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The phosphatidylserine receptor from *Hydra* is a nuclear protein with potential Fe(II) dependent oxygenase activity

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Abstract

Background: Apoptotic cell death plays an essential part in embryogenesis, development and maintenance of tissue homeostasis in metazoan animals. The culmination of apoptosis *in vivo* is the phagocytosis of cellular corpses. One morphological characteristic of cells undergoing apoptosis is loss of plasma membrane phospholipid asymmetry and exposure of phosphatidylserine on the outer leaflet. Surface exposure of phosphatidylserine is recognised by a specific receptor (phosphatidylserine receptor, PSR) and is required for phagocytosis of apoptotic cells by macrophages and fibroblasts.

Results: We have cloned the PSR receptor from *Hydra* in order to investigate its function in this early metazoan. Bioinformatic analysis of the *Hydra* PSR protein structure revealed the presence of three nuclear localisation signals, an AT-hook like DNA binding motif and a putative 2-oxoglutarate (2OG)-and Fe(II)-dependent oxygenase activity. All of these features are conserved from human PSR to *Hydra* PSR. Expression of GFP tagged *Hydra* PSR in hydra cells revealed clear nuclear localisation. Deletion of one of the three NLS sequences strongly diminished nuclear localisation of the protein. Membrane localisation was never detected.

Conclusions: Our results suggest that *Hydra* PSR is a nuclear 2-oxoglutarate (2OG)-and Fe(II)-dependent oxygenase. This is in contrast with the proposed function of *Hydra* PSR as a cell surface receptor involved in the recognition of apoptotic cells displaying phosphatidylserine on their surface. The conservation of the protein from *Hydra* to human infers that our results also apply to PSR from higher animals.

Background

Apoptotic cell death plays an essential role in embryogenesis, development and maintenance of tissue homeostasis in metazoan animals. The culmination of apoptosis *in vivo* is the phagocytosis of cellular corpses. One morphological characteristic of cells undergoing apoptosis is loss in the phospholipid asymmetry of the plasma membrane and exposure of phosphatidylserine on the outer membrane leaflet. Surface exposure of phosphatidylserine is

required for phagocytosis of apoptotic cells by macrophages and fibroblasts [1,2].

A putative receptor for phosphatidylserine binding was cloned from human and mouse macrophages [3]. The receptor (PSR) was identified by phage display as the antigen for a monoclonal antibody generated against stimulated human macrophages. This antibody inhibited uptake of apoptotic cells by macrophages, fibroblasts and epithelial cells.

When cells that do not express the receptor and cannot engulf apoptotic cells normally, like Jurkat T, were transiently or stably transfected with PSR they gained the ability to phagocytose apoptotic cells in a phosphatidylserine specific manner. Additionally it was demonstrated that, dependent on their interaction with phosphatidylserine, phagocytosing macrophages release cytokines to suppress the inflammatory response [3]. In further studies it was shown that the interaction of phosphatidylserine with PSR is not so much involved in tethering the apoptotic cell towards the phagocyte but rather needed for ingesting it [4]. Anti-PSR antibodies prevented the stereospecific phosphatidylserine dependent uptake of artificial target particles. Recently it was also supposed that PSR is involved in annexin-1 mediated apoptotic cell engulfment [5]. New work from *Caenorhabditis elegans* describes that embryonic cell corpses persisted for an average of 55% longer in PSR mutants when compared to wild type animals [6]. Mice deficient in PSR showed a much stronger phenotype. They died shortly after birth due to respiratory failure and exhibited an accumulation of non-ingested dying cells in the lung together with the recruitment of inflammatory leukocytes. They also showed hyperblastic brain malformations that in some aspects were similar to such observed in caspase-9 and APAF-1 knockout mice [7-9].

A very striking feature of PSR is its enormous conservation between mammals, flies and nematodes. This initiated our interest in the question of whether this protein was also present in *Hydra* and if conservation of its function was preserved.

We identified a *Hydra* cDNA encoding PSR with high homology to the mammalian, *C. elegans* and *Drosophila* genes. Absolutely conserved between PSR from all species are three nuclear localisation signals and an AT-hook DNA binding motif. In accordance with this we found *Hydra* PSR localised in the nuclei of transfected *Hydra* cells. Mutation of one of its nuclear localisation signals blocked this localisation. We have also identified a striking structural homology of all PSRs with the enzymatic domain of FIH-1 (factor inhibiting HIF) which has a 2OG-and Fe(II)-dependent oxygenase activity [10]. Our data suggest that the primary function of PSR in *Hydra* is nuclear and not directly linked to phagocytosis. This idea may also apply to mammalian PSR. Very recent work shows nuclear localisation for human PSR in a number of mammalian cell lines [11].

Results and Discussion

Isolation and characterisation of a Hydra *homologue of PSR*

We first isolated *Hydra* PSR using sequence information from an alignment of human and mouse PSR with the predicted ORFs from *Drosophila* and *Caenorhabditis elegans*. Degenerated primers were designed and RT-PCR was carried out with hydra mRNA as template. PCR revealed a 0.5 kb product. It contained an ORF that showed substantial homology with the published PSR sequence. We labelled the PCR product with DIG and used it as a probe to screen a cDNA library. We obtained a full-length *Hydra* PSR clone.

Fig. 1 shows an alignment of *Hydra* PSR with PSR from human, mouse, *Drosophila* and *Caenorhabditis elegans*. The middle part of the molecule (red box) comprises the region with most conservation between species and contains a very ancient structurally conserved domain, the cupin fold. It is characterized by a β-barrel structure and has been found in prokaryotic and eukaryotic proteins including metalloenzymes, sugar binding proteins and a number of seed storage proteins (for review see [12]). More recently this cupin fold was detected in members of the jumonji (JmjC) family of transcription factors [13]. Comparing domain structures of proteins containing JmjC domains revealed striking similarity of PSR with the HIF-inhibitory protein (FIH, factor inhibiting HIF, GenBank accession number Q9NWT6 [14]). Both, PSR and FIH contain only one JmjC domain. The structure of human FIH-1 has recently been solved. The core of FIH-1 forms a β-barrel structure (Fig. 2). It also contains a H-X-D/E dyad and a histidine residue located C-terminally of this motif [15,16]. These are features of a number of Fe(II) dependent enzymes that oxidise peptide substrates and 2-oxoglutarate using O_2. In accordance with this structure it has been shown that FIH-1 is able to hydroxylate a specific asparagine residue within the transactivation domain of its substrate, HIF (hypoxia inducing factor). This allows the regulation of HIF activity in response to oxygen.

We analysed the secondary structure of *Hydra* PSR using the program **PredictProtein** [17]. Figure 3 shows an alignment of the FIH-1 β-barrel with the corresponding region in *Hydra* PSR. Arrows indicate extended β-strands as they were predicted by **PredictProtein** for PSR; extended β-strands for FIH-1 were taken from the published crystal structure [15]. There is an obvious similarity in the arrangement of β-strands between the central domain of

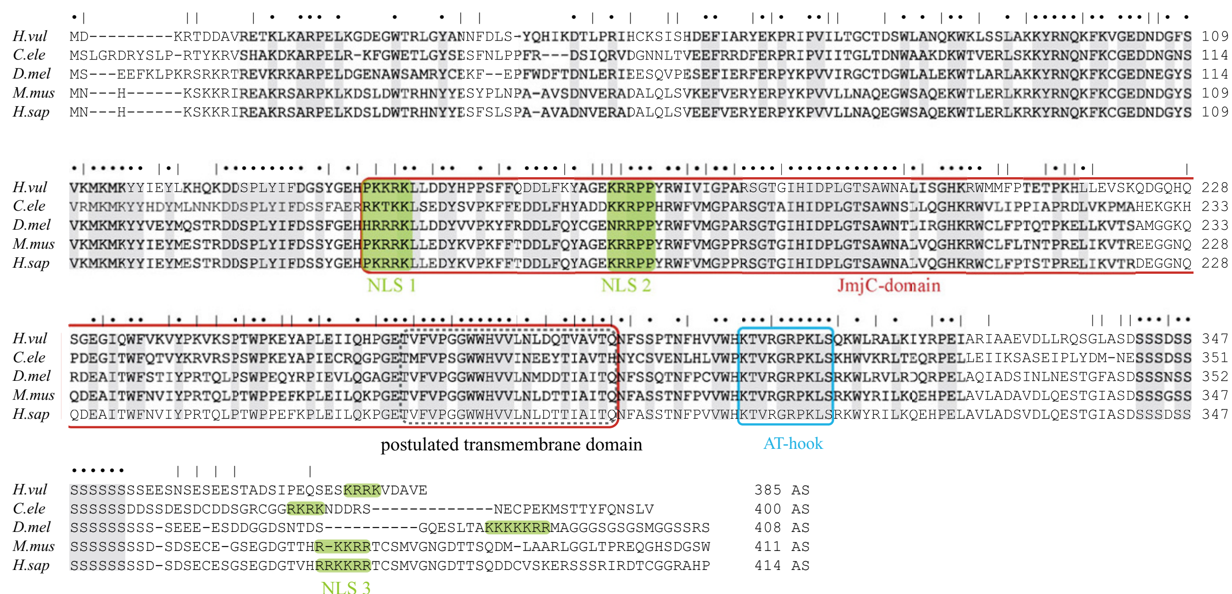

Figure 1

***Hydra* PSR sequence** Clustal alignment of PSR sequences from *Hydra* (H. vul, accession number AY559448), *Caenorhabditis elegans* (C. ele, NP500606), *Drosophila* (D. mel, NP651026), mouse (M. mus, AK122317.1) and human (H. sap, BAA25511). The JmjC domain is labelled with a red box, AT-Hook with a blue box. The putative nuclear localisation signals are depicted in green. A black dotted box encircles the previously postulated transmembrane domain [3].

PSR and FIH-1 (Fig. 3). Moreover, closer analysis of the 3-D structure of the β-barrel that is formed by the depicted β-strands of FIH-1 revealed the hydrophobic residues that stabilise the barrel (Fig. 2 and 3). These are also conserved between PSR and FIH-1, making it very likely that the conserved core domains of both proteins adapt the same fold. Moreover, amino acids that are diagnostic for 2-OG and Fe(II)-dependent oxygenases are conserved (e.g. the H-X-D dyad, that coordinates the catalytic Fe (II) is represented by H199 and D201 in FIH-1 and by H187 and D189 in PSR, and the C-terminal histidine H279 in FIH-1 that corresponds to H272 in PSR, moreover a lysine residue that binds 2-OG, K214 in FIH-1 and K204 in PSR) [18]. In addition, residues T196 in FIH-1 (=T184 in PSR) and N294 in FIH-1 (=N286 in PSR) in the active site are also conserved, whereas the neighbouring residues that line the active site pocket differ and N205 and F207 in FIH-1 correspond to A195 and N197 in PSR. This strongly suggests that the core domain of PSR could catalyse a similar oxidative reaction as FIH-1 or other members of the enzyme superfamily. The differences in the active site pocket, however indicate that PSR most likely has a different substrate specifity. Experimental proof for this prediction has to await the identification of a substrate for such an activity.

PSR shows additional sequence motifs. N-terminally to the central enzymatic domain the motif KTVRGRPKLS is found. It is completely conserved in PSR from *Hydra* to *Homo sapiens* (except an R-K exchange on position 4 in *Caenorhabditis elegans*) and has high similarity with an AT-hook motif. This is a short DNA binding motif first described in the high mobility group of non-histone chromosomal proteins (HMG). Since then it has been discovered in a large number of DNA binding proteins [19]. The motif is centred on the three amino acids GRP that are necessary and sufficient to bind to DNA.

Another conserved feature in PSR from *Hydra* to human are several putative nuclear localisation signals. They are (141) PKKRK (145) and (167) KRRPP (171) in the predicted cytoplasmic domain and (377) KRRK (380) in the predicted extracellular domain (illustrated in Fig. 1).

In Fig. 1 we have also indicated the putative transmembrane domain that was postulated by Fadok [3]. However,

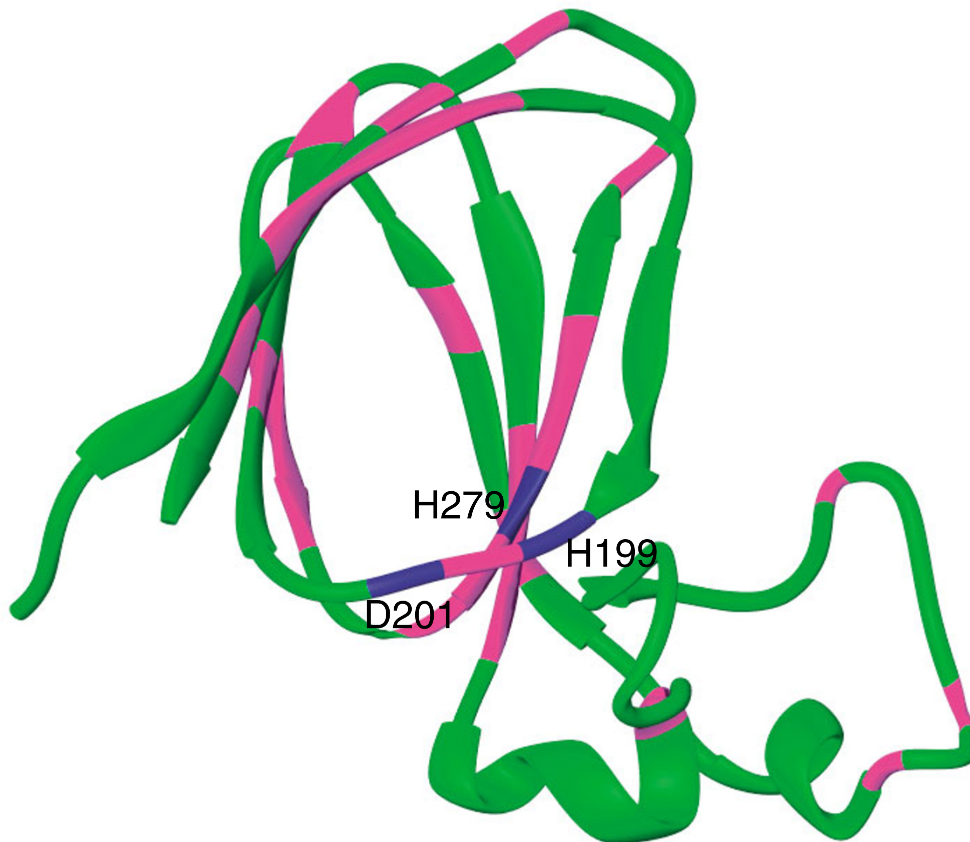

Figure 2
Conserved hydrophobic residues between human FIH-1 and *Hydra*-PSR Ribbon diagram of the conserved core domain (amino acids W179 to K298) of the human FIH-1 structure (structure from [15] using RIBBON (Carson 1997). The active site residues H199, D201 and H272 in FIH are highlighted in blue. The hydrophobic residues that are conserved between *Hydra*-PSR and human FIH-1 are highlighted in magenta.

none of the transmembrane prediction programs we used (see Methods) could identify a transmembrane domain in this region or anywhere else in the hydra PSR molecule.

This prompted us to investigate the subcellular localisation of this protein. Based on the described function of human PSR in phagocytosis a membrane association was expected.

Subcellular localisation of PSR and its NLS-mutants

We expressed both, N-terminal (GFP-PSR) and C-terminal (PSR-GFP) GFP fusion proteins with PSR in hydra under the control of an actin promoter (Böttger 2002). Subcellular localisation of the GFP-fusion proteins was observed in living animals (Fig. 4) and also after fixation (Fig. 5). Both fusion proteins were clearly localised to the nucleus of hydra epithelial cells (Fig. 4A,4B and 4C and Fig. 5A and 5B). For comparison nuclear staining was also applied (SYTO-15 in Fig. 4A and TO-PRO-3 in Fig. 5A and 5B). In order to look for membrane localisation we used the vital membrane stain FM-464 (Molecular Probes) on non-fixed animals (Fig. 4B and 4C). No GFP co-localised with membranes.

As described above, the PSR protein contains 3 putative nuclear localisation signals (Fig. 1). After deletion of NLS

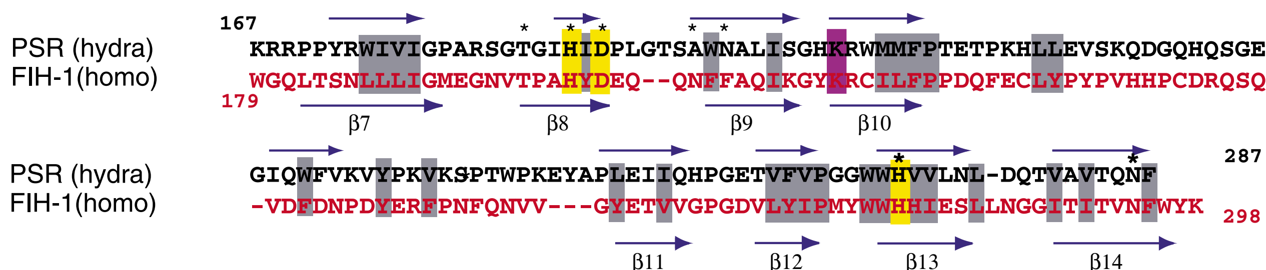

Figure 3

Alignment of the conserved core of human FIH-1 with the homologous region from *Hydra*-PSR Alignment of the conserved core of human FIH-1 forming the central β-barrel structure that harbours the active site with the homologous region from *Hydra*-PSR. Extended β-strands are depicted by blue arrows, for *Hydra*-PSR above the sequence, for human FIH underneath and numbered according to the published crystal structure [15]. Yellow boxes indicate the residues necessary to complex the active site Fe^{2+} ion, violet box indicates the lysine implicated in 2-oxo-glutarate binding. The hydrophobic residues that stabilise the barrel structure are highlighted in grey. Stars indicate residues within 4 Angstrom of the catalytic H-X-D that constitute the active site.

1 or NLS 2, nuclear localisation could still be observed (Fig. 5C and 5D). Deletion of both these NLS sequences did not change the nuclear localisation of the protein either (Fig. 4D). However, deletion of the most C-terminal NLS compromised nuclear localisation of the fusion protein dramatically. This is depicted in Fig. 4E and 4F and in Fig. 5E. In GFP-PSRΔNLS3 GFP is distributed throughout the cell, both in fixed and in non-fixed hydra cells. An additional GFP fusion protein only containing the JmjC domain of PSR is localised in the same way (not shown). Interestingly, although not nuclear, these mutants are not localised to the membrane either.

From these data we concluded that *Hydra* PSR is normally localised in the nucleus. Nuclear localisation depends on a C-terminal nuclear localisation signal. The nuclear function of this protein has still to be established. The presence of an AT-hook in the sequence suggests an attachment to DNA. More strikingly, the putative dioxygenase activity of this molecule that is inferred by comparison of its sequence and secondary structure with FIH-1, could point to a function in the regulation of nuclear proteins by hydroxylation. This regulation would be dependent on the concentration of oxygen, as it has been described for FIH-1 and oxygen dependent activation of HIF.

The question remained, if *Hydra* PSR also has a function in phagocytosis. We followed the fate of GFP-PSR in transfected hydra cells after induction of apoptosis with colchicine or with the PI(3) kinase inhibitor wortmannin. Both treatments lead to massive apoptosis of interstitial cells in *Hydra* [20]. These dying cells display phosphatidylserine on their surface and are phagocytised by epithelial cells. This was demonstrated by staining apoptotic hydra cells with annexin-V-fluorescin (Fig. 6). We could not observe a change in the nuclear localisation of GFP-PSR in epithelial cells that were in the process of phagocytising apoptotic cells. Figure 7 shows an epithelial cell that has ingested two apoptotic cells and appears to be in the process of phagocytising one more that is in its close proximity. GFP-PSR is still present in the nucleus and no GFP-PSR is localised to the membrane.

Conclusions

Our results clearly contradict a primary function for *Hydra* PSR as a membrane receptor involved in phagocytosis. We suggest that it is a nuclear dioxygenase that is capable of modifying nuclear proteins. We can, however, not exclude that the protein excerts an indirect effect on apoptosis and phagocytosis. The implied oxygen dependence of its putative enzymatic activity could interact with apoptotic mechanisms. This would also provide an additional explanation for the dramatic effect on lung development observed in PSR knockout mice [8]. In order to understand the function of PSR its intracellular targets will have to be identified.

Methods

Cloning of Hydra *PSR*

Degenerated primers were designed on the basis of the sequence homologies between PSR from human to *Caenorhabditis elegans*.

Primer 2 (forward): AARATGAARATGAARTAYTA

Primer 5 (reverse): NACNACRTGCCACCANCC

RT-PCR using hydra cDNA as a template revealed a 0.5 kb fragment. After sequencing this fragment was labelled

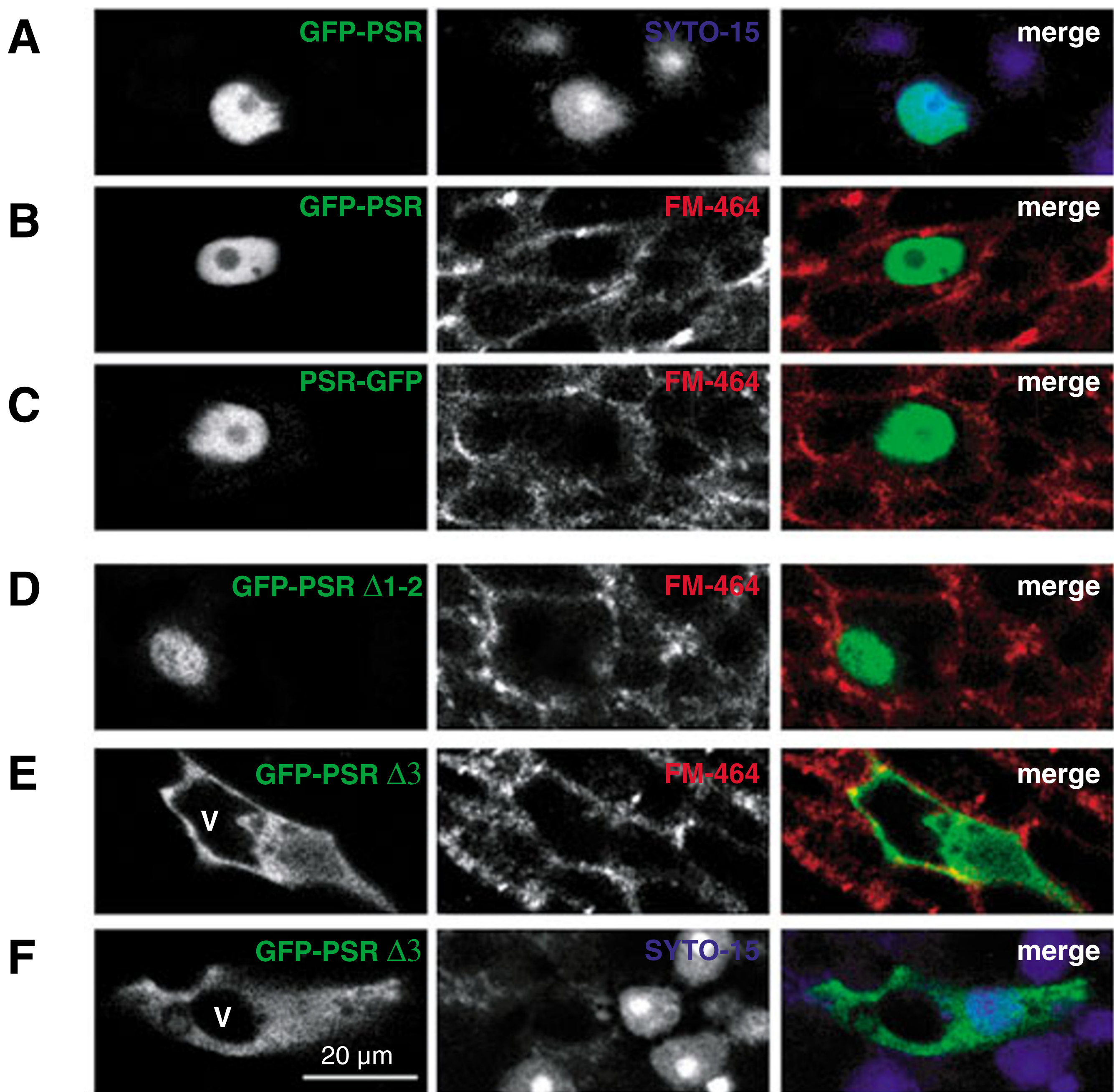

Figure 4
PSR localisation in living animals Single optical sections of GFP expressing cells of living hydra after transfection with GFP-PSR (A and B), PSR-GFP (C), NLS-mutants ΔNLS1-2 (D) and ΔNLS3 (E and F). Left hand panels show GFP, middle panels nuclear staining with SYTO-15 or membrane staining with FM-464 as indicated. Right hand panels are merged images. V indicates vacuoles typically seen in living hydra cells.

with DIG and used to screen a *Hydra* cDNA library as described previously [20]. The sequence has been submitted to GenBank (accession number AY559448).

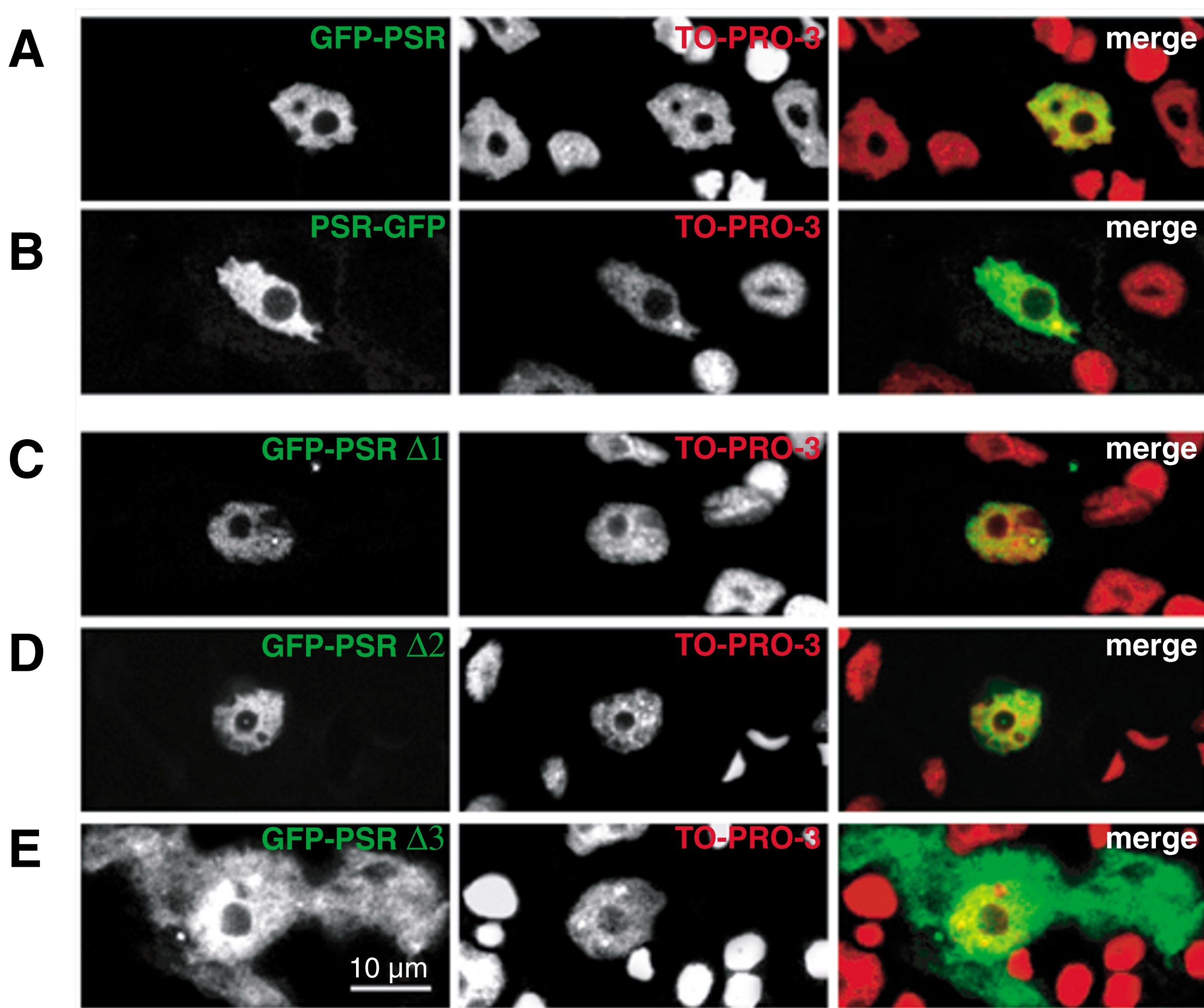

Figure 5
PSR localisation in fixed animals Single optical sections of GFP-PSR (A), PSR-GFP (B) and single NLS-mutants ΔNLS 1, 2 and 3 in single GFP expressing cells after fixation (C-E). Left hand panels detect GFP, middle panels DNA-staining with TO-PRO and right hand panels represent merged images. Nuclear morphology is typical for hydra epithelial cells after fixation. Note that nucleoli are not stained with TO-PRO (middle panel) and also free of GFP (left hand panel).

Cloning of psr-gfp *fusions in the EGFP expression vector [21]*

First a Sma I-site was introduced into the vector 5' to the *gfp*-sequence by site directed mutagenesis. For PSR-GFP fusion proteins the *psr* sequence was introduced into this Sma1-site. For GFP-PSR fusion proteins it was cloned into the EcoR I-site. The STOP-codon 5' of this site was then removed by site-directed mutagenesis. This created a short linker between GFP and PSR encoding for the amino acids IAFVAF.

NLS-mutants were introduced by site directed mutagenesis. In ΔNLS1 amino acids 142–145 (KKRK) were deleted, in ΔNLS2 amino acids 166–168 (KRR) and in NLSΔ3 amino acids 377–380 (KRRK). The double mutant Δ1,2 was constructed by deleting amino acids 166–168 in ΔNLS 1.

DNA was introduced into hydra cells using a particle gun as described previously [21].

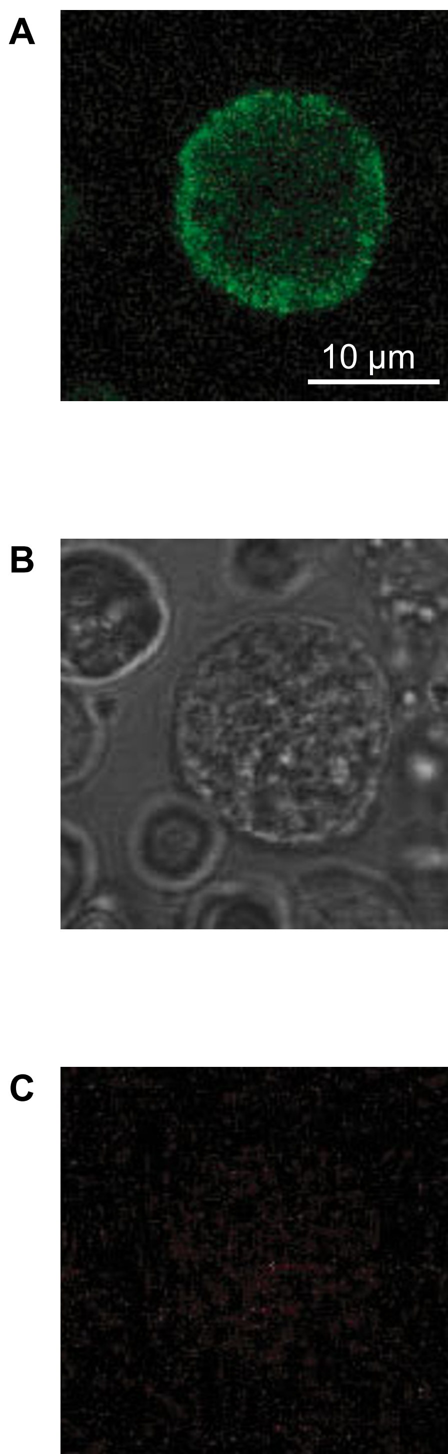

Figure 6
Phosphatidylserine on the extracellular face of apoptotic hydra cells (A) annexin-V-fluorescin label of a hydra cell after induction of apoptosis with colchicine, (B) phase contrast image of the cell, (C) absence of propidium iodid (PI) staining: PI can only enter cells after membrane integrity is lost, absence of PI staining indicates that annexin-V-fluorescin binds the extracellular face of the membrane.

Confocal imaging
Living animals with GFP expressing cells were stained with FM-464 (Molecular Probes) for detection of plasma membranes or SYTO-15 for nuclear staining, treated with urethane and scanned immediately. Fixation was carried out with 2% paraformaldehyde in PBS for 1 h. Fixed animals were counterstained for DNA with TO-PRO-3 (Molecular Probes) and mounted on slides with VECTASHIELD mounting medium (Alexis Biochemicals).

Light optical serial sections were acquired with a Leica (Leica Microsystems, Heidelberg, Germany) TCS SP confocal laser scanning microscope equipped with an oil imersion Plan-Apochromat 100/1.4 NA objective lens. Fluorochromes were visualized with an argon laser with excitation wavelengths of 488 nm, 488 nm, 514 and emission filters 520–540 nm, 550–700 nm and 530–550 for EGFP, FM-464 and SYTO-15 respectively, and with a helium-neon laser with excitation wavelength 633 nm, emission filter 660–760 nm (for TO-PRO-3). Two fluorochromes and the phase contrast image (transmission filter) were scanned sequentially. Image resolution was 512 × 512 pixel with a pixel size ranging from 195 to 49 nm depending on the selected zoom factor. The axial distance between optical sections was 200 nm for zoom factor 4 and 1 µm for zoom factor 1. To obtain an improved signal-to-noise ratio each section image was averaged from 4 successive scans. The 8 bit greyscale single channel images were overlayed to an RGB image assigning a false colour to each channel, and then assembled into tables using Adobe Photoshop 5.5.

Apoptosis was induced with 1 µM wortmannin for 4 hours before cells were analysed. For annexin-V-fluorescin staining apoptosis was induced with 0.4% colchicine for 6 hours. Animals were then dissociated with pronase [20] and incubated with annexin-V-fluorescin and PI. The two fluorochromes and the phase contrast were scanned as described above.

Structural predictions
PSORT II[22] was used to predict the nuclear localisation signals. The same program did not predict an N-terminal signal peptide or transmembrane domain for the threshold 0.5. Furthermore for prediction of transmembrane domains we used Toppred 2 [23] for eukaryotic cells with the default settings (Cutoff for certain transmembrane segments: 1.00, Cutoff for putative transmembrane segments: 0.60, Critical distance between 2 transmembrane segments: 2, Critical loop length: 60.) and TMHMM [24], also with default settings.

Abbreviation
PSR phosphatidylserine receptor

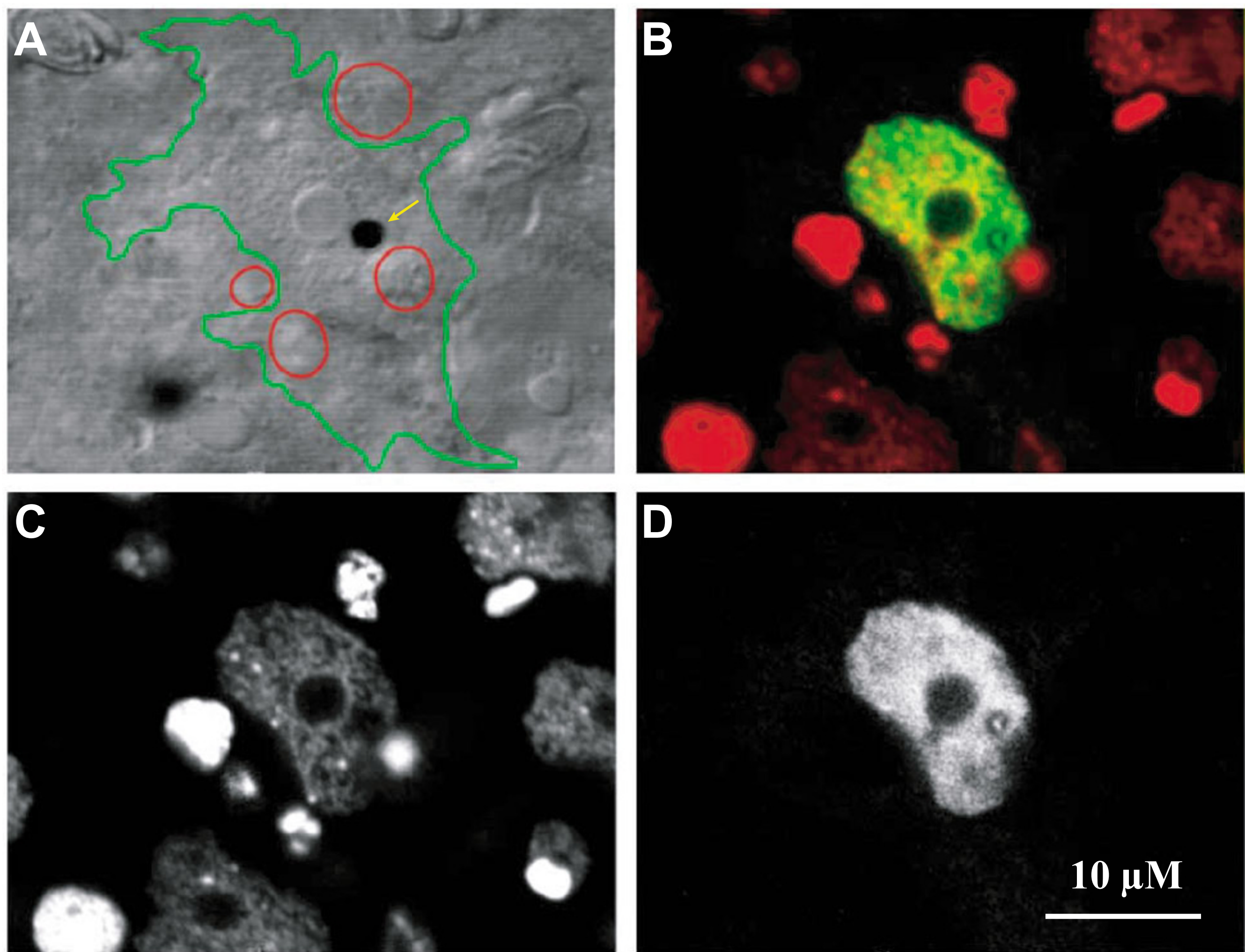

Figure 7
PSR stays in the nucleus of phagocytising cells Hydra epithelial cell expressing GFP-PSR in the nucleus. The cell has engulfed two apoptotic cells with pycnotic nuclei (red circles). Two additional apoptotic cells are still on the outside of the cell surrounded by its membrane (red circles). They are apparently in the process of being phagocytised. Apoptosis was induced with wortmannin. Single sections of confocal images are shown: (A) phase contrast, (B) merged image from (C) and (D), (C) TO-PRO-DNA staining, (D) GFP. In (A) a green line marks the outline of the transfected cell. Yellow arrow indicates the gold particle. Scale bar 10 µm.

GFP green fluorescent protein

EGFP enhanced GFP

ORF open reading frame

NLS nuclear localisation signal

HIF hypoxia inducing factor

FIH-1 factor inhibiting HIF

2-OG 2 oxoglutarate

PI propidium iodid

Authors' contributions

M.C. isolated *Hydra* PSR cDNA and cloned most of the GFP-fusion constructs, O.A. and B.S. carried out transfection experiments and O.A. imaged the fusion proteins in living and fixed animals, M.P. constructed and analysed GFP-JmjC and carried out part of the bioinformatic analyses, P.C. did the structural comparisons between PSR and

FIH-1, C.N.D. participated in the design of the study, A.B. designed and coordinated the study and drafted the manuscript. All authors read and approved the final manuscript.

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