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Characterization of the biochemical and biophysical properties of the phosphatidylserine receptor (PS-R) gene product

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Abstract The PS-R gene product was originally described as a cell surface receptor that interacts with externalized phosphatidylserine (PS) on apoptotic cells, but more recent studies have shown that it plays a critical role in organ development and terminal differentiation of many cell types during embryogenesis. Despite these important developmental functions, the biochemical and molecular properties of PS-R are poorly understood. Here we have used several approaches to show that PS-R undergoes processive post-translational protein cross-linking to form covalent multimers within the nuclear compartment. Although PS-R has a potential Glu-Glu (QQ) duet that is often targeted by transglutaminase TG-2, the oligomerization of PS-R was not effected by QQ → AA mutation, or when PS-R gene product was expressed in TG-2 (-/-) fibroblasts. Pulse-chase experiments with ^{35}S-methionine indicates that the PS-R undergoes an initial proteolytic cleavage, followed by progressive multimerization of the monomeric subunits over time. In summary, we report here that PS-R is modified by an unusual post-translational modification, and we speculate that homomultimer of PS-R might be playing an important function as a scaffolding protein in the nucleus.

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Introduction

The PS-R gene product was originally characterized as a type II transmembrane protein receptor on phagocytes that directly recognizes externalized PS on the apoptotic cell [1]. During physiological cell death or apoptosis, apoptotic cells redistribute PS on their surface which acts as an ''eat-me'' cue for efficient and safe engulfment without inflammation [2–4]. Many serum proteins, such as Milk-Fat Globule Protein-EGF Factor 8 (MFG-E8) [5], Developmental-Endothelial Locus-I (Del-1) [6], and vitamin K-activated proteins and Gas 6 and Protein S [7–8] serve as PS-binding proteins that promote phagocytosis of apoptotic cells. Although recent studies have cast doubt that the PS-R functions as a phagocytic receptor [9–11], its physiological function has not yet been established. PS-R has a predominantly nuclear localization and has sequences homologous to Jumonji-family transcription factors [9–11]. Therefore, PS-R may play a role in the transcription of competence factors regulating terminal differentiation, but not directly in apoptotic cell clearance. Curiously, however, in *C. elegans*, PS-R has been genetically linked to the Ced-12 (ELMO) and Ced-5 (DOCK180) pathway, possibly by direct binding to these proteins [12] Like the PS-R gene product, a functional complex of DOCK180 and ELMO has been reported to reside in the nucleus [13]. Additional studies are required to ascertain how PS-R indirectly influences the phagocytic machinery, either as a binding partner or through regulation of gene expression.

While the exact molecular function of PS-R remains elusive, several groups have reported severe phenotypes of mice deficient in the PS-R gene product, and all point to a critical role in organ development, morphogenesis, and terminal differentiation resulting in perinatal lethality [14–17]. At the organogenesis and tissue level, C57 Bl/6 mice deficient in PS-R manifested organ abnormalities consistent with a defect in apoptotic cell clearance, including a hyperplastic brain phenotype, reduced luminal air space in the lungs, and macrophage dysfunction [16]. Other PS-R knockout studies show defective fetal liver erythropoiesis [15], T cell lymphopoiesis, and defects in cardiac development [17]. Defects in embryonic development and subsequent apoptotic cell clearance have also been reported after disruption of PS-R orthologs in Zebra fish, *Drosophila melanogaster*, and *C elegans*, suggesting that the function of PS-R is evolutionarily conserved [12, 18–19]. What is not entirely clear is whether defects in phagocytosis are secondary to developmental and organogenesis defects that impair the differentiation of lymphoid and myeloid cells into mature phagocytes [14]. Clearly, a better understanding of the biochemistry of the PS-R gene product is warranted to understand its physiological functions.

In the present study, we have begun to characterize the biochemical function of PS-R in mammalian cells. Consistent with previous reports, we find that endogenous or ectopically-expressed PS-R localizes to the nucleus independently of N or C-terminal epitope-tags. Interestingly, however, isolation of nuclear extracts from PS-R-expressing cells shows that nuclear PS-R is characterized by progressive post-translational multimerization to form non-disulfide linked covalent dimers, trimers, tetramers, and pentamers. Although we identified a potential transglutaminase 2 consensus site, covalent cross-linking of PS-R was not abrogated in TG2 (-/-) cells, nor did mutations of the most conserved QQ duet motif affect this post-translational modification. Our present data indicate that the PS-R gene product undergoes an unusual form of covalent cross linking to form protein multimers. We speculate that this may facilitate PS-R function as a scaffold to assemble protein-protein or protein-DNA interactions in the nucleus.

Materials and methods

Cell culture

HEK 293, HeLa and NIH 3T3 cells were maintained in Dulbecco's Modified Eagles' medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Transglutaminase-2 null cells (TG-2(-/-) were generously provided by Dr Gerry Melino (University of Tor Vergata, Rome, Italy) and PS-R (-/-) cells were kindly provided by Dr. Richard Flavell (Yale University School of Medicine). Immunofluorescence of fixed cells was carried as previously described [11].

Antibodies and plasmids

Anti-PSR antibody was purchased from Abcam (Cambridge, MA), anti-HA mAb was purchased from Santa Cruz Biotechnology (San Diego, CA), and anti-Flag mAb was from Sigma Chemical Company (St Louis, MO). HRP conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies were from Jackson Laboratories (Bar Harbor, ME). pRK-HA-PSR was a kind gift from Dr Valerie Fadok and Dr. Peter Henson (National Jewish University, Denver, Colorado). pcDNA-PSR-V5-His or pCX-GFP-*ires*-PS-R were generated by inserting a PCR product of PS-R into pcDNA 3.1/ V5-His-Topo or pCX, to express a C-terminal or a non-epitope tagged protein respectively. The pcDNA-PSR (QQ to AA) - V5-His mutant construct was made by site directed mutagenesis. All DNA constructs were verified by DNA sequencing.

Transfection, immunoprecipitation, and western blotting

A total of 5×10^5 HEK or NIH3T3 cells were plated on 60 mm tissue culture plates. The cells were transfected with expression plasmids as indicated in the figures using Lipofectamine2000 (Invitrogen, Carlsbad, California). After 48 h, the cells were lysed and the nuclei isolated in Sigma buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1 mM EDTA and 1% Triton X-100) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 μg/ml aprotinin. Nuclear protein quantification was determined by the Bradford Lowry method. For immunoprecipitation, 1.0 mg of total nuclear protein was incubated with primary antibody for 2 h at 4°C, followed by incubation with protein A-Sepharose beads (Amersham Biosciences) for 1 h at 4°C. The beads were then washed four times with 0.1% HNTG buffer and samples were separated onto 6% SDS PAGE gel. The gel was then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Blots were blocked in Tris-buffered saline (TBS) containing 5% milk for 30 min, and subsequently incubated with primary antibodies for 1 h. The blots were subsequently incubated with Horseradish Peroxidase (HRP)-conjugated secondary antibodies, and detected by Enhanced Chemiluminescence (ECL).

Metabolic labeling and pulse chase with ^{35}S-methionine

5×10^5 HEK cells were transfected with the pRK-HA-PSR construct using Lipofectamine (Invitrogen, Carlsbad, CA).

After 24 h post transfection, cells were incubated with ^{35}S-labeled-methionine (ISC Biosciences) (100 μCi/ml) for 30 min and following the pulse, cells were incubated in DMEM with excess cold L-methionine and L -cystine for up to 24 h. At the indicated time points, the cells were lysed with Sigma buffer. 1.0 mg of total nuclear protein extract was immunoprecipitated with anti-HA antibody. The lysates were then washed with 0.1% HNTG buffer and samples were boiled with sample buffer and loaded on a 6% SDS PAGE gel. The gel was developed with fluorographic En^{3}Hance followed by autoradiography.

Mass spectrometry analysis

Protein mixture was separated by SDS-PAGE and stained with SyproRuby^{TM} dye. The gel bands were excised and washed with 30% ACN in 50 mM ammonium bicarbonate prior to proteolytic digestion using 25 ng/μl of trypsin for 2 h on a robotic platform (TECAN, Durham, NC). The resulting peptides were extracted with 30 μl of 1% trifluoracetic acid followed by C_{18} Ziptip desalting. The resulting peptides were mixed with 7 mg/ml α-cyano-4-hydroxy-cinnamic acid matrix in a 1:1 ratio and spotted onto a matrix assisted laser desorption/ionization (MALDI) plate. The peptides were analyzed on a 4700 Proteomics Analyzer tandem mass spectrometer (Applied Biosystems, ABI, Framingham, MA). Mass spectra (*m/z* 800–3,600) were acquired in positive ion reflector mode. The 15 most intense ions were selected for subsequent MS/MS sequencing analysis in 1 kV mode. Protein identification was performed by searching the combined MS and MS/MS spectra against the human sequences in Swiss-Prot database (V. 46) using a local MASCOT search engine (V. 1.9) on a GPS (V. 3.5, ABI) server. Proteins containing at least two peptides wereidentified with Confidence Interval (C. I.) values no less than 95% were considered identified.

Results

Mice that carry targeted deletions of PS-R show major defects in organogenesis, resulting in perinatal lethality, growth retardation, and defective terminal differentiation in a multitude of organs, including the brain, heart, and liver [20]. Although immature macrophages isolated from PS-R (-/-) mice showed defects in their ability to induce IL-10 and TNF-α when challenged with apoptotic cells or LPS respectively [14], neither PS-R (-/-) fetal macrophages [14] or PS-R (-/-) embryonic fibroblasts [11] revealed quantitative defects in the engulfment of apoptotic cells. To understand better the molecular and cellular function of PS-R, we expressed the hPS-R gene product in mammalian fibroblasts and epithelial cells. As shown in Fig. 1A (i), HA-PS-R (N-terminal tag) localized predominantly to the nucleus when ectopically expressed, with little or no cytoplasmic or plasma membrane staining detectable. Identical results were obtained Fig. 1A, (ii) when PS-R-V5 (C-terminal tag) was expressed. Interestingly, however, although the native molecular weight of PS-R is approximately 50 kD, when we made nuclear lysates from cells expressing HA-PS-R, PS-R-V5, or GFP-*ires*-PS-R for Western blot analysis, PS-R was detected as a discrete protein ladder of ~50, 100,150, 200, and 250 kD proteins (Fig. 1B, lanes 1–3; indicated as a–d in the figure). The migration of the PS-R-V-5 is larger than that of the HA-PS-R due to the larger size of the epitope tag, although the same pattern was noted. Multimerization did not appear to be an artifact of over-expression of the tagged proteins, since the same pattern was observed in lysates from cells expressing GFP-*Ires*-PS-R (which is not epitope-tagged. Lane 3) or from native cells without over-expression, using an antibody raised against the PS-R protein itself (lane 4). This pattern of PS-R staining was conspicuously absent in PS-R (-/-) MEFs, suggesting that multimerization adducts were derived from, or dependent on, the PS-R gene product (Fig. 1C).

To study the mechanism of covalent modification and multimerization in more detail, we first explored whether this might involve end joining of the N and C regions of the protein. To address this, we coexpressed pRK-HA-PSR (tagged on the N-terminal) with pcDNA-PSR-V5 (tagged on the C-terminus) in HEK cells, followed by coimmunoprecipitation with anti-HA mAb and immunoblotting with anti-V5 antibody (Fig. 2A, lane 1). As indicated, both HA-PS-R and PS-R-V5 mixtures were contained in single immunoprecipitates, suggesting these adducts were mediated by an internal protein cross-linking, possibly by an enzymatic-catalyzed reaction. PS-R adduct formation was not labile to the reducing agents DTT and *β*-mercaptoethanol in the sample buffer and gel, nor if we added iodoacetic acid to the lysis buffer to alkylate SH groups (data not shown).

Since the cross-linked protein adducts appeared to be multimers of ~50 kD, this suggested that PS-R may undergo homomeric polymerization. To address this, we expressed pRK-HA-PSR, and after immunoprecipitation with anti-HA antibodies, proteins in the immune complexes were separated by SDS-PAGE and stained with SYPRO Ruby stain for MALDI-MS mass and MS/MS sequence analysis protein identification (Fig. 2B). Each of the bands at 50, 100, and 150 kD was excised (labeled a, b, and c respectively), and tryptic peptides of the *in-gel* digests were mixed with α-cyano-4-hydroxy cinnamic acid and analyzed by MALDI-TOF MS using a peptide mass mapping method (data not shown). Both MALDI-TOF MS and MS/MS fragmentation analysis confirmed that all signature

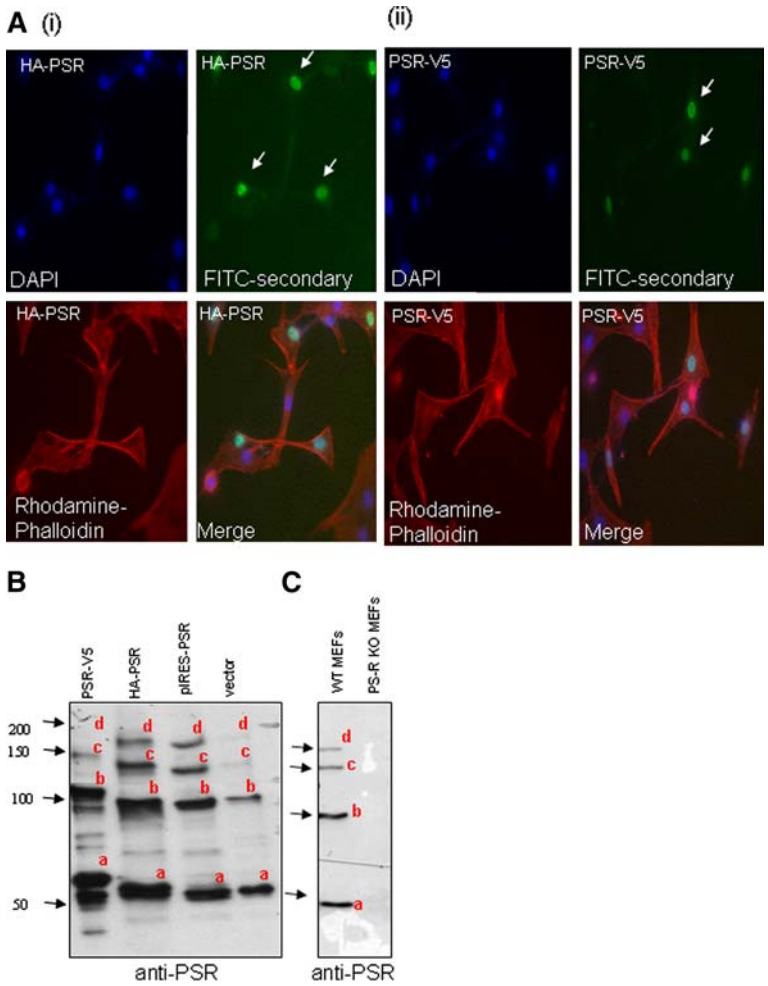

Fig. 1 Nuclear localization and modification of the PS-R gene product. (**A**) NIH3T3 cells were transfected with HA-PSR (N-terminal tagged) or with PS-R-V5 (C-terminal tagged) and cultured on FN-coated glass coverslips prior to staining. About 48 h after transfection, cells were fixed with 4% paraformaldehyde and then stained with DAPI to identify the nuclei (DAPI) or Rhodamine Phalloidin to stain F-actin. Indirect immunofluoresence staining was performed with anti-HA mAb followed by FITC-conjugated secondary antibody (FITC). Rightmost panel shows the merge of nuclear (DAPI), F-actin (Rhodamine Phalloidin), and secondary antibody (FITC) staining. (**B**) Western blot analysis of PS-R in transfected HEK 293T cells. Western blot with anti-PSR antibody from nuclear fractions of cells expressing indicated constructs i.e. PSR-V5 (lane 1), HA-PS-R (lane 2), untagged PS-R (IRES-PSR) (lane 3) or empty vector (endogenous PS-R) (lane 4). The expression of PS-R (MW 50 kD; a) as well as higher molecular weight bands shown by arrows and letters in red (b–d). (**C**) lysates of PS-R (-/-) null MEFs (lane 2) and wild-type MEFs (lane 1) were resolved by SDS-PAGE, and endogenous PS-R detected with anti-PS-R specific antibody (Abcam)

peaks in a, b, and c were identified as PS-R (Fig. 2C). Curiously, purified PSR proteins obtained by expressing pGEX6A-PSR in bacterial cells did not show the post-translational cross-linking suggesting this may be a specialized modification restricted to higher-eukaryotic cells (data not shown).

We next investigated whether PS-R might be a target of transglutaminase activity. TG-ases are a family of enzymes involved in transamidation of polypeptide chains through their glutamine and lysine residues, and participate in a wide variety of signaling reactions [21]. Interest in TG-2, the most ubiquitous member of the TG family of enzymes, in phagocytic functions has been prompted by interesting studies showing that TG-2 participates in the activation of TGF-β production in macrophages during apoptotic cell clearance [22–23] TG-2, as well as other family members, recognize target proteins in the context of conserved QQ residues flanked by acidic residues, mainly E and D [24] ClustalW alignment of human PS-R with typical the TG-2 substrates, i.e., γ -fibrin, fibronectin, crystallin, and osteopontin shows that PS-R has typical motifs found in TG-2 substrates (Table 1). However, when we examined the endogenous pool of PS-R in wild-type versus TG-2 (-/-) MEFs, there was no apparent reduction in PS-R covalent modification as assessed by Western blotting (Fig 3A). Further, to rule out the possibility that other ubiquitous

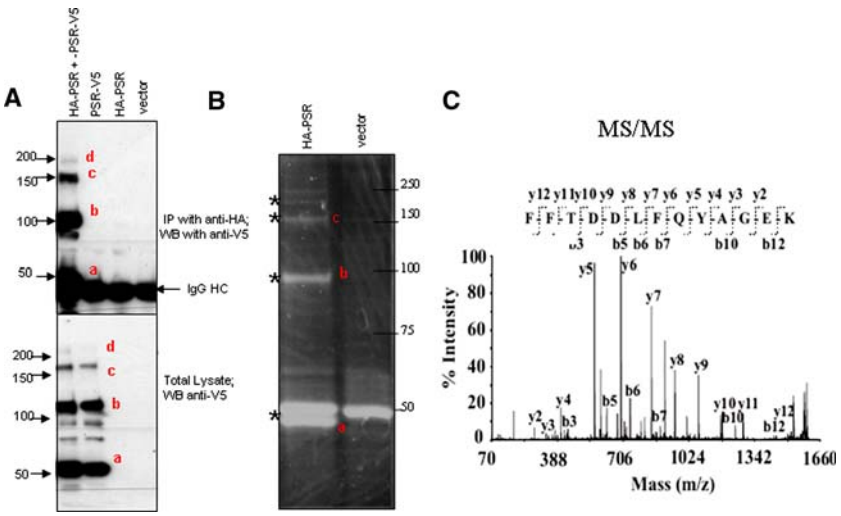

Fig. 2 MS identification of PS-R cross-linked proteins. (**A**) PS-R adducts are not joined ''head to tail''. In panel A, HA-PSR and PSR-V5 were co-expressed in HEK cells, after which nuclear lysates were prepared and immunoprecipitated with anti-HA antibody. The lower panel is a loading control to show equivalent expression of PS-R V5. Similar results were obtained when the IP was performed with anti-V5 and immunoblotted with anti-HA (not shown). (**B**) Cells expressing HA-PS-R were immunoprecipitated with anti-HA as above, after which the SDS-PAGE gel was stained with SYPRO Ruby. Bands a, b, and c were subjected to MALDI-TOF peptide fingerprints of a, b, and c respectively, which were similar among the three MS spectra, all corresponding to PS-R. (**C**). Tandem mass spectrometry was used for peptide sequences confirmation. The MS/MS shows the spectrum of peptide FFTDDLFQYAGEK (m/z 1580.73) in PS-R. All bands were confirmed to be PS-R

Table 1 Alignment of QQ duets in PS-R with various other protiens that are substrates for transglutaminase. The QQ duet is underlined for comparison, and juxtapositioned acidic and basic residues are shown in dots.

γ- fibrin	R L T I G E G Q Q H H L G G A K Q A G D V
fibronectin	E A Q Q I V Q P Q S P L T V S Q S K P G
osteonectin	L A G A L A A P Q Q E A L P D E T E V V E E T V A E V T
IGFBP-1	R C R A L P G E Q Q P L H A L T R G Q
PS-R	V T R D E G G N Q Q D E A I T W F N V I Y

family members, such as TG-5 or TG-6, [25] may compensate for TG-2, we made a mutation to disrupt the conserved QQ duet that makes up the TG consensus sequence (Fig. 3B). As indicated, expression of the QQ → AA PS-R mutant in HEK cells did not prevent subsequent covalent modification.

To explore whether PS-R cross-linking was a post-translation modification, we performed pulse-chase experiments (Fig. 3C). For these experiments, HEK cells were transfected with pRK-HA-PSR and after 24 h were briefly pulsed with 100 uCi/ml ^{35}S-methionine for 30 min, after which the cells were washed and chased with DMEM media (with excess cold L-methionine and L-cystine) at different time points for up to 24 h. Detergent lysates were prepared and immunoprecipitated with anti-HA mAb, after which immune complexes were electrophoretically resolved and assayed by autoradiography. Immediately following labeling, a predominant labeled protein migrated at approximately 54 kD, followed by the appearance of a faster migrating cleavage product of ~50 kD that steadily increased through 24 h suggesting that it is a cleavage product (identified by stars in the figure). In addition to the 50 kD band, we observed progressive multimerization of this cleavage-modified protein into multimers of 50 kD that appeared after 6–24 h. These results may indicate that PS-R undergoes an initial proteolytic cleavage, and possibly required for post-translational processive covalent modification.

Discussion

The results of this study suggest that the PSR-gene product undergoes a processive post-translational covalent modification to generate nuclear aggregated products. Because the function of PS-R is not well understood, we cannot yet predict whether the observed multimerization is important for the biological function of PS-R. As mentioned above, sequence alignment analysis of PS-R sequences from a variety of taxonomic species shows homology to members of the super family of ''Jumonji'' (JmjC) chromatin-remodeling and transcription factors [9] Several members of the JmjC family have been shown to be involved in transcriptional repression and chromatin remodeling, and play important roles in tissue development, some of which are consistent with what is observed in the PS-R null mice

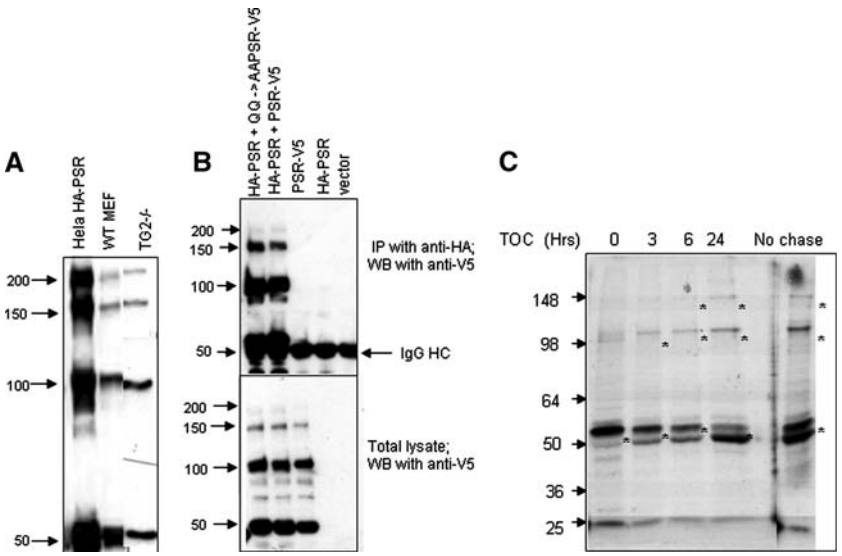

Fig. 3 PS-R multimerization is a post-translational modification independent of TG-2 activity. (**A**). Detergent lysates were prepared from control MEFs (lane 3), or TG-2 (-/-) MEFs (lane 2) and 50 μg/ml of cellular protein was analyzed by immunoblotting with anti-PS-R antibody. As a control, HA-PS-R plasmid DNA was transfected into Hela cells (lane 1) and lysates were compared. (**B**). HEK Cells were transfected with WT pcDNA -PS-R-V5, or a construct having a QQ → AA double mutation (pcDNA-PS-R-V5 (QQ → AA). After transfection, lysates were immunoprecipitated with anti-HA mAb to monitor cross-linking to the WT protein (HA-tagged). (**C**) Pulse-chase labeling of PS-R. HEK cells were pulsed-labeled with 100 μCi of ^{35}S-methionine for 30 min. TOC refers to the time of chase into ''cold media'' containing excess methionine and cystine

[26] Recently, two members of the JmjC family of proteins (Epe1 and HIF1AN/FIH) have been shown to possess intrinsic histone-demethylase activity [27, 28] relating to the regulation of gene expression and/or co-activator-recruitment to chromatin. Moreover, as pointed out by Cikala et al., the sequence of PS-R has considerable sequence homology to HIF1AN/FIH, an enzyme with 2-oxoglutarate-Fe (II)-dependent dioxygenase activity. HIF1A/FIH mediates the hydroxylation of asparagine residues in hypoxia-inducible factor (HIF-1α) as part of a complex oxygen-sensing mechanism in response to hypoxia [29] It will be interesting to observe whether the putative dioxygenase activity of PS-R is related to the covalent cross linking reported in this study.

In our attempts to characterize PS-R as a phagocytic receptor, we have unexpectedly found that PS-R undergoes an unusual and complex form of nuclear covalent cross-linking. Several independent experiments including MS/MS, pulse chase, co immunoprecipitaions with epitope tagged at both N and C termini are consistent with the finding that PS-R undergoes a covalent cross-linking, involving a non-TG-2 mediated, a non-end-to end mediated, sequential aggregation reaction leading to processive post-translational modification. We posit that the multimerization of the PS-R may act as a molecular scaffold for the assembly of higher order protein complexes and to regulate the nuclear function of PS-R.

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