Changing story of the receptor for phosphatidylserine-dependent clearance of apoptotic cells

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The phosphatidylserine receptor (PSR) was originally described as the putative receptor for phosphatidylserine, which is displayed on the outer membrane leaflet of apoptotic cells as a so-called 'eat me' signal. Since then, contradictory findings about this protein have been published. A common characteristic of all PSR loss-of-function experiments in vertebrates has been neonatal lethality accompanied by severe developmental defects. However, impairment of phagocytosis has only been detected in some of these experiments. Furthermore, several groups have shown that PSR localizes to the nucleus. Structural *in silico* analysis of PSR indicates that it has a JumonjiC domain, and the molecular features characteristic of Fe(II)-dependent and 2-oxoglutarate-dependent oxygenases. This review summarizes the current status of research on the PSR protein.

Keywords: dioxygenase; JumonjiC domain; phosphatidylserine receptor

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Introduction

In 2000, Fadok and colleagues suggested that the phosphatidylserine receptor (PSR) is responsible for the recognition of phosphatidylserine on the surface of apoptotic cells. These authors raised an antibody against transforming growth factor- β / β -glucan-treated human macrophages (mAb217), which bound more strongly to stimulated macrophages than to non-stimulated ones. Phosphatidylserine-displaying liposomes inhibited the binding of mAb217 to macrophages and the antibody prevented the uptake of apoptotic cells (Fadok *et al*, 2000). These characteristics suggested that mAb217 interacted with a receptor for phosphatidylserine on the membrane, and prompted Fadok and colleagues to investigate the molecular nature of its antigen. By using phage display, they identified a 48-kDa protein (Fadok *et al*, 2000). When this protein was ectopically expressed in cells that could not be stained with mAb217 and that could not phagocytose apoptotic cells—such as mouse M12.C3 cells and human Jurkat cells—an antibody signal on the plasma membrane was obtained in flow-cytometric analysis.

Furthermore, 25% of M12.C3 cells that were transiently transfected with the *psr* gene gained the ability to bind apoptotic cells and 12.5% even engulfed them (Fadok *et al*, 2000).

On the basis of these results, Fadok and colleagues proposed that the 48-kDa antigen of mAb217 is the receptor for the phosphatidylserine-dependent clearance of apoptotic cells. The protein was highly conserved in mice, *Caenorhabditis elegans* and *Drosophila* (Fadok *et al*, 2000).

Annexin 1 and PSR for apoptotic cell uptake

Arur and colleagues reported that Annexin 1 (anx-1) co-localizes with phosphatidylserine on the outer membrane of dying cells, and is required for their efficient engulfment and clearance (Arur *et al*, 2003). These authors examined whether PSR could recognize anx-1 and support the uptake of apoptotic cells. Overexpression of human PSR in 293T cells—co-cultured with either apoptotic or non-apoptotic Jurkat T cells—resulted in mAb217 signals clustering around apoptotic cells, but not around non-apoptotic ones (Arur *et al*, 2003). This clustering disappeared after RNA interference-mediated silencing of anx-1 in apoptotic Jurkat T cells, and tethering defects between these cells and human umbilical vein endothelial cells (HUVECs) were observed. Comparable results were obtained through PSR silencing in endothelial cells; however, double silencing of anx-1 and PSR did not further increase the effect. These experiments led to the conclusion that PSR and anx-1 both have a crucial role in the same engulfment pathway. *In vivo* experiments in *C. elegans* larvae, carried out by the same authors, did not support these findings. Downregulation of nex-1—the anx homologue in *C. elegans*—resulted in defects in phagocytosis of apoptotic cell corpses, whereas downregulation of the predicted *C. elegans* PSR-1 protein had no significant effect (Arur *et al*, 2003).

Phagocytosis phenotype in *C. elegans*

Wang and colleagues investigated a *C. elegans* PSR loss-of-function mutant (tm469), which has a 968-base pair deletion in the *psr-1* locus and therefore lacks the main part of the PSR-1 protein. These authors detected a relatively mild engulfment defect. Cell corpses in mutant embryos remained for 55% longer than those in wild-type embryos (Wang *et al*, 2003). Overexpression of PSR-1 in the mutant strain tm469 fully rescued this defect. Wang and colleagues also showed that *C. elegans* PSR-1 could bind to

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phosphatidylserine in an enzyme-linked immunosorbent assay (ELISA; Wang et al, 2003).

Developmental defects in zebrafish

Experiments in zebrafish indicated an important role for PSR in development and morphogenesis. Screening a complementary DNA (cDNA) library resulted in the identification of the zebrafish homologue of *psr* (*zfpsr*; Hong et al, 2004). Knockdown of *zfpsr* with morpholino oligonucleotides in embryos produced severe developmental defects in the somites, brain, heart and notochord. Weakly defective embryos displayed a small delay in heart development and a bending of the notochord at three days post-fertilization (dpf). Severely defective embryos were characterized by shrinkage of the brain, loss of posterior somite development and failure to hatch at three dpf; they also had a tube-like heart that lacked atria and ventricles. The most severely affected embryos accumulated numerous cell corpses throughout their bodies at 12 h post-fertilization and died before three dpf; these were classified as death-type embryos. All defective phenotypes accumulated cell corpses between the somite boundaries, close to the notochord, which was not the case in normal embryos (Hong et al, 2004).

Knockout mice

Three research groups independently generated *psr*-knockout mice with different genetic backgrounds (Böse et al, 2004; Kunisaki et al, 2004; Li et al, 2003). Although heterozygous *psr*^{+/-} mice were devoid of anomalies, all *psr*^{-/-} mice showed strong developmental defects during early embryogenesis, and all died perinatally.

The most prominent symptoms observed in PSR-deficient mice included brain malformations, which were sometimes accompanied by exencephaly (Böse et al, 2004; Li et al, 2003). Furthermore, eye development was affected in some of the animals, with several mice lacking one or even both eyes (Böse et al, 2004). In newborn animals, Li and colleagues also noted breathing defects resulting from insufficient lumen formation in the lungs. Böse and colleagues observed delays in differentiation and lumen formation of this organ during embryogenesis; however, at birth the lungs seemed to be relatively normal in development and fully ventilated. By contrast, cardiac muscle differentiation was generally impaired, and more than 50% of the animals suffered from malformations such as double-outlet right ventricle or pulmonary artery hypoplasia (Böse et al, 2004; Schneider et al, 2004). Böse and colleagues therefore concluded that heart defects, rather than respiratory failure, were responsible for the deaths of the knockout mice. Further symptoms diagnosed in PSR-deficient mice included a block of erythroid differentiation at an early erythroblast stage (Böse et al, 2004; Kunisaki et al, 2004), impairment of thymocyte differentiation, and poor development of the thymus (Kunisaki et al, 2004), intestine and kidney (Böse et al, 2004). The observed differences in the precise phenotypes of all three knockout mice are probably attributable to the different genetic backgrounds: Kunisaki and colleagues used a chimeric 129xC57BL/6 background, Li and colleagues used a mixed 129xC57BL/6 background, and Böse and colleagues used a pure C57BL/6 background. Furthermore, the disruption of the gene started upstream of exon 1 and extended into exon 3 in the first two knockouts, but was restricted to exons 1 and 2 in the third knockout.

Böse and colleagues used the monoclonal antibody mAb217 to compare immunofluorescence staining of macrophages from normal mice with staining of cells from knockout mice. They obtained

the predicted membrane pattern in the cells from normal mice, but it was unchanged in the knockout mice. This was an indication that PSR is not the surface antigen for mAb217 (Böse et al, 2004). However, the strong deficiencies of embryonal development that were present in all three mice, as well as in the zebrafish knockdown experiments, indicated that PSR affects principal differentiation pathways.

Controversial observations were reported from the three mouse knockout studies with respect to the impairment of phagocytosis: Li and colleagues, and Kunisaki and colleagues, detected engulfment defects, whereas Böse and colleagues did not. The differences in the genetic backgrounds of the knockouts might account for these variations. Furthermore, the extreme developmental deficiencies displayed by these mice made it difficult to obtain directly comparable results for *psr*^{+/+} and *psr*^{-/-} cells from different knockouts when looking for apoptotic bodies at different stages of development. Differences in the *in vitro* phagocytosis competence of fetal liver-derived PSR-deficient macrophages were originally reported (Böse et al, 2004; Kunisaki et al, 2004; Li et al, 2003), but have been recently re-evaluated (Mitchell et al, 2006). To circumvent potential problems with selectively generated macrophages from the developmentally impaired mouse embryos, Mitchell and colleagues analysed immortalized 3T3 cell lines derived from fibroblasts of murine *psr*^{+/+}, *psr*^{+/-} and *psr*^{-/-} embryos (Li et al, 2003; Mitchell et al, 2006). These 'non-professional' phagocytes are able to recognize and clear apoptotic cells, and also express PSR (Böse et al, 2004; Parnik et al, 2000; Rabinovitch, 1995). The presence or absence of PSR in these fibroblasts did not lead to differences in their abilities to phagocytose apoptotic or necrotic cells. Rac activation in response to $\alpha_v\beta_5$ -integrin engagement, modulation of nuclear factor- κ B (NF κ B)-dependent transcription after treatment with apoptotic target cells, and the induction of specific signalling events, such as activation of protein kinase B after incubation with apoptotic cells, were also unimpaired. It was therefore concluded that PSR—which was detectable as an epitope-tagged form only in the nucleus—was involved in neither the recognition nor the engulfment of apoptotic cells.

Nuclear localization and JumanjiC domain of PSR

Nuclear localization of PSR has been observed in vertebrate cells and in the invertebrate *Hydra vulgaris* (Cikala et al, 2004; Cui et al, 2004). Cui and colleagues showed that PSR fused to the FLAG epitope and the green fluorescent protein (GFP) was exclusively found in the nucleus of different human and murine cell lines. Endogenous PSR was also detectable in the nucleus with a polyclonal PSR antibody. Five potential nuclear-localization signals (NLSs) were identified within the PSR sequence, each of which was sufficient to target overexpressed GFP-PSR to the nucleus of HeLa cells. Similar results were obtained for the PSR homologue in the early metazoan *H. vulgaris* (Cikala et al, 2004). In transfected epithelial cells, the GFP-tagged protein was found only in the nucleus. Detailed sequence analysis of the *Hydra* protein revealed three NLSs. Removal of the most carboxy-terminal NLS abolished nuclear localization of *Hydra* GFP-PSR, indicating that the other two are not sufficient for nuclear targeting. Furthermore, a putative DNA-binding motif (AT-hook) and a central Jumanji (JmjC) domain were identified in the protein. These are conserved in the PSR proteins of species ranging from *Hydra* to humans (Clissold & Ponting, 2001; Cikala et al, 2004).

Fig 1 | A comparison of various types of JumoniC-domain containing proteins. (A) Domain structure of human JumoniC (JmJc)-domain containing proteins. The colours of the domains are indicated in the box. (B) Multiple sequence alignment of the JmJc-domain of the proteins from (A); the amino-acid residues involved in Fe(II)-binding are shown in blue and the amino acids involved in 2-oxoglutarate-binding are shown in yellow. The tyrosine residue typical for histone demethylases next to the H-X-D/E-X_n-H motif is shown in red. The alignment was made by the Multiple Alignment using Fast Fourier Transform (MAFFT) program (Kato et al, 2002). (C) Ribbon diagram of the conserved core domain (amino acids W179 to K298) of the human factor-inhibiting hypoxia-inducible factor-1 (FIH-1) structure (Lee et al, 2003). The active-site residues H199, D201 and H272 in FIH are shown in blue. The hydrophobic residues that are conserved between *Hydra* phosphatidylserine receptor (PSR) and human FIH-1 are highlighted in magenta (adapted from Cikala et al, 2004). ARID, AT-rich interactive domain; C5HC2, zinc finger C5HC2 type; CXXC, zinc finger CXXC type; JARID, Jumoni AT-rich interactive domain; JHDM, JumoniC domain-containing histone demethylases; PHD, plant homeodomain.

The JmJc domain was first identified in the Jumoni family of transcription factors (Balciunas & Ronne, 2000). It represents a subgroup of the cupin superfamily, which is characterized by a barrel-shaped double-stranded β -helix (Clissold & Ponting, 2001). This domain is present in many other proteins, such as the family of Fe(II) and 2-oxoglutarate-dependent dioxygenases (Aravind & Koonin, 2001). Members of this enzyme superfamily couple the oxidative decarboxylation of 2-oxoglutarate to the hydroxylation of specific substrates, and release CO₂ and succinate in the process (reviewed by Hausinger, 2004). They require Fe(II), which is bound by two

histidine residues and an aspartate or glutamate residue. These amino acids occur in the motif H-X-D/E-X_n-H (Fig 1B, blue). The asparaginyl hydroxylase factor inhibiting hypoxia-inducible factor (FIH) belongs to this family. It is responsible for the oxygen-dependent hydroxylation of an asparagine residue of the transcription factor hypoxia-inducible factor (HIF; Hewitson et al, 2002; Lancaster et al, 2004; Lando et al, 2002) and therefore has a crucial role in the hypoxia response of mammalian cells. Cikala and colleagues compared the molecular structure of FIH-1 (Elkins et al, 2003; Lee et al, 2003) with the predicted secondary structure of

Hydra PSR and observed great similarity between the JmjC domains of both proteins (Cikala et al, 2004). The secondary structure elements predicted for the *Hydra* protein are identical to those seen in FIH-1. Furthermore, the hydrophobic residues that stabilize the β -barrel as well as the amino acids that coordinate the two cofactors in FIH-1—the H-X-D/E-X_n-H motif, and the 2-oxoglutarate binding site involving K214 and T196 on FIH (Fig 1B, yellow; Elkins et al, 2003; Lee et al, 2003)—are conserved in *Hydra* PSR. This led to the conclusion that PSR might have dioxygenase activity.

A similar activity was proposed for the *Escherichia coli* DNA-repair demethylase AlkB by means of sequence profile searches for protein fold recognition (Aravind & Koonin, 2001), and was confirmed experimentally (Trewick et al, 2002). In this case, the oxidative decarboxylation of 2-oxoglutarate is coupled to the hydroxylation and subsequent demethylation of 1-methyladenine and 3-methylcytosine in methylated DNA, which produces succinate, CO₂ and formaldehyde (Trewick et al, 2002). Recent work has shown that a new class of JmjC domain-containing histone demethylases (JHDMs) operates through a similar oxidative demethylation mechanism. The oxidase activity was shown to reside within JmjC domains, although these histone demethylases have further protein-interaction domains, including zinc finger, Tudor, plant homeodomain (PHD) and JmjN domains (Klose et al, 2006a,b; Tsukada et al, 2006; Whetstine et al, 2006; Yamane et al, 2006). JHDMs include JHDM1A (previously called FBXL11; Tsukada et al, 2006), which specifically demethylates monomethylated and dimethylated H3K36, and JHDM2A and its close relatives JHDM2B and JHDM2C, which use monomethylated and dimethylated H3K9 as substrates (Yamane et al, 2006). In accordance with its ability to remove H3K9 methylations, JHDM2A stimulated the transcription of several genes in F9 cells and was shown to be an essential co-activator of the ligand-bound nuclear androgen receptor (Yamane et al, 2006). Another group of histone demethylases is able to act on trimethylated histones H3K9 and H3K36 (Klose et al, 2006b; Whetstine et al, 2006), although it also uses monomethylated and dimethylated histones H3K9 and H3K36 as substrates.

Close comparison of the PSR sequence with these histone demethylases reveals two important differences. First, histone demethylases have a tyrosine residue closely following the H-X-D/E motif (Fig 1B, red); this tyrosine residue is not present in FIH and is also absent in PSR. Second, JHDMs have additional protein domains that are important for their enzymatic activity (Fig 1A). The double Tudor domain of JHDM3A is needed for the recognition of methylated H3K4 and H3K20 (Huang et al, 2006; Klose et al, 2006b), and the JmjN domain as well as the zinc finger of JHDM2A are indispensable for enzymatic activity (Yamane et al, 2006). Of the seven classes of JmjC domain-containing proteins, only one has no other recognizable functional domains; both FIH and PSR fall into this group together with three other potential dioxygenases (Klose et al, 2006a).

Conclusions

In summary, PSR has an important role in vertebrate development. However, a growing body of data challenges the idea that PSR is a membrane receptor for phosphatidylserine on the surface of apoptotic cells. These data also suggest that PSR is not the antigen that is recognized by mAb217 on the plasma membrane of macrophages (Williamson & Schlegel, 2004). The PSR protein is localized in the nucleus of vertebrate and invertebrate cells, and the structure of the

molecule indicates that it has Fe(II) and 2-oxoglutarate-dependent dioxygenase activity. However, the role of this enzyme activity remains unclear. Hydroxylation of asparagine or glutamine residues, demethylation of lysines in histones, demethylation of methylated lysine or, more speculatively, of arginine residues in other proteins, and even demethylation of nucleic acids for DNA repair or during RNA modification are among the possibilities. The enzymatic activity of PSR is probably able to modify as yet unknown important regulators in the nucleus. The absence of these PSR-specific modifications in PSR loss-of-function experiments probably accounts for the observed severe developmental defects. The identification of proteins and/or nucleic acids that physically interact with PSR, as well as functional studies of this protein at the cellular level, should shed light on its function in the near future.

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