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argos transcription is induced by the *Drosophila* EGF receptor pathway to form an inhibitory feedback loop

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SUMMARY

Argos is a secreted molecule with an atypical EGF motif. It was recently shown to function as an inhibitor of the signaling triggered by the *Drosophila* EGF receptor (DER). In this work, we determine the contribution of Argos to the establishment of cell fates in the embryonic ventral ectoderm. Graded activation of DER is essential for patterning the ventral ectoderm. *argos* mutant embryos show expansion of ventral cell fates suggesting hyperactivation of the DER pathway. In the embryonic ventral ectoderm, *argos* is expressed in the ventralmost row of cells. We show that *argos* expression in the ventral ectoderm is induced by the DER pathway: *argos* is not expressed in *DER* mutant embryos, while it is ectopically expressed in the entire ventral ectoderm following ubiquitous activation of the DER pathway. *argos* expression appears to be triggered directly by the DER pathway, since induction can also be observed in cell culture, following activation of DER by its ligand, Spitz. Argos therefore functions in a sequential manner, to restrict the duration and level of DER signaling. This type of inhibitory feedback loop may represent a general paradigm for signaling pathways inducing diverse cell fates within a population of non-committed cells.

Key words: receptor tyrosine kinase, EGF receptor, *argos*, *Drosophila*, transcription, inhibitory

INTRODUCTION

During the development of multicellular organisms, determination of cell fates requires communication among cells through neighboring cell-cell interactions or over a distance. Receptor tyrosine kinases (RTKs) mediate a variety of responses to extracellular signals, which result in differentiation of specific cell types. Because of the complexity of the patterning mechanisms and the necessity for accurate timing in development, activation of these receptors must be tightly regulated. One aspect of regulation is the restricted presentation of activating ligand(s). The mouse Steel protein, which is the ligand of the White-Spotting RTK, is expressed in cells adjacent to the tissues expressing the receptor (Motro et al., 1991). Similarly, PDGF-A and its receptor are expressed in neighboring cell layers in the embryo (Orr-Urtreger and Lonai, 1992). In *Caenorhabditis elegans*, activation of the Let-23 receptor in the vulval progenitors is regulated by the expression of the ligand Lin-3 in the neighboring anchor cell (Hill and Sternberg, 1992). In *Drosophila*, activation of the Torso RTK during embryonic development is spatially controlled by presentation of ligand from the terminal regions of the vitelline membrane (reviewed in Sprenger and Nüsslein-Volhard, 1993) while, in the eye, expression of Boss, only by the R8 photoreceptor, regulates the activation of the Sevenless receptor (Krämer et al., 1991).

Spatial restriction of activation is complemented by a variety of mechanisms whose purpose is to modulate the level or duration of signaling. Activation of RTKs triggers the cytoplasmic signaling pathways by transphosphorylation of tyrosine residues on the cytoplasmic domain of the receptor (reviewed in Ullrich and Schlessinger, 1990). Tyrosine phosphatases act specifically to reduce the steady-state level of these phospho-tyrosine residues. Internalization of ligand-receptor complexes is another widely used mechanism to regulate the duration of signaling events of RTKs and other receptors (Ullrich and Schlessinger, 1990). Phosphorylation by protein kinase C has also been postulated as a mechanism to reduce the number of high affinity ligand-binding sites (Collins et al., 1983; Cochet et al., 1984). Finally, reduced transcription of the receptor gene, following activation of the receptor, may represent another mechanism for modulation of signaling (Sturtevant et al., 1994).

In *Drosophila*, activation of the EGF receptor homologue (DER) (Livneh et al., 1985) is tightly regulated. DER is a receptor tyrosine kinase required at different stages in development for processes including midline glial cell differentiation (Klämbt et al., 1991; Raz and Shilo, 1992), wing vein formation (Sturtevant et al., 1993), cell fate determination in the eye disc (Tio et al., 1994; Xu and Rubin, 1993; Freeman, 1994a) and establishment of anterior-posterior as well as dorsoventral polarity in the follicle cells (González-Reyes et al., 1995; Roth et al., 1995; Price et al., 1989). In the follicle cells of the ovary, activation of DER is regulated by the

expression of Gurken, a ligand which is produced as a transmembrane precursor protein containing a single EGF repeat. Localization of the *gurken* transcript at the dorsal-anterior corner of the oocyte is responsible for restricted activation of DER in the follicle cells (Neuman-Silberberg and Schüpbach, 1993).

The earliest zygotic function of DER was shown to be the establishment of cell fates in the embryonic ventral ectoderm (Raz and Shilo, 1993), where graded activation of the DER pathway gives rise to different cell fates along the dorsoventral axis. In this context, DER activation appears to be regulated by processing of the ligand Spitz. The Spitz precursor is produced as a transmembrane protein. Experiments in cell culture and in embryos have shown that only a secreted form of Spitz, presumably generated by cleavage between the EGF and transmembrane domains, is capable of triggering the DER signaling pathway (Schweitzer et al., 1995a).

A novel mechanism for modulation of DER signaling by the secreted protein Argos has recently been described (Schweitzer et al., 1995b). In the eye imaginal disc, photoreceptor recruitment takes place by an elaborate array of cell-cell interactions, leading to ordered induction of cell fates (reviewed in Wolff and Ready, 1993). The *argos* locus encodes an inhibitor of photoreceptor fate determination; in its absence, extra photoreceptors are recruited (Freeman et al., 1992; Freeman, 1994b; Sawamoto et al., 1994). Argos is a secreted molecule with an atypical EGF domain (Freeman et al., 1992). Genetic interactions have identified Argos as an inhibitor of the DER pathway during eye and wing development. Furthermore, in a cell culture assay, addition of Argos completely blocked the activation of DER by its ligand, Spitz (Schweitzer et al., 1995b).

argos is expressed in a variety of tissues from early embryonic stages to the adult fly (Freeman et al., 1992). In this study, molecular markers were used to demonstrate that Argos is a key player in modulating the induction of cell fates in the embryonic ventral ectoderm. In *argos* mutant embryos, ventralization of the ectoderm is observed while overexpression of Argos results in loss of ventral fates. The expression pattern of *argos* in *DER* mutant embryos and in embryos in which DER is ectopically activated, shows that *argos* expression is dependent upon and induced by the DER pathway. Thus, Argos acts sequentially, to provide an inhibitory feedback loop and control the duration and level of DER signaling. Induction of *argos* transcription by DER appears to be direct, since it can also be observed in cell culture, when DER-expressing cells are triggered by Spitz. The implications of the feedback loop generated by Argos as a general mechanism to restrict the duration of signaling pathways are discussed.

MATERIALS AND METHODS

Fly strains

$argos^{l\Delta7}$ and $argos^{W11}$ strains were described in Freeman et al. (1992). 4×*hsp*70-*argos* flies homozygous for the construct on the second and third chromosomes were described in Freeman (1994b). Argos was induced at 2.5 hours AEL by a 20 minute heat shock at 37°C. flb^{1F26} is a temperature-sensitive allele of *DER* that displays the null phenotype at 29°C (Raz and Shilo, 1992). flb^{1F26} mutant embryos were grown at 29°C. The $spitz^{IIT25}$ allele was used. Ectopic activation of DER was achieved by generating embryos in which the *rho-Gal*4 chromosome (obtained from M. Levine) was used to induce the UAS-*secreted spitz* chromosome (Schweitzer et al., 1995a).

Antibody and RNA staining

Rabbit polyclonal antibodies against β-gal were purchased from Cappel. Mouse monoclonal antibodies against Fas III were obtained from T. Volk. All secondary HRP-conjugated antibodies were purchased from Jackson laboratories. The *otd* clone was obtained from R. Finkelstein.

Cell culture assays

DER-expressing S2 cells and their activation by secreted Spitz were previously described (Schweitzer et al., 1995a). To follow induction of Argos protein, a monoclonal anti-Argos antibody was used (Freeman, 1994b). For the DER shut-down experiment, the cells were induced by secreted Spitz medium diluted 1/10 or 1/75. After 5 minutes, the medium was replaced by Argos-conditioned medium supplemented with the corresponding Spitz concentration. Western blots were performed by standard protocols and monitored by ECL (Amersham).

RESULTS

Argos is necessary for fate determination in the ventral ectoderm

In the embryo, ventral ectodermal cell fates are induced by the zygotic DER pathway; absence of DER activity at stages 8-9 leads to disappearance of ventral fates (Raz and Shilo, 1992, 1993), while overexpression of secreted Spitz, a DER ligand, leads to ventralization (Schweitzer et al., 1995a). Argos is expressed during embryonic development (Freeman et al., 1992). Since Argos was shown to be an inhibitor of the DER pathway, the embryonic phenotype of $argos^{l\Delta7}$ null embryos was examined. The molecular marker Fasciclin III (Fas III) was used to monitor the fate of the embryonic ventral ectoderm. Normally, Fas III is expressed at stage 11 in the ventralmost 4-5 rows of cells, most prominently in the thoracic parasegments (Patel et al., 1987; Fig. 1A). In *DER* mutant embryos, Fas III expression in the ventral ectoderm is eliminated (Raz and Shilo, 1993). Analysis of Fas III staining in *argos* mutant embryos was indeed suggestive of a role for Argos in fate determination. Instead of the usual 4-5 cell rows that are Fas III-positive, about 6-7 rows of cells were stained in the *argos* mutant (Fig. 1B).

Another marker, *orthodenticle* (*otd*), which is normally expressed in one or two rows of cells on either side of the midline (Wieschaus et al., 1992; Fig. 1D) was tested. In *DER* mutant embryos, expression of this marker is lost (Kim and Crews, 1993). In $argos^{l\Delta7}$ embryos, *otd* expression is observed across 5-6 rows of cells from the midline (Fig. 1E). This dramatic expansion of the ventralmost fates demonstrates that Argos is normally involved in pattern determination in the ventral ectoderm, presumably by restricting the ventralizing activity of the DER pathway.

The competence of Argos to pattern the ventral ectoderm was also examined by overexpression of Argos. In embryos carrying four copies of the *hsp*70-*argos* construct (Freeman, 1994b), ectopic expression of *argos* was induced early in embryogenesis, by a single heat shock at 2.5 hours after egg lay (AEL). Following this treatment, only the ventralmost row

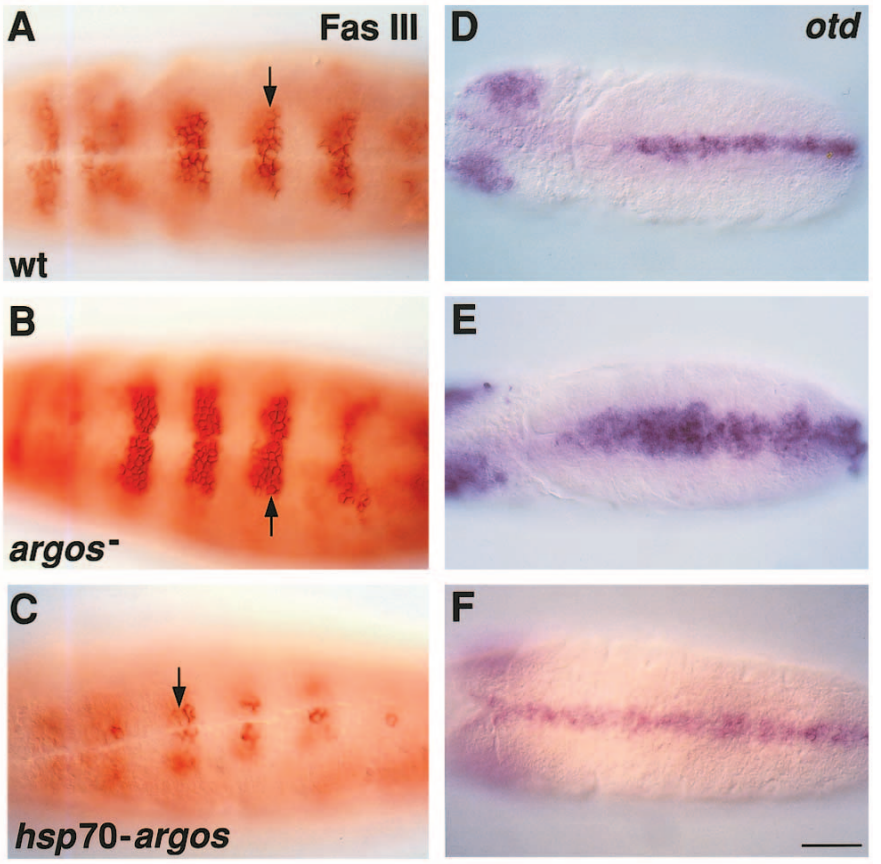

Fig. 1. Argos affects patterning of the embryonic ventral ectoderm. (A) In wild-type embryos the ventral cells can be identified at stage 11 by the expression of Fas III in 4-5 cell rows (arrow) on each side of the midline. (B) In homozygous *argos*$^{l\Delta 7}$ embryos, expansion of Fas III expression to 6-7 cell rows on each side is detected (arrow). (C) Argos overexpression in embryos carrying four copies of *hsp*70-*argos* results in loss of Fas III expression in the ectoderm except in the ventralmost row of cells (arrow). (D) *otd* is expressed at stage 11 in the ventralmost 1-2 cell rows in wild-type embryos. (E) In homozygous *argos*$^{1\Delta 7}$ embryos, expansion of *otd* expression to 5-6 cell rows on each side of the midline is observed. (F) Following Argos induction in *hsp*70-*argos* embryos, no alteration in *otd* expression is observed. Anterior is to the left. Scale bar, 30 μm for A-C and 60 μm for D-F.

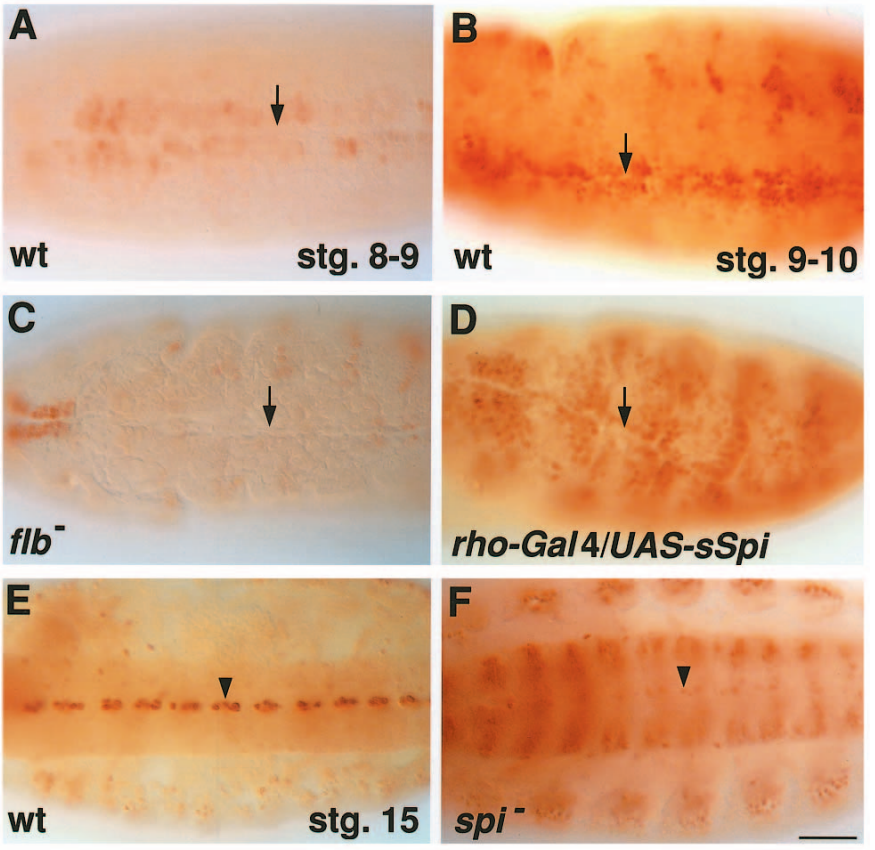

Fig. 2. Argos expression in embryos is induced by the DER pathway. (A) In wild-type embryos carrying the *argos*^{W11} enhancer trap, low expression is observed at stage 8-9, as monitored by anti-β-gal staining. The midline is shown by an arrow. (B) At stage 9-10, expression in the ventralmost 1- to 2-cell rows, as well as in lateral patches and in the head is observed. (C) In homozygous *flb*^{1F26} embryos (grown at 29°C), no expression of Argos in the ventral ectoderm is observed, while normal expression in the head and lateral patches can be detected. (D) Activation of the DER pathway by secreted Spitz, in embryos carrying the *rho-Gal*4 and *UAS-sSpitz* chromosomes, resulted in ubiquitous expression of Argos. (E) In a wild-type embryo at stage 15, expression of Argos in the midline glia cells (arrowhead) is prominent. (F) In homozygous *spitz*^{IIT25} embryos only weak expression of Argos in the midline glia cells is detected, while the expression on the ectoderm appears normal. Scale bar, 30 μm.

of cells expressed Fas III at stage 11 (Fig. 1C). Thus high levels of Argos result in a change of cell fates similar to the one observed in *DER* mutant embryos.

Expression of *otd* is not altered however, following ectopic induction of *argos* (Fig. 1G). This agrees with the observation that Fas III staining is also retained in the ventralmost row of cells. It is possible that the level of *argos* expression under the *hsp*70 promoter is not high enough to completely shut off DER activity. Another option is that the onset of *argos* expression following induction is not early enough, such that the most ventral fates are already determined.

argos is induced by DER activation

The embryonic expression pattern of Argos was previously described (Freeman et al., 1992). Clear Argos expression is first seen at stage 9-10. In the ventral ectoderm, it is restricted to a single row of cells on each side of the midline (Fig. 2B). In addition, expression in lateral ectodermal patches and in the head region is observed. At stage 15, the ectodermal expression is broader and prominent expression in the midline cells is observed (Fig. 2E).

The restricted expression of Argos in the ventral ectoderm, in the region where the activity of DER is highest, suggested that it may actually be induced by this pathway. To further test this possibility, we analyzed the dependence of *argos* expression on DER activation. A line carrying an *argos* enhancer trap in a *DER* (*flb*) mutant background was constructed. In flb^{1F26} null embryos, there is no *argos* expression in the ventral ectoderm as shown in a stage 11 embryo (Fig. 2C), while expression of *argos* in other tissues of the embryo is retained. *DER* mutant embryos display alterations in cell fates in the ventral ectoderm at stage 11, but no cell death (Raz and Shilo, 1992). Therefore, the absence of *argos* expression can not be accounted for by cell death, but rather reflects the dependence of *argos* expression on DER activity.

Argos expression was also monitored following hyperactivation of the DER pathway. Ectopic expression of secreted Spitz in the ventral ectoderm has previously been shown to result in ventralization of the embryo, due to hyperactivation of DER (Schweitzer et al., 1995a). Expression of the $argos^{W11}$ enhancer trap was monitored in embryos where the UAS-*secreted Spitz* construct was induced in the ventral ectoderm by *rho-Gal*4. In these embryos, *argos* expression is observed in multiple rows of ectodermal cells on each side of the midline (Fig. 2D). In addition, the level of Argos expression in each cell appears to be significantly stronger. This expansion demonstrates that the DER pathway is sufficient to induce *argos* expression.

DER is also involved in the process of midline glia cell differentiation (Raz and Shilo, 1992). At stage 15 of embryonic development, *argos* is strongly expressed in the midline glia cells of the CNS (Freeman et al., 1992; and Fig. 2E). It is therefore possible that, in these cells, *argos* transcription is also induced by the DER pathway. In contrast to the experiment in the ventral ectoderm, *argos* expression in the midline glia cells could not be tested in *DER* mutant embryos, since these cells die or fail to develop (Raz and Shilo, 1992). It was therefore necessary to test *argos* expression under conditions in which signaling by the DER pathway would be reduced but not eliminated, thus retaining the viability of the midline glia cells. Elimination of the zygotic contribution of *spitz* was shown to affect the migration of the midline glia cells but not their viability or capacity to express tissue-specific markers (Klämbt et al., 1991). *argos* expression was monitored in *spitz* mutant embryos carrying the *argos* enhancer trap. Indeed, expression of *argos* in the midline glia cells was drastically reduced, while the level of *argos* expression in the ectodermal cells, which is probably not DER-dependent at this phase, remained unchanged (Fig. 2F).

The reliance of *argos* expression on the DER pathway supports a sequential model for the activities of DER and Argos. Further evidence is provided by the fact that, at stage 8-9 of embryonic development, the critical time for DER action in the ventral ectoderm (Raz and Shilo, 1993), there is only low expression of *argos* (Fig. 2A). Thus, the late onset of *argos* expression relative to the time of DER activity is also suggestive of a consecutive mode of action.

argos is induced by the DER pathway in S2 cells

The induction of *argos* transcription in the embryonic ventral ectoderm and midline glia cells may be triggered directly by the DER pathway. Alternatively, *argos* transcription may be a secondary consequence of cell fate changes induced by DER. To distinguish between these possibilities, the ability of DER to induce *argos* transcription in cell culture was tested. Normally, Schneider S2 cells express neither DER nor Spitz. An S2 cell line expressing DER (termed S2:DER2f), in which the DER pathway can be triggered by secreted Spitz (Schweitzer et al., 1995a), was used. Activation of DER was shown to induce not only autophosphorylation, but also association with Drk and activation of MAP kinase (Schweitzer et al., 1995a). It is therefore possible that transcription of relevant downstream genes could also be monitored in the cells.

Secreted Spitz was added to the S2:DER2f cells, and the expression of Argos was followed by western blotting at different times. Extracts of S2:DER2f cells and of S2 cells show no Argos protein. Following addition of secreted Spitz to the DER-expressing cells, low expression levels of Argos can be detected within 4 hours as a 55×10^3 M_r band. At 6 hours, a stronger induction is observed and, by 16 hours, this band becomes very prominent (Fig. 3). Secretion of Argos to the medium is also observed (L. Gabay and B. S., unpublished). No such induction is seen in naive S2 cells exposed to secreted Spitz.

Argos shuts off activated DER

Implicit in the sequential mode of Argos action is the assumption that presentation of Argos to cells in which DER activity has already been triggered by Spitz, has the capacity to shut off DER signaling. This notion could be tested in the cell culture assay. S2 cells expressing DER were incubated with high levels of secreted Spitz for 5 minutes. Under these conditions, maximal activation of DER autophosphorylation is observed and maintained for at least 30 minutes when Spitz is retained in the medium. After the initial incubation with Spitz, Argos was added to the Spitz-containing medium and the level of DER autophosphorylation was followed. A reduction in DER autophosphorylation is observed within 2 minutes and, by 30 minutes, only the basal level is detected (Fig. 4). When the same experiment was carried out using lower concentrations of Spitz to trigger DER, Argos was capable of reducing

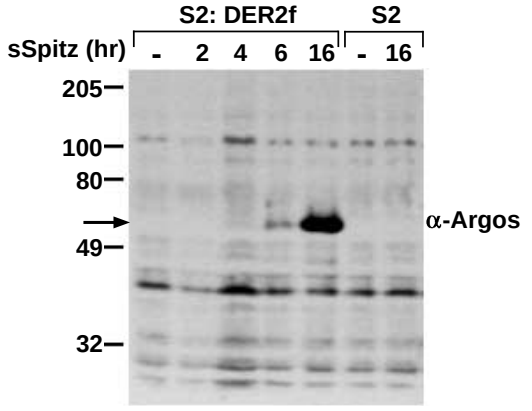

Fig. 3. The DER pathway induces *argos* expression in S2 cells. S2:DER2f cells were incubated with secreted Spitz medium and the level of Argos protein monitored by western blotting with anti-Argos antibodies. Within 4-6 hours, a 55×10^3 M_r band is observed. After 16 hours the induced Argos protein is very prominent. Following incubation with Spitz, no induction of Argos is observed in S2 cells which do not express DER. Molecular weight markers are $\times10^{-3}$ M_r.

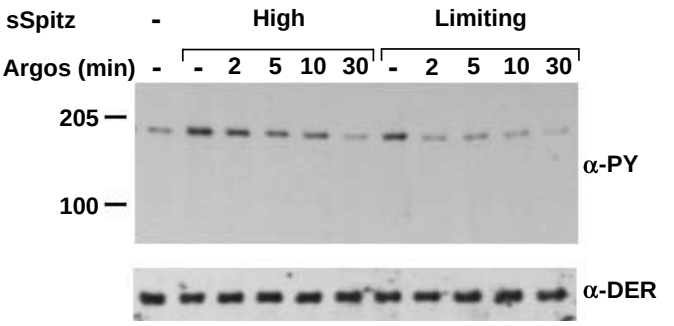

Fig. 4. Argos can shut off activated DER. S2:DER2f cells expressing DER were incubated with a high (1/10 dilution) or limiting (1/75 dilution) concentration of medium containing secreted Spitz. After 5 minutes, the medium was replaced by Argos-conditioned medium with the corresponding Spitz concentration and the cells were lysed at the indicated times. The level of DER autophosphorylation was monitored by blotting with anti-phosphotyrosine antibodies. Following addition of Argos, DER autophosphorylation declines to the basal level within 30 minutes when high levels of Spitz are used to activate DER and within two minutes when limiting Spitz concentrations are present. The level of DER itself remains constant, as monitored by anti-DER antibodies. Molecular weight markers are $\times10^{-3}$ M_r.

DER autophosphorylation to the basal level already within two minutes after its addition (Fig. 4).

DISCUSSION

Argos has been shown to inhibit DER activation in cell culture and in several developmental processes including eye differentiation and wing development (Schweitzer et al., 1995b; Freeman et al., 1992; Sawamoto et al., 1994). In this work, the function of Argos in the process of cell fate determination in the ventral ectoderm was examined. In the absence of Argos, the embryos are ventralized, presumably due to hyperactivation of the DER pathway. Furthermore, it was shown, both in embryos and in cell culture, that *argos* transcription is dependent upon and induced by the DER signaling pathway. Thus, DER and Argos function sequentially: high DER activity induces *argos*, and Argos provides an inhibitory feedback signal to restrict the duration and level of DER signaling.

argos transcription is induced by DER

The embryonic phenotype of *argos* null mutant embryos demonstrates that Argos has a cardinal role in modulating cell fates in the ventral ectoderm. In these embryos, ventralization is manifested by expansion of the ventralmost and ventrolateral cell fates by 3-4 rows. An increased distance between Keilin's organs in the cuticle secreted by the ventral ectoderm was also noted for *argos* mutant embryos (Freeman et al., 1992). The ventralization in *argos* mutants is less pronounced than the one observed following ubiquitous activation of the DER pathway, where expansion of the ventralmost fates by 8-10 cells was monitored (Schweitzer et al., 1995a). However, it is likely that in the absence of Argos, the DER pathway is overactivated, leading to altered cell fates.

Several observations indicated that the actual onset of *argos* transcription is induced by the DER pathway. In *DER* null mutants no expression of Argos in the ventral ectoderm is observed, while ubiquitous expression of Argos is seen following ectopic activation of DER by secreted Spitz. This explains why the normal expression of Argos in the embryo is observed only after the time in which DER signaling is first required to pattern the ventral ectoderm, i.e. at stage 8-9 (Raz and Shilo, 1993). *argos* expression is detected in the ventralmost ectodermal cells, where the highest activity of DER is manifested. The correlation between high levels of DER activity and the sites of Argos expression holds also for other tissues: In the midline glia cells where signaling by DER and the *spitz* group is necessary (Klämbt et al., 1991; Raz and Shilo, 1992), a high level of Argos is observed. Again, in *spitz* mutant embryos, Argos expression in these cells is reduced. Argos is also expressed in the vein precursors of the wing disc (Sawamoto et al., 1994) and in the photoreceptor cells in the eye (Freeman et al., 1992), where DER signaling is required (Sturtevant et al., 1993; Diaz-Benjumea and Hafen, 1994; Xu and Rubin, 1993). In some tissues, however, the expression of *argos* does not appear to be dependent upon the DER pathway. For example, in *DER* mutant embryos, expression in ectodermal lateral patches and in head structures is still observed. We do not know whether the DER-independent expression of Argos has any functional role.

While our results demonstrate that *argos* expression requires the activity of the DER pathway, the mechanism of induction in the embryo is not readily apparent. The simplest model is that activation of the cytoplasmic DER pathway would trigger the transcription of *argos*. However, more complicated options are also available, e.g. DER could induce cell differentiation, which would subsequently result in *argos* expression. The cell culture experiments support the former possibility.

Although S2 cells were originally derived from embryos, they do not normally express specific markers such as DER, Spitz and Rhomboid (Schweitzer et al., 1995a). There is no evidence that they can be induced to differentiate upon activation of the DER pathway. Therefore, changes observed following activation of DER signaling in these cells, are likely to reflect a direct response to this pathway. The components of

the cytoplasmic DER signaling pathway are normally expressed in S2 cells. Activation of DER by secreted Spitz in the S2 cells has been shown to induce the association with Drk and the activation of MAP kinase (Schweitzer et al., 1995ba). Since *argos* is not normally expressed in S2 cells, the induction of *argos* expression was examined. Indeed, within several hours, a dramatic accumulation of the Argos protein was observed. This result strongly supports the possibility that the DER pathway is responsible for triggering Argos expression.

The role of Argos in the ventral ectoderm

How is the spatial activity of Argos tied to patterning of the embryonic ventral ectoderm? In the blastoderm embryo, the nuclear distribution gradient of the Dorsal protein is responsible for subdividing the embryo into distinct domains of gene expression along the dorsoventral axis (reviewed in Chasan and Anderson, 1993). Within each of these domains, the activity of zygotic pathways is responsible for generation of distinct cell fates. The Dpp pathway patterns the dorsal ectoderm. In the ventral ectoderm, patterning is induced by the Spitz/DER pathway. Spitz, the ligand of DER, is ubiquitously expressed as a transmembrane precursor (Rutledge et al., 1992). Only the secreted form of the molecule appears to be biologically active (Schweitzer et al., 1995a). Thus, processing of Spitz is the key for regulating the spatial and temporal pattern of DER activation. According to the level of DER activation, different cell fates are induced in the ventral ectoderm: The ventralmost cells express markers like *otd*, *argos* and Fas III, while more lateral cells express only Fas III.

Argos does not participate in the initial patterning of the ectoderm by DER. Activation of DER induces *argos* expression in the ventralmost cells and the Argos protein diffuses within the ventral ectoderm to shut off DER signaling in the adjacent cells. A minimal estimate for the extent of Argos diffusion may be obtained from the *argos* mutant phenotype. Since the expanded patches of Fas III expression in *argos* mutant embryos includes 6-7 rows and *argos* itself is normally expressed only in the ventralmost 1-2 rows, we would argue the functional diffusion of Argos should cover at least 4-5 cell diameters. The capacity of Argos, which is normally produced as a secreted molecule, to diffuse over several cell diameters has previously been demonstrated in the eye disc by mosaic clones (Freeman et al., 1992).

In the absence of Argos, cells in which the DER pathway is normally activated only at intermediate or low levels, continue to receive the Spitz signal for a longer time and assume the ventralmost fates. Thus, the function of Argos is to provide an inhibitory feedback loop, to shut off or reduce DER signaling and, thus, maintain the gradient of DER activation. A model for the sequential activity of Argos is shown in Fig. 5.

There is indirect evidence that ar*gos* expression is also dependent on DER signaling in the eye and the wing. Clones of cells in which DER signaling is absent show a non-autonomous phenotype, corresponding to over-active DER signaling, adjacent to the clone (Xu and Rubin, 1993; Diaz-Benjumea and Hafen, 1994). This previously confusing result can now be explained: there is a reduction in Argos concentration in the cells around the clone (due to its lack of production in the clone), allowing over-active DER signalling in this neighbouring tissue.

The fact that the biological role of Argos is manifested after its induction by the DER pathway implies that, normally, the consequences of DER signaling are not immediate. In other words, effective signaling by DER normally continues during the period in which Argos protein is synthesized and diffuses to reach the target cells. It is an open question what molecules within the induced cells have to accumulate, or be modified in the time window of signaling, in order to manifest the developmental switch triggered by DER. In the case of Sevenless, it has also been suggested that signaling must be maintained for several hours in order to induce the R7 photoreceptor cell fate (Mullins and Rubin, 1991).

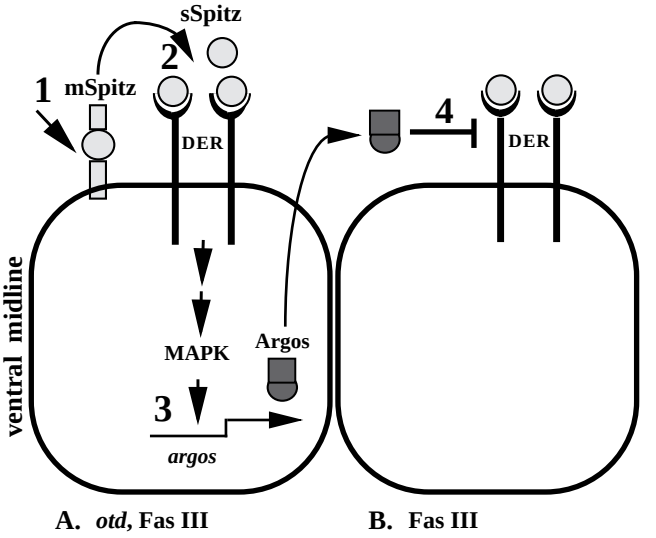

Fig. 5. Induction of *argos* expression by DER, leads to DER shut off in the neighboring cells. The DER ligand, Spitz is expressed as a transmembrane precursor (mSpitz). (1) Cleavage of the precursor to generate secreted Spitz (sSpitz) is the limiting event for activation of the pathway. (2) Secreted Spitz triggers the DER signaling pathway. According to the amount of secreted Spitz, different levels of DER signaling and subsequently of MAP kinase activity will be induced. (3) In cells where the highest level of DER activation is obtained, the transcription of Argos will be induced. (4) Since the Argos protein does not contain a transmembrane domain, it will be secreted, will diffuse to shut off or reduce DER signaling in the neighboring cells and will maintain the fates induced by graded activation of DER. (A) The ventralmost cells, in which Argos is induced, are refractive to the inhibitory effect, and will also express *otd* and Fas III. (B) In the more lateral cells in which intermediate levels of DER activation take place, DER signaling will be shut down by Argos and the cells will express Fas III but not *otd*. Thus Argos is induced by DER and provides an inhibitory feedback loop, to restrict the duration and level of DER signaling in neighboring cells.

'Lateral inhibition' by Argos

It is interesting to note that Argos does not affect the cells in which it is being produced, but rather the neighboring cells. In wild-type embryos, the cells expressing the ventralmost markers like *otd* are the ones which also express Argos. Similarly, when secreted Spitz is expressed ectopically all cells express Argos, yet the ectoderm is ventralized. After being exposed to high levels of DER activation that are required to induce Argos expression, the cells appear to be already committed to the ventralmost fate, and are refractive to the effects of Argos which they now produce. The activity of

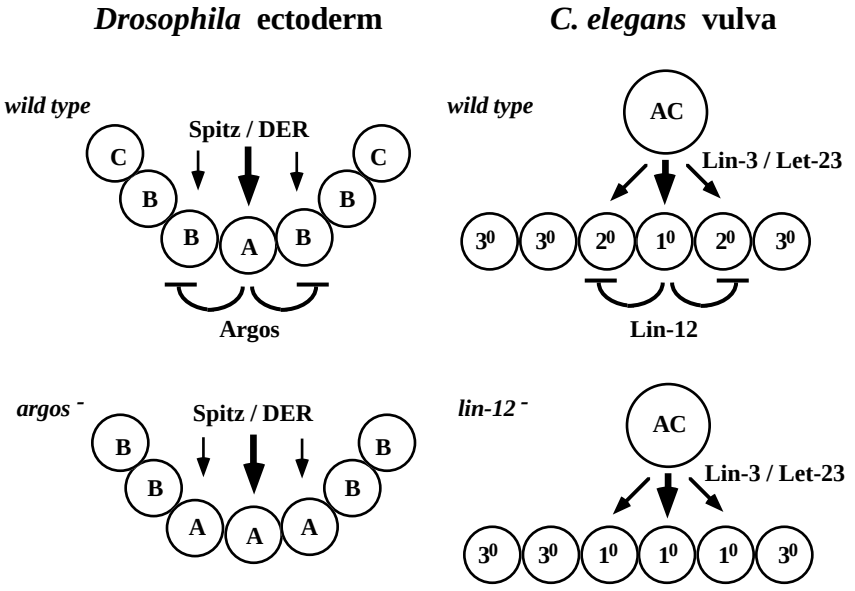

Fig. 6. Analogies between the EGF receptor signaling pathways in the *Drosophila* ventral ectoderm and the *C. elegans* vulva. In *Drosophila* graded activation of DER by secreted Spitz induces ventralmost (A) and ventrolateral (B) cell fates. The ventralmost cells produce and secrete Argos to terminate or reduce DER signaling in the neighboring cells. In *argos* mutants, ventralization is observed. In *C. elegans* the ligand, Lin-3, is produced by the anchor cell (AC). Graded levels of the ligand trigger Let-23 in the vulval precursor cells to induce 1° and 2° vulval cell fates. The 1° cells trigger the Lin-12 pathway to counteract excessive signaling by Let-23 and induce the 2° cell fate. In *lin-12* mutants, 1° fates are also induced in the neighboring vulval precursor cells.

Argos is thus executed non-autonomously, to affect the cells neighboring the Argos-expressing cells. In some ways, the function of Argos is equivalent to 'lateral inhibition', where cells that have adopted a particular fate (in this case as a result of high levels of DER activation) inhibit their neighbors from assuming a similar fate, by shutting off or reducing DER signaling.

We note interesting similarities between patterning the *Drosophila* ventral ectoderm by the DER pathway, and the *Caenorhabditis elegans* vulva by Let-23 signaling (summarized in Fig. 6). In both cases, graded activation of an EGF receptor homologue (DER or Let-23, respectively) is observed (Katz et al., 1995). Cells receiving the highest level of signaling assume a given fate (ventralmost or primary vulval cells, respectively), and inhibit the neighboring cells from assuming a similar fate. Inhibition is achieved through the activity of Argos or the Lin-12 pathway (reviewed in Kenyon, 1995). Both in *argos* and *lin-12* mutants, expansion of the ventralmost or primary cell fates is observed.

Graded activation of receptors leading to the induction of several cell fates has been reported for receptors like Toll and Torso, encoded by maternal transcripts (Chasan and Anderson, 1993; Sprenger and Nüsslein-Volhard, 1993), or by DER and the Dpp receptors, participating in zygotic pathways (Schweitzer et al., 1995a; Ferguson and Anderson, 1992). The duration of signaling by the respective maternal pathways may be restricted, since the receptors are triggered by ligand(s) which should have limited pools. In the case of the zygotic pathways, however, more specific mechanisms appear to be required to limit the duration or extent of signaling by the activating ligands. The expansion of dorsalizing Dpp activity is restricted by Short gastrulation (Sog), an inhibitor of the pathway, which is expressed in the ventral ectoderm (Francois et al., 1994). Overlapping activities of Dpp and Sog may also generate a sharper gradient of Dpp activity in the dorsal ectoderm (Holley et al., 1995). In the ventral ectoderm, graded DER activation is generated by ligand processing, and Argos appears to be crucial for defining the correct time window and level of signaling by the DER pathway.

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