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The proteasome-ubiquitin pathway in the *Schistosoma mansoni* egg has development- and morphology-specific characteristics

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ABSTRACT

Schistosoma mansoni eggs, consisting of an ovum surrounded by nutritive vitelline cells packaged in a tanned protein shell, are produced by paired worms residing in the mesenteric veins of the human host. The vitelline cells are degraded as the larval miracidium matures, the fully developed egg either crossing the gut wall to escape the host or becoming lodged in the host's tissues where it dies and disintegrates, inducing a potentially pathological immune response. Thus, the egg is central to both the transmission of the parasite and the aetiology of the disease. Here we present the first study investigating protein turnover in the egg. We establish that the ubiquitin-proteasome pathway (UPP) changes with egg development and furthermore, that the morphological components of the fully developed egg (the miracidium and the subshell envelope) also exhibit different proteasome subunit expression profiles. We conclude that the UPP is responsible not only for degrading the vitelline cells but is also more highly developed in the envelope than in the miracidium. The envelope is involved in the defence of the miracidium and produces the proteins that the egg secretes, presumably to facilitate its escape from the host, so the UPP probably has a multi-faceted role in the egg's biology.

1. Introduction

The ubiquitin-proteasome pathway (UPP) is an intracellular system in which large, multi-subunit, non-specific proteolytic machines called proteasomes degrade proteins that have been tagged with chains of ubiquitin. The UPP is involved in numerous processes, from the recycling of damaged proteins to the regulation of the cell cycle, apoptosis and transcription [1–3]. Proteasomes are comprised of α and β subunits that assemble as seven-member rings. Four rings stack together to form a barrel-like particle in the conformation α_{1-7}, β_{1-7}, β_{1-7}, α_{1-7}, called the 20S proteasome [4–6]. It has six proteolytic subunits with active sites pointing towards the centre of the ring, a structure ensuring that uncontrolled degradation of proteins is avoided by confining proteolysis to the internal chamber of the proteasome [7]. The proteolytic subunits are located at the β_1, β_2 and β_5 positions and have different substrate specificities (β_1; caspase-like, β_2; trypsin-like and β_5; chymotrypsin-like). Acting in consort, they degrade most proteins to peptides of 3–22 amino acids in length, depending on their amino acid sequence [8]. The post-proteasomal peptides are then further degraded into their constituent amino acids in the cytosol by conserved families of endopeptidases, aminopeptidases and carboxypeptidases [9]. Target proteins enter the 20S proteasome through the aperture in the centre of either outer α-subunit ring, the process being controlled by a multi-subunit component called the 19S regulatory complex (also known as PA700), which caps the 20S proteasome. The 19S regulatory complex recognises the ubiquitin tag, cleaves off the ubiquitins one-by-one (for reuse), unwinds the substrate protein and feeds it into the 20S proteasome. The active proteasome, consisting of the 20S proteasome core plus its 19S regulatory complex is called the 26S proteasome (30S when doubly capped). Both the 20S proteasome core and the 19S regulatory subunits undergo post-translational modifications, including phosphorylation, N-acetylation and O-GlcNAc glycosylation [10–14]. Regulation also occurs via a family of proteins called "proteasome interacting proteins" (PIPs) that associate with the 19S regulatory complex [15].

Ubiquitin is a multi-functional 8.5 kDa protein of 76 amino acids, best known for its role in the UPP. The tag recognised by the 19S proteasome regulatory complex consists of a chain of at least four ubiquitins, where the C-terminus of each ubiquitin is linked to lysine 48 of the preceding ubiquitin [16]. However, other forms of protein ubiquitinylation are commonplace: a single ubiquitin can be attached to the substrate (mono-ubiquitinylation), or several

Abbreviations: ESP, egg secreted proteins; PIP, proteasome interacting proteins; SEA, soluble egg antigen; UPP, ubiquitin-proteasome pathway.

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ubiquitins attached to different residues (multi-ubiquitinylation). A lysine-63-linked poly-ubiquitin chain attached to a substrate protein is associated with non-proteolytic functions such as endocytosis, signal transduction, transcriptional regulation, ribosome function and DNA-repair pathways [17]. Ubiquitin's central structure, the "ubiquitin superfold" is also incorporated into much larger proteins, collectively called "ubiquitons". These are less-well studied, but they include kinases and kinase-interacting proteins, so are likely to be involved in signal transduction [18–20]. Ubiquitons have also been identified as transport proteins that bind to poly-ubiquitinylated proteins and transport them to the 26S proteasome for destruction [21–23].

Schistosomiasis is a tropical parasitic disease caused by trematode worms of the genus *Schistosoma.* Adult *S. mansoni* reside in the mesenteric veins producing eggs, some of which pass through the tissues of the gut wall, enter the gut lumen, exit the host in faeces and rupture when encountering fresh water to release the miracidium larva. However, some eggs fail to adhere to the mesenteric venous epithelium and are washed away in the bloodstream to become lodged in the portal venules of the liver. Here they mature, secrete proteins, then die and disintegrate, inducing a granulomatous response which causes severe pathology in some individuals [24]. Thus, the egg and its development are crucial in the aetiology of the disease.

The newly released schistosome egg consists of an ovum surrounded by vitelline cells encased in a cross-linked protein shell [25,26]. It takes a week to mature, during which time the vitelline cells degrade, providing nutrients for the developing miracidium. A syncytial envelope, known as "von Lichtenberg's envelope" [25] forms between the developing miracidium and the shell, and produces a simple mixture of proteins termed egg secreted proteins (ESP). At least one protease is present, presumably required to facilitate the egg's escape through the host tissues [26,27]. The space between the miracidium and the envelope contains a viscous liquid called hatch fluid and large vesicles that could be the remains of spent vitelline cells. Hatch fluid is enriched in defence proteins which probably offer the miracidium and the envelope protection from toxins derived from host leukocytes [27]. Thus, the fully developed *S. mansoni* egg is multifaceted, with its contents (the miracidium, ESP and hatch fluid) having markedly differing roles. Although Mathieson and Wilson [27] found the miracidial, ESP and hatch fluid proteomes to be markedly different, reflecting their respective functional roles, it is noteworthy that the differences were less clear-cut for those proteins involved in protein turnover, principally the proteins of the UPP.

The UPP has previously been studied in *S. mansoni* cercariae, lung-worms and adults [28,29]. In the mouse model, proteasome inhibitors prevent larval development and reduce adult worm burden [28]. Although each of the fourteen *S. mansoni* proteasomal subunits are encoded by a single copy gene, they separate into 58 spots on a 2D gel, so they must be post-translationally modified [30]. In the spot-pattern for each subunit, a single large intensely staining spot lies in close proximity to several smaller spots which differ slightly in charge but not mass, suggesting that the post-translational modifications consist of different degrees of phosphorylation. When 2D Western blots of adult and cercarial preparations were probed with an antibody that recognises 20S proteasome α-subunits, Castro-Borges et al. [30] were able to show that the spot patterns were different in each preparation, thereby demonstrating that the pattern of post-translational modification differed in the two life-cycle stages.

The work in this paper follows on from the Guerra-Sa et al. [28] and Castro-Borges et al. [30] papers. The extent to which 20S α-subunit expression changes as the egg develops was assessed by comparing 2D Western blots of a vitellaria-enriched preparation (Vit) from adult females (representing the egg's vitelline cells), an undeveloped egg preparation (SEAu) and a fully developed egg preparation (SEAd). *In vitro* fluorogenic substrate assays were used to determine whether the changes in α-subunit expression revealed by blotting could be linked to the proteolytic activity of the proteasome. Western blotting was also used to compare the proteasome α-subunit expression pattern in a miracidial preparation (Ms) and a hatch fluid preparation containing envelope proteins (Hf) to gain insights into how the UPP might be employed by the egg to aid its passage through the gut wall and successfully emerge into the gut lumen. The extent of ubiquitinylation in SEAu, SEAd, Ms and Hf was then established by blotting and linked back where possible to the information obtained with regard to proteasomal expression and activity.

2. Materials and methods

2.1. Parasite material

Male C57BL/6xCBA mice were each infected with 180 cercariae of a Puerto Rican isolate of *S. mansoni* via the shaved abdomen [31]. Seven weeks later the mice were euthanized by CO_2 inhalation and eggs at all stages of development were recovered from their livers by trypsin digestion, then cleaned by washing and sieving [26]. Ethical Approval for this work was granted by the University of York Ethics Committee and all animal procedures were carried out within the Codes of Practice of the United Kingdom's Animals (Scientific Procedures) Act (1986). Egg preparations were made as previously described [27], but briefly, undeveloped eggs were separated from developed eggs using a Percol gradient. SEAu and SEAd were made by grinding either immature eggs or mature eggs in M3 buffer (7 M urea, 2 M thiourea, 4% CHAPS and protease inhibitor cocktail (all Sigma, Poole, UK)), centrifuging them and retaining the supernatant. Ms and Hf were made by hatching mature eggs in a glass test-tube. Transverse illumination attracted the miracidia to the top of the tube; these were collected by filtration then processed as in SEAu and SEAd to make Ms. The bottom 3 ml of test tube sample (consisting of extracellular hatch fluid proteins and intracellular envelope proteins washed from the eggs as the miracidia emerged) was gravity-filtered, concentrated and then solubilised in M3 buffer to produce Hf. Adult worms were recovered from infected mice by portal perfusion with RPMI-1640 (Gibco, Renfrew, UK) to which 10% FCS, 2.5% HEPES and 5 units heparin/ml (all Sigma) had been added. Paired females were separated from males using two fine brushes and then washed three times in RPMI-1640 to remove the FCS. The females were then severed laterally immediately below the ventral sucker and the posterior sections (containing the vitellaria) transferred into 200 μl M3 buffer containing 10% glycerol (Sigma). Once approximately 50 worm posteriors had been collected in this way the 'Vit' preparation was made by grinding and centrifuging the sample in the same way as in SEA and Ms.

2.2. Western blotting

Soluble proteins were fractionated by standard two-dimensional electrophoresis techniques [32]. Samples containing 35 μg of SEAu, SEAd, Ms, Hf and Vit were adjusted to 125 μl with re-hydration buffer (M3 buffer containing pH 3.5–10 resolytes (BDH, VWR International, Dorset) plus DTT and bromophenol blue (both Sigma)). The preparations were then separated according to their charge by isoelectric focusing (IEF) in 7 cm, pH 3–10 IEF strips (Applied Biosystems, Warrington, UK) using the following protocol: 12 h rehydration followed by 500 V (30 min), 1000 V (30 min) and then at 50 μA for 4 h. Focussed proteins were reduced (1% DL-dithiothreitol (Sigma)) and alkylated (4% iodoacetamide (Sigma)) and then the IEF strips were transferred to the top of

7 cm NuPAGE 4–12% Bis–Tris gels (Invitrogen, Renfrew, UK). The proteins were then separated by molecular mass by applying 200 V for 40 min. Immediately after electrophoresis, each gel was placed on 8.3 cm × 7.3 cm Invitrolon 0.2 μm polyvinylidenefluoride (PVDF) membrane (Invitrogen) and installed in an XCell II Blot Module (Invitrogen). The blot module was filled with NuPAGE transfer buffer (Invitrogen) and the proteins transferred from the gel to the membrane by applying a constant current of 30 V for 75 min. Successful protein-transfer was confirmed by staining the membrane with Sypro Ruby protein blot stain (Bio-Rad, Hemel Hempstead, UK) and imaging it using a Versa Doc (Bio-Rad). The blots were probed with mouse anti-human 20S proteasome IgG (Biomol, Exeter, UK), which recognises *S. mansoni* proteasome α-subunits [30], then detected with goat anti-mouse IgG peroxidase conjugate (Sigma). The image was visualised using the ECL Plus Western Blotting Detection System with Hyperfilm ECL paper (both GE Healthcare, Little Chalfont, UK) processed in a Compact X4 automatic film processor (Xograph, Tetley, UK). After imaging, each blot was washed and Restore Western Blot Stripping Buffer (Pierce, Cramlington, UK) was used to strip the blots of their antibodies. The blots were then probed with polyclonal rabbit anti-ubiquitin IgG (Sigma), which recognises mono-, multi- and poly-ubiquitinylated proteins. The detection antibody was goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) and the image was visualised using the chromogenic substrates 5-Bromo-4-Chloro-3-Indolyl phosphate/Nitro Blue Tetrazolium (Sigma).

2.3. Measuring proteasomal activity in the undeveloped and fully developed egg

Aliquots of undeveloped and developed eggs were ground for three minutes in 40 mM Tris–HCl, pH 7.5 containing 5% glycerol and 1 mM DTT, then the protein concentrations adjusted to 350 μg/ml. Chymotrypsin-like activity was measured by incubating 75 μg of each preparation at 37 °C for 1 h in a 96-well microtitre plate (Nunc, Roskilde, Denmark), with 25 μM of the fluorogenic substrate *N*-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Biomol) plus 5 mM $MgCl_2$ (making a total volume of 240 μl per well). In order to distinguish between 20S proteasomal activity and non-proteasomal chymotrypsin-like activity, the assay was carried out in the presence and absence of 0.02% SDS (see Section 3.2 for rationale). After incubation, the reactions were terminated with 240 μl/well of 1% SDS and the fluorescence measured on a Polarstar Optima plate reader (BMG Labtech, Aylesbury, UK) at 320 nm excitation and 460 nm emission wavelengths. The fluorescence of the blank was deducted from the fluorescence of each assay and the results expressed as means (±standard error of the mean) of duplicated assays. Non-proteasomal chymotryptic activity was measured by repeating the SDS^{-ve} assay with crude SEA (made from a mixture of immature and mature eggs) in the presence/absence of the proteasome-specific inhibitor epoxomicin (Biomol).

3. Results

3.1. Proteasomal α-subunit expression changes with egg development

In order to assess how proteasomal subunit expression changed with egg development we compared 20S α-subunit expression in the Vit blot (representing the vitelline cells) with the SEAu blot (made exclusively from undeveloped eggs) and the SEAd blot (made from fully developed eggs). Although α-subunits were present in all the preparations, the spot-pattern on each blot was different. The Vit preparation contained seven spots, which could be grouped into a row of five 30 kDa spots of *p*I 5–7 and two 25 kDa spots of *p*I 4.5–5 (Fig. 1B). Two of the 30 kDa spots were much fainter than all the other spots. This spot pattern was very distinctive, so all the other ECL images in the experiment could be matched to it exactly, thereby enabling between-preparation expression changes in individual isoforms to be seen (Fig. 1C). In the SEAu blot, all the spots had diminished in both size and intensity compared with the Vit blot, except that of the most acidic (25 kDa) isoform, which was now the largest spot by far. The more acidic of the two small, faint 30 kDa spots in the Vit blot was not present in the SEAu blot. Spots continued to diminish in number between the SEAu and SEAd blots, such that only a single 25 kDa isoform and a single 30 kDa isoform were visible in the latter. Although the 30 kDa spot had diminished in size, the 25 kDa spot had not, remaining the same size as it was in the Vit blot.

3.2. Proteasomal activity is greater in the undeveloped egg than in the developed egg

As the SEAu blot contained more proteasomal α-subunit isoforms than the SEAd blot, an assay was performed to compare the levels of chymotryptic activity in the two preparations, using a fluorogenic substrate in the presence and absence of 0.02% SDS. In the SDS^{-ve} assays the substrate is not degraded by 30S proteasomes because they are capped at both ends by 19S regulatory subunits and the substrate is not ubiquitinylated. Instead, the substrate is degraded by a combination of non-proteasomal chymotryptic proteases and 20S/26S proteasomes lacking 19S subunits and/or interacting with PIPs. In the SDS^{+ve} assays however, non-proteasomal chymotryptic proteases are deactivated, PIPs and 19S regulatory subunits disassociate leaving all the proteasomes in the 20S state and therefore capable of degrading the substrate [33,34]. The background level of non-proteasomal chymotryptic protease activity in the SDS^{-ve} assays was controlled for using the proteasomal inhibitor epoxomicin; these accounted for 54% of the total chymotryptic activity in the egg (Supplementary Fig. 1). Net of this, fluorescences in the SDS^{-ve} assays were similar in SEAd (15.8 kU ± 0.4) and SEAu (15.2 kU ± 0.4), suggesting that they had equal concentrations of proteasomes in the 20S and 26S configurations (Fig. 1D). Adding SDS increased fluorescence by 150% in SEAd (to 39.6 kU ± 0.8) and 271% in SEAu (to 56.4 kU ± 1.0), so 26S and 30S proteasomal activity was 1.4 times higher in SEAu than in SEAd.

3.3. Proteasomal α-subunit expression differs in the subshell envelope and miracidium

When the spot-patterns on the Hf blot (representing the subshell envelope) and the Ms blot (representing the miracidium) were compared with each other and with the SEAd blot it was possible to see which of the intra-egg preparations was enriched with proteasome α isoforms (Fig. 2). The Hf spot-pattern was completely different to that of any other blot, with three horizontal rows containing 16 spots of 25, 30 and 50 kDa, 10 of which (including the 50 kDa spots) were not present in any other preparation (Fig. 2B). The Ms blot contained six visible spots, all of which could be matched to hatch fluid spots, and three of which could be matched to the spots on the SEAd blot. Five out of the six Ms spots also matched spots from the Vit blot (Fig. 2C).

3.4. Ubiquitinylation in the egg

When the blots were stripped and re-probed with an anti-ubiquitin antibody (which recognises mono-, multi- and poly-ubiquitinylated proteins) many distinctive spot patterns were

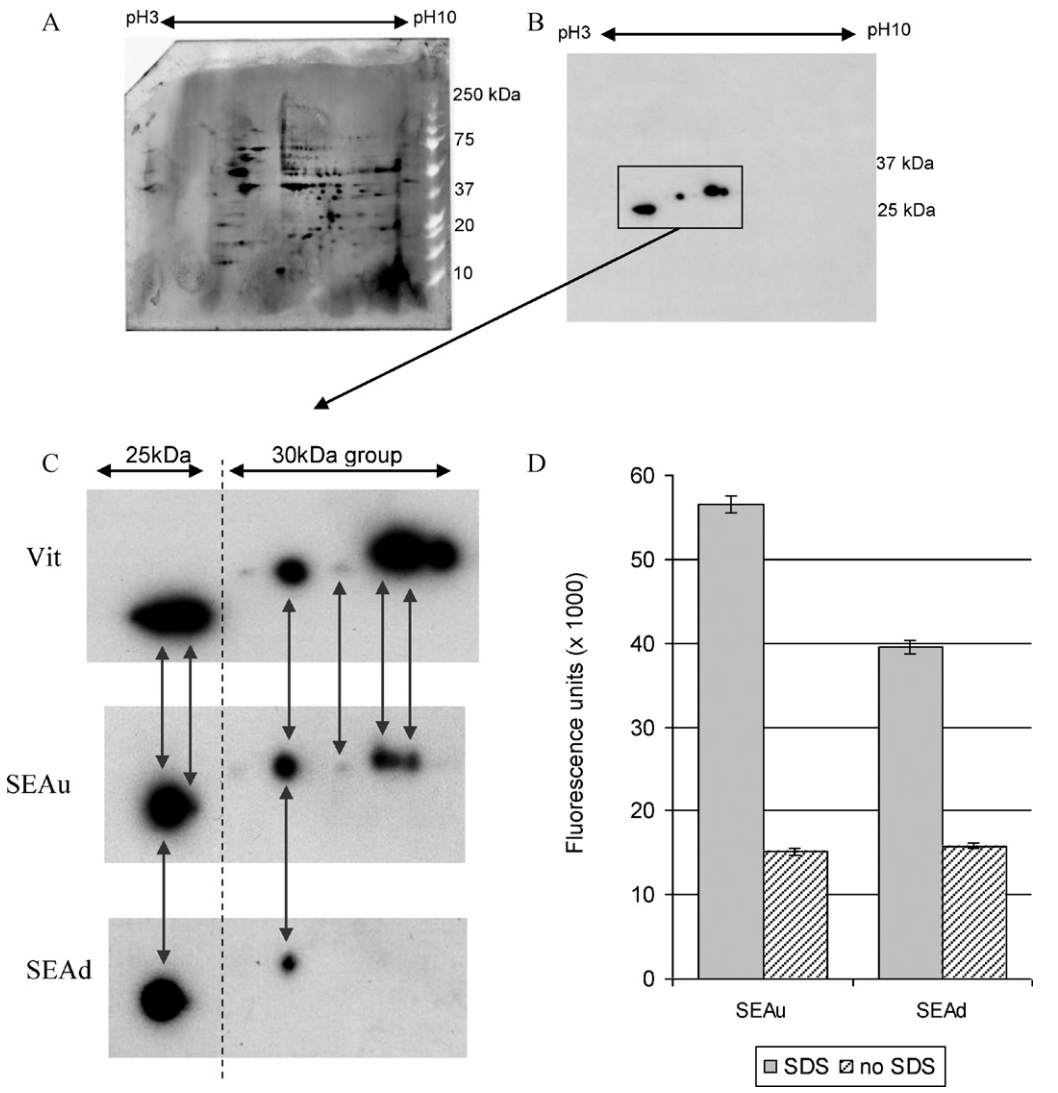

Fig. 1. The 20S α proteasome expression in the developing egg. (A) Blot of 35 μg of Vit, stained with Sypro Ruby protein stain. (B) Blot from A, probed with anti-proteasome-α subunit antibody. (C) Enlarged section of the blot from B, compared with similar blots of SEAu and SEAd. Matching subunits are linked by arrows. The α-subunit isoforms fall into two groups based on molecular weight and *pI*. (D) Proteasomal chymotrypsin-like activity in SEAu compared with that in SEAd. Regulatory subunits prevent proteasomal degradation of the substrate in the SDS^{-ve} assays, but these disassociate in the SDS^{+ve} assays, causing 20S proteasomal degradation of the substrate. Therefore, the 20S proteasomal activity is represented by the difference between the fluorescence in the SDS $^{+ve}$ and SDS^{-ve} assays.

apparent (Fig. 3). For example, the 15 kDa slightly acidic proteins (annotated as "A" in Fig. 3) were in all the preparations, and the "B" series of spots was present in all of the blots except Ms. The very acidic protein "C" of 50–100 kDa was the most heavily reacting protein on the blots in SEAu and SEAd and Hf, appeared as a faint spot in Vit and was not present in Ms. The location, size, shape and pattern of expression of the spot was similar to that of the previously described protein of unknown function Smp_170410 [27]. Some ubiquitinylated proteins were very abundant in Vit and SEAu, but had declined in abundance in SEAd ("D"). Unbound ubiquitin could be seen in the blots of SEAu and Ms ("E"), but not in SEAd or Hf.

4. Discussion

The *S. mansoni* egg needs to recycle proteins for radically different reasons depending on its stage of development. When undeveloped, the egg degrades its vitelline cells to nourish the embryo, but once fully developed and surrounded by host leukocytes, any damaged miracidial and envelope proteins require recycling. However, as far as we know, protein turnover has never been studied in trematode eggs before. In insects, developing eggs consist of the ovum and nurse cells which play a similar nutritive role as the vitelline cells of the schistosome egg [35]. The most detailed studies, involving *Drosophila*, show that cytoplasm is transferred from the nurse cells to the developing oocyte. Then, after the nurse cells have become apoptotic and their DNA has fragmented, their remnants are phagocytosed by somatic follicle cells [36,37]. Although these studies demonstrate that apoptosis is utilised in the final *coup de gras* of the nurse cells, they fall short of establishing what mechanisms are involved in the earlier stages of nurse cell degradation. Therefore, the UPP could be central to the degradation process of both nurse cells (in insects) and vitelline cells (in schistosomes). There is a fundamental difference between the situation in the schistosome egg and that in the insect egg however, in that the vitelline cells of the schistosome egg are not surrounded by follicular cells with a phagocytic capability. Neither the developing miracidium nor the envelope has been demonstrated to undertake phagocytosis, so it is unlikely that apoptosis has evolved to become important in vitelline cell degradation.

Our Western blots have shown that the 20S proteasome is more highly expressed in the undeveloped egg (SEAu) than in

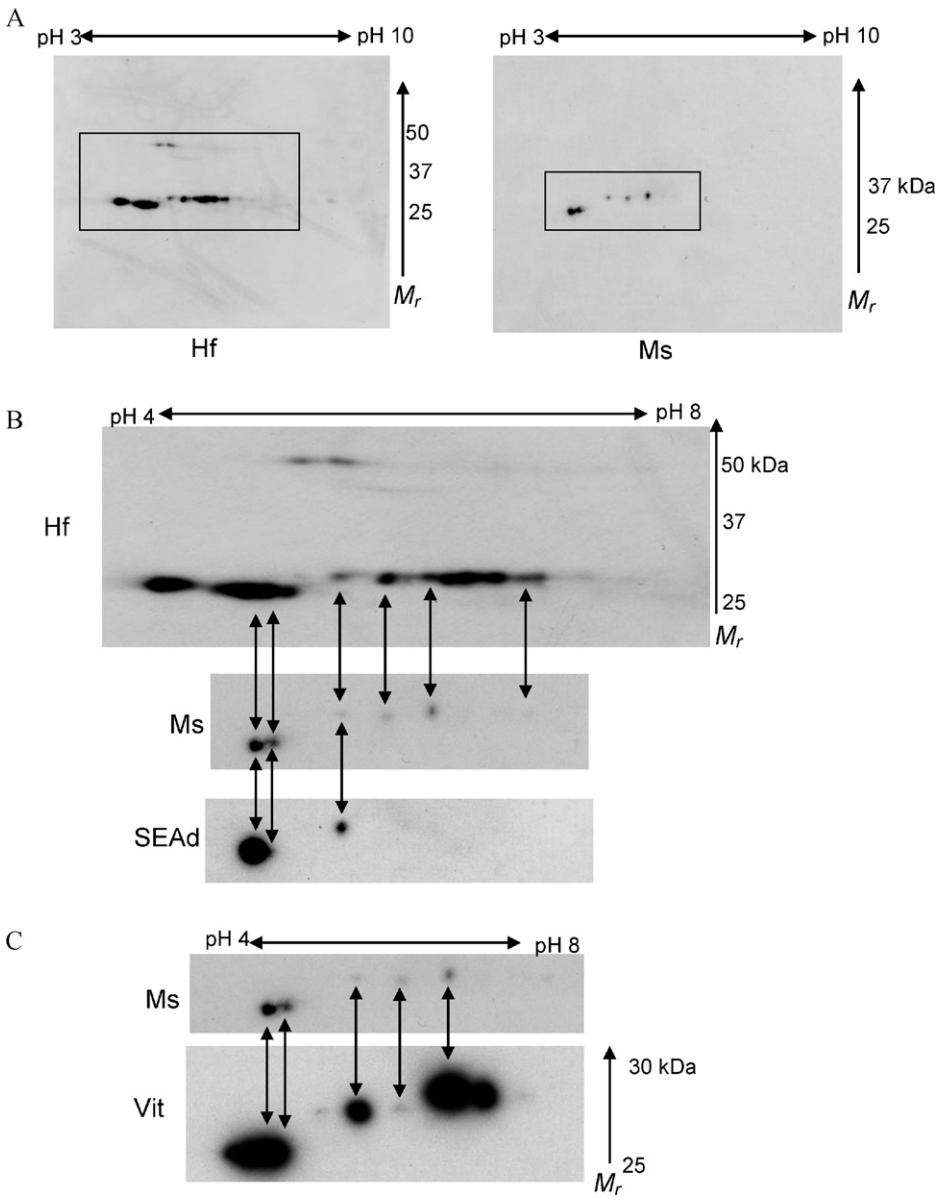

Fig. 2. Proteasome 20S α-subunit isoform expression in the morphological components of the developed egg. (A) Hf and Ms blots were probed with anti 20S proteasome α-subunit antibody and imaged using ECL reagents. The areas inside the boxes are enlarged in (B). (B) The proteasome α-subunit-containing sections of (A) with shared subunit isoforms shown by arrows. For comparison, the SEAd blot from Fig. 1C is also shown. (C) A similar comparison, but between the Ms and Vit blots.

the fully developed egg (SEAd), and our functional assays have demonstrated that proteasomal activity is also higher in the undeveloped egg. This decline in UPP utilisation with egg development could have two alternative explanations: (i) a reduction in damage inflicted by host leukocytes over time or (ii) the immature egg (the composition of which is dominated by vitelline cells) inherently utilizes the UPP more actively than the fully developed egg (containing the miracidium and envelope). Granulomata do not form around undeveloped eggs [38], the leucocytes only accumulating later in egg development, so it will be the more developed eggs that are in an environment with higher concentrations of toxic compounds. Also, the embryo is small in the immature egg, so the thick, protective layer of vitelline cells will buffer it from any host-derived toxins entering through the shell. Thus, the embryo of the undeveloped egg is likely to be at a lower risk of suffering damage from the products of host cells than the miracidium and envelope in the mature egg. Consequently, the more active UPP in the immature egg can most likely be attributed to some factor(s) relating to the function of the vitelline cells. As these cells have been proposed to provide an auto-degrading reservoir of nutrients for the developing ovum [39], we hypothesize that the UPP is relatively active in the immature egg because the UPP is the pathway by which this degradation occurs.

It is logical that the UPP would be used in this way because the process is highly regulated, both at the point where the substrate proteins are ubiquitinylated and at the proteasome itself. At the ubiquitinylation stage, E3 ubiquitin ligases determine which proteins are to be ubiquitinylated. Then, the speed at which ubiquitinylation takes place will depend on the availability of unbound ubiquitin. At the proteasome itself, a further level of regulation exists, not only with PIPs, but also with de-ubiquitinylation enzymes such as Ubp6, which can progressively remove ubiquitin moieties from substrate proteins to delay their proteasomal degradation [40]. Also, as proteasomal degradation is highly ATP-dependent [41], intracellular ATP concentration will also determine the rate of activity. Thus, using the UPP rather than apoptosis or autophagy, the vitelline cells could degrade slowly, forming an amino acid-concentration gradient between themselves and the

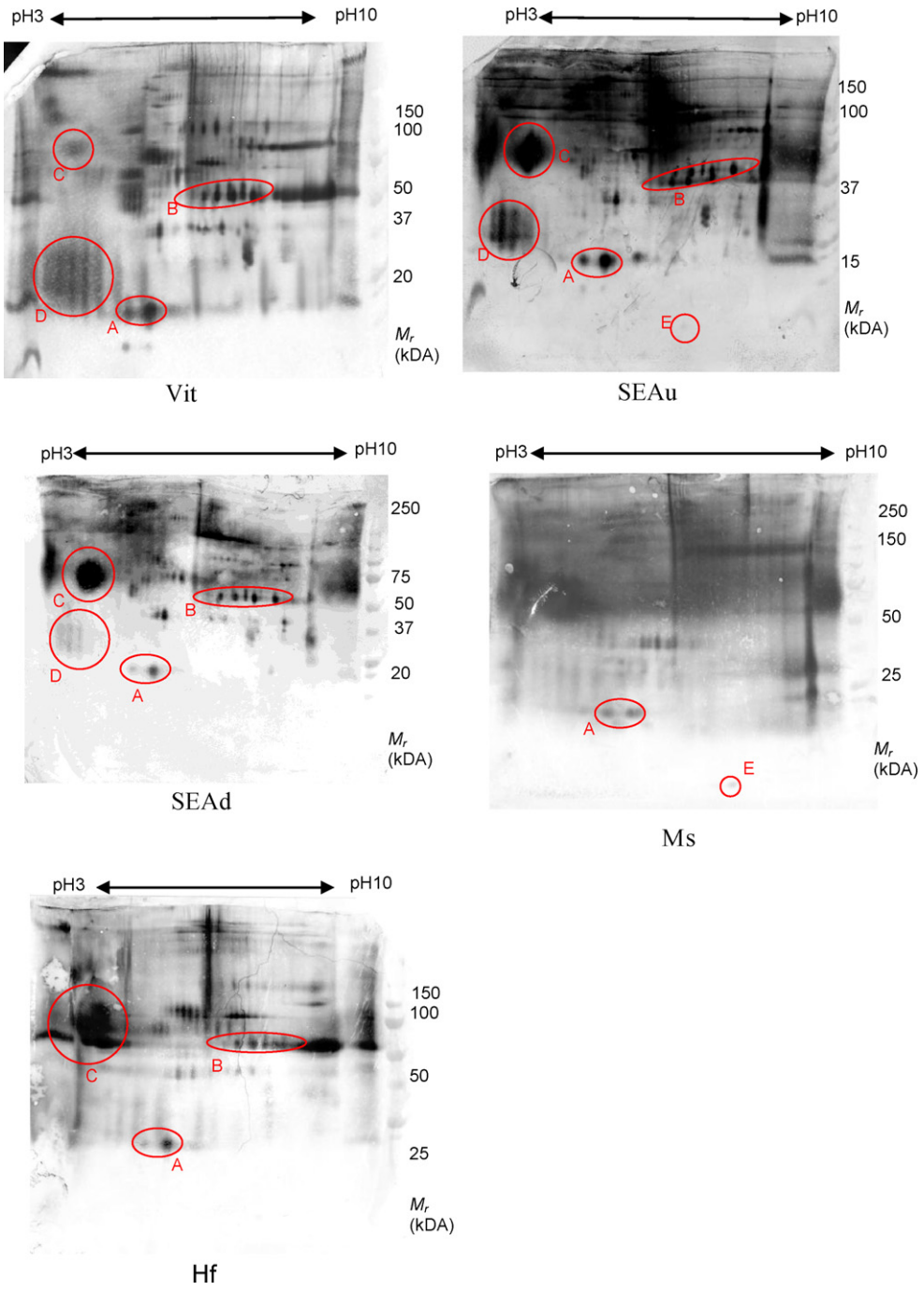

Fig. 3. Ubiquitinylated proteins in the egg. 2D blots of 35 μg of protein from each egg preparation were probed with an anti-ubiquitin mAb that recognises mono-, multi- and poly-ubiquitinylated proteins. The blots were imaged using alkaline phosphatase reagents. The spots annotated A–F are discussed in the text.

developing miracidium, capable of delivering the amino acids that the miracidium requires for its growth.

There is little evidence that apoptosis is particularly active in schistosomes. The *S. mansoni* transcriptome was found to contain some but not all of the components of the apoptotic pathway [42]. Also, the apoptotic pathway is relatively uncontrollable once it has begun, with the process of caspase activation taking minutes to complete and then, once a cell is committed to undergo apoptosis the process is complete within several hours [43]. Thus, even if the apoptotic pathway was utilised in the egg, it is difficult to reconcile such a rapid mechanism of protein degradation with the slow and steady release of amino acids that the developing miracidium would require.

Autophagy is a more feasible process than apoptosis for vitelline cell degradation because almost all of the autophagic components have been found in the transcriptome and autophagy is known to occur in adult schistosomes [42,44,45]. Autophagy eliminates unwanted cells during developmental cell death in various taxa, including the ovarian nurse cells in *Bombyx mori* and *Ceratitis capitata* [46–48]. It is therefore possible that autophagy as well as the UPP is involved in the degradation of vitelline cells, perhaps with the UPP operating at the earlier stages and autophagy commencing at a later time point. The egg does not contain any phagocytic cells, so after their degradation, any vitelline cell remnants would remain inside the egg and possibly go on to form the vesicles of the fully mature egg.

Although more proteasome α subunit isoforms were expressed in SEAu compared with SEAd, more expression was seen in the blots of the Vit preparation, enriched in vitellaria. The vitellaria occupy the posterior two-thirds of the female worm and consist of the vitelline ducts and follicles, the latter where the vitelline cells are produced [49]. Each female *S. mansoni* produces an estimated 200 eggs per day [50,51], each containing 30–40 vitelline cells [25,26], so the vitellaria are clearly very active in terms of protein production. During protein production, upwards of 30% of new proteins in various cell types are misfolded and are degraded by proteasomes [52,53]. It is therefore inevitable that the UPP will be highly active in the vitellaria and consequently, it is not surprising that the Vit preparation contained more proteasome α-subunit isoforms than either SEAu or SEAd.

The mature egg can be sub-divided into the miracidium and the hatch fluid, represented by the Ms and Hf preparations respectively. Hatch fluid consists of extracellular proteins located either side of the envelope, the contents of the vesicles and those intracellular membrane proteins that are water-soluble. As the UPP is an intracellular system, all the proteasomal subunits and ubiquitinylated proteins on the Hf blot must have been derived from the envelope. In the proteasome α-subunit blots, the spot-pattern in Ms is a sub-set of that in Hf. This could be partially due to the methods adopted to make each preparation. Hf only contains water-soluble proteins whilst Ms was made by homogenising miracidia in a buffer containing urea, thiourea and CHAPS. As proteasomal subunits are water-soluble they would have been enriched in Hf. However, the difference between the blots is probably too dramatic to be solely due to the different buffers. It is more likely that hatch fluid contains more 20S α-subunit isoforms than the miracidial body because the UPP is more active in the envelope than in the miracidium. The envelope is responsible for producing ESP [26,54], so it has a high rate of protein production, and consequently needs an active UPP to deal with the mistranslated proteins. The nature of misfolded proteins will be different in hatch fluid compared with the miracidium however: the envelope is much more involved in the production of proteins for secretion, so hatch fluid proteasomal substrates will have originated in the endoplasmic reticulum rather than in the cytosol. In contrast, the miracidium is short-lived and does not secrete proteins whilst it is in the egg. It needs to find and penetrate a snail, and as it does so without feeding it must conserve ATP. It has been calculated that 300–400 ATP molecules are used during the degradation of one substrate protein through the UPP [41], so we hypothesize that operating the UPP would be too expensive in terms of ATP expenditure for the miracidium. Instead, the miracidium could utilize its high levels of the non-ATP-requiring chaperone Sm-p40 [27] to store misfolded proteins rather than degrade them. It is likely that any misfolded proteins associating with Sm-p40 are effectively excluded from the UPP because Sm-p40 does not contain the tetratricopeptide repeat motifs that are found in other common miracidial heat shock proteins such as HSP70 and HSP90 [27,55–57]. The tetratricopeptide repeats are important because they interact with the E3 ubiquitin ligase CHIP (C-terminus of HSP70-interacting protein) and thereby form the bridge linking the chaperones and the UPP [58,59]. Although CHIP has never been localised to the miracidium, it is present in the *S. mansoni* transcriptome (http://www.genedb.org/genedb, accession number Sm03288).

To our knowledge, this is the first study addressing the question of protein turnover in the schistosome egg. We have shown that the undeveloped egg has a more active UPP and a more complex proteasome subunit expression pattern than the developed egg, consistent with the egg adopting the (highly regulated) UPP and not the (uncontrollable and fast) apoptotic pathway to degrade its vitelline cells. We have also established that the sub-shell envelope has a more complex proteasome subunit expression profile than the miracidium, thereby highlighting the crucial importance of the hitherto unstudied envelope in the biology of the egg.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2010.10.005.

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