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The Role of Star in the Production of an Activated Ligand for the EGF Receptor Signaling Pathway

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The *Star* gene is a member of the EGFR signaling pathway which has diverse functions throughout *Drosophila* development. In order to investigate the protein distribution for Star, we have generated a polyclonal antibody. Here, we show that the Star protein is expressed perinuclearly in the early female germline and later is found in the oocyte cytoplasm. Star is expressed at low levels in other tissues. The subcellular localization of the protein has been determined when Star is overexpressed in the eye disc. Star is located in the nuclear and contiguous endoplasmic reticulum membranes. A functional assay in the wing disc demonstrates that Star expression can activate a nonprocessed membrane-bound form of the EGFR ligand Spitz and overexpression of Star in the eye disc promotes the formation of smaller Spitz proteins. Based on these results, we propose that the Star protein is likely to be involved in Spitz ligand processing. © 1999 Academic Press

Key Words: **Star; Spitz; EGFR; *Drosophila;* processing; nuclear membrane.**

INTRODUCTION

The epidermal growth factor receptor (EGFR) signaling cascade has been implicated in a diverse array of developmental processes throughout the life of the fly (reviewed in Schweitzer and Shilo, 1997). Genetic screens in the fly have allowed for the isolation of putative ligands for the EGF receptor. One of these ligands, Spitz, is a 26-kDa membrane-bound protein, with one EGF-like repeat in its N-terminal extracellular portion and a putative dibasic cleavage site which, by analogy to the mammalian TGF-α ligand, may allow for the secretion of a processed N-terminal Spitz protein outside of the cell (Rutledge *et al.,* 1992). Experiments in *Drosophila* embryonic cell lines have established that secreted Spitz (sSpi) can increase EGFR activation, suggesting that the cleaved form of Spitz is a bona fide ligand (Schweitzer *et al.,* 1995). Furthermore, overexpression of sSpi, but not a membrane-bound construct (mSpi), gives an activated phenotype in the embryo and in the eye, supporting the hypothesis that the Spitz ligand is processed *in vivo* (Schweitzer *et al.,* 1995; Freeman, 1996). Two other candidate ligands for EGFR have been described: Gurken and Vein. The Gurken protein contains a single EGF-like repeat and functions during oogenesis (reviewed in Ray and Schüpbach, 1996). The *vein* gene encodes a secreted protein, not found in a preprocessed membrane-bound form, and functions during wing development (Schnepp *et al.,* 1996).

Mutants belonging to the "*spitz* group" (Mayer and Nüsslein-Volhard, 1988) share the same embryonic ventral epidermal defects as *spitz* and interact genetically with *EGFR.* The *Star* gene is a member of this group and encodes a novel 66-kDa protein with a putative transmembrane stretch, but no signal sequence (Kolodkin *et al.,* 1994). Genetic studies in the ovary, wing, and eye suggest that *Star* is a member of the *EGFR* pathway (Sturtevant *et al.,* 1993; Heberlein *et al.,* 1993; Kolodkin *et al.,* 1994). The *Star* phenotype in the embryonic ectoderm can be suppressed by activated sSpi leading Golembo *et al.* (1996) to suggest that the Star protein functions upstream of the secretion of the Spitz ligand.

MATERIALS AND METHODS

Star Antibody Production

A 4-kb *Pvu*II DNA fragment encoding amino acids 15–597 of the Star reading frame was cloned into the pATH1 vector and transformed into XL1 blue cells (Stratagene). Total bacterial lysate of the induced TrpE–Star fusion protein was used to generate antibodies

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in rat (Pocono Laboratory, PA). The serum was affinity purified over TrpE and TrpE–Star Bio-Rad affigel-10 columns. The antibody was used at a 1/250 dilution for Western blots and a 1/10 dilution for tissue staining.

Tissue Staining

Ovary, embryo, and imaginal disc stainings were carried out as described in published protocols (Neuman-Silberberg and Schüpbach, 1996; Ashburner, 1989; Rogge *et al.,* 1995, respectively), except that embryos were hand peeled, rather than methanol devittelinized, and peripodial membranes were removed from eye discs.

For electron microscopic preparations of eye discs, 0.01% saponin was used as a detergent for permeabilization and ABC reagents (Vectastain) were used for the amplification of the signal. The remaining protocol was carried out as described by Liposits *et al.* (1984). Discs were embedded in epon plastic resin (Polysciences) and 0.1-μm sections were examined using a Zeiss 10C electron microscope.

Fly Stocks

sE-*S*10 and $S^{hs.8}$ lines are described in Kolodkin *et al.* (1994). The UAS–*Star,* UAS–*mspi,* and UAS–*sspi* stocks were obtained from B-Z. Shilo and are described in Schweitzer *et al.* (1995) and Golembo *et al.* (1996); *ap*-Gal4 (md544 allele) was obtained from the Bloomington Stock Center.

UAS/Gal4 Wing Disc Experiments

For wing disc experiments, flies were allowed to lay eggs for 24 h at 21°C; eggs were then aged for 48 h and shifted to 27°C. Wing discs were dissected from late third instar larvae.

Western Blot Experiments

Twenty-five eye discs from larvae of respective genotypes were dissected in PBS containing protease inhibitors. Discs were homogenized within 15 min of dissection in loading buffer containing 1% SDS. Cellular debris was removed by centrifugation; samples were boiled for 10 min and then resolved on 10% polyacrylamide/SDS gels. Proteins thus resolved were transferred to Nitrocellulose membranes and probed with anti-spitz antibody as described (Schweitzer *et al.,* 1995). No heat shock was used in these experiments.

RESULTS AND DISCUSSION

In order to investigate the cellular function of the Star protein, we generated rat polyclonal sera against a TrpE–Star fusion protein (see Materials and Methods). The affinity-purified antibody recognizes a protein of the predicted molecular weight, 66 kDa, on a Western blot of wild-type adult tissue (Fig. 1A). The level of this 66-kDa antigen is elevated in $Star^{hs.8}$ and sE-*S10* flies, where *Star* is overexpressed behind either the *hsp70* promoter or the *sevenless* enhancer element, respectively (Basler *et al.,* 1989), suggesting that this antibody does specifically recognize the Star protein. This antibody was used for immunolocalization of Star in various tissues.

In ovaries, Star is expressed in all stages of the developing egg chamber (Fig. 1B). Star expression is first detected in region 2A of the germarium (Figs. 1C and 1D). Sections of the germaria show that there is staining in punctate rings surrounding the nuclei of the germ cells (Fig. 1D). Rings of perinuclear staining can be seen in some stage 2–3 egg chambers, of a size and position that makes them likely to be surrounding nurse cell nuclei (Fig. 1E). In stages 4–7 the Star protein becomes concentrated in the oocyte, which develops from the most posterior of the germ cells. Interestingly, at these later stages Star is distributed uniformly throughout the oocyte cytoplasm (Fig. 1F) and is not concentrated around the germinal vesicle (oocyte nucleus). At late stage 10, the Star protein may be visualized in the nurse cell cytoplasm and throughout the egg (Fig. 1G). This staining may represent a maternal component of Star expression. The oocyte expression pattern is consistent with the results from germline clones which demonstrated a requirement for Star function in the germline (Mayer and Nüsslein-Volhard, 1988). At no stage is Star seen in the plasma membrane, nor is Star protein expression detected in the follicle cells.

The α-Star antibody stains other wild-type tissues, but only at very low levels. For example, in stage 14 embryos, there is weak perinuclear staining in all the nuclei of the ectoderm (Fig. 1H). In the eye disc, all the cells show weak perinuclear staining, but there is higher staining at the posterior edge of the furrow in equally spaced clusters that resemble the expression of *Star* RNA at the furrow (Fig. 1I and Kolodkin *et al.,* 1994). Since the staining is very weak, we were not able to make further inferences about the cell identity or the subcellular location of the Star protein from this experiment. However, when the Star protein is overexpressed using a heterologous promoter, strong staining can be observed (Fig. 1J). Due to the nature of the enhancer, no inference about which cell types ordinarily express Star can be made. However, this genetic background allowed us to investigate possible subcellular compartments to which Star belongs. In eye discs where Star expression is under the control of the *sevenless* enhancer element (Basler *et al.,* 1989) the antibody shows a perinuclear staining pattern (Fig. 1K). This is clearly seen in sections of eye discs (Fig. 1L). This perinuclear localization is consistent with the wild-type staining in the embryo and early ovary described above. Although any interpretation of this result carries the caveat that the protein is being overexpressed and that this localization may not reflect the endogenous distribution, this *sevenless* enhancer element has been widely used in our laboratory and those of others to express many different proteins and this has never led to mislocalization of the protein to a novel cellular compartment. We also used the UAS/Gal4 system (Brand and Perrimon, 1993) to overexpress Star in the R8, R2, and R5 photoreceptor precursor cells in which it is normally expressed at this time (Heber-

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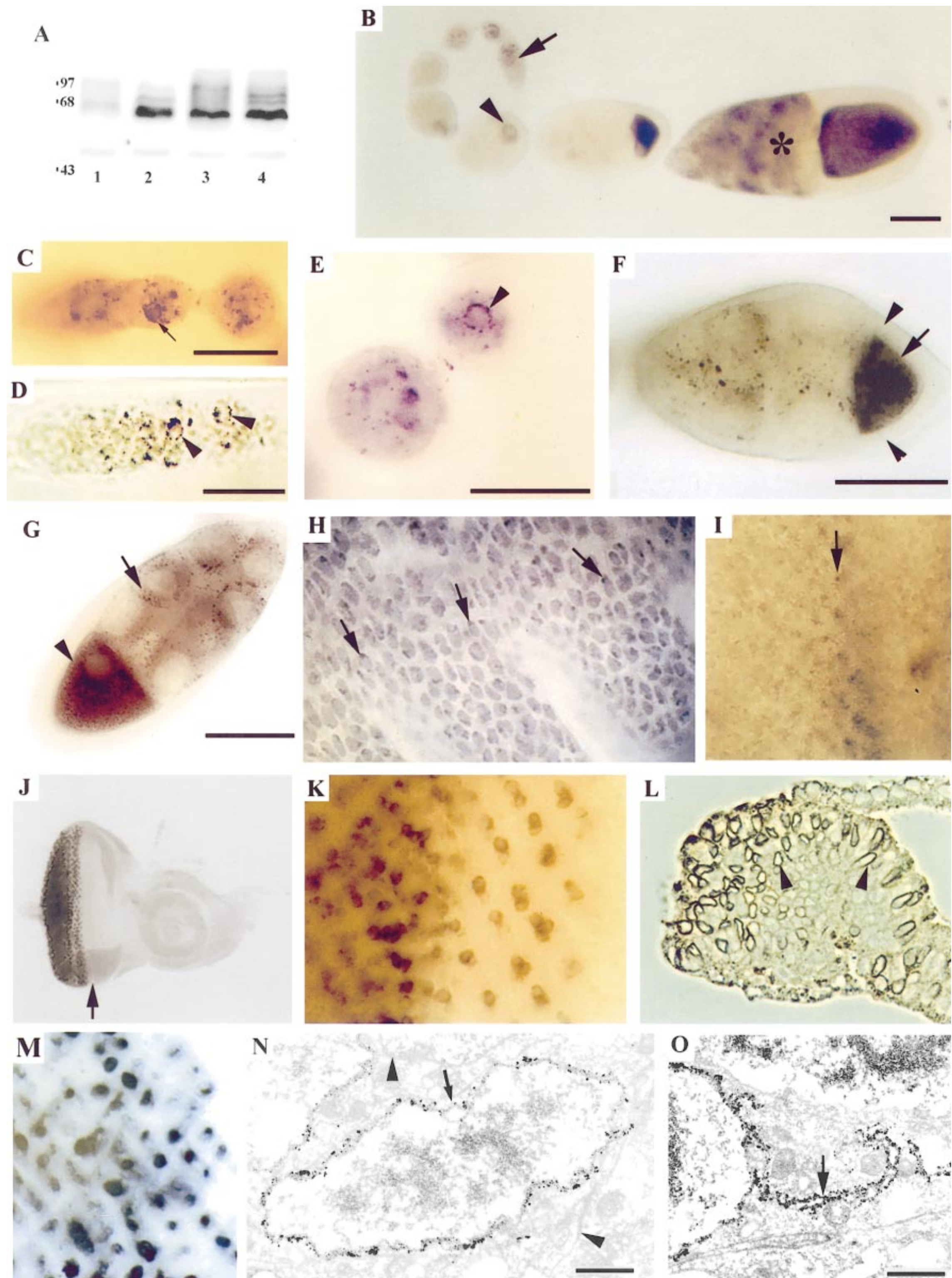

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lein *et al.,* 1993; Kolodkin *et al.,* 1994). Again, the Star protein was found in a perinuclear location (Fig. 1M).

Electron microscopic examination of eye discs of the above genetic background detected Star protein in both the inner and the outer nuclear membranes and occasionally in the contiguous endoplasmic reticulum (ER) membranes (Figs. 1N and 1O). The staining is continuous and is not concentrated in or around the nuclear pores, which are seen as dense areas where the inner and outer nuclear membranes converge, suggesting that Star is probably not involved in nuclear transport functions. This membrane localization is consistent with the predicted sequence of the Star protein, which includes a putative transmembrane domain, but no signal peptide. The nuclear membrane is contiguous with the ER (Alberts *et al.,* 1994) suggesting a processing function for Star. The subcellular localization of Star in eye discs is consistent with the perinuclear localization of the protein seen in the wild-type germarium, as well as in the embryo. Notably, Star protein is not detected in the plasma membrane in any of these tissues. It is interesting to note that the Spitz pro-protein also shows a perinuclear localization in eye discs (Tio and Moses, 1997), supporting our hypothesis that Star has a role in the posttranslational processing of Spitz.

In order to test whether Star overexpression has any effect on Spitz processing *in vivo,* we looked at Spitz expression on Western blots of eye discs where Star was overexpressed. When an α-Spitz monoclonal antibody (Schweitzer *et al.,* 1995) raised against the entire extracellular portion of Spitz was used on a Western blot of wild-type eye discs, three bands of approximately 29, 28, and 27 kDa were detected (Fig. 2A, lane 1). It is not known what form of the protein these three bands represent since both putative cleavage and extensive glycosylation of the Spitz may generate numerous different protein products (Rutledge *et al.,* 1992; Schweitzer *et al.,* 1995). When the Star gene is overexpressed in eye discs, using expression driven by the hsp70 heat shock promoter, there are only two lower molecular weight bands detected with the α-Spitz antibody, as opposed to the three bands seen reproducibly in wild-type discs (Fig. 2A, lane 2). This is the first direct indication that the Star protein may have an effect on Spitz processing. We overexpressed mSpi under the control of the GMR enhancer that causes expression in all cells behind the morphogenetic furrow in the eye disc. On Western blots of these eye discs, the highest (29-kDa) band is seen to be increased in intensity over the wild-type level suggesting that this band corresponds to unprocessed Spitz (not shown).

Schweitzer *et al.* (1995) ubiquitously expressed the sSpi construct (bearing the putative extracellular portion of Spitz) and showed that it is able to ventralize wild-type embryos. Although the mSpi construct is able to rescue the embryonic lethality of *spitz* mutants, it does not confer a ventralization phenotype on the embryo when expressed in a wild-type background. Analogous results have been reported in the eye disc (Freeman, 1996), where the same sSpi construct expressed in *sevenless*-expressing cells can cause the recruitment of additional photoreceptor cells to the clusters, but the mSpi form has no phenotype. These results suggest that the membrane-bound form of Spitz must be activated in some way, in order to give an overexpression phenotype. We have used the developing wing imaginal disc as an assay system to test the hypothesis that Star exerts its

FIG. 1. (A) Western blot of protein extracts from adult flies. Wild type (lane 1), $S^{hs.8}$/Cyo with heat-shock (lane 2) and sE-*S*10 without (lane 3) and with (lane 4) heat shock, probed with an α-Star antibody, showing a signal at the predicted native molecular weight for Star (66 kDa), whose expression level is elevated in flies where *Star* is overexpressed using either the heat shock promoter (*hsp70*) or the *sevenless* enhancer (sE). (B–G) Ovaries stained with α-Star antibody. Anterior is left; ventral is top. Bar, 10 μM. (B) A wild-type (Oregon-R) ovariole showing staining in the germ cells in region 2 of the germarium (arrow), Star accumulation in the oocyte (arrowheads), and, at later stages, Star staining in nurse cells (asterisk) as well as in the oocyte. (C) A whole mount wild-type germarium stage 2 egg chamber, showing punctate perinuclear rings of Star protein in germ cells in region 2A (arrow) and at later stages. (D) This staining can be more clearly visualized (arrowhead) in sections of the germarium. (E) Stage 2–4 egg chambers. Star is expressed in a perinuclear ring (arrowhead) surrounding a nurse cell nucleus. (F) Whole mount stage 8 egg chamber, predominantly showing punctate staining throughout the oocyte cytoplasm (arrow). The Star protein is not concentrated around the oocyte nucleus at this later stage, nor is it found in the follicle cells (arrowheads). (G) Late stage 10 egg chambers showing Star protein is expressed in the nurse cell cytoplasm in granular structures (arrow) and also at high levels in the oocyte (arrowhead). (H) Stage 14 embryo (ventral view) showing that a low level of Star protein is found in all cells of the ectoderm in a perinuclear distribution. There is punctate staining in concentrated patches on the surface of some of the nuclei (arrows). (I) Oregon-R eye disc showing weak Star staining at the posterior edge of the furrow (arrow) in small vesicular structures. (J–M) Light microscope images of α-Star antibody staining of third instar imaginal eye discs where Star is overexpressed. Posterior is to the left. (J) Low magnification picture of whole mount sE-*S*10 disc, showing staining posterior to the furrow (arrow) in Sevenless-expressing cells. (K) Higher magnification, demonstrating that Star is found perinuclearly in photoreceptor precursor cells. (L) Longitudinal section of sE-*S*10 eye disc; apical at top, posterior to right, showing Star protein localized to perinuclear rings (arrowheads) in the photoreceptor precursor cells. (M) *rhomboid*-Gal4/+; UAS-*S*/+, eye disc, where Star expression is driven in the R8, R2, and R5 cells where it is expressed in wild type, showing perinuclear staining. (N, O) Electron micrographs of photoreceptor precursor cells in sE-*S*10 eye discs stained with α-Star antibody. (N) Star protein is located in the nuclear membrane (arrow) and not in the plasma membrane (arrowhead) of the developing photoreceptor cell. (O) Star protein expression can be seen in the nuclear membrane and occasionally in the contiguous ER membrane (arrow). Bar, 1 μM.

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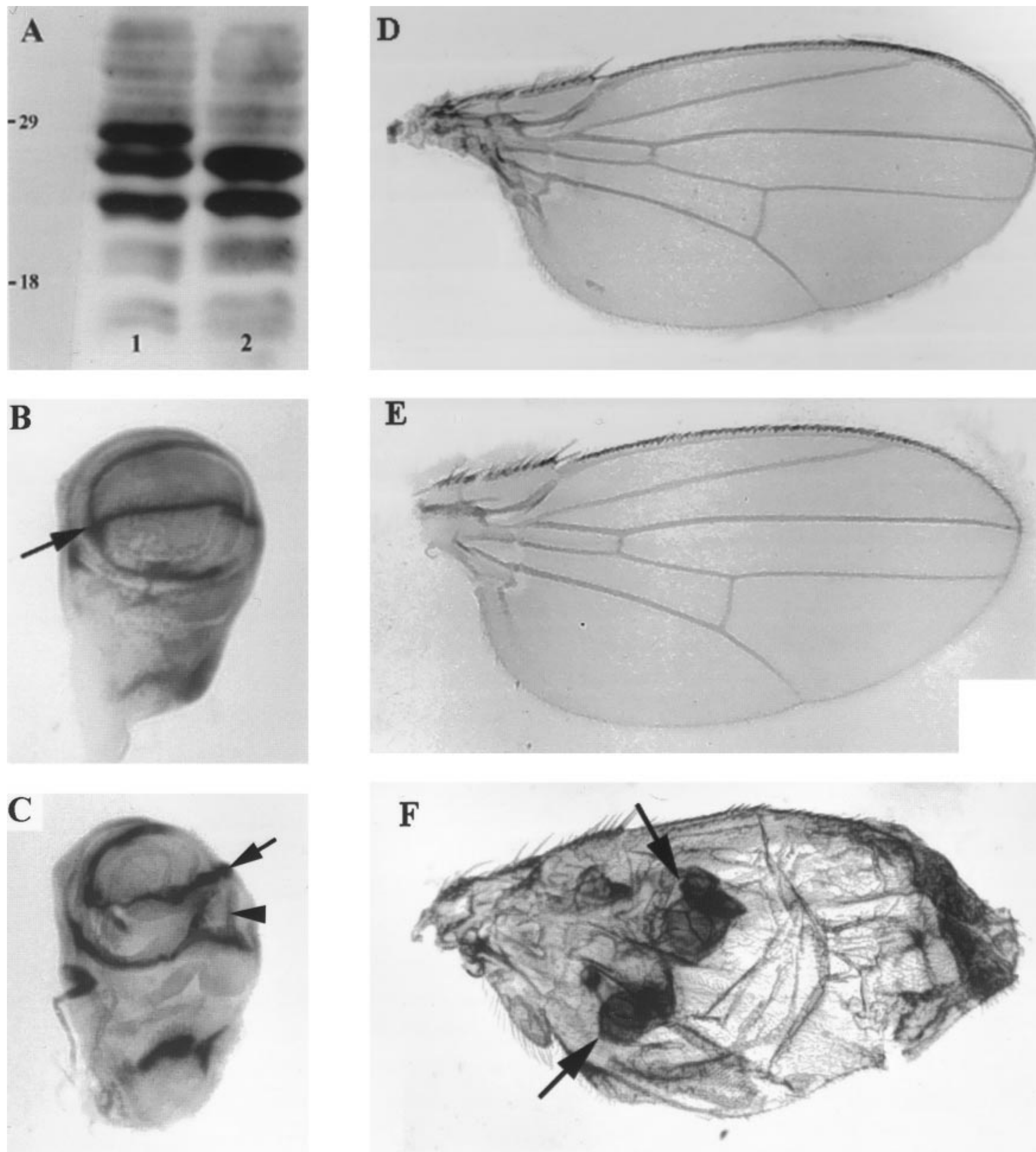

FIG. 2. (A) Western blot probed with α-Spitz monoclonal antibody. Wild-type eye discs (lane 1) express three major protein products that are recognized by this antibody. In discs from $S^{hs.8}$ homozygous escaper larvae (lane 2) only two of these bands are observed. (B, C) Third instar imaginal wing discs, stained with α-Wingless antibody, which labels the D/V margin cells (arrow). (D–F) adult wings. (B) Control *ap*-Gal4/+; UAS-*S*/+ discs show wild-type α-Wingless stain and no overproliferation. (C) UAS-*mspi*/+; *ap*-Gal4/+; UAS-*S*/+ discs are not wild type in appearance. The α-Wingless staining is distorted, but not expanded and the pouch shows uneven growth (arrowhead). (D) Control UAS-*mspi*/+; *ap*-Gal4/+ adult wings are wild type in appearance. (E) Control *ap*-Gal4/+; UAS-*S*/+ adult wings are wild type. (F) UAS-*mspi*/+; *ap*-Gal4/+; UAS-*S*/+ adult wing. The surface has blisters in it (arrow), indicative of uneven growth of the dorsal and ventral sides of the pouch. The wing veins are mostly missing, presumably due to misapposition of the two wing surfaces during pupal phases of wing development.

effects on the membrane-bound form of the Spitz proprotein.

Misexpression of sSpi causes an overproliferation of cells in the wing disc, whereas misexpression of the membrane-bound Spitz has no effect on cell division (Nagaraj *et al.*, 1998). The *apterous*-Gal4 driver can be used to give expres-

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sion in the dorsal portion of the wing pouch during the second and third instar larval stages. When this driver was used to express either a UAS–*Star* construct or the *mSpi* construct alone, neither gives a wing phenotype on its own in the wing disc (Figs. 2B, 2D, and 2E). However when *apterous*-Gal4 is used to drive the expression of both the UAS–*mSpi* and the UAS–*Star* constructs together, this gives rise to ectopic proliferation of cells in the wing pouch (Fig. 2C). The adult flies also show a wing phenotype where misapposition of the dorsal and ventral wing surfaces gives rise to blisters and missing and extra wing vein material (Fig. 2F). These results are consistent with a model where Star regulates the transition of mSpi to its active secreted form. As a control, ectopically expressed sSpi causes similar, but much more extensive growth of the wing pouch in the discs (not shown). However, adults of this genotype fail to eclose.

In summary, the perinuclear localization of the Star protein suggests that Star may be involved in Spitz ligand processing. Support for this hypothesis also derives from the fact that the overexpression of Star can affect Spitz activity in the imaginal discs. The evidence presented here and in Golembo *et al.* (1996) suggests that Star is probably involved in Spitz processing prior to its secretion. Further experiments will be required to understand the biochemical nature of Star involvement in ligand processing. Since it is clear that a mammalian homolog of Spitz, TGF-α, may also be activated prior to receptor binding, we anticipate that this work with Star may help to elucidate similar protein-processing systems in mammals.

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