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Characterisation of Upd2, a *Drosophila* JAK/STAT pathway ligand

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Abstract

The characterisation of ligands that activate the JAK/STAT pathway has the potential to throw light onto a comparatively poorly understood aspect of this important signal transduction cascade. Here, we describe our analysis of the only invertebrate JAK/STAT pathway ligands identified to date, the *Drosophila unpaired*-like family. We show that *upd2* is expressed in a pattern essentially identical to that of *upd* and demonstrate that the proteins encoded by this region activate JAK/STAT pathway signalling. Mutational analysis demonstrates a mutual semi-redundancy that can be visualised in multiple tissues known to require JAK/STAT signalling. In order to better characterise the in vivo function of these ligands, we developed a reporter based on a natural JAK/STAT pathway responsive enhancer and show that ectopic *upd2* expression can effectively activate the JAK/STAT pathway. While both Upd and Upd2 are secreted JAK/STAT pathway agonists, tissue culture assays show that the signal-sequences of Upd and Upd2 confer distinct properties, with Upd associated primarily with the extracellular matrix and Upd2 secreted into the media. The differing biophysical characteristics identified for Upd-like molecules have implications for their function in vivo and adds another aspect to our understanding of cytokine signalling in *Drosophila*.

Keywords: Unpaired; Domeless; STAT; Cytokine; Janus Kinase; *Drosophila melanogaster*; Ligand; Auto-regulation

Introduction

The JAK/STAT signal transduction cascade is named after its two major components, a receptor-associated Janus kinase (JAK) and the signal transduction and activator of transcription (STAT). In the canonical model of JAK/STAT signalling, extracellular ligands bind to a bi-partite transmembrane receptor complex that is itself non-covalently associated with the JAK tyrosine kinase via its intracellular C-terminal region. Upon binding of the ligand to the extracellular domain, a conformational change in the receptor is thought to lead to the activation of the JAK kinases which trans-phosphorylate tyrosine residues within both the receptors and one another. This phosphorylated receptor/JAK complex then acts as a docking site for cyotsolic STAT molecules which associate via their SH2 domain and are themselves phosphorylated on a C-terminally located tyrosine residue. STATs activated in this manner dimerise in a head to tail arrangement via their SH2 and phospho-tyrosine residues and translocate to the nucleus where they bind to DNA activating transcription of target genes (reviewed in Levy, 2003).

Both the major components and many of the functions of the JAK/STAT pathway appear to have been conserved throughout evolution with STAT-like molecules identified in the slime mould *Dictyostelium* (Araki et al., 1998) and the nematode *C. elegans* (Liu et al., 1999). The *Drosophila melanogaster* JAK/STAT pathway, the most intensively studied invertebrate example, contains a 'complete' canonical pathway. The *Drosophila* genome encodes a single STAT homologue, encoded by *stat92E* (Hou et al., 1996; Yan et al., 1996), as well as a JAK homologue *hopscotch (hop)* (Binari and Perrimon, 1994) and a receptor molecule called *domeless (dome)* (Brown

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et al., 2001). In vertebrates, JAK/STAT signalling is known to regulate multiple developmental processes including haematopoietic development, immune responses and cellular proliferation. The *Drosophila* pathway is required for segmentation, tracheal development, and also innate immune responses (Agaisse et al., 2003), cellular proliferation (Bach et al., 2003; Mukherjee et al., 2005), haematopoiesis (Hanratty and Dearolf, 1993; Harrison et al., 1995) and stem cell maintenance (Kiger et al., 2001).

One aspect of JAK/STAT signalling in *Drosophila* that has received less attention are the pathway ligands. In vertebrates, over 40 polypeptide ligands have been identified that function to activate JAK/STAT signal transduction. These include the α- and β-interferons, interleukins and a wide range of cytokines and growth factors (Boulay et al., 2003). This multitude of ligands stimulates a large number of diverse receptors which activate differing subsets of the four JAKs and seven STATs present in vertebrates. The situation in *Drosophila* is significantly more straightforward. By contrast to the rest of the pathway for which homologues can be identified, the fly genome does not appear to encode obvious interferon or interleukin-like proteins. However, the *unpaired* (*upd*) gene (Wieschaus et al., 1984), also known as *outstretched* (Muller, 1930), has been identified on the basis of its distinctive mutant cuticle phenotype that mirrors the segmentation defects produced by loss of other pathway components (Binari and Perrimon, 1994; Harrison et al., 1998; Hou et al., 1996). Genetic analysis of *upd* first indicated that the locus is likely to encode a molecule capable of acting at a distance (Gergen and Wieschaus, 1986) and subsequent molecular characterisation showed that it encodes a glycosylated secreted protein that associates strongly with the extracellular matrix in tissue culture assays and is capable of activating JAK/STAT signalling (Harrison et al., 1998). *upd* is expressed in regions known to require JAK/STAT pathway activity during development, has been visualised extracellularly in vivo (Zeidler et al., 1999) and is capable of stimulating target gene expression at a distance (Karsten et al., 2002; Tsai and Sun, 2004). Retrospective alignments of Type I cytokines have subsequently characterised Upd as being most closely related to the vertebrate leptins (Boulay et al., 2003).

In addition to the original *upd* locus, a recent study has characterised a role for the homologous protein Upd3 which also appears to function via stimulation of the JAK/STAT pathway and is central for the signalling of haemocytes to the fat body in response to septic injury (Agaisse et al., 2003). By contrast, no information regarding the biochemistry or developmental roles of the third *upd*-like gene, termed *upd2*, has been described and its potential functions are as yet unknown.

Here, we present a detailed analysis of the *upd* genomic interval present within the 17A region of the *Drosophila* X-chromosome. We define the extent of existing deletions and mutations in the region and show that the strength of the phenotypes caused by these mutations are related to the number of Upd homologues removed. Tissue culture-based assays show UpdGFP is secreted and associated with the adjacent extracellular matrix (ECM), while only minimal levels of Upd2GFP appear to associate with the ECM. This agrees with in silico analysis suggesting that Upd2 contains an N-terminal anchor-sequence present in membrane inserted, non-secreted proteins. However, the situation seems to be more complex, as both Upd and Upd2 activate JAK/STAT signalling in vivo. Moreover, in a tissue culture reporter assay system Upd2 is capable of strongly conditioning its overlying media suggesting that Upd2 is indeed secreted.

Our analysis therefore reveals an unsuspected level of complexity in the regulation of Upd and Upd2 that explains their mutual semi-redundancy and may affect their signalling potential in vivo.

Materials and methods

Molecular biology and cloning

upd2 expression constructs were generated by amplifying the open reading frame as predicted by flybase (http://fly.ebi.ac.uk:7081/), from genomic DNA using the primers TACGATGGCCAATCCACTAACGC and TCAAGACTCATTGGATCCGCCATC. The resulting 1.8 kb product was cloned into pCR-TOPO (Invitrogen) to give *pCR-upd2.* EGFP tagged Upd2 was generated by amplifying Upd2 from *pCR-upd2* using the SP6 and GGAAGATCTGACTCATTGGATCCGCCATC primers, trimming with *Eco*RI and *Bgl*II and subcloning into *pBS-EGFPA* (MPZ unpublished). Similarly, UpdGFP was generated by amplifying the coding region from the *upd* cDNA (Harrison et al., 1998) with the primers AGAATTCGATATCGGCGATGGCTCGTCCGCTG and CGGATCCGTGCGCTGCACGCGCTTC, trimming with *Eco*RI and *Bam*HI and cloning into *pBS-EGFPA*. The resulting plasmids were sequenced before subcloning into *pUAST* (Brand and Perrimon, 1993).

Signal swap constructs were generated by two step mega-primer amplification of both Upd and Upd2 using the primers GCCACCTGGTCGCGCAAGTGTCGCCCCTCGGCGAGGTGGGGCAAC (for Upd1SS2) and CCGCCGCTGCTGGTGGTGCTGCGCGCCTTGGTGAATGGCATCACG (for Upd2SS1) in conjunction with the existing forward and reverse primers (see above). Details available on request (MPZ).

Plasmids for expression in tissue culture were subcloned into *pAc5.1* (Invitrogen).

The *6x2DrafLuc* plasmid (Müller et al., 2005) is based on a multimerisation of the *2xDrafSTAT(wt)* plasmid (Kwon et al., 2000).

For deficiency break point mapping experiments hemizygous mutant male embryos were identified by use of a GFP expressing balancer chromosome and used for single embryo PCR reactions using primer pairs designed to amplify each predicted gene in the region.

dome-MESO was generated from an *Eco*RI genomic fragment flanking the $dome^{321}$ P-element insertion which was subdivided into a 0.6-kb *Kpn*I/*Not*I fragment containing the untranslated and upstream sequences and a *Not*I/*Bam*HI 2.8 kb fragment containing part of the first exon and most of the first intron. These fragments were subcloned into the *pCaspeR-lacZhs43* plasmid and transformed into flies. The 0.6-kb construct did not drive any consistent embryonic expression while the 2.8-kb construct gave the patterns of expression described in the results section as the *dome-MESO* reporter. Two insertions where kept that gave strong levels of *lacZ* expression in homozygous (for the X and third chromosome insertions) or hemizygous conditions (for the X insertion). In all lines, expression is observed in the pharynx, hindgut and precursors of the longitudinal visceral mesoderm. The expression in the latter is JAK/STAT independent.

upd-like genes present in other *Drosophilids* were identified using *D. melanogaster* Upd as a protein probe in tblastn searches of the available databases. Predictions of signal/anchor sequences were undertaken at http://www.cbs.dtu.dk/services/SignalP/ and prediction of potential N-linked glycosylation sites at http://www.cbs.dtu.dk/services/NetNGlyc/. Protein alignments and phylogenetic trees were constructed using the DNA Star software package.

Tissue culture experiments

For tissue culture experiments involving visualisation of Upd-like fusions to GFP, 8×10^4 S2 cells were seeded into wells of a 6 well plate containing sterile glass microscope cover-slips before transfection with 100 ng pLit-His2AvD-mRFP expressing Histone2aV-mRFP (a kind gift of S. Heidmann) and 100 ng of the indicated Upd-like GFP fusion molecules. After 4 days media was carefully removed and replaced with PBS + 3% EM grade formaldehyde (Polysciences, Inc.) for 10 min. Cells were then washed 2×5 min with PBS + 0.1% Tween-20 (PBT) before incubation for 20 min with PBT+1u phaloidin-Alexa633 (Molecular Probes). Cells were mounted in 70% Glycerol, 1× PBS and visualised on a Leica TCS NT scanning confocal microscope.

For autocrine luciferase assays 5×10^5 Kc_{167} cells were seeded into a 6 well plate and transfected with 1.5μg *pAc-Hop*, *pAc-UpdGFP*, *pAc-Upd2GFP*, *pAc-Upd1SS2GFP* or *pAc-Upd2SS1GFP* together with 500ng of *6x2xDrafLuc* reporter and 25 ng *pAct-renilla*. Cells were resuspended in fresh media and 5×10^4 cells plated into the wells of a 96-well plate in sextuplicate. Cells were subsequently grown for 3 days prior to lysis and measurement of luciferase activities.

For paracrine luciferase assays 5×10^5 Kc cells were seeded into wells of a 24-well plate and transfected with 3 ng *pAct-renilla* and 150 ng of the *pAc5-UpdGFP (Upd2GFP*, *Upd1SS2GFP* or *Upd2SS1GFP)* as well as 150 ng empty *pAc5* vector using Effectene (Qiagen). For conditioned media assays 3×10^6 Kc cells were seeded into wells of a 12-well plate and transfected with 20 ng *pAct-renilla* and 700 ng of the *pAc5* empty vector, *pAc5-UpdGFP* (*Upd2GFP*, *Upd1SS2GFP* or *Upd2SS1GFP*) and incubated for 3 days. Media was then harvested and the cells lysed for renilla luciferase assays. Media was then centrifuged to remove potential non-adherent cells and was normalised by dilution with fresh media based on the *Renilla* luciferase values of the conditioning cells. For experiments to release Upd-like proteins from the ECM, Heparin (Sigma H-9399) at a final concentration of 50μg/ml, was added to cells 1 day after transfection. In parallel 5×10^6 Kc cells were seeded into a 6 well plate and transfected with 500 ng of *6x2xDrafLuc* reporter. 5×10^4 reporter cells were then added in sextuplicate to a 96-well plate, stimulated by addition of 100μl of the conditioned media and incubated for 3 days before lysis and measurement of luciferase activity. Variations in the level of pathway activation observed may result from differences in transfection efficiency or changes in the time over which firefly luciferase was allowed to accumulate in the cells prior to measurement.

Luciferase assays were undertaken using either the Stop and Glow dual luciferase assay system (Promega) or as described in Cortenbosch and Schram, (1971). Activity was measured using a Victorlight 1420 luminomiter (PerkinElmer) and analysed in Excel with error bars representing the standard deviation of the samples.

Genetics and histology

The $upd2^{G1031}$ allele is an insertion of the *p{Mae-UAS.6.11}* P-element (Crisp and Merriam, 1997) generated by the Göttingen X-chromosome mutagenesis project (Peter et al., 2002). To generate additional *upd2* alleles we mobilised the *p{Mae-UAS.6.11}* insertion and isolated an excision allele, $upd2^{\Delta 3-62}$. PCR-based analysis showed that this excision lacks all P-element sequences. Direct sequencing of a PCR product spanning the deleted region identified the loss of 2530 bp of genomic DNA encompassing a region from 266 bp downstream of the Upd2 AUG codon to 2264 bp upstream (Fig. 1A and not shown). This excision removes the 5′UTR and regions upstream of *upd2* as well as DNA encoding the first 89 amino acids of the protein. Even if subsequently transcribed, conceptual translation of the resulting mRNA from the next in frame start codon would result in protein truncated by 114 amino acids lacking the anchor/signal-sequence present in wild type Upd2.

$dome^9$, a previously uncharacterised *dome* allele that does not express *lacZ*, was used to avoid interference between sources of *lacZ* when using the *dome-MESO* reporter. $dome^9$ germ line clones result in strong segmentation defects similar to the molecularly characterised strong $dome^{217}$ and $dome^{468}$ alleles (not shown). $dome^9$ was isolated as a second site mutation found associated to the P{w^+*Actin5C*^{9}} insertion generated in the same Göttingen collection as $dome^{217}$ and $dome^{468}$ (Peter et al., 2002). When $dome^9$ was recombined from the $Actin5C^9$ insertion, the phenotype was shown not to be caused by a P-element enhancer trap insertion as it does not express *lacZ* and does not rescue *white* mutant phenotype.

For in vivo misexpression experiments we used *UAS-upd* (Harrison et al., 1998), *UAS-updGFP* (Tsai and Sun, 2004) and *UAS-upd2GFP* (this study). We find Upd and UpdGFP to function in an indistinguishable manner, indicating that the GFP tag does not interfere with its signalling properties. The *UAS-upd2GFP* line used in this study shows very high levels of expression suggesting that the higher levels of induction when using this line may be insert specific rather than due to Upd2 being a more potent activator.

In situ hybridisation was undertaken as described previously (Lehmann and Tautz, 1994) using probes derived from the T7 and SP6 promoters present within *pCR-upd2,* and as previously described for *vvl* (de Celis et al., 1995) and *trh* (Brown et al., 2003). Cuticle preparations and antibody staining were as described in Hu and Castelli-Gair (1999).

Results

Three *unpaired*-like genes have previously been identified by sequence homology searches within the 17A interval of the *Drosophila* X-chromosome (Fig. 1A and Castelli-Gair Hombria and Brown, 2002). The founding family member *upd* has been molecularly characterised (Harrison et al., 1998) and its activation of the JAK/STAT signal transduction pathway is required for multiple developmental processes (Bach and Perrimon, 2003; Castelli-Gair Hombria and Brown, 2002; Zeidler et al., 2000). In addition, a recent report has identified Upd3 as an infection specific cytokine produced by haemocytes in response to septic injury (Agaisse et al., 2003). However, no function has been proposed for *upd2* and no analysis of the *upd* locus as a whole has been undertaken.

Expression of upd2 and upd3

To investigate the potential developmental roles of *upd2* and *upd3* we analysed their embryonic expression. Although adult haemocytes have been shown to express Upd3 in response to bacterial challenge (Agaisse et al., 2003) the only detectable expression of *upd3* during embryogenesis is observed in the gonads from embryonic stages 14–15 (Fig. 2A). By contrast, *upd2* is expressed in the central region of the blastoderm, segmentally repeated stripes at stage 9 (Fig. 2B), in the tracheal placodes at stage 10 (Fig. 2C), and in a region within the hindgut and posterior spiracles from stage 11 (Fig. 2D and not shown). Given the almost complete overlap between the *upd2* expression pattern and that previously described for *upd* (Harrison et al., 1998; Karsten et al., 2002) these results suggest that both *upd2* and *upd* could be regulating embryonic development.

Mutations in the upd gene region

In order to understand the role of the *upd*-like genes for the different aspects of JAK/STAT signalling we set out to make a detailed characterisation of the defects caused by different mutations in the region.

Molecular characterisation of EMS induced point mutations in the *upd* gene have identified a nonsense mutation (Q55Stop) in upd^{YM55} and a two base pair insertion causing a frame shift mutation after amino acid 144 in upd^{YC43}

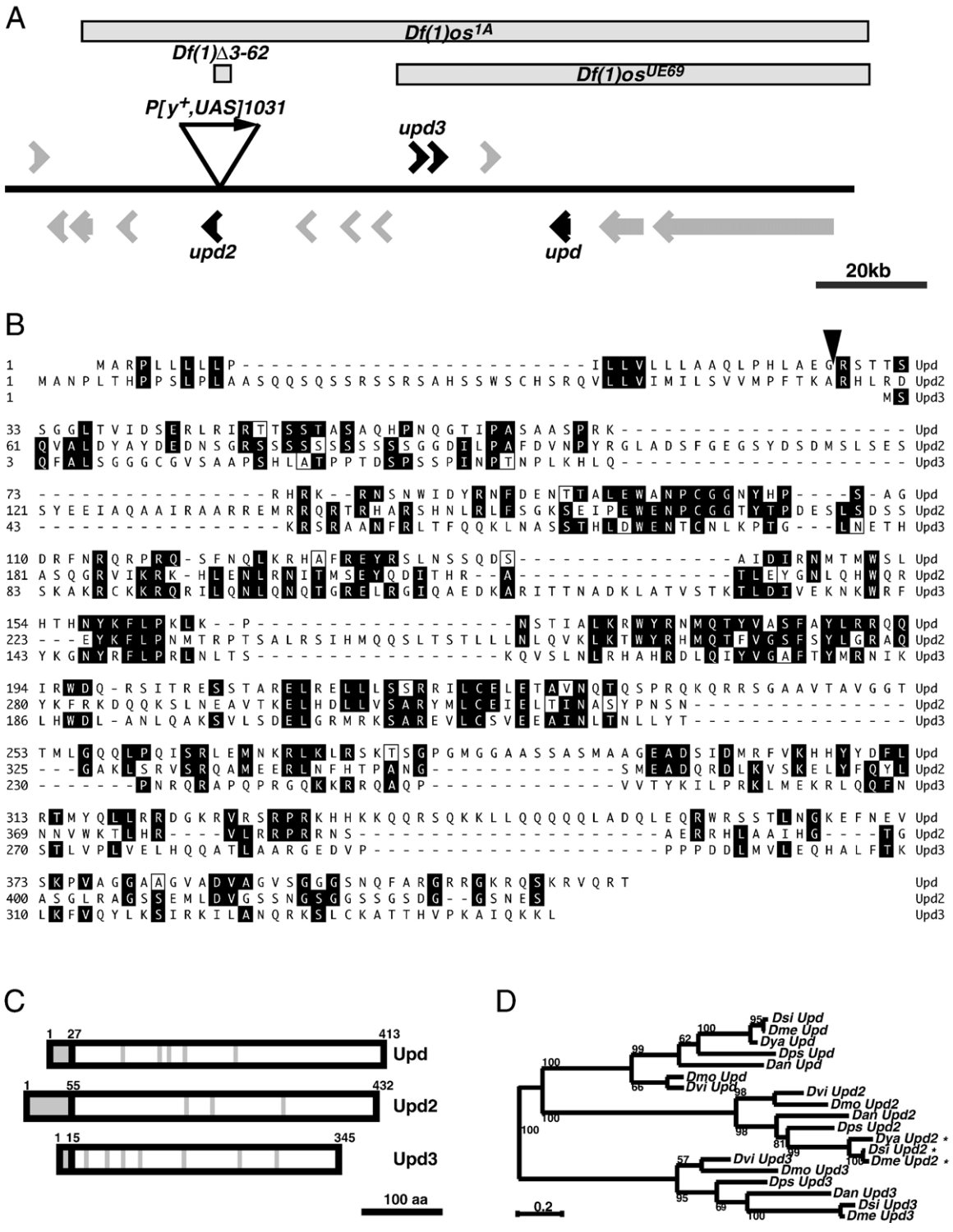

Fig. 1. Genomic organisation of the Upd region and comparison of Upd homologues. (A) The 17A genomic region showing the *upd*-like genes and the extent of the deficiencies characterised in this study. The position and orientation of the *p[y*$^{+}$ *UAS]1031* P-element insertion is shown (not to scale). (B) Alignment of the predicted amino acid sequence of the *D. melanogaster* Upd-like proteins with identical residues shown in black and similar residues boxed. The predicted cleavage site of the Upd and Upd2 signal-/anchor-sequences is indicated by the arrowhead. (C) Schematic representation of the *D. melanogaster* Upd, Upd2 and Upd3 proteins showing their signal-/anchor-sequences (grey box) and potential N-linked glycosylation sites (bars). Numbers indicate amino acid position. (D) Phylogenetic tree showing the relationship of the proteins encoded by the upd-like genes of *D. melanogaster* (Dme), *D. simulans* (Dsi), *D. yakuba* (Dya), *D. ananassae* (Dan), *D. pseudoobscura* (Dps), *D. virilis* (Dvi) and *D. mojavensis* (Dmo). Upd2 homologues with predicted anchor sequences are indicated by an asterisk (*).

(Harrison et al., 1998). Although *upd*^{YM55} and *upd*^{YC43} are likely to represent amorphic alleles of Upd, the segmentation phenotype in these alleles is clearly milder than that derived from maternally and zygotically mutant germline clones of amorphic *stat92E* or *dome* receptor alleles (compare Figs. 3 A–B with E). We therefore set out to quantify the frequency

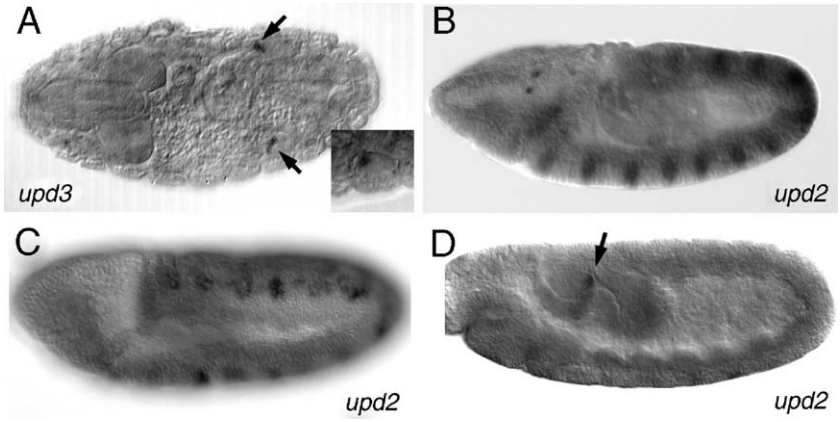

Fig. 2. Expression of *upd3* and *upd2*. (A) *upd3* expression in the gonads of a stage 16 embryo (arrows and inset). (B–D) *upd2* expression. At stage 9 *upd2* is expressed in a striped segmental pattern (B), that is substituted at stage 10 by a transient tracheal expression (C) that disappears by stage 11 leaving hindgut (arrow) and posterior spiracle expression (out of the focal plane).

at which each segment is defective in several *upd* alleles (Table 1). Although the variable fusion and deletion of segments complicates unambiguous segment identification, our analysis confirms that the frequency of segment defects is lower in upd^{YC43} mutant embryos compared to *stat92E* germline clone mutant embryos (Compare first two columns in Table 1 and see Figs. 3A, B and E for representative examples).

We also analysed the less variable posterior spiracle and head skeleton defects associated with alleles of JAK/STAT pathway components (Figs. 3F–K) and observed that the phenotypes caused by the *upd* alleles are consistently less severe than those of germ line clones for null alleles of the *dome* receptor or *stat92E* (compare Figs. 3G–G′ with J–J′ and K–K′). These results indicate that the JAK/STAT pathway is still at least partly active in *upd* mutants.

We then set out to map the molecular extent of two deficiencies previously described as deleting different areas of the *upd* region (Eberl et al., 1992). Using a PCR-based assay, we determined that *Df(1)os1A* removes the genomic region encoding all three *upd*-like genes (Fig. 1A). *Df(1)os1A* embryos have a very similar head and posterior spiracle defects to *stat92E* and *dome* null embryos in which posterior spiracles and trachea are almost missing, and the mandible is extremely abnormal (compare in Figs. 3 panels I, I′ with J, J′–K, K′). The segmentation defects in *Df(1)os1A* are also very similar to those of *stat92E* mutants (Table 1) indicating that all embryonic JAK/STAT pathway ligands are located in the 17A region removed by this deficiency.

A similar analysis of *Df(1)osUE69* shows that it deletes the *upd* and *upd3* loci while *upd2* is unaffected (Fig. 1A). *Df(1)osUE69* mutant embryos have milder spiracle and head skeleton defects than *dome* or *stat92E* null embryos (Figs. 3, compare panels H–H′ with J–J′ and K–K′). Finally, the A8 segment of *Df(1)osUE69* mutants is defective in 8% of the embryos compared to 85% in embryos lacking *stat92E* (Table 1).

To extend this phenotypic analysis to the molecular level we also tested the expression of the pathway target gene *trachealess (trh)* in embryos hemizygous for the *Df(1)osUE69* and *Df(1)os1A* deficiencies. In the wild type embryo, *trh* (Figs. 3L–M) is strongly dependent on JAK/STAT pathway signalling for its expression within the developing tracheal system (Brown et al., 2001). In *Df(1)osUE96* mutants *trh* expression is reduced, but clearly expressed at all stages (Figs. 3N–O), while *Df(1)os1A* mutants show a significantly stronger effect with almost complete loss of *trh* (Figs. 3P–Q), a phenotype similar to that reported for *dome* and *stat92E* (Brown et al., 2001).

Taken together, these results prove that Upd is not the only JAK/STAT ligand functioning during embryonic development and strongly suggest that the *upd*-like genes are mutually semi-redundant with *upd2* able to partly rescue the effects caused by loss of *upd*. However, it should be noted that both *Df(1)os1A* and *Df(1)osUE69* remove other predicted genes in addition to the *upd*-like loci (shown as greyed arrows in Fig. 1A) and it cannot be formally excluded that these may contribute to the phenotypes observed.

Generation of upd2 mutations

To isolate *upd2* alleles, we mobilised a $P[y^{+}UAS]1031$ transposon insertion (Peter et al., 2002) located 702 bp

Fig. 3. Analysis of *upd* alleles. (A–E) Comparison of the segmentation defects of various alleles as indicated on the panels. The arrowheads point at the A8 denticle belt and the bracket at the fused /deleted A4–A5 segment. Note the difficulty to assign a particular denticle belt to a specific segment in the strongly abnormal embryos. The variability of the phenotype is illustrated by the partial fusion affecting only one side of the embryo in panels C and E. (F–K) Comparison of head and spiracle defects in different JAK/STAT pathway mutants. Top row (F–K) heads, lower row (F′–K′) posterior spiracles of (F) wild type, (G) upd^{YM55}, (H) *Df(1)osUE69*, (I) *Df(1)os1A*, (J) *dome* null germ line clone, (*K*) $stat92E^{6349}$ germ line clone. Arrowheads point at the filzkörper of the posterior spiracle. Note the thin remnant filzkörper in H′. (L–Q) Expression of *trachealess (trh)* in various genetic backgrounds at stage 10 (left panels) and stage 14 (right panels). (L–M) Wild type expression in the tracheal placodes and invaginating tracheal system. (N–O) Reduced expression in the *Df(1)osUE69* allele removing *upd* and *upd3* (P–Q) Extremely reduced levels of *trh* expression are detected in *Df(1)os1A* individuals.

upstream of the predicted first codon and obtained *upd2*$^{\Delta 3-62}$, an excision allele that lacks from 266 bp downstream of the Upd2 AUG codon to 2264 bp upstream (Fig. 1A, see Materials and methods). Although *upd2*$^{\Delta 3-62}$ is likely to represent an amorphic allele, homozygous *upd2* mutant flies are viable and fertile. This suggests that loss of Upd2 activity

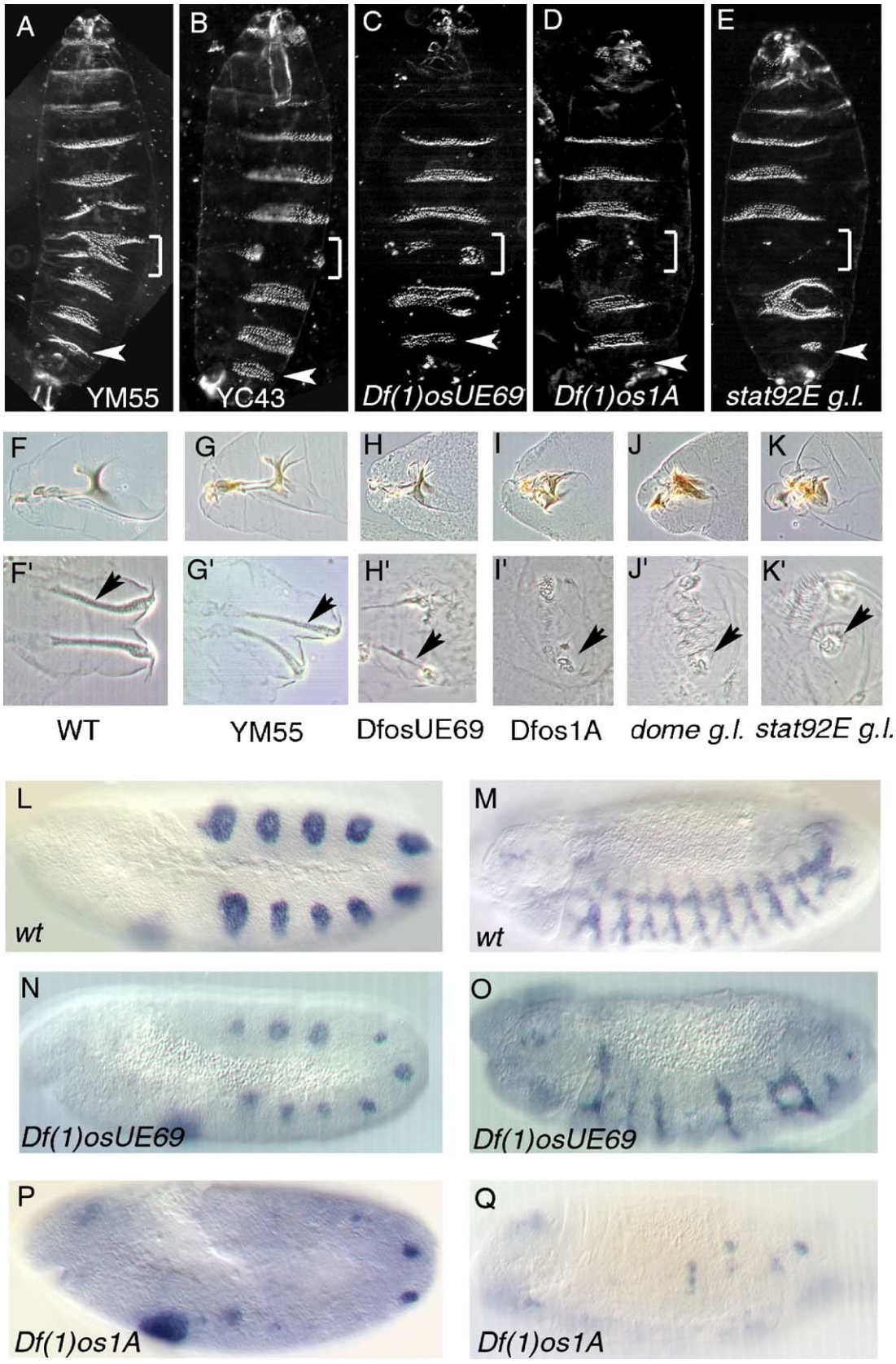

Table 1
Segment defects in various *upd* alleles

SEGMENT	% of segmental defects			
	stat92E (n = 34)	*upd*^{YC43} (n = 27)	*Df(1)os1A* (n = 39)	*Df(1)osUE69* (n = 25)
T1	12	0	33	4
T2	53	56	97	76
T3	62	7	92	32
A1	0	0	7	0
A2	18	0	46	8
A3	15	0	10	44
A4	100	52	100	100
A5	100	78	100	100
A6	38	7	33	44
A7	38	4	15	56
A8	85	26	100	8

during development can be compensated by endogenous Upd.

The Upd-like proteins

Conceptual translation of the *upd*-like genes present in *Drosophila melanogaster* identifies three related proteins (Fig. 1B). Each of these proteins contains an N-terminal region representing a predicted signal- or anchor-sequence and multiple potential N-linked glycosylation sites (grey box and bars in Fig. 1C). Glycosylation of these sites has been previously suggested to mediate the binding of Upd to the extracellular matrix (ECM) (Harrison et al., 1998).

Analysis of Upd2 shows that it contains a strongly hydrophobic region from amino acid 30 to 55. However, using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP-3.0/), a hidden Markov model (Bendtsen et al., 2004) predicts the likelihood of Upd2 containing a signal sequence at only 28%, with a 25% confidence level of cleavage between positions 55 and 56. The same model also predicts an anchor sequence (at a 71% confidence level), a motif required to insert Type II, III and IV trans-membrane proteins into the endoplasmic reticulum (ER) membrane (Wahlberg and Spiess, 1997). By contrast, when Upd is analysed using the same hidden Markov model, a signal peptide is predicted (at 100% confidence level) with an 89% probability of cleavage between position 27 and 28. Strikingly, the Upd2-like molecules present in *D. melanogaster*, *simulans* and *yakuba* contain predicted anchor-sequences while more distantly related Upd2-like molecules include N-termini predicted to act as signal-sequences (Fig. 1D). This prediction therefore suggests that *D. melanogaster* Upd2 cannot be secreted by the 'classical' Golgi/ER-based secretion machinery and may be trapped as a trans-membrane protein within the ER (but see below).

All related *Drosophilid* species for which genome sequence is available, encode clear homologues of all three *upd*-like genes (Fig. 1D ; Juni et al., 1996). However, no unambiguous *upd*-like homologues are identifiable in more distantly related species including the fellow dipteran *Anopheles gambiae*. It therefore appears that the *upd* gene family is evolving rapidly in the *Drosophilids*.

Secretion of Upd and Upd2

Although the N-terminus of *D. melanogaster* Upd2 is predicted to function as an anchor-sequence, the true nature of this signal ultimately requires experimental validation. We therefore generated C-terminal fusions of both Upd and Upd2 to enhanced GFP to allow the direct visualisation of the resulting proteins. When expressed in S2 *Drosophila* tissue culture cells (Schneider, 1972; Yanagawa et al., 1998), UpdGFP appears to be present extracellularly around transfected cells (identified by their co-expression of nuclear localised mRFP) (Fig. 4A). This extracellular GFP is only detectable in the most basal confocal sections and appears to be associated with the substrate on which the cells grow (Fig. 4B). This is consistent with previous results that identify Upd as an ECM-associated protein (Harrison et al., 1998). By contrast, fluorescence associated with Upd2GFP expressing cells appears to be intracellular with particular accumulations surrounding the nucleus in structures that may represent the endoplasmic reticulum. Although no pattern comparable to the surrounding ECM-associated halo of UpdGFP protein is detected (Fig. 4C), very low levels of extracellular Upd2GFP are occasionally detected adjacent to Upd2GFP transfected cells (Fig. 4D). It therefore appears that Upd2 cannot associate with the ECM in a manner comparable to Upd.

We next set out to determine if the dissimilar signal-/anchor-sequences could explain the observed differences of Upd and Upd2 secretion. We therefore undertook domain swap experiments to determine the individual contributions of the signal-/anchor-sequences for the activity of Upd and Upd2. GFP-tagged fusion molecules consisting of the secreted portion of Upd2 joined to the signal sequence of Upd (termed Upd2SS1) and another comprising the Upd2 anchor sequence attached to the secreted portion of Upd (called Upd1SS2) were constructed. Expression in S2 cells showed that Upd2SS1 can now be visualised as an ECM-associated halo surrounding transfected cells while Upd1SS2 appears to be located exclusively intracellularly (not shown). These results appear to indicate that the nature of the signal-/anchor-sequences present is responsible for the differential ECM interactions of UpdGFP and the low levels of extracellular Upd2GFP detected in Figs. 4C–D. In addition, the extracellular halo of Upd2SS1 indicates that the secreted region of Upd2 is capable of associating with the ECM under these tissue culture conditions.

Cell culture-based Upd/Upd2 activity assays

Upd and Upd2 induced JAK/STAT activation can be assayed using transcriptional reporter systems in tissue culture cells. To undertake such experiments, we used a *6x2xDrafLuc* reporter transgene containing twelve STAT92E binding sites located upstream of the gene encoding firefly Luciferase in the haemocyte-like Kc_{167} cell line (Cherbas et al., 1977). Stimulation of endogenous JAK/STAT pathway activity by ectopic expression of Upd has been shown to result in a strong *6x2xDrafLuc* response dependent on endogenously expressed pathway components (Müller et al., 2005).

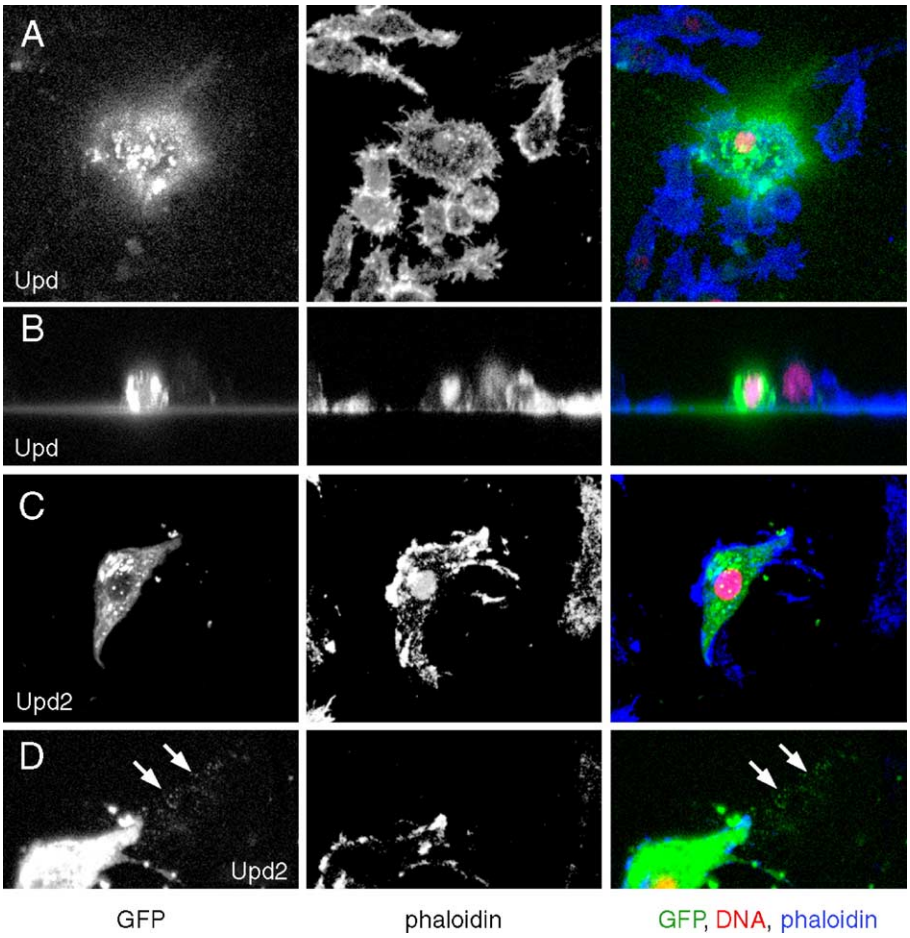

Fig. 4. Secretion of Upd-like GFP fusion proteins. S2 tissue culture cells transfected with the indicated GFP fusion protein (white in left hand panels and green in right), co-transfected with Histone2v-mRFP (red) to identify transfected cells and stained with phaloidin (blue) to show the morphology of the cells (right hand panels). (A) A projection through an XY confocal series shows extracellular UpdGFP fluorescence visible as a 'halo' surrounding the UpdGFP expressing cell. (B) A projection of an XZ series scan of the cells in (A) showing that GFP fluorescence is associated with the substrate on which the cells are growing and forms a gradient of intensity centred on the UpdGFP expressing cell. (C) A projection through XY confocal series shows Upd2GFP fluorescence localised almost exclusively intracellularly with bright structures adjacent to the nucleus visible that may represent the endoplasmic reticulum. (D) A close up of the cell shown in C obtained using increased laser power and detector sensitivity visualises very low levels of extracellular Upd2GFP fluorescence (arrows).

We first set out to test the activity of the Upd variants by co-transfecting cells with the *6x2xDrafLuc* reporter and Upd, Upd1SS2, Upd2 or Upd2SS1. This autocrine assay showed that Upd is capable of stimulating reporter activity over 80-fold, while Upd1SS2 shows considerable lower levels of activity, indicating that the anchor sequence of Upd2 reduces the activity of Upd in this assay (Fig. 5A). While Upd2 generates lower levels of activation, it is clearly capable of stimulating the JAK/STAT pathway, an activity that is not enhanced by the presence of the Upd signal sequence (Fig. 5A).

We then assayed the ability of the Upd-like molecules expressed in one population of cells to stimulate the *6x2xDrafLuc* reporter transfected into a separate population of co-cultured cells—a test for paracrine activity which may more accurately model in vivo signalling. Expression of Upd is clearly capable of activating JAK/STAT signalling at high levels, a result consistent with the known functions for this ligand in vivo (Harrison et al., 1998; Tsai and Sun, 2004). By comparison, the level of activation elicited by Upd1SS2 is reduced and Upd2 also induces pathway activation indicating that the molecule can stimulate JAK/STAT signalling non-autonomously (Fig. 5B). These results demonstrate that both Upd and Upd2 are capable of activating JAK/STAT signalling non-autonomously and further show that the nature of signal-/anchor-sequences play a role in modulating the activity of these molecules.

Finally, we harvested media overlying cells transfected with the Upd variants and, following normalisation for transfection efficiency, used these to stimulate an independent population of cells transfected with only the *6x2xDrafLuc* reporter. This allowed us to assay the activity of secreted Upd-like molecules not associated with the extracellular matrix. Strikingly, while media freshly conditioned by Upd expressing cells is capable of stimulating reporter activity, this effect is far weaker than the activation elicited by media conditioned by Upd2 or Upd2SS1 expressing cells (Fig. 5C). Thus, indicating that Upd2 can act as a potent activator of JAK/STAT signalling.

It has previously been shown that heparin treatment is sufficient to release Upd from the ECM in tissue culture

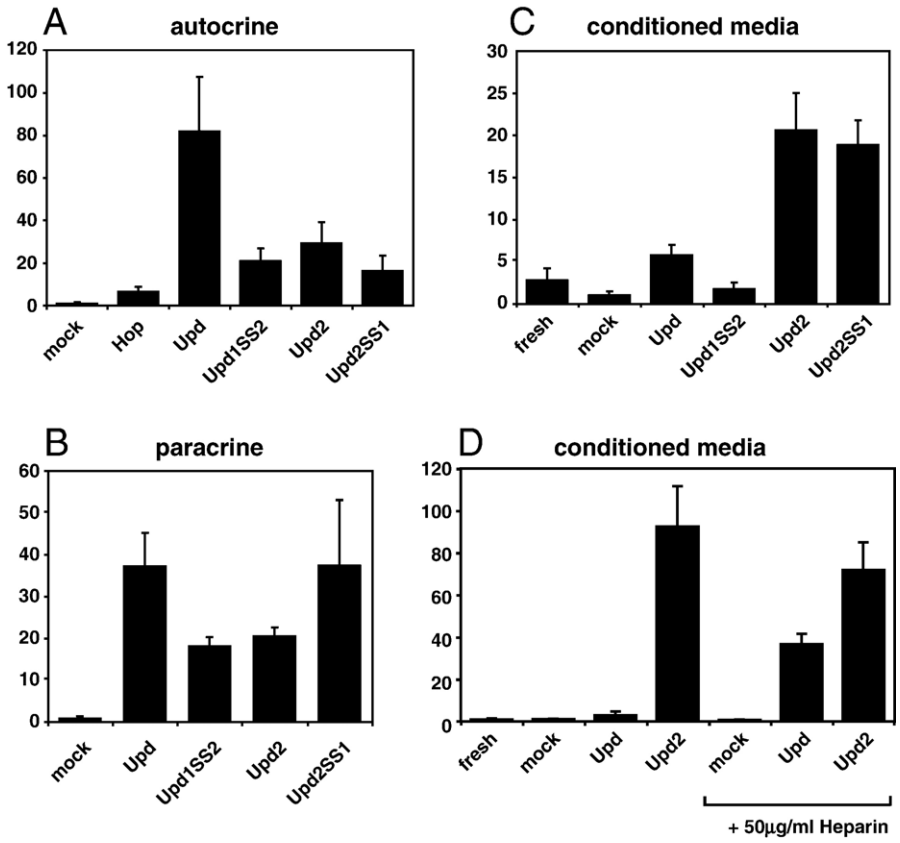

Fig. 5. Luciferase reporter assays for Upd-like in Kc_{167} cells. JAK/STAT activity, as reported by the *6x2DrafLuc* reporter in Kc_{167} cells, following stimulation by the indicated Upd molecules and experimental conditions. In each case, firefly luciferase activity was normalised with respect to co-transfected, constitutively expressed renilla luciferase and is expressed as a multiple of the signal generated by mock transfected cells. Each bar represents the average fold activation and standard deviation of 6 samples from one representative experiment. Experimental conditions used are: Autocrine assay in which all components are transfected into the same cells (A). A paracrine assay in which Upd-like molecules and *renilla* luciferase are expressed in one population of cells with the reporter present in another (B). A conditioned media assay in which cells containing the reporter and *renilla* luciferase expressing plasmids are stimulated by either fresh media or media conditioned by cells expressing the indicated Upd-like genes (C and D).

experiments (Harrison et al., 1998). We therefore set out to utilise this effect in our conditioned media assay system. Media conditioned by mock transfected, Upd and Upd2 expressing cells was assayed with and without the addition of 50µg/ml heparin (Fig. 5D). While heparin treatment increased the level of signalling elicited by Upd by over 10-fold, the ability of Upd2 to stimulate pathway signalling was not statistically significantly altered (Fig. 5D) suggesting Upd2 is secreted, but is unlikely to bind strongly to the ECM. Considering the microscopic and tissue culture-based assays together, it appears that Upd2 is unlikely to be ECM associated and is probably a freely diffusible pathway ligand.

Development of JAK/STAT pathway activation reporter in vivo

To test if *upd2* can activate the JAK/STAT pathway in vivo, we then searched for reporters of JAK/STAT activation. The only rigorously analysed target of STAT, the *even-skipped* stripe 3 enhancer (Yan et al., 1996), is not useful to test induced STAT activation, as it is under negative regulation by gap genes (Small et al., 1996).

While searching for an alternative reporter we observed that the *dome* gene might contain enhancers regulated by STAT. First, *dome* mRNA expression increases after stage 11 in areas where *upd* is expressed: the pharynx, the hindgut, the tracheae and the posterior spiracles (Fig. 6A). Second, lacZ expressed by heterozygous P-element enhancer traps inserted in the *dome* 5′UTR show the same areas of elevated expression (Fig. 6B). Third, when hemizygous (and therefore causing a *dome* mutation), these same P-elements no longer up-regulate expression in these areas indicating that JAK/STAT activation through *dome* is required for *dome* up-regulation (not shown). Consistent with these observations, potential STAT binding sites were found in the 5′UTR and first intron of *dome*. Reporter constructs made using a 2.8-kb genomic fragment containing part of the first exon and most of the first intron activate lacZ expression in the pharynx and hindgut mesoderm, two of the areas expressing increased *dome* mRNA levels (Fig. 6C). Double RNA in situ/antibody staining shows that *upd* RNA is expressed in the ectoderm of the pharynx and in the hindgut, in a region adjacent to the mesoderm cells expressing the reporter construct (Fig. 6D). Given its mesoderm specific expression, we will refer to the 2.8-kb construct as *dome-MESO*.

To determine if *dome-MESO* is regulated by JAK/STAT signalling, we studied its expression in different JAK/STAT mutant backgrounds. In $dome^{9}$ mutant embryos (see Materials

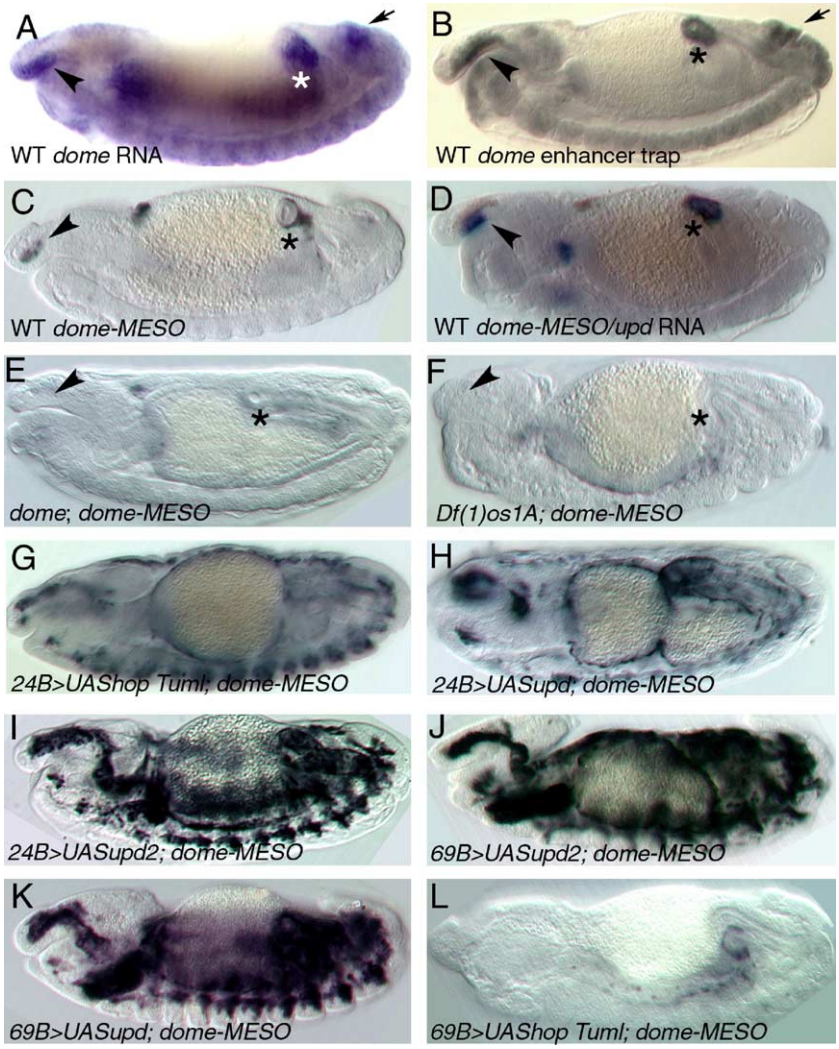

Fig. 6. A natural JAK/STAT reporter assay to test the in vivo activation of Upd2. (A) Expression of *dome* RNA in a wild type embryo. RNA is expressed ubiquitously, but higher levels of expression can be observed on the pharynx (arrowhead), anterior hindgut (asterisk) and posterior spiracles (arrow). (B) Expression of a P-element inserted in the 5′ *dome* region shows a similar spatial distribution of β-galactosidase expression as the endogenous *dome* RNA. (C) The *dome-MESO* reporter gene reproduces the pharynx and hindgut mesoderm expression of *dome* but lacks ectoderm expression in these organs and in the posterior spiracle. (D) A *dome-MESO* embryo stained for *upd* RNA (blue) and β-Gal (brown) showing that the reporter is expressed in the mesoderm cells adjacent to the ectoderm cells expressing the *upd* ligand. (E) In a zygotic $dome^{9}$ mutant embryo homozygous for *dome-MESO,* the β-galactosidase expression in the pharynx and hindgut are absent. (F) A *Df(1)os1A* embryo homozygous for *dome-MESO* showing absence of β-galactosidase expression in the pharynx and hindgut. (G) A *24B-Gal4* embryo driving hop^{Tuml} results in ectopic *dome-MESO* expression in the mesoderm. (H) Ectopic *dome-MESO* induction after ectopic expression of *upd* with *24B-Gal4*. (I) *Ectopic dome-MESO* expression in a stage 14 after ectopic expression of *upd2* with *24B-Gal4*. Ectopic *dome-MESO* induction after ectopic expression of (J) *upd2* or (K) *upd* with the ectoderm specific driver line *69B-Gal4.* (L) No ectopic activation of *dome-MESO* is observed after *69B-Gal4* driven expression of Hop^{Tuml} in the ectoderm. The scattered cells that can be observed anterior to the hindgut correspond to the longitudinal visceral mesoderm precursors which also express β-galactosidase in *dome-MESO* (see Materials and methods). Arrowheads point to the pharynx, asterisks mark the anterior hindgut and arrows the posterior spiracles. A–D, F, I–L are stage 14 embryos, E, G–H are stage 15 embryos. Anterior is left, dorsal up. C and D embryos have *dome-MESO* inserted in the third chromosome. This insertion has also some unrelated expression in the lymph gland primordium that serves as an internal control for the presence of the insertion. F–L embryos have the construct inserted on the X chromosome.

and methods), *dome-MESO* is not activated in either the pharynx or the hindgut (Fig. 6E), showing that *dome-MESO* regulation is comparable to that of the endogenous *dome*. The same result is observed in *Df(1)os1A* embryos lacking all Upd-like ligands (Fig. 6F). We next tested whether pathway activation is sufficient to drive *dome-MESO* expression. Gal4 mediated expression of *upd* or the activated form of the JAK kinase Hop^{Tuml} (Luo et al., 1995) using the *24B-Gal4* or the *twi-Gal4* mesodermal drivers results in ectopic *dome-MESO* expression both in the visceral and somatic mesoderm (Figs. 6G and H). We also tested if signalling from the ectoderm could non-autonomously activate mesoderm specific expression of *dome-MESO* by using the ectodermal specific line *69B-Gal4* to express *upd*. Under these conditions, *dome-MESO* was activated in the mesoderm, with the salivary glands being the only non-mesodermal tissue induced (Fig. 6K). As a control we also expressed Hop^{Tuml} with *69B-Gal4*. In these conditions *dome-MESO* was not ectopically activated in the mesoderm (Fig. 6L), confirming the ectodermal specific expression of this Gal4 line. These results show that the *dome-MESO* enhancer is

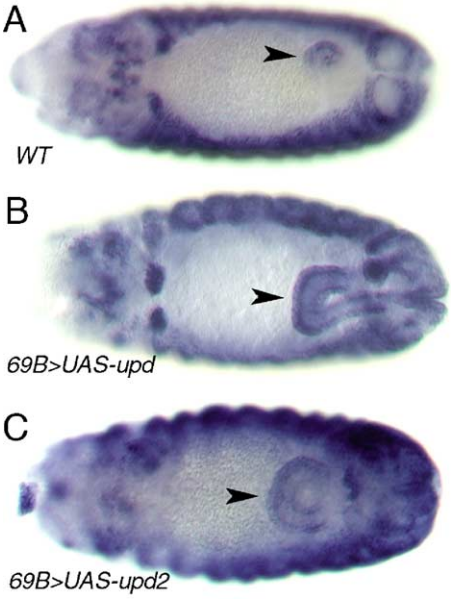

Fig. 7. Upd and Upd2 induced activation of *vvl*. (A) Expression of *vvl* in the anterior hindgut (arrowhead) of a wild type embryo. (B) Ectopic *upd* expression with the *69B-Gal4* line induces a greatly enlarged domain of *vvl* expression along the hindgut. (C) Ectopic *upd2* expression by the *69B-Gal4* line also induces ectopic *vvl* expression along the hindgut.

a mesodermal specific reporter for JAK/STAT activation that mimics the behaviour of endogenous *dome*. These results also provide evidence of JAK/STAT signalling across germ layers and demonstrates the existence of a positive feed back loop in *dome* regulation.

In vivo consequences of upd2 expression

Having established the *dome-MESO* reporter as a useful tool to show the status of JAK/STAT pathway activity in mesodermal cells we then set out to test if both Upd and Upd2 have similar functions in vivo. As would be expected of a bona fide pathway ligand, Upd2 expression in the mesoderm using *24B-Gal4* or in the ectoderm using *69B-Gal4* results in strong ectopic mesodermal *dome-MESO* activation (Figs. 6I–J). Upd2 is therefore able to non-autonomously activate JAK/STAT signalling in vivo.

We next studied the expression of *vvl*, a gene whose expression in the anterior part of the hindgut ectoderm (Fig. 7A) has been shown to depend on JAK/STAT pathway activity (Brown et al., 2003). We observed that pathway induction driven by expression of either Upd or Upd2 with the *69B-Gal4* line, is sufficient to expand the domain of *vvl* within the hindgut (Figs. 7B and C). We also studied the morphological effect of Upd2 activation on germ band movements and observed that, as reported for Upd (Brown et al., 2003), Upd2 is also sufficient to block germ band retraction (data not shown).

Finally, we tested if *upd* and *upd2* expression can exert the same effects when expressed in the imaginal discs. Recently, it has been reported that JAK/STAT pathway activation is sufficient to modulate cellular proliferation during the development of the wing imaginal disc (Mukherjee et al., 2005). Using the *Bx-Gal4* (also known as *1096-Gal4*) driver line, we expressed either *upd* or *upd2* in wing imaginal discs. In both cases, the resulting adults developed reduced size wings (Figs. 8A and D).

All these studies taken together indicate that ectopic expression of Upd2 is sufficient to reproduce all effects caused by misexpression of Upd and suggest that both molecules are likely to activate the same pathway.

Upd and Upd2 are redundant ligands for the Dome receptor

To analyse if both ligands activate the JAK/STAT pathway through the Dome receptor, we studied if the wing defects

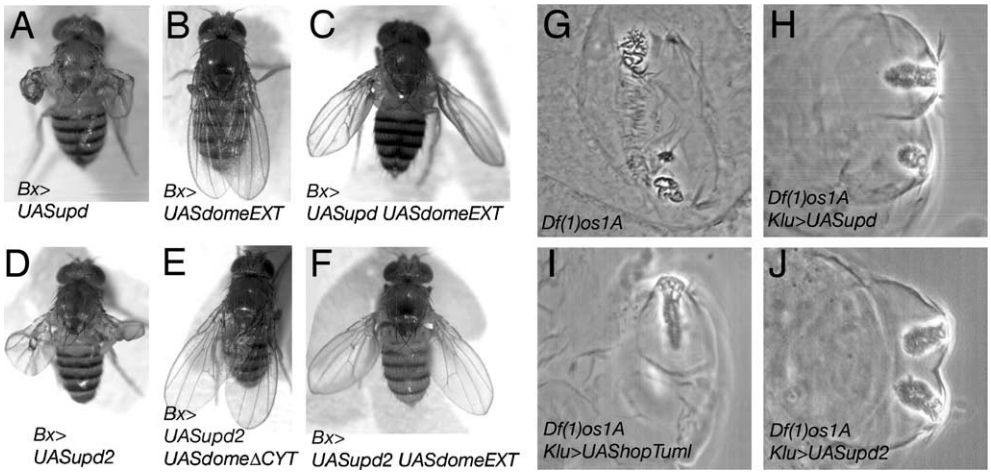

Fig. 8. Upd and Upd2 signal redundantly through Dome. Adult flies (A–F) and close up of posterior spiracles in late embryos (G–J) expressing different UAS constructs with the wing specific line *Bx-Gal4* (A–F) or *Klu-Gal4* to express in the posterior spiracles (G–J). Expression of Upd (A) or Upd2 (D) results in reduced wing size. (B) Expression of *UAS-domeEXT*, a dominant negative receptor, has a very mild wing phenotype. (C) Simultaneous expression of *UAS-domeEXT* rescues the effect of ectopic Upd expression (compare to panel A). (E–F) Rescue of the ectopic Upd2 phenotype by simultaneously expressing the dominant negative receptor *UAS-domeΔCYT* (E), or *UAS-domeEXT* (F). The cuticles of *Df(1)os1A* mutant embryos develop only the most external parts of the Filzkörper (G). By contrast, those from *Df(1)os1A; Klu-Gal4/UAS-upd* (H), *Df(1)os1A; Klu-Gal4/UAS-hop^{Tuml}* (I) and *Df(1)os1A; Klu-Gal4/UAS-upd2* (J) all show a partial rescue of the Filzkörper and stigmatophore.

caused by the ectopic misexpression of Upd and Upd2 could be rescued by simultaneous expression of dominant negative versions of the Dome receptor (Brown et al., 2001; Ghiglione et al., 2002). Having established that misexpression of dominant negative Dome alone has only mild effects (Fig. 8B and not shown), we co-expressed Upd or Upd2 and the dominant negative receptors. Expression of both transgenes resulted in an almost complete rescue of the Upd/Upd2 mediated wing defects (Figs. 8C, E–F), indicating that both Upd and Upd2 induce their effect via Dome. A similar blockage of Upd2-induced signalling is also observed in tissue culture-based assays by RNAi-induced knock down of *dome* mRNA (not shown).

As both the loss-of-function analysis (Fig. 3) and the ectopic expression tests (Figs. 5–8) indicate that Upd2 and Upd can act as partially redundant JAK/STAT ligands, we analysed the capacity of each ligand to rescue the absence of all other *upd*-like ligands. For that purpose, we concentrated on the requirement of the JAK/STAT pathway for spiracle morphogenesis. Mutations in any of the components of the JAK/STAT pathway result in a highly abnormal posterior spiracle (Brown et al., 2001 and Fig. 8G). We therefore used the *Klu-Gal4* spiracle driver line to test rescue of the *Df(1)os1A* spiracle defect. As a positive control, we induced the expression of the activated JAK kinase and found that under these conditions, *UAS-hop*^{Tuml} results in a partial rescue of the spiracle phenotype (Fig. 8I). Very similar rescues where obtained when expressing either *UAS-upd* (Fig. 8H) or *UAS-upd2* (Fig. 8J) thus confirming that Upd and Upd2 represent redundant ligands in vivo.

Discussion

Here, we have presented a detailed molecular analysis of the *upd*-like family of JAK/STAT ligands and show that Upd2 acts as a semi-redundant activating ligand during development. These results add to our understanding of this important signal transduction pathway and provide insights into alternative processing/secretion of pathway ligands.

The role of upd-like genes during development

Our analysis of mutations and deficiencies in the 17A region of the *Drosophila* X-chromosome present evidence that *upd* is not the only positively acting JAK/STAT pathway ligand signalling through the Dome receptor. While loss of downstream pathway components and the simultaneous loss of all *upd*-like genes lead to very similar phenotypes, amorphic *upd* alleles lead to relatively weak phenotypes and amorphic *upd2* alleles develop normally. When considered in conjunction with the very similar expression patterns of *upd* and *upd2* it seems likely that the Upd and Upd2 proteins are semi-redundant, whereby endogenous Upd can fully rescue *upd2* mutations, while endogenous Upd2 (whose mRNA is expressed at very low levels) is able to partly rescue amorphic *upd* alleles.

Given the sequence similarity of the *upd*-like genes and their clustering within the genome, it appears that this region has undergone two genomic duplication events; a similar scenario proposed to explain the functional redundancy of the proneural *ac* and *sc* genes (Marcellini et al., 2005). As all available *Drosophilid* genomes encode all three *upd*-like genes, it is likely that the duplication events occurred before the radiation of the *Drosophila* family. However, no plausible *upd*-like genes were identified in the genome of the fellow dipteran *Anopheles gambiae* suggesting that the Upd ligand is undergoing a phase of rapid evolutionary change. This conjecture is supported by the restriction of the *upd3* expression pattern and its apparent specialisation to roles in immune signalling (Agaisse et al., 2003) as well as the apparent divergence of the N-terminal signal-/anchor-sequences of Upd2 molecules in the closely related *D. melanogaster, simulans* and *yakuba* (Fig. 1C).

JAK/STAT autoregulation

In addition to our analysis of Upd and Upd2, we have also presented evidence that the expression of *dome* is upregulated in response to pathway signalling. Although negative autoregulation of *dome* in the follicle cells has been suggested to occur through two STAT binding sites 12 kb upstream of *dome* (Ghiglione et al., 2002), we show that positive *dome* autoregulation in the mesoderm is driven by intronic regulatory sequences used to generate the *dome-MESO* reporter gene. Expression of *dome-MESO* requires the function of the JAK/STAT pathway and can be induced by its ectopic activation. The reporter includes several putative *Drosophila* STAT binding sites and future experiments should confirm molecularly if the autoregulation is direct. In any case, our results show that *dome-MESO* is a useful tool to test the state of activity of the pathway in embryos and that, in the mesoderm, JAK/STAT induces positive autoregulation of *dome*. We have also observed that in addition to the mesodermal up-regulation, *dome* mRNA expression also increases in the posterior spiracles and in the ectoderm of the pharynx and hindgut close to where *upd* is expressed (Fig. 6A), suggesting the existence of another ectoderm-specific positive autoregulatory element. It is interesting to speculate that several *dome* tissue-specific enhancers exist to modulate the strength of JAK/STAT signalling by increasing or decreasing the amount of receptor. These changes in Dome levels would either amplify the reception of ligands in the embryonic mesoderm and ectoderm (pharynx and hindgut), or act to down-regulate the signal in cases where only transient pathway activity is desired (such as in the follicle cells).

The Upd2 molecule

Although tissue culture-based secretion assays indicate that Upd2 is only weakly associated with the ECM immediately surrounding expressing cells, the ability of Upd2 to condition media indicates that the molecule is secreted and active under these conditions. This result is further supported by the mesodermal induction of the *dome-MESO* reporter following ectoderm specific expression of Upd2.

However, when fused to the signal sequence from Upd1, high levels of the Upd2SS1 molecule are detected surrounding transfected cells. Although not addressed directly in this study, this effect could be explained by the glycosylation state of the Upd and Upd2 molecules. It has previously been shown that Upd is both glycosylated and associated with the ECM (Harrison et al., 1998). In addition, our own observations regarding the localisation of UpdGFP surrounding transfected cells, as well as the signal-sequence and predicted N-linked glycosylation sites within the secreted region of Upd, all argue that the protein is processed via the classical secretion pathway. In this process, N-linked glycosylation of Asn residues of secreted proteins occurs within the endoplasmic reticulum (ER) into which the nascent polypeptide chain of signal-sequence containing proteins are targeted during translation. Although it appears that Upd2 can also strongly associate with the ECM when fused to the Upd signal-sequence, the predicted anchor-sequence appears to result in secretion into the media. Indeed, Upd2 is able to condition media more effectively than Upd. Furthermore, the addition of heparin to release ECM-associated ligand does not appear to affect the degree of Upd2 mediated conditioning.

Although these results suggest that Upd2 may stimulate pathway activity at greater distances from its source of expression than Upd, this remains to be proven definitively in vivo.

Note added in proof

It has come to our attention that Gilbert et al. have recently published a study on Upd2 that supports the conclusions presented here. (Gilbert et al., 2005).

Acknowledgments

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