrates by using *Drosophila* genetics, and whether components identified in flies are molecularly conserved throughout evolution.

RESULTS AND DISCUSSION

Phenotype of *adipose* mutant flies

The *adipose* (*adp*) mutation of *D. melanogaster* that was used in this study, *adp*⁶⁰, was derived from a natural population in Kaduna, Nigeria (Doane, 1960a). Homozygous *adp*⁶⁰ flies are viable, and can be propagated in the same way as wild-type flies. However, *adp*⁶⁰ mutants develop an obese phenotype if environmental factors (the food supply) allow. After one week of *ad libitum* feeding, the fat cells of *adp* mutant flies contain greatly enlarged lipid droplets (Fig. 1A) compared with wild-type flies (Fig. 1B). This cellular phenotype correlates well with the mutant's twofold higher accumulation of triglycerides (Fig. 1D; see also Teague *et al.*, 1986), which serve as lipid stores in the fly (Keith, 1966). Concomitantly, a variable, slight reduction of glycogen levels to 70–80% of normal levels occurs (data not shown).

Homozygous *adp*⁶⁰ flies generally show good viability, but most individuals fall behind wild-type flies in flight endurance tests (Doane, 1980). However, when well-fed *adp*⁶⁰ flies encounter starvation, they significantly outlive wild-type flies (Fig. 1E; see also Doane, 1960b) by making use of their accumulated fat resources. The finding that *adp*⁶⁰ mutants can survive starvation better than wild-type flies is consistent with the 'thrifty genotype' hypothesis (Neel, 1962) that is proposed for human populations. This hypothesis proposes that the persistence of obesity-causing genes in the human gene pool is due to historical advantages that human populations had at times when food supplies were limiting or unstable. The loss of physical fitness of homozygous *adp*⁶⁰ mutants is counterbalanced by their extended lifespan during starvation, providing an argument for the maintenance of *adp* mutations in fly populations that are naturally exposed to unstable food resources.

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Identification of an *adipose* candidate gene

Genetic analysis has defined a region of 70 kb that contains essential sequences of the *adp* gene, which is flanked proximally by *pABP* and distally by the deficiency breakpoint of *In(2R)Pc1¹¹* (Fig. 2A). We sequenced transcription units within the 70-kb DNA segment of the

Fig. 1 | Phenotypes of *adipose* mutant flies. (A–C) Haematoxylin-stained sections of the fat bodies of *adipose*⁶⁰ (*adp*⁶⁰) flies (A), wild-type flies (B) and an *adp*⁶⁰ mutant that contains a transgene with a wild-type copy of the *adp* gene (rescued flies; C). Note the enlarged lipid droplets of *adp*⁶⁰ flies (arrow in (A)). (D) Triglyceride content of well-fed flies six days after hatching. The triglyceride content of *adp*⁶⁰ mutant flies was approximately twice that of wild-type flies (Ore R). In rescued flies, the triglyceride content was reduced to wild-type levels. Errors bars represent the s.d. for three independent assays. (E) Starvation resistance of well-fed flies six days after hatching. The increase in starvation resistance was similar in male and female flies. ww, wet weight.

*adp*⁶⁰ mutant chromosome and compared them to the sequence of the corresponding wild-type DNA (Adams *et al.*, 2000; our sequencing data (not shown)). A single transcription unit showed a 23-bp deletion. This deletion causes a frameshift that would result in premature termination of the predicted protein in *adp* mutants (Fig. 2B). We isolated a full-length complementary DNA clone of the presumptive *adp* transcription unit to determine the structure of the *adp* candidate gene (Fig. 2A). Sequence analysis, and comparison of this cDNA with wild-type genomic DNA sequences, revealed two exons separated by a small, 66-bp intron in the candidate gene (Fig. 2A; GenBank accession number AJ556168).

The candidate gene rescues the *adipose* mutant phenotype

To show that the candidate transcription unit encodes *adp*⁺ activity, we performed P-element-mediated germline-transformation experiments (Rubin & Spradling, 1982). We introduced into the *adp*⁶⁰ host genome a 3.6-kb wild-type genomic DNA fragment (Fig. 2A), which contains only the 1.8-kb open reading frame (ORF) of the *adp* candidate gene, and less than 700 bp of sequences upstream of its translational start site. On transformation, flies derived from the host stock failed to develop an obese phenotype, and their fat cells were indistinguishable from those of wild-type flies (Fig. 1C). Furthermore, the lipid content (Fig. 1D) and starvation resistance (Fig. 1E) of transformants were reduced to wild-type levels. These results show unambiguously that the candidate transcription unit encodes the wild-type *adp* function. Northern blot analysis of developmental stages revealed a 2.7-kb transcript that was seen in wild-type embryos, larvae, pupae and adults (Fig. 2C). In addition, *in situ* hybridization of the cDNA to sections prepared from adult flies indicated that *adp* transcripts are not only expressed during all stages of the *Drosophila* life cycle (Fig. 2C), but are also expressed at a low level in all tissues (data not shown).

Tissue-specific expression of *adipose* gives lower fat content

We then examined the effect of ectopic *adp* activity in flies by generating transgenic animals carrying wild-type *adp* cDNA under the control of the *Gal4/UAS* system (Brand & Perrimon, 1993). Overexpression of *adp* in the fat body was achieved using an enhancer-trap line (FB) that directs Gal4 expression in larval and adult fat-body cells. Animals with transgenic *adp* activity developed into larvae with reduced levels of triglycerides, as compared with wild-type control larvae (Fig. 3A). Thus, the *adp* gain-of-function phenotype (decreased fat content) is consistent with its loss-of-function phenotype in *adp*⁶⁰ mutants (increased fat content). In addition, staining with Oil Red O, which specifically marks lipids in fat-containing cells (Lillie, 1944), indicated that the amount of fat-body tissue in which *adp* was overexpressed was significantly reduced in the third-instar larval stage (Fig. 3B). Such individuals continued to develop until pupation, but died in their pupal cases. By contrast, *adp* overexpression in cells of the nervous system, under the control of the panneuronal *elav* (*embryonic lethal, abnormal vision*) promoter (Campos *et al.*, 1987; The FlyBase Consortium, 1999), did not significantly alter total triglyceride content (Fig. 3A). These individuals developed into normal-looking, healthy, fertile adults. Similarly, larvae receiving ectopic expression of *adp* under the control of the panmuscular *how* (*held out wings*) promoter (Fyrberg *et al.*, 1997) showed normal triglyceride levels (data not shown). As the fat body is a polytene tissue, we tested whether *adp* affects the development of other polytene tissues, such as salivary glands. Here too, overexpression of *adp* under the control of the *F4*

Fig. 2 | Cloning and expression of the *Drosophila adipose* gene. (A) Schematic representation of the *adipose* (*adp*) genomic region. See main text for details. One potential transcription unit lies within the 3.6-kb rescue fragment, which was used to transform the *adp⁶⁰* host strain, thus confirming its identity as the transcription unit of the *adp* candidate gene. (B) Sequence of the *adp* gene. In the mutated *adp* gene (*adp⁶⁰*), 23 bp are deleted, resulting in two changed amino acids followed by a stop codon in the predicted protein (Adp⁶⁰). (C) Northern blot analysis of the expression of *Drosophila adp* transcripts in various developmental stages (E, embryos; L1–L3, larval stages 1–3; P, pupae; A, adults). A major 2.7-kb band is present in all stages (upper blot). A weak band of 1.2 kb can be detected after prolonged exposure (not shown). A probe for the ribosomal gene *L9* was used as a loading control for equal amounts of RNA (lower blot).

promoter (Weiss *et al.*, 1998) did not produce visible defects in the salivary glands (data not shown). These results show that *adp* functions in fat-body cells, and has no general effect on cell viability.

adipose encodes a protein with a novel structure

Sequence analysis of wild-type *adp* cDNA revealed a single ORF of 1,887 nucleotides, corresponding to a predicted polypeptide of 628 amino acids, with a calculated molecular weight of 72 kDa (Fig. 4). The protein encoded by *adp* (Adp) has a novel structure. It combines

Fig. 3 | Overexpression of *adipose* in the fat body and nervous system.

(A) Triglyceride levels in third-instar larvae. Animals overexpressing *adipose* (*adp*) in the fat body (FB-Gal4, UAS-*adp*⁺) show a reduction of lipid levels compared with control animals (FB-Gal4). Expression of *adp* in the nervous system (elav-Gal4, UAS-*adp*⁺) does not reduce lipid levels. (B) Fat-specific staining with Oil Red O shows a significant reduction of fat-body tissue in the larva overexpressing *adp* (bottom) compared with a control animal (top; the asterisk indicates the intensely stained (red) fat-containing tissue). See main text for details.

two previously identified protein–protein interaction motifs, WD40 repeats (Fong *et al.*, 1986) and tetratricopeptide repeats (TPRs; Blatch & Lassle, 1999), which are arranged in a specific pattern (Fig. 4). The amino-terminal region of Adp contains at least four WD40 motifs. Two more WD40 motifs are located in the carboxy-terminal portion of the protein. This motif was first described in the β -subunit of heterotrimeric G-proteins (the G_{β} subunit), which transduce signals generated by heptahelical receptors across the plasma membrane (Sondek *et al.*, 1996). The two WD40 repeats of Adp are interrupted by a WD40-unrelated protein–protein interaction module composed of three TPR motifs. This type of motif is found in several functionally diverse intracellular proteins (Blatch & Lassle, 1999). Secondary-structure analysis suggests that TPR motifs form two antiparallel α -helices (Das *et al.*, 1998). They can function as a ligand-interacting surface, as has been shown for protein phosphatase 5, the activity of which is regulated by binding of long-chain fatty acyl-coenzyme-A esters to its TPR domain (Ramsey & Chinkers, 2002). In addition to WD40 and TPR motifs, Adp contains a

Fig. 4 | Homology between the *Drosophila*, mouse and human Adipose proteins. The human (Hs), mouse (Mm), and *Drosophila* (Dm) amino-acid sequences of the predicted Adipose (Adp) proteins are aligned. Sequences containing WD40-like and tetratricopeptide repeat (TPR)-like motifs are indicated by bars above the alignment. The ADP domain is also indicated by a bar. The end of the truncated *adp*⁶⁰ mutant protein is marked by an asterisk (compare with Fig. 2B).

conserved region N-terminal to the TPR domain (Fig. 4; and see below), which we refer to as the Adp domain (ADP). Comparison of this domain to a WD40-repeat profile (Smith *et al.*, 1999) suggests that the two may be distantly related.

adipose is evolutionarily conserved

Recent studies have shown that the majority of genes involved in human disorders are conserved throughout evolution, and share sequence homology with genes in the *Drosophila* genome (Rubin *et al.*, 2000; Kornberg & Krasnow, 2000). We therefore asked whether *adp*, which has a crucial role in *Drosophila* obesity, is also conserved in vertebrates. We identified cDNAs from human and mouse genes that encode Adp proteins (Fig. 4). Northern blot analyses of the two mammalian cDNA homologues revealed that mammalian *adp* genes, as in *Drosophila*, encode transcripts that are expressed in an unrestricted manner (data not shown). The predicted Adp proteins show a linear conservation of the characteristic WD40/ADP/TPR/WD40 domain structure. The mammalian amino-acid sequences are 96% identical to each other, and

share 37% sequence identity (50% sequence similarity) with *Drosophila* Adp (Fig. 4). On the basis of this conserved secondary structure, and by using known three-dimensional structures of comparable proteins as a template, a model for the presumed three-dimensional structure of the human Adp protein was constructed. The proposed molecule contains a seven-bladed β -propeller linked to antiparallel α -helices (Fig. 5).

SPECULATION

Our findings provide evidence that loss of *adp* activity causes obesity in *Drosophila*. The target organ, the fat body, is the mesodermally derived, main energy-storage organ of insects (Ritzki, 1978), and has morphological, physiological and developmental similarities to mammalian adipose tissue (Tong *et al.*, 2000). The novel arrangement of protein-protein interaction domains in Adp suggests that Adp might be an adaptor component of an intracellular signalling pathway or network that is involved in determining susceptibility to the development of obesity in *Drosophila*. Therefore, the newly

Fig. 5 | Modelled structure of the human adipose protein. Model building suggests that the adipose (ADP) protein can form a seven-bladed β -propeller. The six α -helices of the tetratricopeptide repeat (TPR) domains are shown on the right; 'linker' regions between the β -propeller and the TPR motifs are represented by dashed lines.

identified obesity gene in the fly, and its conservation in mammals, suggest an entry point into the identification of additional key determinants for the control of fat storage and energy homeostasis in both *Drosophila* and mammals.

METHODS

Fly stocks and P-element-mediated transformation. *adipose* mutants and wild-type fly strains have been described previously (Doane, 1960a; Clark & Doane, 1983). P-element-mediated transformation was performed using *white*⁻ flies (Rubin & Spradling, 1982).

Phenotypic analysis. To examine the phenotype of *adp*⁶⁰ mutants, newly emerged flies were kept in uncrowded conditions on fresh fly food. The phenotypes were analysed by the following assays: to examine the size of lipid droplets, 8- μ m paraffin sections of paraformaldehyde-fixed male flies, fed for 6 days, were rehydrated, stained with Harris haematoxylin for 10 min, washed, and mounted in Aquamount (Merck). The lipid content of ten flies was determined using the Triglyceride (INT) kit (Sigma) in accordance with the manufacturer's instructions. One hundred and fifty flies were starved at 25 °C in 3 food-free vials, with an unlimited water supply.

Genetic and molecular localization, cloning and expression of *adipose*. *adp* was uncovered by a deficiency that is associated with the proximal breakpoint of the inversion *In(2R)Pcl*¹ (Doane, 1994), and maps distally to *pABP* within the deficiency (Doane, 1999). The distal breakpoint of the deletion in *In(2R)Pcl*¹ was determined by Southern blot analysis using a PCR probe that was made using the primers 5'-AGTCGGAGAAGCTGCATCATGAGGC-3' and 5'-TGC TATGCCTTATTTGTCGCTGCGG-3'. Transcription units between *pABP* and the distal breakpoint of the deficiency were analysed for mutations by comparing the sequence of PCR-amplified genomic DNA from wild-type and *adp* mutant flies. The deletion resulting in the mutation in *adp*⁶⁰ homozygotes was detected by sequencing PCR fragments that were generated with the primers 5'-CCG CCGTCGCCTGCTGTTTG-3' and 5'-GCGCGTATCTTGCCCGT GTCTCC-3'. The 3.6-kb *EagI*-*EcoRI* fragment was isolated from the

Cosmid 15B10 (The FlyBase Consortium, 1999) and cloned into the *NotI* and *EcoRI* restriction sites of the pCaSper4 vector, which was then used for germline integrations. The identified transcription unit of *adp* is identical to the annotated gene CG5124 (Berkeley *Drosophila* Genome Project (BDGP); see website at <http://www.fruitfly.org>). Sequencing of the EST (expressed sequence tag) clone GH10933 (BDGP) that covers the full-length wild-type *adp* cDNA revealed the genomic organization of *adp*. *adipose* messenger RNA distribution was analysed by northern blotting and by performing *in situ* hybridizations on sections from adult flies. For northern blots, digoxigenin-labelled mRNA probes were generated using the wild-type *adp* cDNA clone following standard protocols (supplied by Roche Diagnostics).

The human homologue of *Drosophila adp* was derived from an adipocyte cDNA library (Stratagene) using a PCR probe that was amplified from this library with the primers 5'-GCCGACTCT AAGGTGCATGT-3' and 5'-GCAGGACAGTCCCTGAAGAC-3'. The mouse homologue of *adp* was derived from an embryonic cDNA library, using a PCR probe that was amplified from this library with the primers 5'-ACCACAAGAAGCTGCTGTCC-3' and 5'-GGCCTG TGAGGTCTTCACTC-3'.

Overexpression of *adipose* in transgenic animals. The wild-type *adp* cDNA was expressed in transgenic animals under the control of the *Gal4/UAS* system (Brand & Perrimon, 1993). Triglyceride content was determined as described above. The *white*⁻ background (The FlyBase Consortium, 1999), in which the *adp* transgenic animals were generated, was used as a control (Rubin & Spradling, 1982).

Model building. Searching the WD40-domain-family alignment from the SMART server (Schultz *et al.*, 1998) using the HMMER programme suite (Eddy, 1998) revealed six unambiguous repeats with expectation values between 5.3×10^{-4} and 2.3×10^{-13} . A seventh repeat is suggested using the WD40-repeat profile created by Smith *et al.* (1999). The corresponding HMMER search with the TPR-family alignment (Eddy, 1998) gave three TPR repeats with E-values between 1.3×10^{-3} and 3.4×10^{-5} . This result suggests that Adp is related to the seven-bladed WD40-repeat proteins. As the β -propeller folds are considered to be suitable folds for reliable predictions, due to their highly conserved features, G_{β} (Protein Database code 1gp2; Wall *et al.*, 1995) was used as a template to build a model for Adp. Seven repeats of G_{β} were used to build a seven-bladed propeller using the MODELLER software (Sali & Blundell, 1993). The final model has a packing-quality score of -1.7 (Vriend, 1990), thus indicating a potentially correct fold. The TPR repeats were modelled using protein phosphatase 5 (Protein Database code 1a17; residues 36–149) as a template, and are positioned next to the point of insertion between WD40 repeats 5 and 6 of the Adp model in Fig. 5.

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