EP45 accumulates in growing *Xenopus laevis* oocytes and has oocyte-maturation-enhancing activity involved in oocyte quality

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Summary

The capacity of oocytes to fully support meiotic maturation develops gradually during oocyte growth. Growing oocytes accumulate proteins and mRNAs required for this process. However, little is known about the identity of these factors. We performed a differential proteomic screen comparing the proteomes of growing stage-IV oocytes, which do not undergo meiotic maturation in response to progesterone, with fully grown stage-VI ones, which do. In 2D gels of stage-VI oocytes, we identified a group of four protein spots as EP45 (estrogen-regulated protein 45 kDa), which belongs to the family of serine protease inhibitors and is also known as Seryp or pNiXa. Western blot analysis after mono- and bi-dimensional electrophoreses confirmed the accumulation of certain forms of this protein in oocytes between stages IV and VI. *EP45* mRNA was not detectable in oocytes or ovaries, but was expressed in the liver. A low-mobility isoform of *EP45* was detected in liver and blood, whereas two (occasionally three or four) higher-mobility isoforms were found exclusively in oocytes, suggesting that liver-synthesized protein is taken up by oocytes from the blood and rapidly modified. Alone, overexpression of RNA encoding either full-length or N-terminally truncated protein had no effect on meiotic resumption in *proliferating* oocytes. However, in oocytes moderately reacting to low doses of progesterone, it significantly enhanced germinal-vesicle breakdown, showing a novel and unsuspected activity of this protein. Thus, EP45 accumulates in growing oocytes through uptake from the blood and has the capacity to act as an ‘oocyte-maturation enhancer’ (‘Omen’).

Key words: EP45, pNiXa, Seryp, Oocyte-maturation-enhancing activity, Oocyte growth, Oocyte quality, M-phase induction, GVBD, Serpin, Bet-hedging

Introduction

Oocytes of vertebrates are arrested in the first meiotic prophase for weeks, months or years. During this long-lasting period, they undergo considerable growth, which occurs through the accumulation of different types of molecules (proteins, lipids, mRNAs) of maternal origin. Some of them are synthesized in ovo (through mRNA transcription and translation), others are incorporated from blood. Both oocyte transcription and protein uptake are extremely active during oocyte growth. In mesolecithal amphibian oocytes, including those of *Xenopus laevis*, nutrients are certainly the most abundant constituents of the oocyte mass accumulated during the growth period. However, regulatory molecules, which are less abundant, are certainly of key importance for the functionality of female gametes (Gosden, 2002). When fully grown amphibian oocytes enter meiotic maturation, they become transcriptionally silent and this state persists after fertilization, until the mid-blastula transition (MBT) (Maller, 1998). Thus, both oocyte maturation and early-embryo development depend entirely on the maternal genetic information. As such, to generate an oocyte suitable for fertilization and embryonic development, all regulatory molecules ensuring the normal course of oocyte maturation and early development must properly accumulate during oocyte growth. Oocyte maturation is a highly regulated process, leading to the formation of fully mature female gametes with an appropriate number of chromosomes and the full potential to undergo successful fertilization and embryonic development until the MBT. Thus, the quality of mature oocytes depends largely on the quality of stockpiles accumulated during oogenesis. These processes are of key importance for embryonic developmental and reproductive success (Marteil et al., 2009).

Oogenesis and oocyte maturation are well characterized in *X. laevis*. The ovary of sexually mature females contains oocytes in all stages of oogenesis (stages I to VI according to Dumont’s classification; Dumont, 1972). Oocytes at all stages are arrested in meiotic prophase I and undergo continuous growth by accumulating yolk proteins, among which the large conserved phosphoglycolipometalloprotein vitellogenin plays a major role (Stifani et al., 1990). Interestingly, only stage-VI postvitellogenic oocytes are responsive to progesterone, the major steroid that induces meiotic resumption and leads to a mature oocyte. Progesterone stimulation induces not only the inhibition of adenylate cyclase, leading to a rapid drop in cAMP levels and a reduction in protein kinase A (PKA) activity, but also de novo synthesis of protein (such as the Mos proto-oncogene) (Tunquist and Maller, 2003). These events trigger a cascade of protein
phosphorylation events, culminating in the abrupt activation of M-phase-promoting factor (MPF), also known as the cyclin-dependent kinase 1 (CDK1)-cyclin B complex (Masui and Markert, 1971). Active MPF induces germline-venusicle (oocyte nucleus) breakdown (GVBD), chromatin condensation and meiotic-spindle formation (Jessus et al., 1991). The first meiotic division terminates with the first polar-body extrusion followed by arrest in meiosis II (MII) under the control of the cytokinetic factor (CSF) (Masui and Markert, 1971). At this step, the mature oocyte is now ready for ovulation and fertilization.

As mentioned above, smaller oocytes cannot resume meiosis upon progesterone stimulation (Reynhout et al., 1975). Indeed, progesterone is not able to release prophase I arrest in stage-IV oocytes, despite the fact that these small pigmented oocytes express functional progesterone receptors that are able to inhibit PKA (Mulner et al., 1983; Sadler and Maller, 1983). Even upon PKA inhibition, stage-IV oocytes are unable to activate MPF (Masui and Markert, 1971) and enter the first meiotic M phase (Sadler and Maller, 1983). However, injection of low doses of active MPF in stage-IV oocytes is sufficient to initiate MPF activation followed by GVBD. This experiment clearly indicates that part of the molecular machinery responsible for meiotic maturation is already present in a silent form in these oocytes (Wasserman et al., 1984; Rime et al., 1991), but that the coupling between PKA and CDK1 activation is not functional (Jessus and Ozon, 2004).

To understand the molecular mechanisms leading to the acquisition of the capacity of an oocyte to proceed to GVBD, comparison of oocyte morphology and functional changes between stages IV and VI is particularly interesting. The best-known factor essential for CDK1 activation and lacking in stage-IV oocytes is Plx1 (a polo-like kinase) (Karauskou et al., 2004). However, Plx must be one of further regulatory proteins that accumulate following stage IV, because providing stage-IV oocytes with Plx1 does not confer the capacity to respond to progesterone. Other proteins might not necessarily be involved in the core mechanism of CDK1 activation. To gain insight into the nature of the molecules potentially involved in these processes, we performed a proteomic screen in which we compared the proteomes of stage-IV oocytes before they acquire the ability to undergo meiotic maturation, we compared the proteomes of small stage-IV oocytes and fully grown stage-VI oocytes (Dumont, 1972) by 2D electrophoresis. Among proteins less abundant in stage-IV than in stage-VI oocytes, we found a family of four spots in the same region (molecular weight 45 kDa; pHi 7.8) that was always clearly present in stage-VI oocytes, was reported to accumulate following stage IV, because providing stage-IV oocytes with Plx1 does not confer the capacity to respond to progesterone. Other proteins might not necessarily be involved in the core mechanism of CDK1 activation. To gain insight into the nature of the molecules potentially involved in these processes, we performed a proteomic screen in which we compared the proteomes of stage-IV and -VI oocytes. We focused on cytoplasmic proteins that were absent or only weakly present in stage-IV oocytes and became particularly abundant in stage-VI oocytes. Such behavior suggests potential involvement in the development of the capacity of stage-VI oocytes to undergo GVBD. EP45 (estrogen-regulated protein 45 kDa), a member of the superfamily of serine protease inhibitors (serpins), was one such protein identified in our proteomic screen as a cytosolic protein that accumulates in oocytes between stages IV and VI raised an intriguing question of why the endogenous protein does not induce GVBD by itself without progesterone stimulation. Recently, EP45/pNiXa was found to also be an important component of yolk platelets and was named Seryp (for Serpin yolk protein) (Jorgensen et al., 2009). Here, we characterize in detail the accumulation of cytosolic EP45/pNiXa/Seryp in growing X. laevis oocytes. We show that it is not synthesized in oocytes, but rather in the liver and then delivered to oocytes from the blood. Moreover, we show that cytoplasmic expression of this protein does not induce GVBD in either stage-IV or -VI oocytes, but enhances GVBD in oocytes responding moderately to progesterone. This protein is therefore a candidate for membership of a novel class of positive regulators of oocyte maturation, which we propose to name ‘oocyte-maturation enhancers’ (or ‘Omen’) and which are involved in non-core pathways stimulating CDK1 activation, GVBD and the process of oocyte maturation.

Results

EP45 is a novel protein that accumulates in the cytoplasm of growing oocytes

Accumulation of proteins during oocyte growth participates in the acquisition of the capacity of the oocyte to undergo meiotic maturation. To identify novel proteins that accumulate in X. laevis oocytes before they acquire the ability to undergo meiotic maturation, we compared the proteomes of small stage-IV oocytes and fully grown stage-VI oocytes (Dumont, 1972) by 2D electrophoresis. Among proteins less abundant in stage-IV than in stage-VI oocytes, we found a family of four spots in the same region (molecular weight 45 kDa; pHi 7.8) that was always clearly more abundant in stage-VI oocytes (Fig. 1A, females 1-5). All four spots were identified as the same protein, EP45, by mass spectrometry (MS) (Fig. 1B).

To validate our screen, polyclonal antibodies raised against two different regions of EP45 (Fig. 2A) were used to detect the expression of EP45 in stage-IV and -VI oocytes by western blotting (WB) following mono-dimensional PAGE (Fig. 2B). In stage-VI oocytes obtained from most females, EP45 migrates as two bands: a more abundant slow-migrating one (band 1, corresponding to a
protein of 45 kDa) and a less abundant fast-migrating one (band 2, corresponding to a protein of 41 kDa) (Fig. 2B). Interestingly, in oocytes obtained from some females, we found one additional fast-migrating band (band 3, corresponding to a protein of about 36 kDa; Fig. 3A, female e, stage VI), illustrating the high degree of heterogeneity of the WB profile of EP45. Apart from the two (or three) major bands, each antibody detects only a faint higher molecular weight band (around 91 kDa; Fig. 2B), which might correspond to a covalent complex between the EP45 serpin and a target protease (Lawrence et al., 1995). The comparison of stage-IV and -VI oocytes shows that both the 45 and 41 kDa bands, as well as the 36 kDa band present in some pools of oocytes, are less abundant in stage-IV than in stage-VI oocytes (as normalized to tubulin WB; Fig. 3A,B). However, the most striking accumulation is observed for the EP45 form corresponding to band 2 (41 kDa), with a threefold increase in stage-VI oocytes compared with stage IV (Fig. 3B). Thus, EP45 accumulates extensively in the cytoplasm of stage-VI oocytes and its quantity increases in stage-VI oocytes in comparison to stage IV, confirming the results of our proteomic screen. In addition, EP45 shows an important heterogeneity in respect to its migration pattern in oocytes from different females. Because the proteomic screen was carried out by 2D electrophoresis, we compared the proteomes obtained from stage-IV and -VI oocytes by 2D PAGE followed by 2D WB with EP45 antibody (Fig. 4). 2D WB revealed the presence of multiple spots corresponding to EP45 bands 1, 2 and 3 (Fig. 4A,C) in oocytes from the two stages, suggesting complex regulation of this protein. Numerous spots were absent or less abundant in stage-IV oocytes compared with stage VI (Fig. 4A,C). These results confirm that EP45 accumulates between stages IV and VI as multiple spots, not just as the four spots previously identified by silver staining of 2D gels (Fig. 1). Fast-migrating isoforms of EP45 (bands 2 and 3) are mainly responsible for the accumulation of EP45 in stage-VI oocytes (Figs 3 and 4). The heterogeneity of EP45 was also confirmed in 2D WB (compare the patterns of EP45 in Fig. 4A and 4C).

**Accumulation of EP45 in oocytes**

To obtain more information about the behavior of EP45 during oogenesis, we compared the WB profiles of this protein during all stages of oogenesis and throughout oocyte maturation. EP45 is barely detectable in the smallest stage-I and stage-II oocytes, and starts to accumulate in stage-III oocytes as the slow-migrating form (Fig. 5A). The levels of EP45 increase through stages IV, V and VI. The quantification of the WBs of five different pools of oocytes from stages IV and VI shows that the intensity of the two major bands (1 and 2) increases, and that the fast-migrating form accumulates in the most dynamic manner between stages IV and VI (Fig. 3A). Generally, apart from the changes in abundance of

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**Fig. 2. Characterization of EP45 antibodies.** (A) Two polyclonal antibodies produced in rabbits were raised against synthetic peptides (1 and 2, underlined) selected from the sequence of X. laevis EP45. (B) WB showing the specificity of antibodies (antibodies 1 and 2 raised against peptides 1 and 2, respectively) used to detect X. laevis EP45: both antibodies (1/2000) give the same detection pattern for stage-VI oocyte extracts (two major bands are visible at 45 and 41 kDa and one minor at 91 kDa).

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**Fig. 3. Proteomic screen validation: 1D electrophoresis and WB analysis of EP45 in stage-IV and stage-VI oocytes.** (A) EP45 is less abundant in stage-IV than in stage-VI oocytes. High heterogeneity of the EP45 protein pattern is observed among pools of oocytes. Extracts from stage-IV and -VI oocytes of five different females (females a–e, different to those used for the screen; Fig. 1) were immunoblotted with antibody 1 against EP45. (B) The high-mobility isoform of EP45 (band 2 of 41 kDa) is the main form of EP45 that accumulates between stage-IV and -VI oocytes. Histograms correspond to quantification of the WB shown in A normalized to β-tubulin signal (shown in A). Quantification of WB from A was performed using Image-Quant 5.2 software.
each band, no other change in the migration pattern of EP45 was found during oocyte maturation (Fig. 5A). However, some minor differences could be observed in pools of oocytes with atypical patterns of EP45 (Fig. 3A, females d and e). Similarly, there was no change in the WB pattern of EP45 during oocyte maturation (data not shown). Reverse transcriptase (RT)-PCR showed that EP45 mRNA is highly present in liver, but remains undetectable both in oocytes, as recently reported by Jorgensen and colleagues (Jorgensen et al., 2009), and in the whole ovary (Fig. 5B). The protein was found by WB both in liver and in blood plasma, but as a slightly less mobile single band (around 50 kDa; Fig. 5C). Interestingly, this lower-mobility form of EP45 was never detected, even in small proportions, in oocytes or in embryos (Fig. 5C), suggesting that EP45 is taken up by oocytes from blood and immediately modified in oocytes to the faster-migrating forms.

EP45 overexpression enhances GVBD only in stage-VI oocytes, which are partially responsive to progesterone

Injection of pNiXa alone into stage-VI oocytes was shown to induce GVBD (Haspel et al., 1993). Paradoxically, we show in this paper that such oocytes already contain large amounts of EP45. In addition, we have not observed any change in the PAGE mobility of EP45 after oocyte maturation, indicating that there is no major change in the state of EP45 between meiotic prophase I and metaphase II oocyte stages (Fig. 5A). These data suggest that the stockpile of endogenous EP45 is not sufficient to induce GVBD in stage-VI oocytes. To gain information on the potential role of EP45 in oocytes arrested in prophase I, we injected mRNA encoding EP45 in order to overexpress this protein in stage-IV and stage-VI oocytes, and to assess the effect on oocyte maturation.

First, we injected EP45 mRNA into stage-IV oocytes. These oocytes do not undergo GVBD following progesterone treatment, partly because of the absence of Plx (Karaiskou et al., 2004). In this series of experiments, stage-IV oocytes were injected with EP45 mRNA and treated with 1 µM progesterone (saturating dose for stage-VI oocytes) to facilitate any changes triggered by EP45 overexpression. Such a combination could reveal a potential synergistic effect of progesterone and EP45. Despite this double treatment, as expected, no stage-IV oocyte underwent GVBD, as judged by the absence of white spot appearance (Fig. 6A). In addition, no changes in cell-cycle markers of oocyte maturation, such as cyclin B2 and ERK2 phosphorylation states, were detected by WB (Fig. 6B). We also followed the level of EP45 overexpression with anti-EP45 antibody (Fig. 6B). In all cases, the levels of overexpression were moderate (Fig. 6B). These results show unequivocally that EP45 expressed in stage-IV oocytes does not induce GVBD.

Next, we injected stage-VI oocytes using a very similar protocol (with the injection of mRNA encoding β-galactosidase as a control, without or with progesterone). The level of EP45 overexpression was detected using anti-EP45 antibodies or anti-myc antibodies in experiments in which a myc-tagged form of EP45 was expressed (Fig. 6D; Fig. 7B). In experiments using stage-VI oocytes, we reduced the concentration of progesterone to a non-saturating dose of 50 nM. Similarly to stage-VI oocytes, EP45-injected stage-VI oocytes that were not treated with progesterone did not undergo GVBD (Fig. 6C) and did not show changes in cyclin B2 or ERK2 phosphorylation states (Fig. 6D). Moreover, oocytes treated with 50 nM progesterone matured with very similar dynamics and rate, regardless of EP45 or β-galactosidase expression (Fig. 6C,D).
These results show that EP45 expression does not induce GVBD and does not modify the response of stage-VI oocytes to 50 nM progesterone.

Because 50 nM progesterone elicits almost the maximum response of oocytes – such treatment usually induces GVBD in about 90% of oocytes (Fig. 6C) – we assessed the reactions of stage-VI oocytes injected with full-length EP45 mRNA and treated with 10 nM progesterone. In non-injected oocytes, as well as oocytes injected with β-galactosidase mRNA, 10 nM progesterone induces a whole spectrum of oocyte reactions, depending on the batch and characteristics of each female. Some batches did not react to such treatment (no GVBD; non-responsive), some pools of oocytes underwent almost 100% GVBD (maximally responsive), and an intermediate group varied between these two extremes (mid-responsive) (Fig. 7A). Non-responsive batch oocytes (no GVBD in control) injected with EP45 mRNA still did not undergo GVBD. Maximally responsive batch oocytes (almost 100% GVBD in control) remained at the highest level of response to 10 nM progesterone (Fig. 7A). However, the percentage of maturation was clearly increased in the mid-responsive batches of oocytes (Fig. 7A). Entry into oocyte maturation was also confirmed by cyclin B2 and ERK2 phosphorylation in these oocytes (Fig. 7B). The EP45 WB pattern (the presence of two or three bands) did not correlate with either the responsiveness of oocytes to 10 nM progesterone or their response to exogenous EP45 expression (Fig. 7B). These results show that EP45 overexpression enhances oocyte maturation without provoking GVBD by itself.
Fig. 6A. Histograms show the percentage of maturation of oocytes microinjected with RNAs encoding oocytes that are partially responsive to 10 nM progesterone (females 5, 6, 9, 10, 12-15 within dotted frame). Experimental procedures are the same as those described in Fig. 6A. Histograms show the percentage of maturation of oocytes microinjected with EP45 or β-gal mRNA or left untreated in the presence or absence of either 10 nM or 1 μM progesterone (additional positive control) using specific antibodies (the same as in Fig. 6B). Examples of WB of typical oocytes with two bands of EP45 (female 14), and two pools of oocytes possessing three bands of EP45 (females 7 and 9) are shown. Note that the two atypical oocyte pools react differently to EP45 expression: those from female 7 are not stimulated by EP45, whereas those from female 9 are. Exogenous unprocessed EP45 is labeled with an asterisk.

Discussion

We show here that EP45 accumulates during X. laevis oocyte growth, and that some isoforms of this protein accumulate specifically and in large amounts between stages IV and VI of oogenesis (1D gels). EP45 in oocytes is extensively modified, forming a complex pattern as revealed in 2D gels. We confirm that EP45-encoding mRNA is absent in oocytes and ovaries, but is present in liver. EP45 protein was found in both liver and blood of females (absent in liver of males), as well as in oocytes, confirming the exogenous origin of EP45 in oocytes by means of uptake from blood (Holland and Wangh, 1987; Holland et al., 1992; Beck et al., 2003). Moreover, isopycnic centrifugation of gently lysed eggs shows the presence of Seryp in two different fractions: a high-density one accompanied by LV1 and LV2 vitellogenin derivatives, clearly corresponding to Seryp in yolk platelets; and a second, vitellogenin-free, one at the top of the density gradient, corresponding to Seryp in the cytoplasmic compartment. The presence of the latter fraction is interpreted by the authors as artefactual contamination from yolk platelets (Jorgensen et al., 2009). However, in a proteomic experiment with a cytoplasmic fraction of MI oocytes, unrelated to the current study, we found EP45 to be among the most abundant proteins, whereas vitellogenin is barely detectable (identified by a single peptide). This confirms by another method the presence of high levels of vitellogenin-free EP45, supporting the hypothesis that two distinct pools of EP45/Seryp are present in oocytes: one associated with yolk platelets and the other cytoplasmic. However, the translocation process of EP45 to the cytoplasm remains unknown.

EP45 isoforms

We discovered several isoforms of EP45 in the liver, plasma and oocytes, and an intriguing heterogeneity between pools of oocytes obtained from different females. It is possible that certain isoforms correspond to covalent complexes of EP45 with proteinases (e.g. the 91 kDa band; Fig. 2B). Indeed, serpins inhibit proteinases by acting as pseudosubstrates. The interaction between a serpin and a protease leads to two different pathways: the ‘inhibitory pathway’, with the formation of an inactive serpin-proteinase covalent complex, and the ‘substrate pathway’, delivering a cleaved serpin and an inactive protease. Therefore, the inhibitory pathway delivers higher molecular weight complexes of different molecular weights depending on the mass of the protease, whereas the substrate
pathway produces lower molecular weight forms of cleaved EP45. However, novel biological functions for serpins have also been demonstrated: they can act as hormone carriers (serpin A6), neurotrophic factors (serpin F1) and chaperones (serpin H1) (for a review, see van Gent et al., 2003). These various functions might also be linked to formation of the different forms of EP45 detected in oocytes.

In liver and plasma, an isoform of 50 kDa is found, whereas two or three forms are present in oocytes (45, 41 and 36 kDa). We have never found even traces of the precursor p50 protein or EP45 mRNA in oocytes [this paper and Jorgensen et al. (Jorgensen et al., 2009)]. These results show that the three fast-migrating isoforms correspond to post-translation modification of the p50 precursor that occurs immediately after uptake from the blood. 2D analysis of oocyte EP45 shows a high number of additional spots corresponding to the two bands (1 and 2) found in 1D gels of stage-VI oocytes compared with stage IV. The stage-VI-specific spots appear on 2D gels both at lower and at higher pH with respect to stage IV, and we cannot rule out that some modifications could also result in vertical mobility shift in 2D gels. Some of these changes might be, at least in part, attributed to differential phosphorylation. However, stage-IV and -VI oocytes remain in the same cell-cycle stage; thus, it is certainly not a cell-cycle-dependent process. EP45 also has an amino acid signal for N-linked glycosylation (Beck et al., 1992) and we found that it is potentially ubiquitinated during embryonic mitosis (Bazile et al., 2008) (protein number 62 in the latter reference). Identification of oocyte-specific post-translational modifications of EP45 will be of great value for a full understanding of the role of EP45 in oogenesis and oocyte maturation.

**Oocyte-maturation-enhancing function of EP45**

Microinjection of EP45-encoding mRNA in stage-IV and -VI oocytes, with or without progesterone stimulation, shows that EP45 alone is not able to induce oocyte maturation. This contradicts data showing that MII-oocyte-purified pNiXa induces GVBD in stage-VI oocytes (Haspel et al., 1993). Moreover, in our hands, expression of EP45 mRNA in stage-VI oocytes stimulated with 50 nM progesterone has no effect. Haspel and colleagues (Haspel et al., 1993) observed at least three events provoked by pNiXa microinjection into oocytes: GVBD induction in non-stimulated stage-VI oocytes; a clear decrease in GVBD rate upon 10 μg/ml progesterone treatment (in comparison to progesterone alone); and synergy in the rate of GVBD upon coinjection of pNiXa with ras-p21 (compared with ras-p21 injection alone). Apparent discrepancies between these and our results could have different explanations. Firstly, pNiXa purified from MII-arrested oocytes is post-translationally modified in a MII-oocyte-specific manner, whereas the modifications of experimentally expressed EP45 are specific for meiotic prophase. Secondly, contaminants could have been co-injected with pNiXa, inducing GVBD, because MII oocytes contain all components of the M-phase-inducing molecular machinery in a highly active state, including MPF and its substrates in fully phosphorylated and active forms. To fully elucidate the role of each isoform of EP45 in oocyte physiology, further analysis will be necessary to understand the nature of post-translational modifications of this protein following its uptake.

Most interestingly, in stage-VI oocytes stimulated with 10 nM progesterone and injected with EP45-encoding mRNA (this paper), maturation of the mid-responsive oocytes was clearly enhanced. In accordance with the absence of GVBD induction without progesterone stimulation in stage-VI oocytes, the low-responsive oocytes treated with 10 nM progesterone were not stimulated to undergo GVBD upon EP45 overexpression. For the highly responsive oocytes, which respond similarly to low and high doses of progesterone, no increase in the GVBD rate upon EP45 overexpression was observed. Thus, we show that EP45 expressed in stage-VI oocytes has a previously unexpected capacity to enhance, but not to induce, GVBD. This is in agreement with the presence of high quantities of cytoplasmic EP45 in stage-VI oocytes, which do not undergo GVBD without stimulation. Further studies will be required to elucidate how EP45 enhances oocyte maturation. As an example, kallistatin, another serpin, stimulates cell proliferation through activation of the ERK1/2 pathway (Miao et al., 2000). As ERK2 mitogen-activated protein (MAP) kinase pathway activation is crucial to GVBD, one hypothesis might be that EP45 could enhance oocyte maturation by modulating this pathway.

Intriguingly, an activity-enhancing mitotic exit was recently reported (Garnett et al., 2009). Namely, UBE2S, an anaphase-promoting complex/cyclosome (APC/C) auxiliary protein, is dispensable for physiological mitotic exit, but crucial for spindle assembly checkpoint (SAC) slippage (Garnett et al., 2009). Mitotic exit enhancement of UBE2S therefore seems to be analogous to the Omen function of EP45 in M-phase entry (this paper). Thus, the existence of activity enhancing M-phase entry not restricted to meiosis could be envisaged. It might depend on various pathways independent of the core mechanism triggering M-phase entry, for example, upon mobilization of energy sources necessary for cell-cycle transitions. EP45 is probably not the only protein with Omen activity and numerous proteins acting in synergy might enhance M-phase entry. In oocytes, they could collectively contribute to the quality of oocytes, without individually affecting the core pathway leading to CDK1 activation (Marteil et al., 2009). We have recently found other potential Omen proteins as well as potential oocyte-maturation repressors (Omere) in additional proteomic screens (D’Inca et al., 2010).

The Omen activity of EP45 linked to the responsiveness of oocytes to progesterone could be involved in an evolutionary bet-hedging strategy (Kussel and Leibler, 2005). This strategy, well studied in bacteria (Beaumont et al., 2009), relies on the stochastic presence of molecules and processes in cells and organisms that are unnecessary in a normal environment, but become essential for survival in the face of drastic environmental changes. Various environmental factors could induce hormonal disorder, including decrease in progesterone. In such cases, oocytes with higher EP45 levels and activity (equivalent to mid-responsive oocytes expressing exogenous EP45 in our experiment) will mature in a higher proportion than those with lower EP45 levels and will deliver functional gametes supporting relative reproductive success of females. To our knowledge, EP45 is the first gene product identified in vertebrates that could play a role in such a bet-hedging strategy.

**Materials and Methods**

**Oocyte preparation**

*X. laevis* females were purchased from NASCO (Fort Atkinson, WI, USA). All stages of oocytes were manually isolated from the ovary after digestion in calcium-free OR2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 5 mM Heps pH 7.4) containing 257 units/ml collagenase (type 1A, Sigma).

**Sample preparations**

Oocytes and embryos were homogenized in MPF (80 mM β-glycerophosphate, 50 mM NaF, 20 mM EGTA, 20 mM Heps, 15 mM MgCl₂, 1 mM DTT, pH 7.5),
supplemented with aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml), AEBSF (1 μM), EDTA (1 mM), AEBSF (0.5 mM), and sonicated in EB buffer (20 mM Hepes, 100 mM NaCl, 0.05% Triton, pH 7.5), supplemented with DTT (1 mM), β-glycerophosphate (5 mM), Na orthovanadate (0.5 mM), and supplemented with DTT (1 mM), β-glycerophosphate (5 mM), Na orthovanadate (0.5 mM). After dissection, oocytes were cultured in Malter and Koontz buffer (83 mM NaCl, 0.5 mM CaCl2, 1 mM MgCl2, 10 mM Hepes, pH 7.8). Full-length EP45 cDNA and β-galactosidase cDNA (control) were subcloned into pCS2+ vector and transfected using the Ambion Message Machine kit. Typically, 32.2 ng RNA (EP45 or β-galactosidase) was injected into oocyte. About 14 hours post microinjection, oocytes were stimulated with 10 nM, 50 nM or 1 μM progesterone, or left untreated. The time course of maturation was followed by calculating the percentage of GVBD, as measured by the appearance of a white spot centered on the animal pole of the oocyte.

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References


