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Positive and negative regulation of Easter, a member of the serine protease family that controls dorsal-ventral patterning in the *Drosophila* embryo

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SUMMARY

The sequential activities of four members of the trypsin family of extracellular serine proteases are required for the production of the ventrally localized ligand that organizes the dorsal-ventral pattern of the *Drosophila* embryo. The last protease in this sequence is encoded by *easter*, which is a candidate to activate proteolytically the ligand encoded by *spätzle*. Here, we demonstrate biochemically that the zymogen form of Easter is processed in vivo by a proteolytic cleavage event that requires the three upstream proteases. Processed Easter is present in extremely low amounts in the early embryo because it is rapidly converted into a high molecular mass complex, which may contain a protease inhibitor. Easter zymogen activation is also controlled by a negative feedback loop from Dorsal, the transcription factor at the end of the signaling pathway. Each of these regulated biochemical processes is likely to be important in generating the ventral-to-dorsal gradient of Dorsal protein that organizes cell fates in the early embryo.

Key words: Easter, Serine protease, Zymogen, Dorsal-ventral patterning, Serpin

INTRODUCTION

The extracellular serine proteases of the trypsin family are well-known for their functions in blood in the formation and dissolution of blood clots, the regulation of blood pressure and the activation of complement. In all of these processes, it is crucial that protease activity be localized to the site where it is needed and that the protease be rapidly inactivated after it has performed its function (Hecht and Anderson, 1992). Serine proteases are initially synthesized as inactive zymogens containing an N-terminal pro-domain and a C-terminal catalytic domain, and activation requires proteolytic clipping of the zymogen at a defined site by a specific activating protease. Activation is frequently localized to the surface of specific cells by the requirement for a membrane-bound cofactor. Localized activation is often amplified through a positive feedback loop and terminated through a negative feedback loop. Further, specific protease inhibitors prevent the spatial and temporal spread of proteolytic activity away from the localized activation site.

The best-studied example of the role of serine proteases in development is the requirement for four members of the trypsin family during development of the early *Drosophila* embryo (Morisato and Anderson, 1995). These proteases are encoded by four of the twelve maternal-effect genes that are known to encode components of a signaling pathway that establishes the first dorsal-ventral asymmetry of the embryo. A key step in this pathway is the ventral activation of the membrane receptor encoded by *Toll*, apparently by a novel ligand encoded by *spätzle*. Spätzle is secreted as a freely soluble protein into the extracellular space between the plasma membrane of the syncytial embryo and the eggshell, and is proteolytically processed to generate an active ligand, apparently only on the ventral side of the embryo (Morisato and Anderson, 1994; Schneider et al., 1994). Proteolytic activation of Spätzle requires the sequential action of four different members of the trypsin family. The first protease in this pathway is encoded by *nudel*, which is expressed in the somatic follicle cells of the ovary that secrete the eggshell (Hong and Hashimoto, 1995), whereas the other three proteases are expressed by the germline cells. *gastrulation defective* (*gd*) is closely related to the trypsin family, but the protein encoded by *gd* lacks a number of amino acid residues crucial for protease activity, so its biochemical function is not clear (Konrad and Marsh, 1990). Downstream of *gd* is the protease encoded by *snake* (DeLotto and Spierer, 1986; Smith and DeLotto, 1994), which acts upstream of the final protease known in this pathway, Easter (Chasan and Anderson, 1989; Chasan et al., 1992). The Easter protease is likely to be the direct proteolytic activator of Spätzle.

The proteases in this pathway have been ordered genetically using constitutively active forms of the Snake and Easter proteases that consist of only the catalytic domain and should

therefore bypass the requirement for activation of the zymogen (Chasan et al., 1992; Smith and DeLotto, 1994; Smith et al., 1994). However, it has not been demonstrated biochemically that zymogen activation of any of the proteases occurs in vivo, and the events that regulate zymogen activation and subsequent activity of the proteases have not been characterized.

Here we show that the Easter zymogen undergoes proteolytic processing in vivo to generate the cleaved form of the protein that should correspond to the active protease. The active form of Easter is present in extremely low amounts in the embryo because it is converted into a high molecular mass covalent complex. The identification of this complex as a form of Easter that has gone through the activation process has allowed us to demonstrate directly that the Nudel, Gastrulation defective and Snake proteases are required for activation of the Easter zymogen and has also revealed the existence of a feedback loop that can regulate the amount of Easter that is activated in the embryo.

MATERIALS AND METHODS

Drosophila strains and transformants

Oregon R was used as the wild-type strain. The dorsal group mutants used have been described previously: ndl^{111}/ndl^{133}, gd^{7}/gd^{7}, snk^{073}/snk^{229} and spz^{rm7}/Df(3R)Ser^{+R82f} (Anderson and Nüsslein-Volhard, 1984); $ea^{4}/ea^{5022rx1}$ and $ea^{8}/ea^{5022rx1}$ (Chasan and Anderson, 1989); Tl^{5BREQ}/Tl^{9QRE} (Anderson et al., 1985); Df(3R)hkb^{XM3}/Df(3R)$tub^{R5.6}$ and pll^{074}/pll^{385}(Hecht and Anderson, 1993); $cact^{99}/cact^{PD74}$ (Roth et al., 1991); and dl^{1}/dl^{1} (Nüsslein-Volhard, 1979).

The intron-less *eaΔN* cDNA (Chasan et al., 1992) was placed downstream of the 0.4 kb fragment of genomic DNA that lies 5′ to the start site of transcription of *easter*, which had previously been shown to direct normal levels of expression of *easter* (Jin and Anderson, 1990) and then cloned into the CaSpeR3 vector for transformation. The construct was injected into w^{-} flies, and w^{+} transformants were selected. Similar transformants containing the wild-type *easter* cDNA were also constructed; these fully rescued the ea^{-} phenotype, demonstrating that there is no essential regulatory information present in the intron sequences. The P[*eaΔN*] allele used in the experiments described here is an insertion onto the third chromosome. The construction of the *ea* S338A mutation was described previously (Jin and Anderson, 1990).

RNA injections and immunohistochemical staining of embryos

Synthetic SP6 RNA transcripts were generated by in vitro transcription and injected into the center of 0-1 hour embryos (Chasan and Anderson, 1989). Embryos were allowed to develop until the time of gastrulation and harvested by aspirating into a pipette. Expression of the Twist protein was assayed with a rabbit anti-Twist antibody (Roth et al., 1989), a gift from Siegfried Roth; injected embryos were fixed as described by Stein et al. (1991).

Preparation and immunoblotting of embryo extracts

Embryo extracts of dechorionated embryos were prepared as described at 4°C (Morisato and Anderson, 1994), but without PMSF and at lower protein concentration (1-5 mg/ml). Unless otherwise noted, all embryo extracts were prepared from 0-4 hour embryos (collected at 22°C). Extracts of injected embryos were prepared by homogenization in gel loading buffer. Extract proteins were boiled for 5 minutes with SDS loading buffer at a final concentration of either 100 mM dithiothreitol (DTT; reducing) or 20 mM N-ethylmaleimide (nonreducing) and spun for 1 minute at 13,000 revs/minute. Samples were separated on SDS-polyacrylamide gels and transferred to nitrocellulose (S&S) in 25 mM Tris, 190 mM glycine, 0.1% SDS, and 20% methanol (Towbin et al., 1979). Western blots of gels run under nonreducing conditions were incubated 20 minutes in 20 mM DTT in 1× TBS (30 mM Tris pH 7.5/150 mM NaCl). Membranes were incubated with 1:1000 dilution of rat anti-Easter antibody (Chasan et al., 1992) and then goat anti-rat HRP-conjugated antibody (Jackson Immunoresearch) in 5% nonfat dry milk in TBS/0.1% Triton X-100. The immunoblots were visualized using ECL (Amersham).

RESULTS

A form of Easter that does not require zymogen activation promotes development of the most ventral cell types in the embryo

Embryos that lack maternally provided *easter* RNA and protein are completely dorsalized: they lack all ventrally and laterally derived cell types including the mesoderm and ventral nerve cord (Anderson and Nüsslein-Volhard, 1984). We previously demonstrated that an N-terminal deletion mutant *easter, eaΔN*, in which the pro-domain is deleted and the signal sequence is fused directly to the catalytic domain, is biologically active and bypasses the requirement for zymogen activation (Chasan et al., 1992). When *eaΔN* transcripts were synthesized in vitro and injected into dorsalized $easter^{-}$ embryos, the embryos developed laterally derived cell types all around the embryonic circumference, but the ventral-most cell type, the mesoderm, was not induced. This could either indicate that the *eaΔN* RNA did not produce enough active protein to fully activate the pathway or that some other component is required, in addition to the Easter catalytic domain, to promote the most ventral fates.

We constructed females homozygous for a P[*eaΔN*] transgene and lacking wild-type Easter activity; these females produced lateralized embryos, similar to those seen in the *eaΔN* RNA injection experiments (Fig. 1A). To test whether high concentrations of *eaΔN* would be sufficient to induce more ventral fates, we injected *eaΔN* RNA into embryos produced by these P[*eaΔN*], ea^{-} females. Cells at all dorsal-ventral positions near the injection site developed like the most ventral cell type, the mesoderm, as assayed by the expression of the Twist protein (Fig. 1B). Thus activation of the Easter zymogen appears to be an essential step in the production of all lateral and ventral cell types in the embryo.

Proteolytic processing of catalytically inactive Easter can be detected in vivo

The product of the maternally expressed *easter* gene is required during the syncytial blastoderm stage, from about 1.5-3 hours after fertilization, as judged by the time at which it is possible to rescue the dorsalized phenotype of mutant embryos by injection of the transcript of the *easter* gene and by the temperature-sensitive period of temperature-sensitive alleles (Anderson and Nüsslein-Volhard, 1984, 1986). Western blot analysis of embryo extracts separated on reducing gels using antibodies to the Easter protein showed that 0-4 hour wild-type embryos contained protein the size of the 50 kDa Easter zymogen (Fig. 2). Proteolytic activation of the Easter zymogen should produce two smaller protein fragments, the catalytic

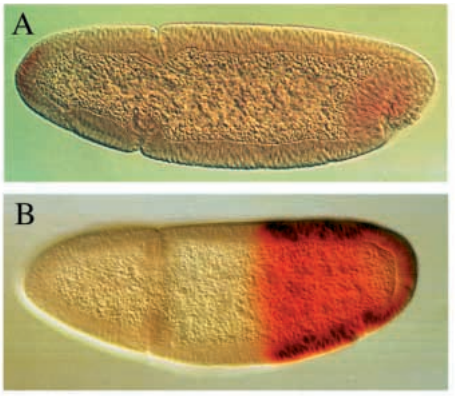

Fig. 1. (A) Gastrulation pattern and expression of Twist in an embryo laid by a transformant female homozygous for the P[*eaΔN*] transgene and homozygous mutant for *gastrulation defective*(*gd*), a gene that acts upstream of *easter* in the pathway. In these embryos, wild-type Easter is not active because active *gd* is required for its function; as a result, the only active Easter is EaΔN. This embryo shows the typical lateralized pattern of gastrulation, with a dorsoventrally symmetrical head fold (Anderson et al., 1985). This embryo does not express the mesoderm-specific Twist protein, except at the anterior and posterior poles of the embryo. (B) Expression of the catalytic domain of *easter* (*eaΔN*) is sufficient to induce Twist expression in nuclei around the entire dorsal-ventral circumference. Transcripts of the *eaΔN* cDNA were injected into the posterior region of embryos produced by females homozygous for the P[*eaΔN*] transgene and mutant for *gd*. Cells at all dorsoventral positions near the injection site embryos express Twist, showing that they have acquired the most ventral cell fate.

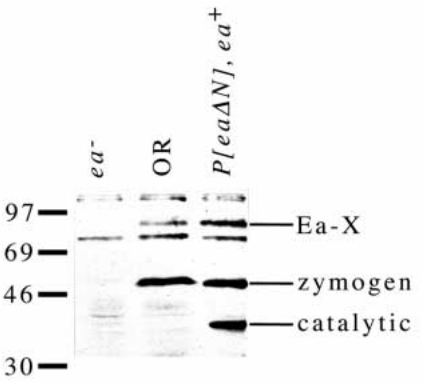

Fig. 2. In addition to the Easter zymogen, a higher molecular mass form of Easter protein, Ea-X, is present in extracts of young wild-type embryos. Extracts from 0-4 hour embryos laid by ea^{-} females ($ea^{4}/ea^{5022rx1}$) (both of these alleles make no detectable *easter* transcript), wild-type females (Oregon R), or females carrying a transgene encoding only the catalytic domain of Ea, P[*eaΔN*] (in a genetic background wild type for *easter*, P[*eaΔN*] ea^{+}/ea^{+}) were separated on a 13.5% SDS-polyacrylamide gel under reducing conditions. Protein immunoblotting using α-Ea antibodies (Chasan et al., 1992) detected the 50 kDa Ea zymogen, as well as an 80-85 kDa band, Ea-X, in both wild-type and P[*eaΔN*] transformant embryos. The P[*eaΔN*] embryos also contain the 35 kDa EaΔN catalytic domain. Molecular masses (kDa) are shown along the left.

domain and the pro-domain. The catalytic domain should co-migrate with the 35 kDa product encoded by the P[*eaΔN*] allele present in the transformants, but no band the size of the Easter catalytic domain or the smaller pro-domain was detected in wild-type embryo extracts (Chasan et al., 1992; Fig. 2).

Although no processed Easter was detected in wild-type embryos, we did detect processing of certain mutant forms of Easter. The ea^{8} null allele is a mis-sense mutation that changes the amino acid at the entrance of the binding pocket from glycine to glutamic acid (Jin and Anderson, 1990). This mutation should preclude the binding of the basic amino acid of either a substrate or protease inhibitor that would fit into the binding pocket of wild-type Easter protein. The ea^{8} allele produces a normal amount of a normal-sized zymogen (Jin, 1991). In contrast to wild type, we detected a form of Ea^{8} protein that co-migrated with the Easter catalytic domain produced by P[*eaΔN*] transformants (Fig. 3A), revealing that this Easter protein is processed to generate a protein the same size as the active catalytic domain, as predicted for a typical serine protease. The processed N-terminal pro-domain was not detected, apparently because the polyclonal antibody used did not recognize this region of the protein. Another *easter* mutant in which the active site serine was replaced by alanine (S338A) (Jin and Anderson, 1990) showed the same pattern of proteins: the Easter zymogen was produced and, again, a protein the size of the processed catalytic domain was present (Fig. 3B). These results present a conundrum: we could detect proteolytically activated Easter only in mutants of *easter* that lack catalytic activity and not in wild type.

Activated Easter forms a stable high molecular mass complex

The lack of accumulation of processed Easter in wild-type embryos could reflect rapid degradation of the processed protein (dependent on its own catalytic activity) or conversion of processed Easter to another form. In wild-type embryos, in addition to the 50 kDa Easter zymogen band, a higher molecular mass band of 80-85 kDa was recognized by the Easter antibody (Fig. 2). This high molecular mass band (which we call Ea-X) was not present in embryos that lacked *easter* RNA, so it appeared to be a novel form of the Easter protein. The Ea-X complex was very stable; it was not disrupted by boiling in SDS for 30 minutes (data not shown).

Neither the Ea^{8} nor the Ea^{S338A} protein made an Ea-X complex (Fig. 3). Thus the Ea-X complex forms only with catalytically active Easter protein. The results suggest a sequence of events that occur in vivo: Easter zymogen is activated by proteolytic cleavage and then is rapidly converted into Ea-X, but only if the Easter protein has the potential for catalytic activity (Fig. 4).

The Ea-X complex contains proteolytically processed Easter protein

It should be possible to determine whether the wild-type Easter in Ea-X has been proteolytically cleaved at the zymogen activation site based on the size of the Easter protein in Ea-X. Embryos laid by transformants carrying the P[*eaΔN*] construct as their only source of *easter* (ea^{-} P[*eaΔN*]/ea^{-}) lacked the 50 kDa Easter zymogen, as expected, and also contained both the 35 kDa catalytic domain (EaΔN) and a 80-85 kDa EaΔN-X complex (Fig. 5A). EaΔN-X comigrated with the Ea-X complex from wild-type embryos on reducing gels, as predicted if the wild-type Easter in Ea-X has been

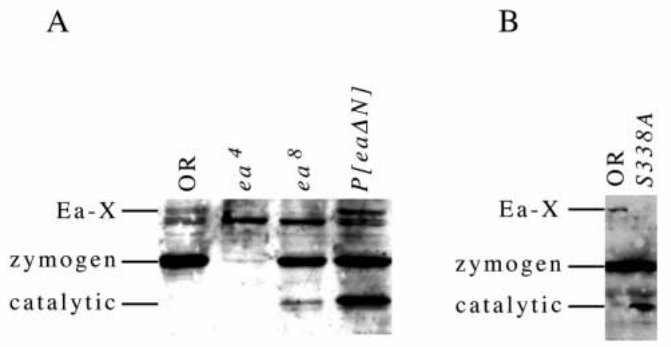

Fig. 3. Processing of the Easter zymogen is observed in catalytically inactive Easter mutants. (A) The *ea*^{8} missense mutation in the binding pocket of Easter allows the detection of the processed catalytic domain of Easter, which comigrates with the product of the P[*ea*Δ*N*] transgene and the prevents formation of the Ea-X complex. The processed N-terminal pro-domain is not detected, apparently because the polyclonal antibody used does not recognize this region of the protein. Extracts were prepared, analyzed and immunoblotted with α-Ea antibodies as in Fig. 2, from embryos laid by females of the following genotypes: lane 1, wild type (Oregon R); lane 2, *ea*^{4}/*ea*^{5022rx1}; lane 3, *ea*^{8}/*ea*^{5022rx1}; lane 4, P[*ea*Δ*N*] *ea*$^{+}$/P[*ea*Δ*N*] *ea*$^{+}$. (B) A null mutant form of *easter* in which the active site serine-338 is replaced with alanine (*ea* ^{S338A}) (Jin and Anderson, 1990) is processed to give the catalytic domain-sized fragment and fails to make Ea-X. Embryos laid by *ea*^{4}/*ea*^{5022rx1} females were injected with transcripts encoded by wild-type *ea* cDNA (lane 1) or *ea* S338A mutant cDNA (lane 2). Extracts were prepared from 15 injected embryos and immunoblotted with α-Ea antibodies as in Fig. 2.

proteolytically clipped at its activation site and X is tightly bound to the catalytic domain of Easter.

Based on the distribution of cysteines in the wild-type Easter protein, the N-terminal pro-domain is expected to remain disulfide bonded to the C-terminal catalytic domain after zymogen activation (Chasan and Anderson, 1989). If the activated Easter in Ea-X is a two-chain protease with the pro- and catalytic domains associated by a disulfide bond, then wild-type Ea-X would migrate more slowly on non-reducing gels, where the pro-domain should remain associated with the complex, than on reducing gels, which should contain only the Easter catalytic domain and X (see Fig. 4). Under non-reducing conditions, Ea-X migrated more slowly than the EaΔN-X, confirming that the wild-type Ea in Ea-X had been proteolytically cleaved at the zymogen activation site and demonstrating that the pro-domain of the wild-type enzyme remained disulfide-bonded to the catalytic domain after zymogen cleavage (Fig. 5B).

Upstream genes are required for activation of the Easter zymogen

We previously found that the *eaΔN* mutation bypasses the requirement for the activity of five other components of this signaling pathway, *pipe*, *nudel*, *windbeutel*, *gastrulation defective (gd)* and *snake* (Chasan et al., 1992), which suggested that those genes are normally required for the activation of the Easter zymogen. Since Ea-X contains proteolytically activated Easter, we can use Ea-X as a biochemical indicator of Easter activation. We examined extracts made from *nudel*, *gd* and *snake* embryos and found that, in each case, the Easter zymogen was present at the same level as in wild-type embryos, but neither activated Easter nor Ea-X was detected (Fig. 6). Because *nudel*, *gd* and *snake* are required for formation of Ea-X, these experiments provide biochemical evidence that three genes act by controlling the activation of the Easter zymogen.

The activation of Easter appears to be regulated by a negative feedback loop from the end of the pathway

As predicted from the genetic analysis, mutations that block this signaling pathway at steps downstream of Easter do not block the formation of Ea-X (Fig. 6). In fact, all these downstream mutants contain 3- to 5-fold more Ea-X than wild-type embryos (Figs 6, 7). Even mutant embryos that lack *spätzle* RNA and protein showed increased levels of Ea-X (data not shown), indicating that Ea-X is not a stable complex of Easter with its putative substrate Spätzle. In ventralized *cactus*$^{-}$ embryos, we observed that the amount of Ea-X was less than half the amount present in wild-type embryos (Fig. 6). Since the amount of Ea-X reflects the amount of activated Easter, these results suggest that a feedback loop regulated by nuclear Dorsal acts back across the plasma membrane of the syncytial embryo to regulate activation of the Easter zymogen.

The accumulation of Ea-X in downstream mutants occurs at the same time that signaling through the pathway occurs. While the zymogen form of Easter was present at fertilization, Ea-X did not appear until 1 hour after fertilization, at approximately the time that the Easter protein is active in the embryo (Fig. 7), and increased during the next 2 hours of development. As soon as Ea-X was detectable, there was more Ea-X in *Toll*$^{-}$ than in wild-type embryos (Fig. 7). Thus, well before the final gradient of Dorsal is achieved at about 2.5 hours after fertilization (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989), the level of Easter processing appears to be modified by this feedback loop.

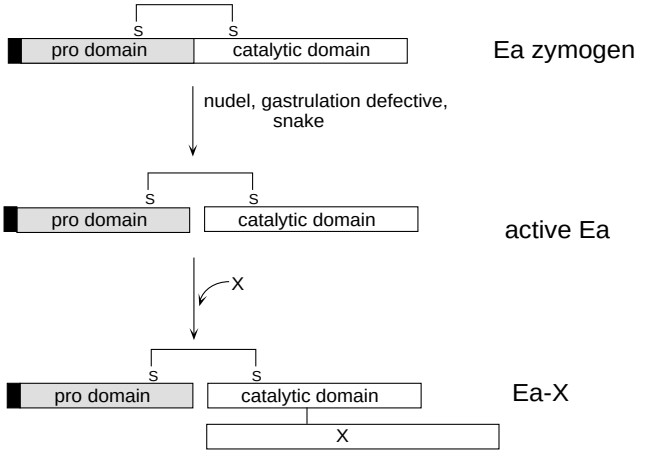

Fig. 4. The sequence of proposed events in Easter activation. Processing of the Easter zymogen by proteolytic clipping at the activation site leads to the formation of the active Easter protease. Association with another molecule, X, occurs after processing and requires a functional Easter active site.

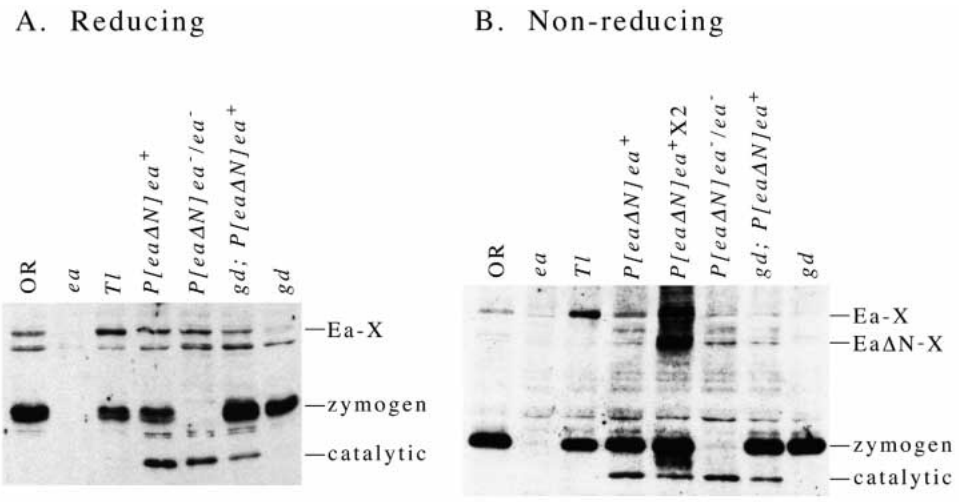

Fig. 5. The Ea-X complex forms after zymogen cleavage, based on migration of the complex on reducing and non-reducing gels. (A) The Ea-X complex in embryos encoded by the wild-type Easter gene and the EaΔN-X complex encoded by a P[*ea*Δ*N*] transgene comigrate under reducing conditions. This suggests that the Ea protein in the Ea-X complex is cleaved between the pro and catalytic domains, and that X is complexed with the catalytic domain of Ea. Note that in the P[*ea*Δ*N*] ea^{-}/ea^{-} lane, no endogenous Ea protein is expressed (no zymogen), but a band comigrating with Ea-X is present, corresponding to EaΔN-X. The doublet band below Ea-X is a cross-reacting band variably recognized by the anti-Ea antibodies; compare to the ea^{-} lane in Fig. 2. Note that in the P[*ea*Δ*N*] transformants a substantial amount of the EaΔN protein does not enter the EaΔN-X complex; this is consistent with biochemical observations that the proteolytic activity of EaΔN is substantially less than that of the properly activated wild-type Ea (Hecht, 1993). Embryo extracts were prepared as in Fig. 2, incubated with dithiothreitol (DTT) as described in the Materials and methods, and analyzed by immunoblotting a 7.5% SDS-polyacrylamide gel with α-Ea antibodies. Extracts were prepared from embryos laid by females of the following genotypes: lane 1, wild type (Oregon R); lane 2, $ea^{4}/ea^{5022rx1}$; lane 3, $Toll^{5BREQ}/Toll^{9QRE}$; lane 4, P[*ea*Δ*N*] ea^{+}/P[*ea*Δ*N*] ea^{+}; lane 5, P[*ea*Δ*N*] $ea^{4}/ea^{5022rx1}$; lane 6, gd^{7}/gd^{7}; P[*ea*Δ*N*]/+; lane 7, gd^{7}/gd^{7}. (B) The Ea-X complex migrates more slowly than the EaΔN-X complex under non-reducing conditions. The pro-domain of Ea is expected to remain disulfide bonded to the catalytic domain of wild-type Ea, causing the Ea-X complex to migrate more slowly than under reducing conditions, where the pro-domain is not bound. Embryo extracts were incubated with N-ethylmaleimide instead of DTT (as described in Materials and Methods) and analyzed as in part A. Extracts were prepared from embryos laid by females of the following genotypes: lane 1, wild type (Oregon R); lane 2, $ea^{4}/ea^{5022rx1}$; lane 3, $Toll^{5BREQ}/Toll^{9QRE}$; lane 4, P[*ea*Δ*N*] ea^{+}/ea^{+}; lane 5, P[*ea*Δ*N*] ea^{+}/P[*ea*Δ*N*] ea^{+}; lane 6, P[*ea*Δ*N*] $ea^{4}/ea^{5022rx1}$; lane 7, gd^{7}/gd^{7}; P[*ea*Δ*N*]/+; lane 8, gd^{7}/gd^{7}. Both Ea-X and EaΔN-X can be seen in the P[*ea*Δ*N*] $ea^{+}/ea^{+}$and P[*ea*Δ*N*] ea^{+}/P[*ea*Δ*N*] ea^{+}lanes. EaΔN-X but not Ea-X is present in the P[*eaΔN*] ea^{-} lane, which lacks wild-type zymogen, and in the gd^{-}, P[*eaΔN*] lane, in which the wild-type Easter zymogen is present but not activated.

DISCUSSION

Regulated activation of the Easter zymogen

Previous results on Easter activation were somewhat paradoxical. Site-directed mutagenesis experiments had shown that the identities of the particular amino acids at the zymogen activation site are critical for Easter activity, arguing that proteolytic processing at that site is necessary for activity (Chasan et al., 1992). Furthermore, the pre-processed catalytic domain of Easter was active in the absence of upstream genes, indicating that those genes are required for activation of the zymogen (Chasan et al., 1992). Despite this molecular genetic evidence, no cleaved, activated protease was detected in the embryo.

We have now demonstrated biochemically that the Easter zymogen is proteolytically processed in vivo to form a catalytic domain of the predicted size and we have discovered that the proteolytically activated protein is rapidly sequestered into a high molecular mass complex, Ea-X. Using the presence of Ea-X to monitor the activation process, we find that activation of the Easter zymogen depends on the serine protease family members encoded by *nudel*, *gastrulation defective* and *snake*, as predicted from the genetic experiments. In contrast, Easter zymogen activation does not depend on Easter activity, since the proteins encoded by the null alleles ea^{8} and ea^{S338A} are activated. Based on the ratio of Ea-X protein to Easter zymogen, we estimate that less than 10% of total Easter protein in the wild-type embryo is processed. Therefore, the amount of Easter activity in the embryo is limited by the amount of zymogen activation allowed by the upstream proteases.

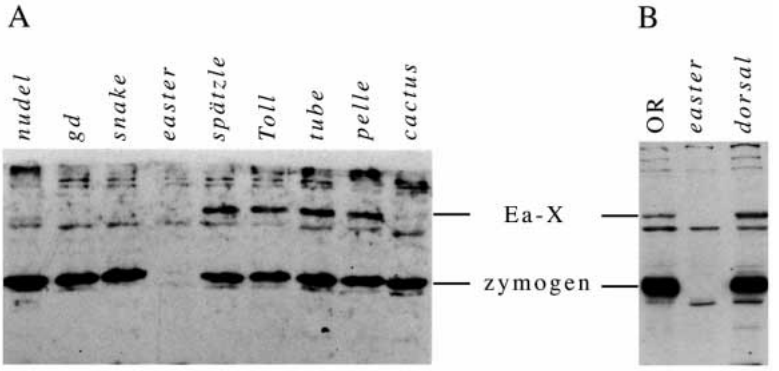

Fig. 6. Mutants that act upstream of *easter* in the genetic pathway prevent the formation of the Ea-X complex, and mutants that block the pathway downstream of *easter* produce more Ea-X. Embryo extracts were separated and blotted as described in Fig. 2 and probed with α-Ea antibodies. (A) Extracts were prepared from 0-4 hour embryos laid by females of the following genotypes: lane 1, ndl^{111}/ndl^{133}; lane 2, gd^{7}/gd^{7}; lane 3, snk^{073}/snk^{229}; lane 4, $ea^{4}/ea^{5022rx1}$; lane 5, spz^{rm7}/Df(3R)spz^{Ser}; lane 6, Tl^{5BREQ}/Tl^{9QRE}; lane 7, Df(3R)tub^{XM3}/Df(3R)$tub^{R5.6}$; lane 8, pll^{074}/pll^{385}; lane 9, $cact^{99}/cact^{PD74}$. (B) Extracts were from embryos laid by females of the following genotypes: lane 1, wild type (Oregon R); lane 2, $ea^{4}/ea^{5022rx1}$; lane 3, $dorsal^{1}/dorsal^{1}$.

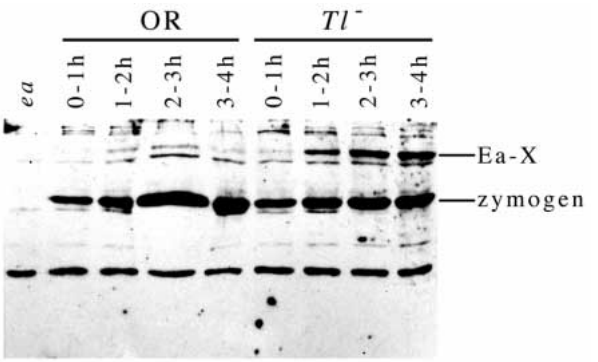

Fig. 7. Ea-X accumulates to higher levels in *Toll*$^{-}$ embryos than in wild-type embryos, even before the final Dorsal gradient is defined at about 2.5 hours after fertilization. Immunoblotting with α-Ea antibodies of extracts from embryos collected 0-1 hours, 1-2 hours, 2-3 hours and 3-4 hours after fertilization at 22°C, laid by wild-type (Oregon R) or Tl^{5BREQ}/ Tl^{9QRE} females. The amount of Ea-X peaks in wild type at 2-3 hours, and subsequently decreases. However, in the dorsalized *Tl*$^{-}$ mutant where no signaling is occurring, more Ea-X is observed by 1-2 hours after fertilization than in wild type, and the level does not decrease after 3 hours.

Activation of the Easter zymogen is first detected at about 1 hour after fertilization. Therefore there must be some event that begins after fertilization that triggers the activity of the proteases upstream of Easter. The Toll receptor is first detected on the surface of the embryo at about 90 minutes after fertilization (Hashimoto et al., 1991). The ligand for Toll, presumably the proteolytically processed Spätzle protein, appears to be made ventrally, but can diffuse freely in the perivitelline space if it is not sequestered by binding to Toll (Stein et al., 1991). If the ligand were activated by proteolysis before the receptor were present, it could diffuse to the dorsal side of the embryo and activate Toll ectopically. It therefore seems likely that timing of Easter activation is important for patterning.

The high molecular mass Easter complex appears to contain a protease inhibitor

Ea-X is a very stable complex between the activated catalytic domain and another component. The appearance of Ea-X correlates with activation of the Easter zymogen, suggesting that Ea-X is a complex of Easter with another protein that either activates or inhibits Easter activity. The formation of the Ea-X complex depends on previous proteolytic activation of the zymogen and on residues essential for catalysis, indicating that the X protein binds specifically to the activated enzyme through its active site. The most likely molecules to bind the active site are substrates and inhibitors. Because Ea-X is extremely stable and because Ea-X does not include Spätzle, its likely substrate, we favor the hypothesis that Ea-X is an inhibited complex and that the other component of Ea-X is an inhibitor. A serine protease inhibitor could play an important role in patterning by preventing the spread of Easter activity away from its initial site of activation.

The Ea-X complex has the properties expected for a complex with a protease inhibitor of the serpin family. The serpins are a family of proteins that act as suicide substrates, with the active site serine of activated serine proteases, thereby irreversibly inactivating the protease (Potempa et al., 1994). Most serpins are 40-60 kDa in size, which would be consistent with the size difference between the predicted masses of the catalytic domains of Easter and Ea-X. As with other serpin-protease complexes, Ea-X formation requires the active site serine and the protease-binding pocket (Laskowski and Kato, 1980; Potempa et al., 1994). Also, like other serpin complexes, the Ea-X complex is extremely stable. Only a single cloned *Drosophila* serpin-encoding gene has been well characterized; this serpin is transferred from the male to the female during copulation and its function is not known (Coleman et al., 1995). Other serpin genes have been identified because of their proximity to cloned genes (FlyBase: http://cbbridges.harvard.edu:7081/). Additional serpins have been identified in other insects (e.g. Jiang et al., 1994), making it very likely that *Drosophila* will have a number of different serpin-encoding genes. The reverse genetic approach of purifying the high molecular mass complex, microsequencing and gene cloning will ultimately make it possible to test whether the other component of Ea-X is a serpin or other protease inhibitor.

A feedback loop may modulate Easter zymogen activation

Recent data from a variety of signaling pathways suggests that secreted inhibitors play key roles in defining the realm of action of extracellular signals. For example, activity of the *Drosophila* EGF receptor induces the secretion of the Argos protein, which inhibits EGF receptor activity and thereby limits the domain of activity of the receptor, apparently by competing with the ligand for the receptor (Golembo et al., 1996). Our data suggest that a negative feedback loop may also operate in the Toll signaling pathway. Mutations in any one of the downstream genes required for pathway activity, *spätzle*, *Toll*, *tube*, *pelle* or *dorsal*, lead to an increase in the amount of Easter that is activated in the blastoderm embryo, as detected by the increase in the amount of Ea-X present. A similar phenomenon was previously reported with Spätzle: more proteolytically processed, activated Spätzle was detected in extracts from embryos in which the pathway was blocked downstream of Spätzle than in wild-type embryos (Morisato and Anderson, 1994). Such a feedback loop could regulate the activation of the Easter zymogen directly, or it could act at any step upstream of Easter in the pathway. Because mutations in *dorsal* lead to increased Easter activation, we propose that the initial component of the feedback loop is a transcriptional target of Dorsal. This hypothesis suggests that there is a more dynamic interaction between maternal and zygotic products in initial dorsal-ventral patterning than previously thought, a possibility that should be experimentally testable.

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