Multiple isoforms of the *Drosophila* Spätzle protein are encoded by alternatively spliced maternal mRNAs in the precellular blastoderm embryo

Received: 2 May 2000 / Accepted: 9 August 2000 / Published online: 21 October 2000 © Springer-Verlag 2001

**Abstract** The *spätzle* gene is required for proper specification of positional information along the dorsal-ventral axis of the *Drosophila* embryo and for induction of the innate immune response to fungal infection. It has been shown to encode a precursor of a Nerve Growth Factor-like ligand which is also a member of the cysteine-knot protein superfamily. In dorsal-ventral patterning, the most widely accepted model of the pathway places Spätzle at the end of a ventrally restricted protease cascade that results in the proteolytic processing of the precursor form of Spätzle to an active ligand which is thought to bind to the Toll receptor. Here we show that the *spätzle* gene encodes at least ten different protein isoforms as a result of complex alternative splicing in precellular blastoderm embryos. Multiple transcripts are clearly present up until the time of cellularization, at which point most transcripts can no longer be detected. Nine isoforms were expressed and at least five are efficiently secreted in a heterologous protein expression system. RNA microinjection experiments demonstrate that three isoforms completely rescue embryos from *spätzle* null mothers, while most of the others rescue to a lesser extent. The phenotypic rescue activities of several isoforms and the relevance of these isoforms to the generation of the ventralizing signal are discussed.

**Key words** Spätzle · Dorsal-ventral · Pattern formation · *Drosophila* embryogenesis

**Introduction**

Specification of cell fate along the dorsal-ventral axis of the *Drosophila melanogaster* embryo during embryogenesis requires the generation of an extracellular signal shortly before the blastoderm cellularizes (reviewed by Roth 1994; Morisato and Anderson 1995). This ventralizing signal leads to the translocation of Dorsal protein, a NF-κB-rel type transcription factor, from the cytoplasm into nuclei on the ventral side of the embryo (Steward 1987). By the cellular blastoderm stage, Dorsal protein is distributed in a ventral to dorsal gradient, with high concentrations in ventral nuclei and progressively diminishing amounts in nuclei located at more dorso-lateral positions (Roth et al. 1989; Rushlow et al. 1989; Steward 1989). Failure to produce the ventralizing signal results in the failure of Dorsal protein to translocate to the nuclei, with the result that all blastoderm cells adopt an extreme dorsal cell fate.

The ventralizing signal appears asymmetrically in the perivitelline space (PVS), a fluid filled extracellular compartment bounded by the outer vitelline membrane and the inner plasma membrane that contributes to the cell membranes of the cellular blastoderm embryo (Stein et al. 1991). Within the PVS reside several proteins which are required for the generation of the signal (Stein and Nüsslein-Volhard 1992). These include three serine proteinase precursors (zymogens) encoded by the genes *gastrulation defective*, *snake* and *easter*, and the product of the *spätzle* gene, which encodes the precursor of a dimeric NGF-like, cysteine-knot growth factor molecule (Morisato and Anderson 1994; Schneider et al. 1994; DeLotto and DeLotto 1998). Studies of genetic epistasis, and molecular and biochemical data, are all consistent with a model in which ventrally restricted proteolytic processing of Spätzle by the activated form of the Easter proteinase results in the generation of an “NGF-like”...
C-terminal fragment of Spätzle (Roth 1994). This C-terminal fragment probably corresponds to the transplantable polarizing activity originally described by Stein et al. (1991), and it is this fragment which is most likely to be the ventral-specific ligand which signals via the Toll plasma membrane receptor (Stein et al. 1991).

The molecular characterization of the spätzle gene revealed the DNA sequence of two spätzle cDNAs, pspz1.9 and pspz2.1, the transcripts of which were capable of completely rescuing embryos derived from mothers which carried a spätzle null allele following microinjection (Morisato and Anderson 1994). The only difference between these two cDNAs was the presence in pspz2.1 of a 219-bp insertion that resulted in a 73-amino acid insertion in the predicted protein. Analysis of Spätzle protein in embryos by immunoblotting revealed numerous Spätzle polypeptides in the range of 32 to 60 kDa, and one at 23 kDa, under reducing conditions in wild-type embryos but not in embryos from spz mothers. Thus far there has been no clear explanation for the large number of cross-reacting polypeptides detected by anti-Spätzle antibodies in early embryos.

We wished to examine further the transcriptional complexity of the spätzle gene, in order to determine whether it could provide an explanation for the multiple polypeptides detected in early embryos. Here, we report the isolation and characterization of ten unique spätzle-encoded cDNAs from 0–3 h embryos, eight of which are novel in their nucleotide sequence. We show that they are all generated by alternative splicing of the primary spätzle transcript and that each cDNA encodes a different protein isoform. These multiple cDNAs are present as maternal RNA in pre-cellular blastoderm embryos and all transcripts, with the exception of one form, appear to be degraded rapidly at the time the blastoderm cellularizes. Expression of each of the isoforms using the baculovirus expression system shows that five isoforms are secreted into the culture medium. Phenotypic rescue by microinjection of RNAs into embryos revealed different biological activities for the various isoforms in spätzle- and wild-type backgrounds. Three rescue embryos to hatching, many others rescue partially, one fails to rescue and one which lacks the putative “NGF-like ligand” still has a weak phenotypic rescue effect in embryos from spz null mothers. Finally, the significance of these isoforms for the generation of the dorsal-ventral pattern is discussed.

Materials and methods

RT-PCR

RNA and DNA was prepared from the wild-type strain Oregon R. The following oligonucleotides were used: z (5'-GATCTTGG-AGATCCAGACGTCCTCTCCTCCG-3'), β (5'-GACCTGTCA-AGATCCATGATGACGGCCATGTGGATA-3'), γ (5'-GATCA-AGCTTGGTTAACCTCACCAAGCTCTTACTCCACGCACT-3') and δ (5'-GATCAAGCTTGACCTCTTCCGACAATTACTTTGCACT-3'). Taq polymerase, AMV and MuLV reverse transcriptases and dNTPs were from Roche Molecular Biochemicals. Conditions for RT-PCR were similar to those previously described, with the exception that the PCR program was as follows: 10 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 2 min at 50 °C and 2 min 72 °C, and a final extension step at 72 °C for 10 min (Smith et al. 1994). Reaction products were purified by extraction with phenol, ethanol precipitated and electrophoresed on Agarose DNA gels (FMC) in TRIS-borate buffer. Stage-specific RT-PCR reactions were conducted on pools of 60 wild-type Oregon R embryos, which were rendered transparent by treatment with 0.14% benzalkonium chloride in water, examined under a stereo microscope, staged and placed in PBS on ice to stop development. Embryos were then homogenized in water/acid phenol (1:1); nucleic acids were ethanol precipitated and DNA was eliminated by treatment with DNase I (Roche). The subsequent procedure for RT-PCR was as described above. PCR products were digested with BamHI and HindIII and subcloned into BamHI + HindIII-digested pGEM4 (Promega). The primary DNA sequence of the inserts was determined by the dideoxy method using primers that annealed to the SP6 or T7 promoter regions and data analysis was done using Lasergene software.

Baculovirus expression

Recombinant baculovirus was generated by subcloning cDNA inserts (as BamHI-KpnI fragments) into BamHI + KpnI-digested pBacPAK8 (Clontech). Recombination of the pBacPAK construct with BacPAK6 viral DNA was performed by lipofection, but otherwise as previously described (Smith et al. 1995). High-titer viral stocks were used to infect 25-ml cultures of IZD-MB0503 cells growing in Hyclone CCM-3 serum-free medium (Hyclone) at 27 °C, and the expressed protein was assayed 72 h post infection. Cells were centrifuged at 900 rpm in a Du Pont RT6000B centrifuge with an H1000B rotor. Cell pellets were resuspended in an equal volume of fresh Hyclone CCM-3 medium and 25 µl of cell pellet suspension and conditioned cell culture medium was resolved on a 12.5% SDS-polyacrylamide gel. Expression of recombinant protein was assayed by transfer of the fractionated proteins to Immobilon membrane (Millipore), followed by Western analysis with a polyclonal rat spz8 antiserum directed against the C-terminal portion of Spätzle as previously described (DeLotto and DeLotto 1998).

Microinjection assays

Phenotypic rescue assays were conducted with eggs derived from e spz+ ca e spz+/− ca females (Tübingen stock No. M367); pGEM4 subclones were linearized at the 3’ end by digestion with KpnI and RNA was synthesized with SP6 polymerase as previously described (Smith and DeLotto 1994). RNA was resuspended at 500 µg/ml in DEPC-treated water, and centrifuged to remove particulate material. A volume equivalent to approximately 2% of the egg volume was microinjected into stage 2 embryos, centrally at about 20% egg length. Embryos were allowed to develop for 3 days at 20 °C in a humid chamber supplemented with oxygen, and cuticle preparations were made as previously described (Wieschaus and Nüsslein-Volhard 1986). Phenotypic rescue was scored as described in Table 1.

DNA sequence data

Primary DNA sequences have been deposited in GenBank. The accession numbers of the spz cDNAs are as follows: AF237964, AF237965, AF237966, AF237967, AF237968, AF237969, AF237970, AF237971, AF237972, AF237973, and the spätzle genomic sequence is available under AF237974.