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ORIGINAL PAPER

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Fluorescently labeled inhibitors detect localized serine protease activities in *Drosophila melanogaster* pole cells, embryos, and ovarian egg chambers

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Abstract Serine proteases are typically synthesized as proteolytically inactive zymogens that often become activated in a limited and highly localized manner. Consequently, determination of the spatial and temporal activation pattern of these molecules is of great importance to understanding the biological processes that they mediate. Until only recently, the tools to conveniently address the question of where and when serine proteases are active within complex tissues have been lacking. In order to detect spatially restricted serine protease activities in *Drosophila* embryos and ovaries we introduce a technique using fluorescent synthetic and protein-based inhibitors. With this approach we have detected a novel serine protease activity with a relative mobility of 37 kDa, localized to the surface of pole cells, the germ-line precursors, in embryos between nuclear cycles 11 and 14 in development. A second novel cell-specific protease activity was localized to the tissues of early gastrulating embryos. Microinjection of inhibitors into the perivitelline space of stage 2 embryos perturbed normal embryonic development. Fluorescein-conjugated chymotrypsin inhibitor and Bowman-Birk inhibitor labeled protease activity localized to the oocyte–somatic follicle cell interface of the developing egg chamber. Our results suggest that this technique holds promise to identify new spatially restricted activities in adult *Drosophila* tissues and developing embryos.

Keywords Oogenesis · Embryonic patterning · Chloromethyl ketone · Phosphonate · Zymogen activation

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Introduction

Proteases are recognized to play fundamental roles in virtually every aspect of an organism's biology. Limited proteolysis provides an effective way of modifying the properties of proteins and consequently the way in which they interact amongst themselves and with other molecules in cells and tissues (Neurath 1991). The proteolytic regulation of activation and assembly is well documented for many biological processes such as blood coagulation, complement activation, hormone production, and apoptosis (Neurath 1986). In *Drosophila melanogaster*, the high representation of serine proteases in the genome suggests that the serine protease superfamily plays an important role in its biology (Adams et al. 2000; Rubin 2000). Serine proteases have been found to be required for the specification of dorsal-ventral cell fates during embryonic development, imaginal disc development, apoptosis, and development of the nervous system (Hecht and Anderson 1992). One of the best-characterized functions of serine proteases is during early embryonic development when an extracellular cascade of ventrally restricted serine protease activities results in the ventrally restricted processing of a ligand which specifies global dorsal-ventral cell fates as a function of position (Dissing et al. 2001; LeMosy et al. 2001). There is evidence that they may be involved in extracellular signaling of the terminal system specifying the cell fates of the anterior and posterior extremes of the embryo and in activation of the humoral immune response (Furriols and Casanova 2003; Williams 2001). They are required for the evagination of leg imaginal discs probably at the level of extracellular matrix remodeling and tissue reorganization (Pino-Heiss and Schubiger 1989). Most of what we do

know about the roles of these enzymes in these pathways to date has come from genetic and molecular biological approaches which, although extremely powerful, possess inherent limitations when studying protease activity and function since the biologically interesting questions are often inherent of the dynamics of the system. For instance serine proteases with regulatory functions are typically synthesized as inactive precursors or zymogens that are often secreted and bound to membrane receptors or activation complexes. Zymogens provide a way of regulating the activity of the protease and enable extremely precise spatial and temporal refinement of the enzyme activation. A common property of protease regulated systems is that the conversion of the protease from its inactive zymogen form to the active form is the rate-limiting event (Milner et al. 2001). Thus, although protease zymogens may be relatively abundant and broadly distributed, the proteases may only be activated in a much more restricted spatial and temporal pattern. A classic example of this would be the blood coagulation system of higher vertebrates in which all of the components of the clotting cascade are present throughout the circulatory system, however activation of the cascade and clotting are highly localized and normally restricted to the wound site (Kisiel and Fujikawa 1983). Consequently, some of the more interesting questions of biological activity do not concern where and if a protease is expressed or even distributed but rather where and when it is activated. These questions cannot be addressed through conventional genetic or molecular biological approaches and require techniques which permit the localization of protease activities in situ (Lojda 1996).

A major way in which protease activities are regulated in vivo is through the action of proteins called serpins (SERine Protease INhibitors) (Travis et al. 1990). Serpins can bind preferentially to active proteases and form an essentially irreversible non-covalent adduct with the protease rendering it unable to further hydrolyze its substrate (Gettins et al. 1992). Specificity of the serpin is determined by the amino acid sequence of the molecule in the bait region near the scissile peptide bond.

In addition to naturally occurring protease inhibitors, small molecule protease inhibitors have been synthesized and these have been highly useful in characterizing the biochemical properties of proteases (Powers et al. 2002). Many different serine protease inhibitors have been chemically synthesized as transition state analogs facilitating study of protease reaction mechanisms. Due to the fact that they are transition state analogs derivatives, inhibitors such as the phosphonates and chloromethyl ketones are highly specific for the serine proteases. Furthermore the inhibitors can be synthesized as peptide derivatives enabling one to distinguish between subclasses of serine proteases with different substrate specificities such as trypsin-like or elastase-like serine proteases. One interesting application of fluorescently tagged derivatives of these mechanism-based inhibitors has been to detect, localize, and isolate serine proteases (Abuelyaman et al. 1994).

Here we describe studies that address the spatial distribution of activated serine proteases in living or fixed tissues and embryos of *Drosophila melanogaster*. Using fluorescently tagged serine protease inhibitors we have detected and characterized a novel serine protease activity localized to the pole cells, the germ line precursors in early embryos. A novel cell-specific activity is also localized to early gastrulating embryos. Two serpins with covalently attached Fluorescein reveal a novel zone of protease activity localized to the oocyte–somatic follicle cell interface of the developing egg chamber within the ovary. This activity changes spatial distribution dramatically during maturation of the developing oocyte.

Materials and methods

Stocks and reagents

The following flystocks were used: *tud*^{1} *bw*^{1} *sp*^{1}/CyO, l(2)DTS1001, *vls*^{1} *cn*^{1} *bw*^{1}/CyO, *cn*^{1} *stau*^{1} *bw*^{1}/CyO, *b*^{1} *vas*^{4} *pr*^{1} *cn*^{1}/CyO (Bloomington). P(*ry+, osk-bcd*)42; *ry* and P(*osk*+ AK65); *ry* were kind gifts of A. Ephrussi (Heidelberg).

The serine protease inhibitors used are listed in Table 1. The first three inhibitors were synthesized and the last two are commercially available (Haematologic Technologies). Serine protease inhibitor activity assays were done as described (Abuelyaman et al. 1994). The enzymatic hydrolysis rates of peptide *p*-nitroanilides (pNA) catalyzed by Chymotrypsin, PPE, and Trypsin were measured at 410 nm [e410=8,800 M cm^{-1}; (Erlanger et al. 1961)] on an LKB Ultraspec II. A buffer (0.1 M HEPES, 0.5 M NaCl, pH 7.5) containing 1–2% DMSO was used for Chymotrypsin (final concentration 2 μM) and PPE (final concentration 2 μM), and for Trypsin (final concentration 4 μM) a buffer (0.1 M HEPES, 0.01 M $CaCl_2$, pH 7.5) was used. Chymotrypsin was assayed with 0.24 mM Suc-Val-Pro-Phe-pNA (Bachem), Trypsin was assayed with 0.24 mM Z-Arg-pNA (Bachem), and PPE with 0.24 mM Suc-Ala-Ala-Ala-pNA (Bachem). Pseudo-first-order inactivation rate constants (k_{obs}) were obtained from plots of $\log(v_t/v_0)$, and the correlation coefficients (R^2) were greater than 0.98.

Table 1 Synthetic serine protease inhibitors used in these studies. Fla and Fitc are fluoresceinyl, Adp is hexanedioc acid, Aca is 6-aminocaproyl, AEBSF is 4-(2-aminoethyl) benzenesulfonyl fluoride, the three letter code is used for amino acid residues, AmPhGly is 4-amidinophenyl-glycyl (an arginine analog), $P(OPh)_2$ is diphenylphosphonate. Specificities are based upon either theoretical predictions or experimental data. Final concentrations represent the concentration present during incubation after embryo permeabilization

Inhibitor	Specificity	Type	Molecular weight	Final concentration (μM)
Fla-Adp-AEBSF	General	Sulfonyl fluoride	659.6	200
Fla-Adp-4-AmPhGlyP $(OPh)_2$	Trypsin	Phosphonate	875.3	200
Fitc-Aca-Phe-Leu-PheP $(OPh)_2$	Chymotrypsin	Phosphonate	1,116.2	200
Fitc-Glu-Gly-Arg-CH_2Cl	Trypsin	Chloromethyl ketone	936.6	160
Fitc-Phe-Pro-Arg-CH_2Cl	Trypsin	Chloromethyl ketone	882.0	120

Permeabilization and labeling of embryos

To allow inhibitors to penetrate the embryos, the chorion was first removed using standard procedures by dissolving the chorion in 3.75% sodium hypochlorite (Aldrich) at room temperature (RT) for about 90 s. After thorough rinsing in deionized water, the vitelline membrane was permeabilized by shaking the embryos for 14 s in isopropanol (HPLC grade; Aldrich) followed by 18 s in heptane (HPLC grade; Aldrich), washing three times quickly in PPS (54 mM NaCl, 12 mM Na_2SO_4, 35 mM K_2SO_4, 4 mM $CaCl_2$, 14 mM $MgSO_4$, 2 mM NaH_2PO_4, 1 mM Na_2HPO_4; van der Meer and Jaffe 1983) and washing 3×5 min in PPS/0.05% Tween 20. Following this treatment embryos continued developing for at least 4 h. Synthetic inhibitors were kept as 20 mM stock solutions in DMSO (HPLC grade; Fluka) at −80°C. Final dilutions in PPS of fluorescently labeled serine protease inhibitors are indicated in Table 1 and the incubation time was 30 min at 25°C with gentle rocking on a Nutator (Clay-Adams). As control, unlabeled embryos were incubated in parallel with Fluorescein at a final concentration of 200 μM. Developmental stages were determined by co-staining the permeabilized and labeled embryos or ovaries by incubating them for 4 min with Höechst (1 μg/ml; Polysciences) in PPS/Tween at RT and afterwards washing three times in PPS/Tween. Fluorescence microscopy was conducted on an Olympus BX50 microscope [100 W Hg lamp, ×40 and ×10 objectives, WIBA-filter cube (excitation 460–490 nm, barrier 515–550 nm), NG-filter cube (excitation 530–550 nm, barrier 590 nm), and WU-filter cube (excitation 330–385 nm, barrier 420 nm].

Immunoprecipitation

To immunopurify the labeled serine protease of pole cells 100–300 mg permeabilized and labeled embryos were washed twice in ice-cold Chan and Gehring buffered medium (55 mM NaCl, 40 mM KCl, 15 mM $MgSO_4$, 5 mM $CaCl_2$, 10 mM Tricine pH 7.0, 20 mM glucose, 50 mM sucrose, 1 mg/ml BSA) with protease inhibitors (EDTA-free Complete; Boehringer Mannheim). Embryos were homogenized in 20 vol buffer using a 7-ml Dounce homogenizer with a loose-fitting pestle on ice. The homogenate was filtered successively through 300-, 100-, and 60-μm nylon meshes and a crude cell pellet was obtained by centrifugation at 450 *g* for 10 min at 4°C. Isolated pole cells were dissolved in 100 μl RIPA (50 mM TRIS-HCl pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS) including EDTA-free Complete and vortexed for 2–3 s at maximum speed. The mix was boiled for 5 min and 100 μl non-denaturing lysis buffer (NON; 1% Triton X-100, 50 mM TRIS-HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, EDTA-free Complete) was added before drawing the liquid through a G27 syringe five to ten times (to break DNA). The tube was placed for 5 min on ice before it was spun for 5 min at 15,000 *g* and 4°C, and the supernatant transferred to a new tube containing an additional 500 μl RIPA buffer.

Immunoprecipitation by magnetic beads was done as per the manufacturer's instructions using 26 μg rabbit anti-FITC antibodies (Dako; V0403, 2.6 mg/ml) incubated with the pole cell lysate for 30 min at 0°C before adding 40 μl washed protein A magnetic beads (Dynabeads; Dynal). The beads were gently rotated for 30 min at 4°C and collected using a magnet (Dynal MPC), washed five times in ice-cold buffer (50 mM TRIS-HCl pH 7.4, 300 mM NaCl, 0.1% Triton X-100, 5 mM EDTA), and antibodies were released using 30 μl 0.1 M citrate for 2 min twice. This procedure was done three times and the citrate collections pooled with the magnetic beads of the last collection before being boiled in 2×SDS loading buffer for 5 min.

As controls we used unlabeled wildtype embryos, mock-purified samples from labeled pole cell-less embryos from *tud1/tud1* females, and immunoprecipitation from labeled wildtype embryos using rabbit anti-goat antibodies instead of the rabbit anti-FITC antibodies. To determine whether pole cells actually had been isolated, SDS-PAGE and western blots were performed as described using an antibody against the pole cell-specific protein Vasa.

SDS-PAGE [10%, 12.5%, or 15% acrylamide (30%/0.8%)] was performed using either the Hoefer Scientific Instruments minigel system or standard 12×14 cm gels. Western blots were made using PVDF membrane (0.45 μm, Immobilon-P; Millipore), 1×transfer buffer (25 mM TRIS-HCl pH 8.4, 1.4% glycine, 15% MeOH), and a BioRad electrophoretic blotter. The PVDF membrane was incubated for 1–2 h at RT in blocking buffer (PBS, 0.1% sodium azide, 0.25% Tween 20, 5% non-fat dry milk) with gentle rotation overnight at RT with primary antibody. Primary antibodies were either alkaline phosphatase-coupled goat anti-FITC antibody (Vector) 1:2,000 or rabbit anti-Vasa antibody (#103 IB5, generous gift from Ruth Lehmann, NYU) 1:5,000. Bound anti-FITC antibody was detected using a color reaction; 100 ml carbonate buffer (2 mM $MgCl_2$, 50 mM $Na_2CO_3/NaHCO_3$ pH 10.2) with 0.005% BCIP and 0.01% NBT which was allowed to develop for at least 1 h. Bound anti-Vasa antibody was revealed with alkaline phosphatase goat anti-rabbit IgG (Jackson Laboratories) 1:2,000 followed by color reaction.

Whole mount in situ hybridization

To test if pole cells had a role in specification of the terminal cell fates mutant embryos completely lacking pole cells were collected and dechorionized. The vitelline membrane was removed by shaking the embryos, 300 rpm (Kühner minishaker) for 3 min at RT, in a scintillation vial containing 4 ml heptane and 4 ml PPS/4% paraformaldehyde. The paraformaldehyde layer (bottom) was removed with a Pasteur pipette, 4 ml MeOH (HPLC grade; Fluka) was added, and the flask was shaken violently for 20–30 s. Devitellinized embryos were collected from the bottom and washed in MeOH before rehydration in PPS/Tween through an ethanol series (75%, 50%, and 30%) for 10 min each step. Afterwards the fixed and rehydrated embryos were washed, 3×5 min in PBT (PBS, 0.2% DEPC, 0.1% Tween 20; Sigma), and treated for 6–7 min with proteinase K (50 μg/ml; Boehringer Mannheim). Digestion was stopped by first 2 min and then 5 min incubation with PBT, 2 mg/ml glycine, and 10 mM phenylmethanesulfonyl fluoride (ultrapure; Boehringer Mannheim), and embryos were washed for 5 min in PBT before a second fixation for 20 min with 4% paraformaldehyde/PTW. Washing and hybridization were done as described (Tautz and Pfeifle 1989) using a slightly modified hybridization buffer, HYB [50% formamide (ultrapure; Schwarz/Mann Biotech), 5×SSPE (Sambrook et al. 1989), 100 μg/ml single-stranded DNA, 0.1% Tween 20], and incubating at 45°C with 2 μl DGG-RNA probe (the probes were made as described by Boehringer Mannheim digoxigenin labeling kit) in 48 μl HYB. Embryos were washed for 4×20 min in HYB at 45°C, 10 min in 1:1 PBT:HYB at RT, and 10 min in PBT. Incubation with anti-digoxigenin-coupled alkaline phosphatase (1:2,000; Boehringer Mannheim) and staining followed the original protocol.

Injections

To test if the serine protease inhibitors perturbed embryonic development, perivitelline injections of the inhibitors were done using 100–1,000 times recommended working concentrations and approximately 1% egg volume. The procedure was as described (Stein et al. 1991) aligning dechorionized stage 2 (just prior to pole cell formation) embryos along the edge of a coverslip under 10S Voltalef oil (Elf Aquataine) and injecting them (Femtotip; Eppendorf) using a Zeiss stereo microscope and a micromanipulator (Narishige). As control a BSA solution (1 mg/μl) was injected.

Labeling of serpins by succinimidyl esters

To create new fluorescent serine protease probes we labeled serpins using fluorescein-aminohexanoyl succinimide esters as recom-

mended by the manufacturer (Molecular Probes). The reagent was dissolved in MeOH and 0.25-mg aliquots were dried and kept at −20°C. Reactions were done in 0.15 M $NaHCO_3$ at pH 8.4 or 9.5 and molar ratios were 1:12 serpin:ester. The conjugated serpins were separated from the unconjugated dye on a Sephadex G25 column (bed volume of 9 ml; Pharmacia) equilibrated to PBS. A clearly visible band was collected in about 1 ml.

Labeling of ovaries

To allow inhibitors to detect serine protease activities in ovaries, these were dissected from about 20 well-fed 5- to 7-day-old females, as described (Verheyen and Cooley 1994) in a nine-well dish under PPS/Tween, pooled, split to single ovarioles, washed twice in PPS/Tween, and treated as above for embryos. The labeled serpin inhibitors FITC-chymotrypsin inhibitor 1 and the FITC-Bowman-Birk inhibitor due to unknown concentration were submitted as 10–100 μl PPS/Tween and incubated for 30–40 min. This dilution was sufficient to measure near complete inhibition of Chymotrypsin (2 μM) after 6 s using the pNA assay described above. As control we labeled the ovaries with hydrolyzed fluorescein-aminohexanoyl succinimide esters. The labeled ovaries were observed in the same way as embryos.

Results

Detection of serine protease activities in pole cells and in gastrulating embryos

To determine whether any serine protease activities might be localized to parts of the embryo during early *Drosophila* embryogenesis, we tested whether fluorescently conjugated serine protease inhibitors labeled specific tissues or cells. Stage 1–8 embryos (nomenclature of Wieschaus) were permeabilized with heptane and incubated with one of five different fluorescently tagged serine protease inhibitors in an isotonic buffer (see Table 1). After incubation, unbound inhibitor was removed by repeated washes. For several inhibitors, localized, tissue-specific labeling patterns were observed. The predominant labeling pattern was a strong fluorescence specifically localized to the pole cells (Fig. 1A, B) of embryos after incubation with Fla-Adp-AEBSF, Fla-Adp-4-AmPhGlyP-$(OPh)_2$, Fitc-Aca-Phe-Leu-PheP$(OPh)_2$, and Fitc-Phe-Pro-Arg-CH_2Cl. Although this pole cell-specific labeling was common to four inhibitors, fluorescence was most intense in embryos incubated with either Fla-Adp-4-AmPhGlyP-$(OPh)_2$ or Fitc-Phe-Pro-Arg-CH_2Cl. In any given experiment, most but not all embryos having pole cells prominently visible at the posterior pole exhibited fluorescence. Later, stage 8 embryos exhibited a symmetric pattern of fluorescence that colocalized with individual cells and groups of cells when incubated with the inhibitors Fla-Adp-AEBSF, Fla-Adp-4-AmPhGlyP$(OPh)_2$, Fitc-Aca-Phe-Leu-PheP$(OPh)_2$, and Fitc-Phe-Pro-Arg-CH_2Cl (Fig. 2A, B). As a control, under similar incubation conditions and concentrations, Fluorescein did not show any specific pole cell labeling or clustered cell labeling, only weak non-specific labeling of the yolk (data not shown). Since the inhibitors Fla-Adp-4-AmPhGlyP$(OPh)_2$ and Fitc-Phe-Pro-Arg-CH_2Cl associate with serine proteases

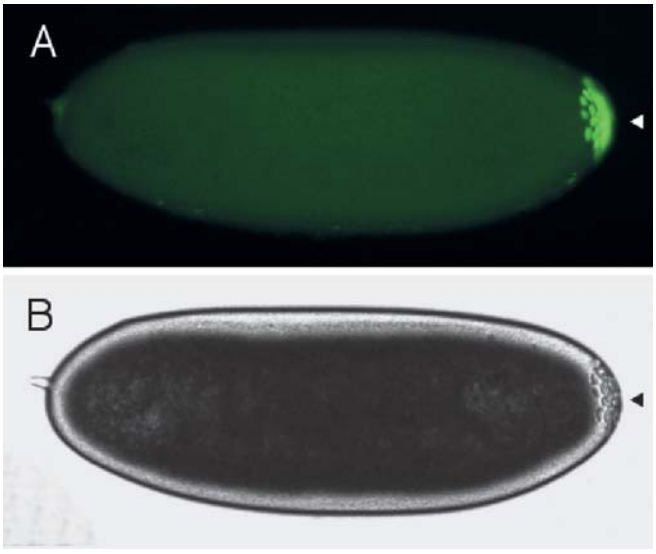

Fig. 1A, B Serine protease activity localized to the pole cells of a stage 4 embryo. **A** An embryo which was permeabilized and incubated with Fla-Adp-4-AmPhGlyP$(OPh)_2$ exhibiting pole cell-specific fluorescence indicating serine protease activity (*arrowhead*). The *Drosophila* embryo is oriented with anterior *left* and dorsal *up*. **B** The embryo in **A** shown by phase contrast optics. The dark material in the middle is yolk, the micropyle is seen at the anterior, the clear area along the rim is where the nuclei are and cellularization is about to take place. Pole cells are visible as discrete cells at the *far right* (*arrowhead*)

by different chemical mechanisms and free fluorescein does not label, we conclude that the fluorescence observed is due to covalent association of both inhibitors with active serine proteases and not due to a non-specific association with the pole cells. Both the pole cell labeling as well as the later pattern were detected by inhibitors with specificities toward chymotrypsin- and trypsin-like serine proteases suggesting that either one serine protease activity of broad specificity is detected or that more than one serine protease activity is present in or on the pole cells. The inhibitor Fitc-Glu-Gly-Arg-CH_2Cl did not specifically label any cells or tissues. Since pole cell labeling was most intense after incubation with Fla-Adp-4-AmPhGlyP$(OPh)_2$, we decided to further characterize the labeling properties of this inhibitor.

Serine protease activity is specific to the pole cells

To determine if the serine protease activity detected was an independent property of the pole cells as opposed to a property of the posterior region of the embryo, embryos laid by females homozygous for the posterior group mutation *tud1* were incubated with inhibitor. *tud1/tud1* females produce embryos which lack pole cells. In these embryos, no fluorescence was detected at the posterior pole where the pole cells emerge, however the later symmetric clustered cell staining seen in wildtype embryos was still observed at stage 8 and in older embryos. This result demonstrates that the protease activity de-

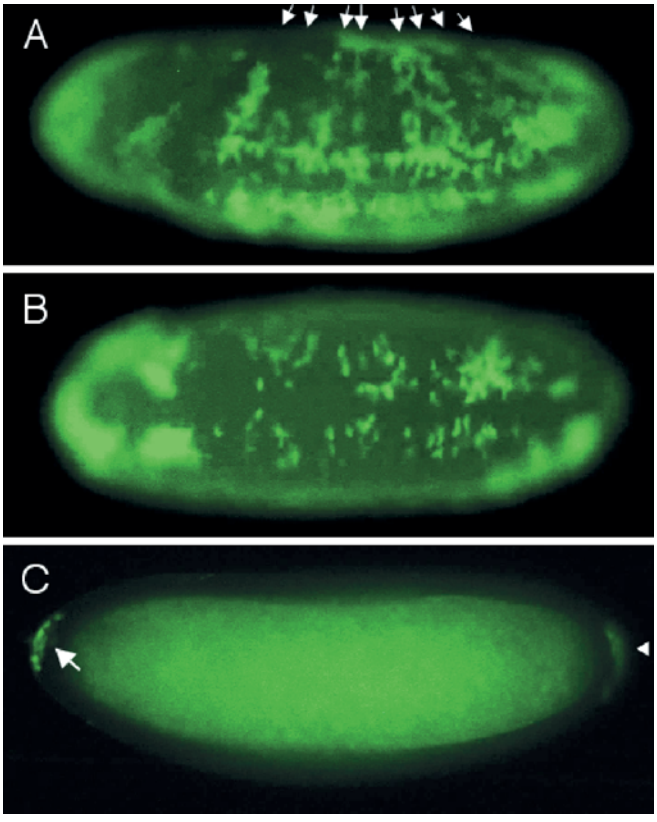

Fig. 2A–C Serine protease activity localized in symmetric patterns of stage 8 and later embryos. The embryos have been permeabilized and probed with the fluorescently tagged serine protease inhibitor Fla-Adp-4-AmPhGlyP$(OPh)_2$. All embryos are seen by fluorescent microscopy and are oriented with anterior *left*. **A** Lateral view of stage 8 embryo, slightly twisted so the ventral furrow is visible along the lower side. Stripes are seen to emanate from the ventral furrow toward the dorsal side (*arrows*). **B** Dorsal view. The embryo is seen from the dorsal side where the outline of several circular patterns can be distinguished. It thus seems as if a serine protease activity is localized to single cells or groups of cells localized in symmetric patterns of later embryos. **C** Serine protease activity is specific to the pole cells. A transgenic *osk-bcd* embryo has been permeabilized and probed with the fluorescently tagged serine protease inhibitor Fla-Adp-4-AmPhGlyP$(OPh)_2$. The serine protease activity is detected on the ectopic pole cells at the anterior (*arrow*) as well as the pole cells at the posterior (*arrowhead*). Nonspecific fluorescence is seen in the yolk, but not associated with any cells except pole cells. The serine protease therefore seems to be specifically localized to the pole cells

tected at the pole cells is indeed a property of pole cells and not a property of the plasma membrane from which the pole cell membranes bud. To further address this question, transgenic *osk-bcd* embryos, which develop functional pole cells both at the posterior and at an ectopic anterior position were incubated with inhibitor (Ephrussi and Lehmann 1992). In these embryos fluorescence was detected in pole cells both at the normal posterior position as well as the ectopic anterior position (Fig. 2C). Although most embryos exhibited fluorescence of both posterior and anterior pole cells, several of the *osk-bcd* embryos exhibited fluorescence only at the anterior and differences in intensity at anterior versus posterior sites were also seen. This suggested that the serine protease activity only appears some time after pole cells are formed and that expression of the activity involves an independent activation event.

Pole cell-specific serine protease activity is detected from nuclear division 11–14

To determine when the pole cell activity appears, consecutive collections of embryos using 15-min egg-laying windows were incubated with Fla-Adp-4-AmPhGlyP-$(OPh)_2$. The earliest detection of pole cell-specific labeling was shortly after pole cell formation (embryonic stage 3) and persisted up until about 215 min of development when the pole cells begin migrating dorsally along the rapidly elongating germ in what is thought to be a passive ride. During this the pole cells are seen situated in a dorsal pocket which is internalized as part of the epithelial sheet that will eventually form the midgut at about 225–270 min of development and marking the beginning of embryonic stage 8. Pole cells, which had begun to migrate to the dorsal side of embryos were never seen to exhibit any fluorescence. However, since the nuclei of early embryos divide in a precise temporal sequence, a biologically more precise method would be to correlate the pole cell activity with the nuclear division cycle. Therefore, the nuclear division cycle was determined by double labeling of embryos with Höechst 22358 to stain DNA and Fla-Adp-4-AmPhGlyP$(OPh)_2$ to visualize protease activity. Embryos were precisely staged by counting nuclei (Fig. 3; Zalokar and Erk 1976). Pole cell fluorescence was first detected at nuclear division 11, shortly after the first nuclear division following pole cell formation and as late as nuclear division 14 when the embryo has cellularized. Therefore, the protease activity had a maximal activity window of about 2 h, terminating at the end of embryonic stage 6 just before the pole cells move dorsally. The fluorescence was observed to cover each pole cell entirely and clearly cover a larger area than the decondensed chromosomes within pole cells suggesting the serine protease activity is localized to the pole cell plasma membrane (data not shown).

Immunoprecipitation of a Fla-Adp-4-AmPhGlyP$(OPh)_2$-labeled 37-kDa polypeptide from 0- to 2-h embryos

To determine the relative mobility of the protease or proteases which we had detected, we used immunoprecipitation. Since the inhibitors bind covalently to serine proteases it is possible to immunoprecipitate the labeled protease using antibody directed against the fluorescein moiety of the inhibitor. We tested this approach by labeling Trypsin with Fla-Adp-4-AmPhGlyP$(OPh)_2$ and found that trypsin could be efficiently immunoprecipitated and detected (data not shown). Pole cells from permeabilized and labeled wildtype embryos were par-

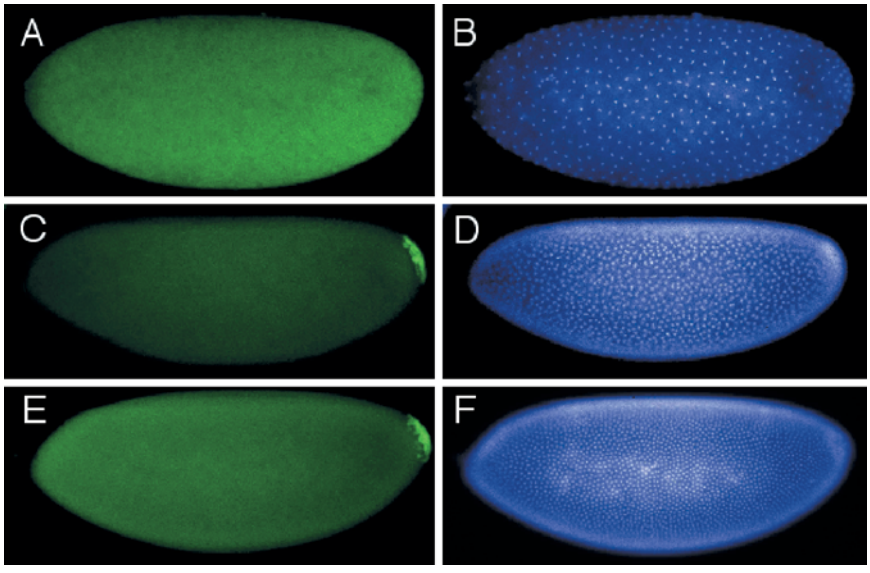

Fig. 3A–F Pole cell-specific serine protease activity is detected in nuclear cycle 11–14. The embryos have been double stained with the serine protease inhibitor Fla-Adp-4-AmPhGlyP(OPh)$_2$ (**A**, **C**, **E**) and the DNA stain Höechst 33258 (**B**, **D**, **F**). All embryos are oriented with anterior *left* and dorsal *up*. Representative embryos of three different nuclear cycles are shown beginning with a stage 2 embryo not yet having pole cells in **A** and **B** (nuclear cycle 9), showing fluorescent pole cells in **C** and **D** (nuclear cycle 12), and progressing to a nuclear cycle 14 embryo in **E** and **F**. The number of the nuclei increases accordingly and the exact stage can be determined by counting the nuclei (Zalokar and Erk 1976). In **F** one can distinguish polyploid yolk nuclei in the center of the embryo. In **A**, **C** and **E** various degrees of unspecific uniform fluorescence are seen

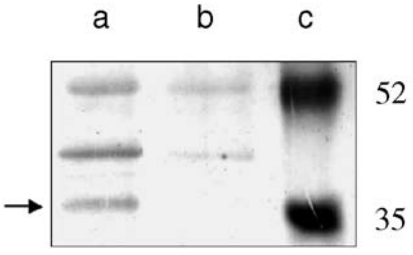

Fig. 4 Immunoprecipitation of a pole cell-specific protease via conjugated fluorescein. Western blot comparing immunoprecipitation using anti-Fluorescein antibodies from Fla-Adp-4-AmPhGlyP(OPh)$_2$-labeled wildtype pole cells (*lane a*) and a mock-purification from pole cell-less *tud1* embryos (*lane b*). A polypeptide with a molecular weight of 37 kDa (see *arrow*) is present in *lane a* but not in the mock purification in *lane b* indicating a pole cell-specific polypeptide is associated with the inhibitor. *Lane c* shows molecular marker weight markers

tially purified and lysed using an immunoprecipitation buffer. After immunoprecipitation and immunoblotting with anti-Fluorescein antibody, a 37-kDa polypeptide was consistently found to be associated with labeled pole cells (Fig. 4 *lane a*). This band was not detected in controls (Fig. 4 *lane b* and data not shown). The single 37-kDa band suggests that Fla-Adp-4-AmPhGlyP(OPh)$_2$ primarily reacts with a single serine protease.

Pole cell protease activity is not required for pole cell migration or terminal pathway signaling during embryogenesis

To address the question of possible roles of the Fla-Adp-4-AmPhGlyP(OPh)$_2$-labeled pole cell protease activity in embryonic patterning, we introduced Fla-Adp-4-AmPhGlyP(OPh)$_2$ into the perivitelline space by microinjection and followed the immediate developmental consequences in living embryos by observing gastrulation. Since the serine protease activity disappears just before pole cell migration, a possible function of the protease might be to release the pole cells from some form of tether at their posterior position allowing them to move dorsally. To test this hypothesis, Fla-Adp-4-AmPhGlyP(OPh)$_2$ was microinjected into the perivitelline space of stage 2 and 3 embryos and the position of the pole cells determined by antibody staining for Vasa, a pole cell-specific antigen, after 4 h of further development. The position of the pole cells in the injected embryos was indistinguishable from that of the injected control embryos (data not shown). None of the injected embryos (n=200) showed any immediate deviations from the expected position of the pole cells such as embryos having developed past germ band elongation (stage 7) which still had pole cells located at the posterior pole or stage 7–8 embryos with an empty dorsal pocket. We conclude that the pole cell-specific serine protease activity was not required for the initial phase of pole cell migration.

A second possibility was that the pole cells provide a localized posterior determinant for a signaling pathway. Localized protease activities within the perivitelline space

Table 2 Perivitelline injections of serine protease inhibitors and their effects upon embryonic development. Concentration represents the concentration present in the injected fluid. Injected volumes were that amount sufficient to noticeably widen the perivitelline space between the vitelline and plasma membranes with minimal perturbation of the fluid volume of the embryo. Number of cuticles indicates the number of embryos which produced clearly interpretable cuticular features. (*vd* Ventral denticles, *mh* mouth hooks, *fk* filzkörper)

Inhibitor	Concentration	Number	Number of cuticles	Percent developed	Appearance
BSA control	1 mg/ml	196	92	50	Wildtype
α-1 Antitrypsin	200 μM	93	16	20	Absence of vd, mh, and fk
Aprotinin	2 mg/ml	111	28	30	Most wildtype, few ventral holes
Bowman-Birk inhibitor	100 mg/ml	147	3	0	No vd, few or no mh and fk
Chymotrypsin inhibitor type I	10 mg/ml	205	4	0	No fk or mh, very narrow vd
Elastatinal	2 mg/ml	104	52	50	Wildtype
Trypsin inhibitor type II-T	50 mg/ml	133	11	10	Wildtype
Trypsin inhibitor type I-S	100 mg/ml	94	44	50	Wildtype
Trypsin inhibitor type IV-O	100 mg/ml	91	15	20	Minor narrowing of vd at injection site

have been proposed in the terminal signaling pathway specifying the cell fates at the posterior part of the embryo via the processing of Trunk (Casali and Casanova 2001). To determine whether the pole cell-specific protease activity is involved in terminal signaling, pole cell-less embryos of homozygous posterior flies lacking the function of posterior group genes (*stau1*, *tud1*, *vas1*, and *vls1*) were in situ hybridized to look for expression of *tailless* (*tls*) and *hückebein* (*hkb*), two zygotic genes which are activated in response to the terminal pathway output. In embryos lacking the terminal group gene *trunk*, *tls*, and *hkb* are not expressed at the posterior. Only about 5% of embryos produced by *vls1/vls1* females cellularized, however all which cellularized exhibited expression of *tls* and *hkb* in spite of the fact that they lacked pole cells (data not shown). The other posterior group embryos also exhibited expression of *tls* and *hkb* mRNA at normal to slightly reduced levels. We determined that the pole cell-specific serine protease is not absolutely required for terminal signaling. Our results are in essential agreement with previous studies which showed that in embryos lacking *oskar*, *tls* and *hkb* are still expressed albeit the expression is slightly reduced (Bronner and Jackle 1991).

Perivitelline injections of serine protease inhibitors and the synthesis of novel fluorescently tagged inhibitors

To address the role of localized protease activities in embryonic development and to generate reagents that would be of potential use for studying these protease activities, we asked whether any of a number of commercially available serine protease inhibitors were capable of interfering with embryonic development. We chose a microinjection assay to introduce these inhibitors into the perivitelline space of early embryos. We chose inhibitors for these experiments that were effectively irreversible and relatively soluble in aqueous solution. The inhibitors were microinjected into the perivitelline space of stage 2 embryos and the embryos were allowed to develop for 24 h; cuticle preparations were done to display any developmental phenotypes (Table 2). Inhibitors which interfered with development included: α-1 antitrypsin, Bowman-Birk inhibitor, chymotrypsin inhibitor I, and trypsin inhibitor type II-T. We chose to further investigate the activity of Bowman-Birk inhibitor and the chymotrypsin inhibitor 1 by conjugating Fluorescein to these inhibitors. The conjugates were purified, concentrated, and tested to have retained inhibitory activity upon a commercial chromogenic substrate by spectrophotometric assay (data not shown).

Fluorescein labeling of serine protease activity at the oocyte-follicle cell interface of stage 7 and later egg chambers

We were unable to test Bowman-Birk inhibitor and chymotrypsin inhibitor 1 in permeabilized embryos since the permeabilization technique should only enable smaller molecules to penetrate embryonic tissues. However, when we incubated these inhibitors with dissected ovarian egg chambers from adult female flies we found tissue-specific patterns of labeling. Using either tagged inhibitor, fluorescence was detected beginning in stage 7 egg chambers [stages reviewed by King (1970) where it appeared at the posterior of the oocyte; Fig. 5A *left*]. In stage 8 and 9 egg chambers, the labeling extended anteriorly into an open cup-like pattern (Fig. 5A *right*). In all cases the activity localized to the apical surface of the follicle cells in the zone of contact between follicle cells and the growing oocyte. Stage 10 egg chambers exhibited strong labeling over the entire oocyte at the follicle cell oocyte interface including the anterior region facing the nurse cells (Fig. 5C). As a control, we treated ovarian egg chambers with hydrolyzed fluorescein succinimide ester and found neither fluorescence nor weak non-specific overall fluorescence, indicating that the labeling was specific. We are unaware of any previously described protease with such an expression pattern in the developing egg chamber. Our results suggest that a posterior to anterior wave of proteolytic activity is generated during oocyte development specifically within the zone of contact between the follicle cells and the developing oocyte.

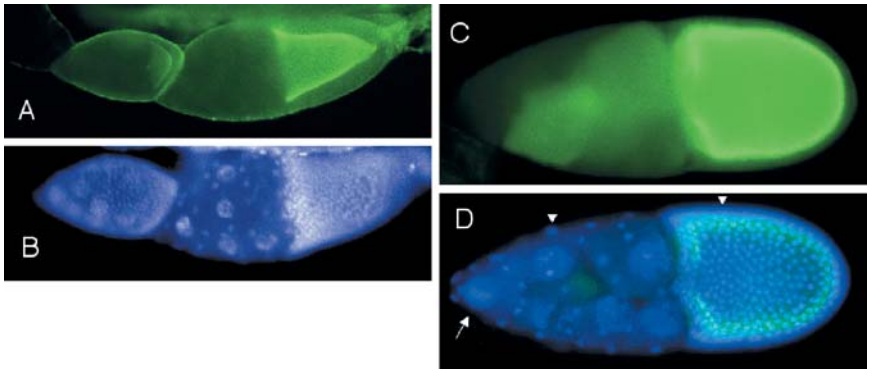

Fig. 5A–D Serine protease activities localized to the oocyte–follicle cell interface. Dissected egg chambers were stained with Höechst (**B**, **D**) and incubated with either fluorescein-conjugated chymotrypsin inhibitor 1 (**A**) or fluorescein-conjugated Bowman-Birk inhibitor (**C**). Egg chambers are oriented with anterior *left*, each egg chamber consists of a thin sheet of follicle cells surrounding 1 large oocyte at the posterior and 15 large nurse cells. This is best seen in the Höechst stainings (**B**, **D**) where the nurse cells can be easily distinguished by their giant polyploid nuclei (*arrow* in **D**) which are much larger than the follicle cell nuclei (*arrowheads* in **D**). The follicle cell sheet covering the oocyte and nurse cells is seen to withdraw from the nurse cells to cover the oocyte densely in later stage egg chambers. Along with the size of the oocyte the position of the follicle cells is used to determine the developmental stage of the egg chamber. In **A** and **B** two consecutive egg chambers from the same ovariole stage 7 (*left*) and stage 9 (*right*) are seen, and in **C** and **D** a stage 10 egg chamber is seen. The oocyte–follicle cell fluorescence is seen to arise in early stage 7 egg chambers as a fluorescence in the posterior third of the oocyte (**A**, left egg chamber). In the later oocytes the fluorescence is forming in a cup-like shape which follows the oocyte–follicle cell interface and finally surrounds the oocyte entirely. The same pattern was seen for both inhibitors and the activities are continually detected in more mature egg chambers as a fluorescence which localizes to the oocyte–follicle cell interface

Discussion

The technique described here has revealed the presence of novel localized serine protease activities associated with three distinct tissues during the development of *Drosophila*. A pole cell protease activity is expressed in a time window of less than 2 h beginning shortly after pole cells bud from the plasma membrane and ending just before the pole cells are carried by gastrulation movements along the dorsal midline and ventrally into the midgut (Campos-Ortega and Hartenstein 1985). This pole cell-specific activity is associated primarily with a polypeptide of 37 kDa, a size which is within the typical range for active serine protease catalytic chains. For example, the Easter serine protease catalytic chain exhibits a relative mobility of 35 kDa on SDS polyacrylamide gels (Chasan et al. 1992). Although we cannot rule out the possibility that additional serine proteases also react with the inhibitor in the early embryo, the 37-kDa protease would appear to be the major target of Fla-Adp-4-Am-PhGlyP$(OPh)_2$ in pole cells.

The window of expression may provide a clue to the function of the protease in pole cells. We can first rule out several possible roles for this protease based upon our data. It does not appear to be required for release of the pole cells from their posterior position within the perivitelline space since microinjection of Fla-Adp-4-Am-PhGlyP$(OPh)_2$ as well as several other inhibitors fails to prevent initial movement of pole cells from their initial location at the posterior pole across the dorsal midline. The protease activity does not appear to provide a localized posterior cue for processing the Trunk protein precursor, a Cys-knot growth factor ligand which must be specifically activated at the posterior pole prior to the cellular blastoderm stage in order to specify terminal cell fates (Furriols and Casanova 2003). One possible function of the protease activity is to facilitate cell proliferation of the pole cells after they are formed by budding off from the plasma membrane (Swanson and Poodry 1980). Pole cells are known to undergo two rounds of cell division after budding off and prior to dorsal displacement. We addressed this question by counting pole cell number in embryos incubated with Fla-Adp-4-AmPhGlyP$(OPh)_2$. Based upon a sample of n=100 embryos, we have not seen any difference in pole cell numbers in treated versus untreated embryos. We therefore have no evidence indicating that the protease activity is required for cell proliferation.

Another possibility is that the pole cell protease activity is involved in respecification of the pole cell membrane in order to differentiate pole cells from the embryonic plasma membrane from which they are derived. Since certain plasma membrane-derived surface proteins on newly budded pole cells would not be required by pole cells, the protease activity could be involved in eliminating plasma membrane proteins from pole cell membranes. Yet another possibility is that it is involved in the processing of Spaetzle ligand precursor on the surface of pole cells enabling the nuclear accumulation of Dorsal in pole cells under regulation independent of the dorsal/ventral protease cascade (DeLotto et al., manuscript submitted). Further studies will be required to resolve this issue.

Several of the inhibitors, upon microinjection, were capable of interfering with embryonic development. Dorsal-ventral patterning in *Drosophila* is a process known to involve a protease cascade and prior studies have indicated that microinjection of soybean trypsin in-

hibitor type I can dorsalize the embryo (Dissing et al. 2001; Stein and Nusslein-Volhard 1992). Although none of the reagents which we tested specifically labeled any activities which might be arguably involved in dorsal-ventral patterning, we feel that this approach may still be useful to identify the spatially restricted activation of this system. This may require us to find an inhibitor with the correct substrate specificity in order to selectively label components of the dorsal-ventral protease cascade.

The later staining, observed at stage 8 with Fla-Adp-AEBSF, Fla-Adp-4-AmPhGlyP$(OPh)_2$, Fitc-Aca-Phe-Leu-PheP$(OPh)_2$, and Fitc-Phe-Pro-Arg-CH_2Cl, appears to correlate with symmetric clusters of cells. The clusters are distributed in a commissure-like pattern along the body as well as in the head region and therefore may correspond to parts of the developing peripheral and central nervous systems. Further analysis using nervous system markers will be necessary to resolve this issue.

The appearance of protease activity at the follicle cell–oocyte interface in the developing egg chamber occurs at a time in which the oocyte undergoes a transition from the previtellogenic to the vitellogenic phase. Stage 7 is characterized by the elongation of the oocyte and the transition of follicle epithelial cells to a columnar shape. Yolk formation and uptake begins in stage 8. In stage 9, the follicle cells begin to move over the oocyte surface, undergoing a change of shape and the deposition of vitelline membrane begins. The localization and timing suggest that the protease activity we detect may be involved in some form of interaction between the follicle cells and the growing oocyte. An interesting correlation exists with the region of the follicle cell oocyte interface expressing the protease activity and that part of the follicle cell undergoing a transition to a columnar shape. It has been shown that follicle cells at the posterior begin to adopt a columnar shape arguably to facilitate the gross movement of the follicle cells to surround the growing oocyte. A mechanism has been proposed for the migration and shape change suggesting that either a new high-affinity ligand or a new receptor appears on the surface of the follicle cell membranes at the posterior of the oocyte during stage 8 (Spradling 1993). We propose that the protease activity might be involved in the generation of a signal for shape change by locally processing an extracellular ligand to which follicle cells respond. Alternatively, the protease activity may be involved in extracellular matrix remodeling as part of the process of follicle cells adopting the columnar shape. Relatively little is known about extracellular matrix proteins in the egg chamber apart from the fact that they are present (Fessler and Fessler 1989). However, based upon the general role of the extracellular matrix in tissue patterning, it would not be unreasonable to test for a role of the protease activity in ECM-mediated follicle cell differentiation.

We have reported the use of fluorescently tagged serine protease inhibitors in developing embryos and oocytes of *Drosophila*. Our results suggest that localized protease activities can indeed be detected in defined temporal and spatial patterns. The technique may have certain limitations for the observation of rapid phenomena and we cannot rule out that incubation with protease inhibitors has deleterious effects upon further development. Nonetheless, this approach is a useful and viable method to find localized activities with cellular or subcellular resolution. Three novel patterns of activity were described and their location and timing are most consistent with a role in the interaction of cells with their local environment and in their differentiation. Further work will be required to define the unique biological roles of these proteases, however the data presented here demonstrate that the overall technique provides a powerful new approach to detect localized protease activities in the context of both adult *Drosophila* tissues and within developing embryos.

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