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Novel level of signalling control in the JAK/STAT pathway revealed by in situ visualisation of protein-protein interaction during *Drosophila* development

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SUMMARY

It is commonly accepted that activation of most signalling pathways is induced by ligand receptor dimerisation. This belief has been challenged for some vertebrate cytokine receptors of the JAK/STAT pathway. Here we study whether DOME, the *Drosophila* receptor of the JAK/STAT pathway, can dimerise and if the dimerisation is ligand-dependent. To analyse DOME homo-dimerisation, we have applied a β-gal complementation technique that allows the detection of protein interactions in situ. This technique has been used previously in cell culture but this is the first time that it has been applied to whole embryos. We show that this technique, which we rename βlue-βlau technique, can be used to detect DOME homo-dimerisation in *Drosophila* developing embryos. Despite DOME being ubiquitously expressed, dimerisation is developmentally regulated. We investigate the state of DOME dimerisation in the presence or absence of ligand and show that DOME dimerisation is not ligand-induced, indicating that ligand independent cytokine receptor dimerisation is a conserved feature across phyla. We have further analysed the functional significance of ligand-independent receptor dimerisation by comparing the effects of ectopic ligand expression in cells in which the receptor is, or is not, dimerised. We show that ligand expression can only activate STAT downstream targets or affect embryo development in cells in which the receptor is dimerised. These results suggest a model in which ligand-independent dimerisation of the JAK/STAT receptor confers cells with competence to activate the pathway prior to ligand reception. Thus, competence to induce the JAK/STAT signalling pathway in *Drosophila* can be regulated by controlling receptor dimerisation prior to ligand binding. These results reveal a novel level of JAK/STAT signalling regulation that could also apply to vertebrates.

Key words: JAK/STAT, Signalling competence, Receptor dimerisation, Protein interaction

INTRODUCTION

The activation of major signalling pathways is triggered by ligand-induced dimerisation of inactive cell surface receptors. This has been thought to be the case for several types of receptors including EGF, TGF-β, T cell, TNF and cytokine receptors (Heldin, 1995). However, several reports studying the IL-2 α, β and γ subunits (Damjanovich et al., 1997), the growth hormone (Gent et al., 2002) and the erythropoietin (Livnah et al., 1999) receptors have questioned this assumption for the cytokine receptors of the JAK/STAT pathway. Given the fundamental roles played by the JAK/STAT signalling pathway in development and disease, it is important to clarify whether ligand-independent dimerisation may be a common feature of all cytokine receptors. To shed light on this issue we have investigated whether dimerisation of the *Drosophila* JAK/STAT receptor DOMELESS (DOME) is ligand induced (Brown et al., 2001).

The *Drosophila* JAK/STAT pathway is functionally identical to the vertebrate pathway (Heinrich et al., 1998) but has less components to each level (Castelli-Gair Hombría and Brown, 2002; Luo and Dearolf, 2001). There is only one confirmed element to each level of the signalling pathway. The ligand is encoded by the *unpaired* (*upd*) gene (Harrison et al., 1998), the receptor by *domeless* (*dome*) (Brown et al., 2001), the JAK kinase by *hopscotch* (*hop*) (Binari and Perrimon, 1994), and the transcription factor by the signal transduction and activator of transcription (STAT) *stat92E* gene (Hou et al., 1996; Yan et al., 1996). UPD directly binds DOME (Chen et al., 2002) and this is thought to bring into proximity the receptor associated JAK tyrosine kinase HOP. The JAK proteins are thought to phosphorylate the receptor, creating a docking site for the inactive cytoplasmic STAT proteins. The STATs become phosphorylated (Chen et al., 2002), dimerise and translocate to the nucleus where they induce gene transcription. The identical mutant phenotypes of these genes suggest that most of the basic elements of this pathway have been isolated (Castelli-Gair Hombría and Brown, 2002). This view is reinforced by the failure to find other JAK or STAT proteins in the *Drosophila* genome databases (Dearolf, 1999).

In vertebrates, the active form of the cytokine receptors of the JAK/STAT pathway have been shown to function as homo- or hetero-dimeric complexes (Stahl et al., 1994). As DOME is the only confirmed receptor in *Drosophila* and is required for

all JAK/STAT functions analysed (Brown et al., 2001; Ghiglione et al., 2002; Johansen et al., 2003), we suspected that it might be forming homo-dimers. To test whether homo-dimerisation of DOME occurs in *Drosophila*, and to find how it is controlled during development, we decided to use a technique that would allow us to observe dimerisation in situ. The technique that we have applied is a β-galactosidase (β-gal) complementation assay developed for the study of protein interactions in vertebrate cell cultures (Blakely et al., 2000; Mohler and Blau, 1996). We show that this technique, which we rename βlue-βlau to distinguish from other β-gal complementation assays, allows detection of protein interactions in whole embryos. Using this technique we present evidence that DOME homo-dimerises in the ectoderm in tissues where the JAK/STAT pathway is active. In agreement with vertebrate results, DOME dimerisation is not ligand induced. Our results indicate that there is a correlation between the tissues where DOME dimerises and those that can respond to ligand activation, suggesting that receptor dimerisation confers competence for ligand activation. These results are very important as they reveal a novel level of control for cytokine receptor activation.

MATERIALS AND METHODS

Fly strains

The following Gal4/UAS lines were used: *UAS-upd* (Zeidler et al., 1999); *UAS-dome-TMCYT* (referred to in the text as *UAS-domeDN)* (Brown et al., 2001); *Klu-Gal4*; *h-Gal4*; *69B-Gal4*; *da-Gal4*; *24B-Gal4*; *twi-Gal4*, *twi-Gal4* (containing two inserts); *matα4-Gal4VP16* (referred as *maternal-Gal4*); *c381.661-Gal4*; *LE-Gal4*; *hs-Gal4*; *arm-Gal4 and arm<stop<Gal4VP16* (recombination induced in *arm<stop<Gal4VP16; KB19* males) (Sanson et al., 1996).

Df(1)os1A was used as a null allele of *upd*. Searches of the genome databases indicate the presence in *Drosophila* of three other genes with related sequence to *upd* clustered in the 17 region of the X chromosome: *cg5988*, *cg5963* and *cg15062* (Castelli-Gair Hombría and Brown, 2002). Using in situ and PCR we have determined that *Df(1)os1A* deletes all four genes (data not shown). On the basis of the identical phenotype of this deficiency to loss of function for JAK/STAT signalling, *Df(1)os1A* can be considered as a null mutant for all JAK/STAT ligands.

Constructs

UAS-domeΔω or *UAS-domeΔα* were made by using the 5′ Nco1 site in the *domeless* cDNA (Brown et al., 2001) fused to the Nco1 site in the β-galactosidase Δα or Δω mutant genes (Mohler and Blau, 1996). This fusion was subcloned into the fly transformation vector pUAST and transgenic stocks made. The fusion proteins are missing the last 60 amino acids of the carboxyl terminal end of DOME. We tested the functionality of the hybrid proteins in $dome^{217}$; *Klu-Gal4/UAS-domeΔα* or $dome^{217}$; *Klu-Gal4/UAS-domeΔω* flies. On some occasions, as well as rescuing the mutant phenotype, high levels of ectopic *UAS-dome* (Brown et al., 2001) as well as the *UAS-domeΔα* and *UAS-domeΔω* lines gave dominant negative phenotypes.

Stainings

Complementation was visualised by incubating embryos with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal). X-gal stainings were done by fixing dechorionated embryos in a mixture of heptane/1% glutaraldehyde in PBS for 2 minutes. The glutaraldehyde solution was then removed and the embryos were devitelinised by adding 70% ethanol and shaking vigorously in a glass vial. Devitelinised embryos were transferred to a fresh vial and washed in 70% ethanol, rehydrated in PBS and transferred to X-gal buffer (Ashburner, 1989). After adding X-gal substrate from a 20 mg/ml solution in dimethylformamide to a final concentration of 1.2 mg/ml, the reaction was developed by incubating at 37°C for 1-3 hours depending on the driver line. Cuticle preparations, RNA in situ hybridisation and antibody stainings were performed as previously described (Brown and Castelli-Gair Hombría, 2000). Only embryos upto stage 15 (0-14 hours) were analysed; these are the stages for which JAK/STAT requirement during development is well understood.

Analysis of protein stability

hs-Gal4; UAS-domeΔω UAS-domeΔα embryos were heatshocked for 15 minutes, 30 minutes or 1 hour to test whether some tissues accumulate more hybrid protein than others. The embryos were allowed to develop for different periods and stained with anti-β-galactosidase (anti-β-gal) to detect the expression of the hybrid proteins. The protein disappeared from all tissues simultaneously with the exception of the amnioserosa where the protein could be detected at stages when the rest of the embryo had no detectable β-galactosidase. Embryos were analysed upto stage 14. Later stages were not studied because of the existence of cryptic promoters in the *hs-Gal4* line.

RESULTS

The βlue-βlau β-gal complementation assay allows the detection of protein interactions in whole embryos

Classically, biochemical techniques have been used to detect protein-protein interactions. As these techniques analyse protein interactions in vitro, they perturb the functional integrity of the system under study. Recently, several techniques have been developed to analyse protein interactions in situ but they have not been tested in whole organisms. To study protein interactions directly in whole embryos, we have applied the βlue-βlau β-gal complementation technique that allows the detection of protein-protein interactions in vertebrate cell cultures (Blakely et al., 2000; Rossi et al., 1997). This technique uses two β-gal mutants (Δα and Δω) that are enzymatically inactive, but can complement if brought into proximity by fusing them to proteins that physically interact (Rossi et al., 1997). Dimerisation of the hybrid proteins does not depend on the β-gal portion of the hybrid protein, but is directed by the proteins that have been fused to the β-gal mutants (Blakely et al., 2000).

We fused the Δα or the Δω mutants to the DOME carboxyl end and expressed the hybrid receptors using the GAL4 system (Brand and Perrimon, 1993). The hybrid receptors are functional as they can rescue *dome* mutant phenotypes (Fig. 1A-C). To determine the sub-cellular localisation of the fusion proteins, we expressed them in the large, polarised, ectodermal cells of the hindgut and the salivary gland using the *h-Gal4* line. The hybrid receptors localise mainly to the apical membrane, although some protein can be detected in the cytoplasm (Fig. 1D-E).

To test whether the fusion constructs homo-dimerise, we expressed both proteins with *h-Gal4* in the same conditions as above and assayed for β-gal complementation by incubating the embryos in X-gal. The blue coloration localised exclusively to the apical region of the cells (Fig. 1F-G), suggesting that

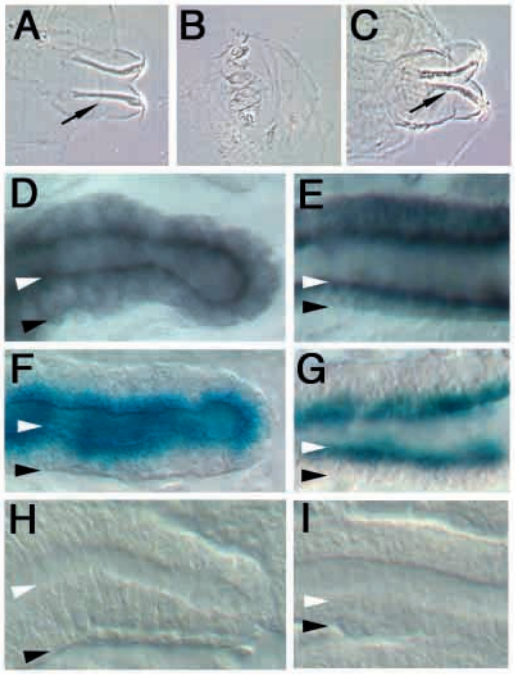

Fig. 1. Homo-dimerisation of DOME as detected by the βlue-βlau technique. (A) Wild-type posterior spiracles with an elongated filzkörper (arrow). (B) Zygotic phenotype of *dome*^{217}, the filzkörper is not formed. (C) Rescue of *dome*^{217} with *UAS-domeΔα*. Expression of the hybrid receptor in the spiracles using *Klu-Gal4* rescues the filzkörper, showing that the hybrid protein is functional. Optical sections through the salivary gland (D,F,H) and the hindgut (E,G,I). White arrowheads point to the apical membrane, black arrowheads to the basal membrane on one side of the tube. Note that the salivary gland is closed at one end whereas the gut tube is open at both ends. (D-E) Anti-β-gal staining of *UAS-domeΔα, UAS-domeΔω* expressed using *h-Gal4*. The hybrid receptors localise mainly to the apical membrane, although the proteins are also detected in the cytoplasm. (F-G) X-gal staining of the same genotype as in D-E. The blue precipitate is formed at the apical side of the cell in both the salivary glands and the hindgut showing that the product of the reaction has a limited diffusion through the cell. (H-I) X-gal staining of *UAS-domeΔα* expressed using *h-Gal4*. No coloured precipitate is seen when only one of the two fusion proteins is expressed, proving that the reaction requires complementation of the hybrid receptors.

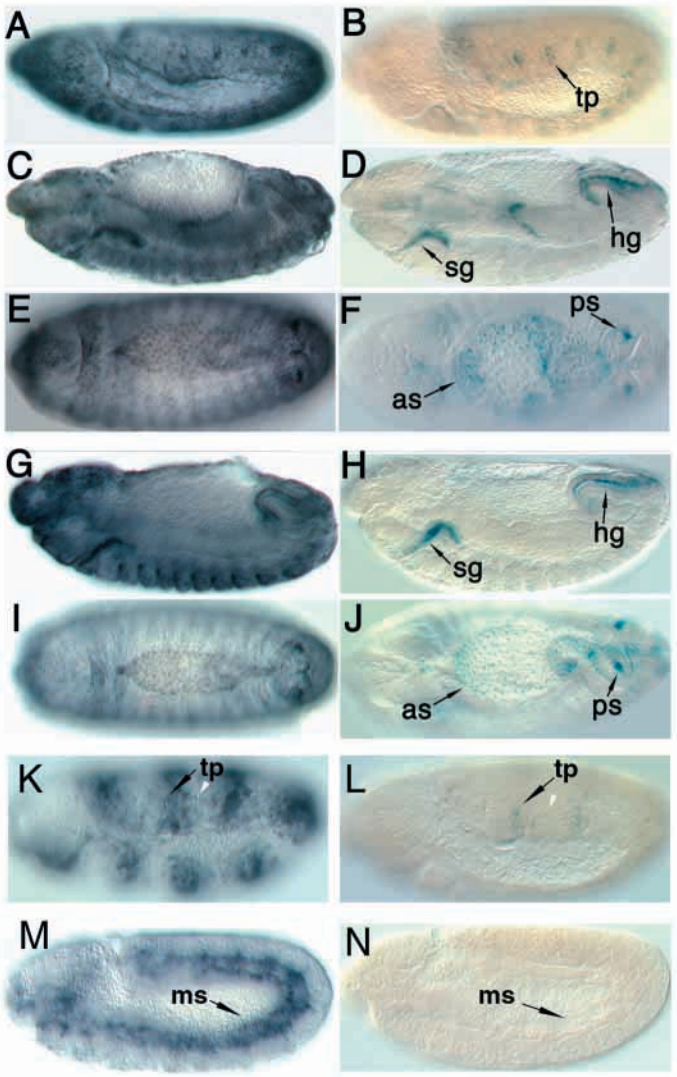

Fig. 2. Developmental regulation of receptor dimerisation. Dimerisation in embryos simultaneously expressing *UAS-domeΔα* and *UAS-domeΔω*, driven by either *da-Gal4* (A-F), *69B-Gal4* (G-J), *h-Gal4* (K-L) or *24B-Gal4* (M-N). Left panels show expression of the hybrid protein detected using an anti-β-gal antibody, right panels show the X-gal patterns induced in those embryos by hybrid protein dimerisation. *da-Gal4 and 69B-Gal4* embryos express β-gal proteins in all ectodermal derivatives but they show X-gal staining in a subset of those cells (Right panels). *h-Gal4* drives expression in stripes (K) but only the tracheal pits in each stripe show X-gal staining (L). Arrows in K-L point to tracheal pits where the hybrid proteins are expressed, and white arrowheads to a tracheal pits without expression. The *24B-Gal4* line drives high levels of expression in the mesoderm (M) but no X-gal expression is observed in this tissue (N). (A-B,K-N) Stage 11 embryos, (C-J) stage 13-14 embryos. When comparing embryos at the same developmental stage, all ectoderm Gal4 lines induce comparable X-gal staining (compare B with L, D with H and F with J). as, amnioserosa membrane; hg, hindgut; ms, mesoderm; ps, posterior spiracle; sg, salivary glands; tp, tracheal pits.

both hybrid proteins are transported to the apical membrane where they dimerise and complement. This result also suggests that the cytoplasmic protein is present as monomers. In control experiments, in which either of the fusion proteins was expressed separately, no blue staining developed (Fig. 1H-I and not shown).

The above experiments show that the βlue-βlau technique can be used to detect protein interaction in whole animals with sub-cellular resolution. Our results also indicate that in the embryonic ectoderm, DOME is differentially transported to the apical membrane where it homo-dimerises.

DOME homo-dimerisation is developmentally controlled

To investigate the regulation of DOME homo-dimerisation during embryonic development we compared the expression of the hybrid proteins as detected by anti-β-gal antibodies (Fig. 2, left column) with the regions where they complement as tested by X-gal staining (Fig. 2, right column). As *upd* is expressed in the ectoderm (Harrison et al., 1998), we first used the ubiquitous ectodermal drivers *da-Gal4* and *69B-Gal4*

(Brand and Perrimon, 1993; Wodarz et al., 1995) to express the tagged DOME proteins. At early stages, both Gal4 lines have similar spatio-temporal dynamics and they revealed identical developmentally regulated patterns of DOME homo-dimerisation. *da-Gal4* drives expression of the fusion proteins in most ectodermal cells (Fig. 2A,C,E). However, not all of these cells developed the blue coloration after X-gal staining (Fig. 2B,D,F). *69B-Gal4*, which drives expression slightly later (Fig. 2G,I), revealed very similar patterns of dimerisation to *da-Gal4* (Fig. 2, compare D with H and F with J).

We tested other Gal4 lines to see if they all provide consistent results. *h-Gal4*, *arm-Gal4* and *hs-Gal4* gave consistent patterns of X-gal staining (Fig. 2K-L and not shown). We also tested the strong mesodermal-specific lines *24B-Gal4* and *twi-Gal4; twi-Gal4* to test whether high levels of hybrid protein would lead to dimerisation in the mesoderm. No X-gal staining was observed at the stages studied (Fig. 2 M-N and not shown), indicating that high levels of protein expression do not result in unspecific dimerisation.

Recently, Gal4VP16 lines have been generated that are stronger inducers of expression. We have used *arm< <Gal4VP16* (see Materials and Methods) and compared it to *arm-Gal4*. Although dimerisation in *arm-Gal4* embryos is observed in the same patterns as 69*B*- and *da-Gal4*, the VP16 line initially has the same patterns of dimerisation, but at later stages results in ubiquitous dimerisation.

The above results show that the pattern of dimerisation is not dependent on the Gal4 line used to express the hybrid proteins, and confirm that the β-gal part of the hybrid protein is not driving dimerisation (Blakely et al., 2000). However, care should be taken when using very strong VP16 inducer lines, as they can generate artefactual staining.

Homo-dimerisation of DOME as revealed with the βlue-βlau technique occurs in the salivary glands, the tracheal pits, the posterior spiracles, the foregut, the hindgut, the oenocytes, the Malpighian tubules, the midline, the anal pads and the amnioserosa (Fig. 2 and not shown). The JAK/STAT pathway is required for the development of the trachea, the posterior spiracles (Brown et al., 2001), the Malpighian tubules, the foregut and the hindgut (Johansen et al., 2003), showing that the regions where DOME homo-dimerisation is detected coincide with the regions where the receptors are active. To date, a requirement for the JAK/STAT pathway has not been reported in the salivary glands, amnioserosa, oenocytes, midline or anal pads. This may reflect that dimerisation of the receptor in certain tissues does not automatically lead to the activation of the pathway, or perhaps that a function for the pathway is still to be discovered in these tissues.

Given that excessive levels of hybrid protein expression can result in artefactual dimerisation, we studied whether the hybrid proteins have the same stability in all cells. We used a *hs-Gal4* line to drive homogeneous levels of expression and tested for protein perdurance. After heat-shock induction, we allowed the embryos to develop and monitored the presence of the proteins in different tissues with an anti-β-gal antibody (Materials and Methods). The protein disappeared with a similar dynamic from all tissues studied except from the amnioserosa where it lasted for a longer period (data not shown). Because the amnioserosa is one of the tissues where we observe X-gal staining, it is conceivable that protein stability in the amnioserosa leads to protein accumulation and artefactual dimerisation. To test whether increased expression of normal Gal4 proteins results in the appearance of novel areas of X-gal staining, we have studied embryos in which the hybrid proteins are expressed with both *da-Gal4* and *69B-Gal4* lines simultaneously. Despite the increased level of protein expression driven by these two strong Gal4 lines, no new areas of expression were detected (not shown). Thus, a wide range of strong Gal4 lines can be used safely with the βlue-βlau technique without obtaining artefactual protein dimerisation.

JAK/STAT pathway activation is also required for segmentation during early development. Expression of the fusion proteins using *maternal-Gal4* lines failed to show any complementation (data not shown). This failure may be because of aberrant folding or insufficient expression of the hybrid proteins driven by the *maternal-Gal4* line or reflect a difference in DOME complex formation at the blastoderm stage. Whatever the reasons for not being able to detect dimerisation at early stages during embryogenesis, the above results show that the βlue-βlau technique can be used to detect protein-protein interactions in whole embryos from, at least, the extended germ band stage. Our data show that DOME homo-dimerises. The dimerisation is not ubiquitous, but is developmentally controlled in space and time. Among the tissues in which we detect dimerisation are the ones that have been shown to require JAK/STAT function during embryonic development, proving that the dimerisation we detect is functionally significant.

DOME homo-dimerisation is ligand independent

To determine whether ectopic ligand can induce ectopic homo-dimerisation of the receptor, we used the same drivers to simultaneously express UPD and the receptor fusions in the ectoderm. No changes in the pattern of X-gal staining were observed (Fig. 3A,B compared to Fig. 2B,D), showing that ectopic UPD cannot induce DOME dimerisation even though UPD has been shown to bind DOME in cell culture and activate the JAK/STAT pathway (Chen et al., 2002).

To test whether DOME dimerisation is independent of ligand binding, we used *Df(1)os1A*, a deficiency of *upd* and related genes that creates a complete loss of function for the ligand activity (Harrison et al., 1998; Castelli-Gair Hombría and Brown, 2002) (see Materials and Methods). When the complementation assay is used in *upd*-deficient embryos, no differences in X-gal staining can be seen compared to the wild type (Fig. 3C,D), indicating that receptor homo-dimerisation is independent of endogenous ligand activity.

To further test whether *upd*, or any unknown ligands not deleted by *Df(1)os1A*, could be responsible for the observed receptor dimerisation, we have expressed a dominant negative DOME receptor with the cytoplasmic and transmembrane domains deleted (*domeDN*). As inferred from its vertebrate equivalent, this construct encodes a soluble protein (Narazaki et al., 1993). DOME receptors lacking the cytoplasmic domain behave as a dominant negative (Brown et al., 2001; Ghiglione et al., 2002; Silver and Montell, 2001), probably because they sequester the ligand(s), making them unavailable for the endogenous receptor. Expression of *domeDN* does not affect receptor dimerisation in any of the tissues studied (Fig. 3E-F), confirming that DOME homo-dimerisation is ligand independent.

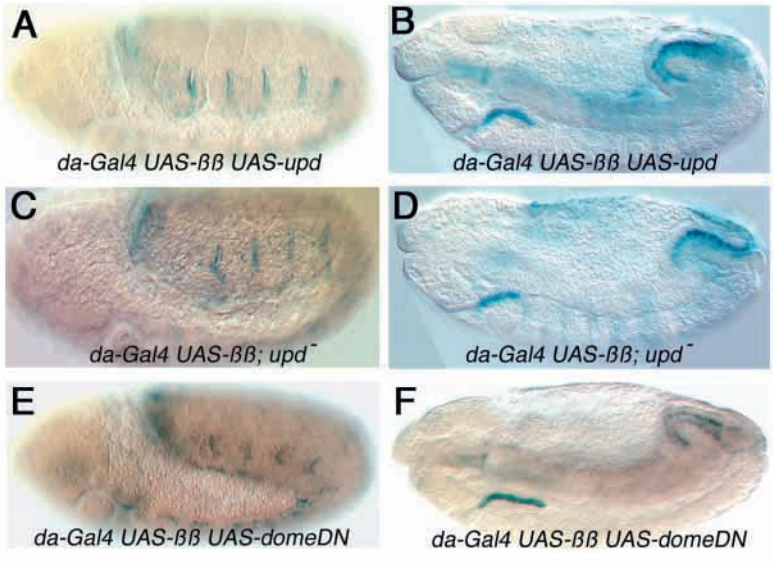

Fig. 3. Effects of altered ligand expression on receptor dimerisation. Expression of *UAS-domeΔα* and *UAS-domeΔω* (abbreviated as UAS-ββ in the Fig.) was induced by *da-Gal4*. X-gal stainings show patterns of receptor dimerisation. (A-B) Embryos in which ectopic ligand expression has been induced using *UAS-upd* do not show ectopic X-gal staining (compare with Fig. 2B,D). (C-D) Patterns of X-gal staining are not affected in embryos deficient for *upd*. (E-F) Expression of a dominant negative receptor does not affect patterns of dimerisation.

Correlation between receptor dimerisation and competence for JAK/STAT activation

To analyse whether the state of receptor dimerisation affects the sensitivity of the cells to the ligand, we compared the effects of ectopic UPD expression on cells that have the receptor in a dimerised form as judged by the βlue-βlau technique, with its effects in cells where it is not dimerised. DOME dimerises in the amnioserosa (Fig. 2F,J), an extra embryonic membrane required for germ band retraction and dorsal closure during development (Lamka and Lipshitz, 1999). However, in wild-type embryos, UPD is not expressed in the amnioserosa (Harrison et al., 1998) and mutants in the JAK/STAT pathway have no defects in dorsal closure (Binari and Perrimon, 1994; Brown et al., 2001; Harrison et al., 1998; Hou et al., 1996; Yan et al., 1996), suggesting that the pathway is not required for amnioserosa development. Despite this, ectopic ligand expression using *da-Gal4* results in embryos with a dorsal hole and incomplete retraction of the germ band (Fig. 4A arrowhead). These phenotypes are similar to those in mutants for *hindsight*, a gene required for amnioserosa development (Lamka and Lipshitz, 1999). Both the amnioserosa and the cells of the abutting leading edge are required for dorsal closure. To distinguish which cells are responsible for the observed phenotype, we expressed UPD with the amnioserosa-specific line *c381.611-Gal4* and the leading edge-specific line *LE-Gal4*. Only expression of UPD with the amnioserosa line resulted in dorsal hole phenotypes (not shown). These results show that the cells of the amnioserosa are competent to receive

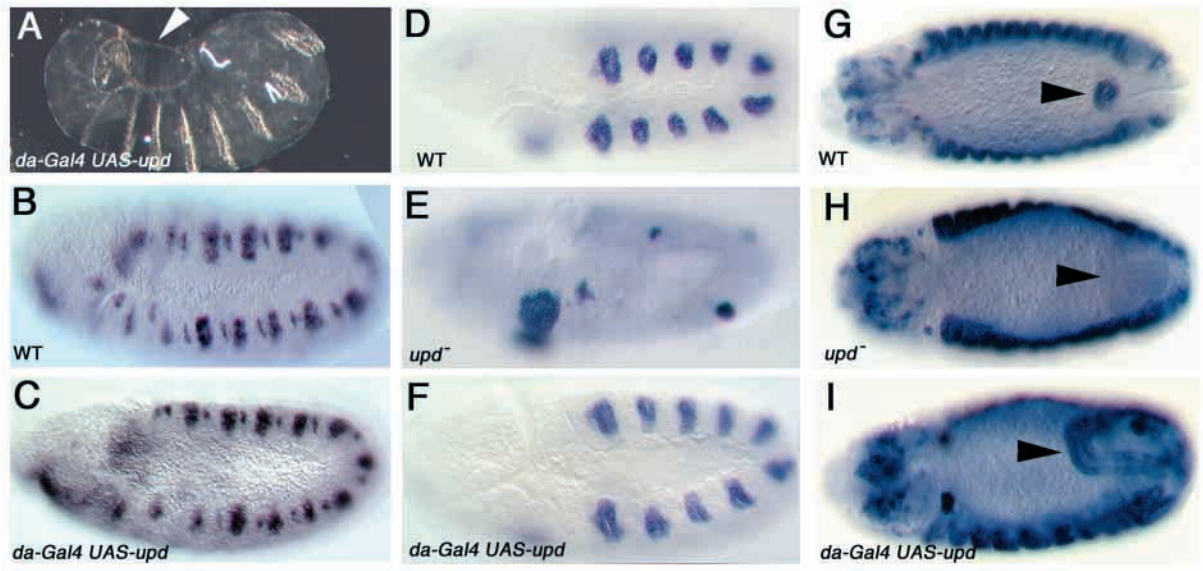

Fig. 4. Effects of altered ligand expression on embryo development. (A) Cuticle of a late embryo showing the result of ectopic UPD expression. There is abnormal retraction of the germ band and a dorsal hole (arrowhead) indicating abnormal development of the amnioserosa, whereas there is little effect on the denticle belts. (B-C) Expression of *kni* in the wild type (B) and in embryos with ectopic UPD expression (C). (D-F) Expression of *trh* in the wild-type (D), *upd*$^{-}$ (E) and ectopic UPD embryos (F). Although the JAK/STAT pathway is required for *trh* expression (E), ectopic *upd* cannot activate *trh* expression outside the tracheal pits (F). (G-I) Expression of *vvl* in the wild-type (G), *upd*$^{-}$ (H) and ectopic UPD embryos (I). In the wild type, expression of *vvl* in the hindgut is restricted to the small intestine (arrowhead in G). *vvl* is not expressed in the hindgut (arrowhead) of ligand-deficient mutants (H). Ectopic *upd* results in ectopic *vvl* activation in the hindgut (arrowhead in I). *Df(1)os 1A* was used as a deficiency for *upd* and related genes. (A-F) Lateral views, (G-I) dorsal views. Anterior is left in all panels. (A) Dark field image of a late embryo cuticle. (B-F) Stage 11 embryos. (G-I) Stage 13 embryos.

the UPD signal even though they do not activate the JAK/STAT pathway during normal development. Also, the capacity of the amnioserosa cells to respond to UPD expression confirms that the dimerisation observed in this tissue using the βlue-βlau technique is physiological.

The JAK/STAT pathway is required in the tracheal pits for the activation of *knirps* (*kni*) and *trachealess* (*trh*) (Brown et al., 2001). We have observed that in the ectoderm the tracheal pits are the major site for DOME dimerisation (Fig. 2B), whereas there is no dimerisation in the adjacent ectodermal cells. Ectopic UPD expression throughout the ectoderm does not result in ectopic *kni* or *trh* expression (Fig. 4C,F). This failure to activate *trh* and *kni* outside the tracheal pits could be because of the presence of repressor genes in these regions, or alternatively because the cells with pre-dimerised receptors are the only ones capable of activating the pathway.

Looking for other putative targets of JAK/STAT signalling, we found that *ventral veinless* (*vvl*) (Anderson et al., 1995; de Celis et al., 1995) is expressed in a small region of the hindgut in a similar pattern to that of *upd* (Fig. 4G). In *upd* or *stat92E* mutant embryos *vvl* is not activated in the hindgut (Fig. 4H and not shown), suggesting that *vvl* is a target of the JAK/STAT pathway. Given that all the cells of the hindgut show DOME dimerisation (Fig. 2D,H), we tested whether they could all respond to the ligand. Ectopic *upd* expression results in ectopic *vvl* activation throughout the hindgut (Fig. 4I). Taken together, the above results suggest that UPD expression is only capable of activating JAK/STAT function in cells in which the receptors are in a pre-dimerised state as detected by the βlue-βlau technique.

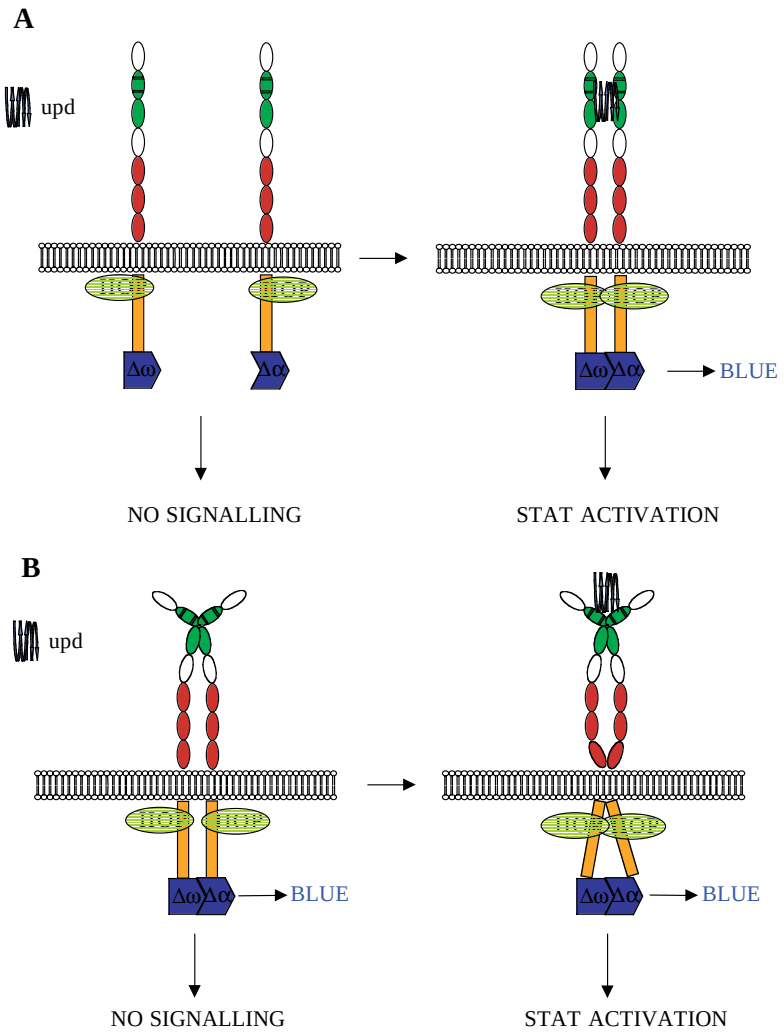

Fig. 5. Alternative models for JAK/STAT receptor complex-ligand interaction. In A the monomeric receptors exist in the inactive state. Ligand binding induces receptor dimerisation and activation. If this model was correct, ectopic ligand expression should result in ectopic blue staining because of ectopic dimerisation, whereas deletion of the ligand should abolish the blue staining. In the alternative model (B), the inactive receptors are dimerised. Ligand induction activates the receptors through conformational modification. If this model is correct, blue staining would be independent of the expression patterns of the ligand. Experiments in Fig. 3 support the second model. Green ovals, cytokine binding module; red ovals fibronectin type III repeats of DOME. The intracellular receptor domain (orange) binds to HOP (JAK). Blue boxes represent Δα and Δω β-gal mutants. Complementation of the mutant proteins occurs when receptor dimerisation brings them into close proximity.

DISCUSSION

Vertebrate cytokine JAK/STAT receptors function as homo- or hetero-dimers. Using a technique that allows visualisation of protein interaction in vivo, we provide evidence that the *Drosophila* cytokine JAK/STAT receptor also homo-dimerises. Our results show that in the ectoderm dimerisation is observed in all tissues known to require JAK/STAT function. However, we have been unable to detect dimerisation during early embryogenesis at stages at which it is known that the pathway is active. This failure could be because of a technical problem (insufficient levels of protein expressed at early stages or incorrect protein folding) or because of the existence in some cell types of a different signalling complex. Analysis of the *Drosophila* genome sequence uncovered the existence of a gene similar to *dome* (Castelli-Gair Hombría and Brown, 2002). Although DOME is required for JAK/STAT signalling at all stages analysed, it could be possible that in some tissues DOME forms hetero-dimers with this other receptor subunit. Future work should resolve which of these two reasons is responsible for the absence of dimerisation observed in our experiments.

Two different hypotheses have been proposed for the induction of dimerisation in vertebrates. Our results are not consistent with the view that JAK/STAT receptors become activated by ligand-induced dimerisation (Fig. 5A), but suggest that the receptors dimerise prior to receptor activation (Fig. 5B). The patterned expression of X-gal shows that pre-dimerisation is a developmentally regulated process that occurs mainly, but not exclusively, in cells that activate the pathway. Previous reports with the IL-2 α, β and γ subunits (Damjanovich et al., 1997), the growth hormone (Gent et al., 2002) and the erythropoietin (Livnah et al., 1999) receptors

have presented evidence for preformed receptor complexes using three different techniques: FRET, immunoprecipitation and crystallography. Crystallographic evidence indicates that the erythropoietin receptor can dimerise in the absence of ligand (Livnah et al., 1999). In this case, only the extracellular domains of the receptor are apposed. Ligand binding changes the extracellular conformation and, as a result, the intracellular domains come together allowing JAK phosphorylation (Fig. 5B). Although these results suggest that ligand binding induces an extracellular conformational change in the pre-associated receptor complex allowing receptor activation, they do not provide information about when and where pre-association occurs or whether it has a physiological role. Our experiments allow us to visualise receptor pre-association in situ and suggest that pre-association is essential for functionality.

Control of ligand-independent dimerisation of cytokine receptors as a novel mechanism for JAK/STAT regulation

We have shown that ligand expression is not capable of inducing dimerisation, and that receptor dimerisation does not require ligand expression. As activation of the JAK/STAT pathway requires receptor dimerisation (Davis et al., 1993; Taga et al., 1989), the discovery that DOME homo-dimerisation is developmentally controlled reveals that there is yet another level of regulation within the JAK/STAT pathway. At least in *Drosophila*, in which all the effectors of the JAK/STAT signalling pathway are ubiquitously expressed in the embryo (Binari and Perrimon, 1994; Brown et al., 2001; Hou et al., 1996), not all cells are activated by ectopic ligand. Although other models are possible, based on our data we would like to propose that signalling-competent cells are those that have the pre-associated receptor complex and it is only these cells that are able to respond to the extracellular signal.

Pre-association could be mediated by unknown ligands, membrane-spanning proteins or by intracellular proteins. Given that a deficiency deleting *upd* and three other homologous genes has an identical phenotype in the ectoderm to the inactivation of *dome*, *stat92E* or *hop*, it is unlikely that there are any other ectodermal ligands. This strongly indicates that other proteins play a role in the formation of the receptor pre-associated complexes. Recent results show that cytoplasmic scaffold proteins are fundamental for specificity of the signaling responses (Harris et al., 2001). We propose that cytoplasmic 'clamp' proteins expressed in a developmentally regulated pattern are responsible for cytokine receptor pre-association, and thus for deciding the competence of a cell to respond to a particular ligand.

The βlue-βlau technique efficiently detects protein interactions in whole organisms

In recent years several techniques have been developed to detect protein interactions in vivo (Ayoub et al., 2002; Hu et al., 2002; Mochizuki et al., 2001; Pelletier et al., 1998; Rossi et al., 1997) but have only been used in cell culture. Three main issues should be taken into account when considering the usefulness of these techniques. First, sensitivity; second, the physiological significance of the results obtained; and third, whether the technique can detect protein interactions in all cellular compartments.

It could be argued that the areas in which we observe dimerisation are those where higher levels of hybrid protein are expressed. The experiments we present discard this possibility because not all strongly expressing areas lead to dimerisation. For example, in Fig. 2A the head expresses high levels of hybrid protein but there is no dimerisation in this area in Fig. 2B; and the same can be said for the head and segmental grooves in Fig. 2G and Fig. 2H. Moreover, lines of very different origin give consistent results regardless of them being strong (*da-Gal4*) or weak ectodermal inducers (*h-Gal4*) (compare tracheal pits of Fig. 2B and Fig. 2L). The lack of correlation between high levels of hybrid protein expression and dimerisation is further confirmed by the absence of dimerisation when using strong mesodermal drivers (Fig. 2M and Fig. 2N). Finally, increasing the levels of expression by simultaneously expressing with *69B-Gal4* and *da-Gal4* does not result in novel areas of dimerisation (not shown). This supports that, as has been described in cell culture experiments (Blakely et al., 2000; Rossi et al., 1997), the βlue-βlau technique reveals the intrinsic ability of the proteins tested to dimerise. However, it should be pointed out that when using a very strong Gal4VP16 activator line we have observed unspecific dimerisation. This result shows that precaution should be taken to use different inducing lines, as unspecific dimerisation can be observed with unusually high levels of hybrid protein expression.

The use of X-gal for the detection of protein interactions makes this technique highly sensitive as even very faint levels of blue staining can be detected against the unstained background. This also provides the advantage of not requiring any special type of microscopy. Although our results show that βlue-βlau can detect in whole embryos interaction between transmembrane proteins, future experiments should test whether the technique can be used for testing protein interactions in other cellular compartments.

In summary, we have shown that βlue-βlau is an inexpensive technique to view protein interactions in whole organisms. By applying the technique to DOME we have provided direct visual evidence that the JAK/STAT receptor homo-dimerises, and that the dimerisation is not ligand induced. It will be interesting to see whether developmental control of receptor pre-association is a peculiarity of JAK/STAT receptors or if it is a more general strategy to control receptor activation in other signalling pathways (Moriki et al., 2001; Yu et al., 2002).

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