

Secreted Phospholipases A₂ – not just Enzymes

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Abstract

Secreted phospholipases A₂ (sPLA₂s) constitute, physiologically and pathologically, a very important family of enzymes. Most actions of sPLA₂s have been explained by their phosphatidylglycerol *sn*-2 hydrolytic activity. However, since pharmacologically active sPLA₂ molecules without enzymatic activity have been discovered, first in snake venom and then also in human, it has become increasingly evident that, on many occasions, the action of these proteins has to be considered as arising from the interplay of their receptor-binding and enzymatic functions. The number of known sPLA₂-interacting molecules is growing and, with the development of more sensitive biochemical techniques, further discoveries are expected. In this paper we are reviewing all the currently known sPLA₂-binding proteins. The structural versatility of these molecules establishes sPLA₂s as ligands with a broad interaction spectrum, in agreement with the already recognized multifunctional nature of these proteins. Mechanistic descriptions of the multitude of actions of sPLA₂s is today one of the most exciting and promising research areas, and the description of the sPLA₂-interactome is for sure one of its vital parts.

Keywords: Secreted phospholipase A₂; receptor; physiological function; pathological effect

1. Introduction

Phospholipases A₂ (PLA₂s; EC 3.1.1.4) are enzymes that catalyze the hydrolysis of ester bonds at the *sn*-2 position in phosphoglycerides. Representatives of this fundamental superfamily of enzymes are spread from viruses to mammals. Some of them function in the cytosol of cells, some are extracellular, some require Ca²⁺-ions to exert their biological activity while others do not. These were the criteria for the division of PLA₂s into four main types – cytosolic Ca²⁺-dependent PLA₂s (cPLA₂s), cytosolic Ca²⁺-independent PLA₂s (iPLA₂s), platelet activation factor-acetyl hydrolase (PAF-AH) and secreted PLA₂s (sPLA₂s). A more exact classification of PLA₂ molecules is based on their structural features. At the moment, sixteen different structural groups of PLA₂s (GI to GXVI) have been defined.¹ The focus of the present review is on the low molecular mass (13–19 kDa), Ca²⁺-dependent, disulfide-rich sPLA₂ molecules found in abundance in, and as important components of, animal and in particular sna-

ke venoms.² In animal venoms GI, GII and GIII sPLA₂ molecules have been characterized. Besides enzymatic activity, these venom components also express versatile pharmacological activities. They can be neurotoxic, myotoxic, anticoagulant, procoagulant, cardiotoxic or cytotoxic. Orthologues of the venom sPLA₂s have been discovered in other animals including humans. To date, eleven paralogues of the low molecular mass sPLA₂s have been described in mammals: GIB, GIIA, GIIC, GIID, GIIE, GIIF, GIII, GV, GX, GXIIA and GXIIB sPLA₂. In human, the GIIC sPLA₂ gene appears to be a non-functional pseudogene. sPLA₂s are differentially expressed in human tissues, demonstrating their non-redundant functional roles.³ Venom sPLA₂s are multifunctional proteins and, similarly, their mammalian counterparts are obviously implicated in multiple physiological and pathological settings. These molecules participate, for example, in innate immunity, neurotransmitter release, neuritogenesis, angiogenesis, embryogenesis, pain perception and apoptosis. Moreover, sPLA₂s are implicated in acute respiratory distress

syndrome (ARDS), endotoxic shock, pancreatitis, rheumatoid arthritis, psoriasis, ischemia, Alzheimer's disease, asthma, atherosclerosis, inflammatory diseases, proliferation and, related to the latter, in different forms of cancer.⁴ Although only a few of the known sPLA₂-related processes are mechanistically established, in most cases the phospholipolytic activity is of vital importance. However, following the discovery of pharmacologically active but catalytically inactive sPLA₂ homologues, some functions of sPLA₂s have been demonstrated to depend on their specific binding to other biomolecules and not on their enzymatic activity. sPLA₂s should therefore also be considered as ligands for membrane or soluble receptors. Snake venom sPLA₂s have proved excellent models in the discovery and characterization of sPLA₂-binding molecules. We will survey the most thoroughly described ones.

2. Plasma Membrane Receptors for sPLA₂s

2.1. Muscle-type sPLA₂ Receptor (M-type sPLA₂R)

The M-type sPLA₂R is the most studied sPLA₂-binding protein. It was discovered in the plasma membrane (PM) of skeletal muscle cells, hence also the name, muscle or M-type receptor. The receptor was identified using radioiodinated OS₂ (¹²⁵I-OS₂), a neurotoxic GIA sPLA₂ from the venom of *Oxyuranus s. scutellatus* snake.⁵ Binding of sPLA₂ to the M-type sPLA₂R was found to be independent of Ca²⁺ ions. Later, isoforms of the M-type sPLA₂R were found by their high affinity for ammodytoxin (Atx), a GIIA sPLA₂ in the venom of *Vipera a. ammodytes*, in the porcine cerebral cortex and in liver membranes.⁶ Since only one gene for the M-type sPLA₂R exists in the genomes, multiple isoforms of the M-type sPLA₂R on the protein level are the result of different posttranscriptional and/or posttranslational processing.^{6,7} A soluble form of the M-type sPLA₂R was also found.^{8,9} This was the first example of a circulating sPLA₂ inhibitor (PLI) that acts as an endogenous inhibitor regulating the enzymatic activity and receptor-mediated functions of sPLA₂s.^{8,9} The sPLA₂-binding specificity of the M-type sPLA₂Rs, originating from either neuronal or non-neuronal tissues, appears to be very similar and is rather broad.^{5,10} However, the M-type sPLA₂Rs from different animal species differ in their binding affinities.¹¹ For the mouse system, the study of Rouault et al.¹² revealed that the M-type sPLA₂R has a high affinity for the endogenous GIB, GIIA, GIIE, GIIF and GX sPLA₂s, low affinity for the GIIC and GV, and no affinity for the GIID, GIII, GXIIA and GXIIB sPLA₂s.

An important role of the M-type sPLA₂R appears to be the neutralization and clearance of sPLA₂s. This receptor is constitutively endocytosed.^{13,14} By binding and clea-

rance of GIB, GIIA and GX sPLA₂s – endogenous ligands for the M-type sPLA₂R – from the extracellular space by degradation in lysosomes, the functions of these enzymes are down regulated. In the circulation, the soluble form of the M-type sPLA₂R is present constitutively as an endogenous inhibitor for mammalian sPLA₂s. Binding of GIB sPLA₂ to this receptor cell induces proliferation and migration, hormone release, a lipid mediator or cytokine production, by way of the MAPK, and probably also ERK1/2, pathway.^{15,16} GIIA sPLA₂ induces NO production by involving M-type sPLA₂R which then mediates signal transduction events that lead to PI3K/Akt pathway activation.¹⁷ Analysis of M-type sPLA₂R-deficient mice suggested a role of the GIB sPLA₂/M-type sPLA₂R signalling system in the production of pro-inflammatory cytokines during endotoxic shock.¹⁸ The sPLA₂/M-type sPLA₂R system probably also plays a role in cell senescence¹⁹ and in renal glomerular podocyte homeostasis.²⁰ It was presumed that the neuronal form of the receptor may possess some unique physiological properties besides those already assigned to the non-neuronal M-type sPLA₂R.²¹ It appears though that the interaction of AtxA with the neuronal M-type sPLA₂R may not be essential for the neurotoxicity of this snake venom sPLA₂.²²

The M-type sPLA₂ receptor is a member of the mannose receptor family (the C-type lectin superfamily) which includes the macrophage mannose receptor (which shares only 29% sequence identity with the M-type sPLA₂R), the receptor DEC-205 found in dendritic cells, and endothelial cell lectin lambda receptor (Endo180). It is a monomeric, single transmembrane domain glycoprotein (type I membrane receptor). It has a short cytoplasmic tail containing no apparent signalling motif and a very large extracellular region comprising an N-terminal Cys-rich domain, a fibronectin-like type II domain, and a tandem repeat of eight distinct C-type carbohydrate recognition domains (CRD)-like domains. The CRD-like domain 5 binds sPLA₂s directly.²³

CRD or CRD-like domains have been identified in several dozens of proteins including hepatic lectins, selectins and lymphocyte receptors. Other CRD-containing and sPLA₂s binding proteins found are α-type snake serum sPLA₂ inhibitors (α-PLIs) and pulmonary surfactant protein A (SP-A). The macrophage mannose receptor, DEC-205, Endo180 and the chicken yolk sac IgY receptor, a functional equivalent of the mammalian MHC-related Fc receptor and the M-type sPLA₂R homologue, did not show affinity for sPLA₂s tested.²⁴

2.2. Heparan Sulphate Proteoglycans (HSPG)

In an attempt to clarify the role of GIIA sPLA₂ in the regulation of prostaglandin (PG) biosynthesis, Murakami et al.²⁵ identified an HEK293 cell PM sPLA₂-binding component. This sPLA₂ receptor was identified as glypican I, a GPI-anchored heparan sulphate proteoglycan

(HSPG). Glypican I resides in the caveolae. The interaction between GIIA sPLA₂ and glypican I was showed to be electrostatic in nature.²⁶ GIIA sPLA₂ is a very basic protein while heparanoids are anionic. Glypican I also binds other cationic heparin-binding sPLA₂s – GIID and GV. Other proteoglycans, biglycan and perlecan, components of the extracellular matrix (ECM), have also been found to bind GII sPLA₂s.²⁷

In the process of potocytosis of the glypican I–sPLA₂ complex, the sPLA₂ was found to be translocated to intracellular vesicular compartments of HEK293 cells. This was essential for the sPLA₂'s augmenting role in arachidonic acid (AA) metabolism, its PGE₂-biosynthetic effect²⁵ and for its implication in the progression of inflammatory diseases.²⁸ GIID and GV sPLA₂s can also augment cellular AA release following their binding to heparanoids.^{29,30} In some cell types, HSPG binding can also contribute to the clearance of cationic heparin-binding sPLA₂s, besides GIIA also GIID and GV, through internalization and lysosomal degradation. Binding of GII sPLA₂ to biglycan and perlecan in the extracellular matrix was suggested as modulating the enzymatic activity of sPLA₂ towards LDL, thus contributing to atherosclerosis.²⁷

2. 3. Integrins

Saegusa et al.³¹ demonstrated in several different assays, including FITC-labelled GIIAsPLA₂, surface plasmon resonance (SPR) and binding of soluble $\alpha\beta$ 3 to immobilized GIIA sPLA₂, that human GIIA sPLA₂ specifically and avidly binds to integrins $\alpha\beta$ 3 and α 4 β 1. The integrin-binding site in sPLA₂ does not include the catalytic site or the M-type sPLA₂R-binding site. GIIA sPLA₂ competed with vascular cell adhesion molecule (VCAM)-1 for binding to α 4 β 1. *Macrovipera lebetina transmediterranea* (MLT) snake venom acidic GIIA sPLA₂ was however found to interact with integrins α 5 β 1, $\alpha\beta$ 3 and $\alpha\beta$ 6.³²

Integrins constitute a family of cell adhesion receptors that recognize ECM ligands and cell surface ligands. They are transmembrane $\alpha\beta$ heterodimers, and 18 α and 8 β subunits have been characterized in mammals. By binding to integrins, GIIA sPLA₂ induces proliferative signals in an integrin-dependent manner. Like the wild type, the catalytically inactive H47Q mutant of GIIA sPLA₂ also induced cell proliferation and ERK1/2 signalling pathway activation in monocytic cells. In contrast, the integrin binding-defective GIIA sPLA₂ mutant (R74E/R100E) did not elicit these effects. This indicates that the integrin binding and not the enzymatic activity of the sPLA₂ is required for GIIA sPLA₂-induced proliferative signalling. Integrins $\alpha\beta$ 3 and α 4 β 1 are thus extracellular PM receptors for GIIA sPLA₂, through which they mediate its pro-inflammatory action. MLT snake venom acidic GIIA sPLA₂ completely abolished cell adhesion and migration of various human tumour cells. This anti-tumour effect was ba-

sed solely on the interaction of the sPLA₂ with integrins, being independent of the enzymatic activity.³² Further study demonstrated a potent anti-angiogenic property of MLT-GIIA sPLA₂. As a consequence of its association with integrins, it affected the actin cytoskeleton and the distribution of $\alpha\beta$ 3 integrin, a critical regulator of angiogenesis and a major component of focal adhesions. MLT-GIIA sPLA₂ treatment significantly increased microtubule dynamicity in cells, explaining the alterations in the formation of focal adhesions that lead to inhibition of cell adhesion and migration.³³

2. 4. Vascular Endothelial Growth Factor Receptors (VEGFR)

In screening for the VEGFR-1/Flt-1 and VEGFR-2/KDR antagonists in snake venoms, Yamazaki et al.³⁴ detected a strong inhibitory activity of VEGF binding to its receptor in the venom of *Agkistrodon p. piscivorus*. The inhibition was produced by a Lys49 sPLA₂ in the venom. This Lys49 sPLA₂ is enzymatically inactive, strongly myotoxic and structurally a GIIA molecule. Besides *Agkistrodon p. piscivorus* Lys49 sPLA₂, some other myotoxic snake venom sPLA₂ molecules also bind to these receptors, for example Lys49 PLA₂s from the venom of *Trimeresurus flavoviridis* (basic proteins I and II), Ser49 sPLA₂ ammodytin L from the *Vipera a. ammodytes* venom, but also enzymatically active Asp49 sPLA₂ from *Agkistrodon p. piscivorus*.^{34,35} An important observation was that binding of Asp49 sPLA₂ to KDR did not inhibit its enzymatic activity.³⁵

It is unclear whether the VEGFR-binding property is directly involved in the myotoxicity of sPLA₂ molecules or not. The Lys49 sPLA₂-mediated apoptosis in leukemic cells could be explained by their VEGFR-blocking property.

VEGFR-1/Flt-1 and VEGFR-2/KDR are each comprised of seven tandem extracellular immunoglobulin (Ig)-like domains, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. The VEGFR family belongs to the receptor tyrosine kinases.

Besides VEGFR-1/Flt-1 and VEGFR-2/KDR, DM64 is also an Ig-like domain-containing sPLA₂-binding molecule, an anti-myotoxic protein discovered in the serum of the Southern opossum (see section 4.2.). The sPLA₂-binding site is thus probably located in the Ig-like domain of these receptors.

2. 5. Vimentin

An auto-antigen on the surface of apoptotic human T cells that binds human GIIA sPLA₂ was characterized as vimentin.³⁶ Vimentin, partially exposed on the surface of apoptotic primary T lymphocytes, binds sPLA₂ via its rod domain in a Ca²⁺-independent manner. Bound vimentin does not inhibit the catalytic activity of human GIIA sPLA₂.

When primary human T lymphocytes were induced to undergo apoptosis they expressed, in addition to HSPG-

s glypican (see section 2.2.), vimentin on the surface of the cells that is not present on the surface of non-apoptotic cells. Vimentin is otherwise an intracellular protein, a member of the intermediate filament family of proteins. It is the cytoskeletal component responsible for maintaining integrity of the cell. Vimentin is a dynamic structural component of the cytoskeleton and, when under mechanical stress *in vivo*, provides cells with the resilience lacking in the microtubule or actin filament networks. Since association of the sPLA₂ with vimentin does not abrogate its enzymatic activity, it was suggested that vimentin could play an anchoring or docking function that enables or enhances sPLA₂'s catalytic activity toward cellular phospholipids.³⁶

2. 6. K⁺-channel

Detected by the means of ¹²⁵I-β-Butx, a neurotoxic sPLA₂ from *Bungarus multicinctus* snake venom, Scott et al.³⁷ isolated a membrane receptor from bovine brain synaptic PMs and characterized it structurally as a voltage-dependent K⁺ channel. The ¹²⁵I-β-Butx binding site was shown to be one of the α-dendrotoxin-binding K⁺ channels.³⁸ α-Dendrotoxin is a bovine pancreatic trypsin inhibitor-like neurotoxin from the green mamba *Dendroaspis angusticeps*. β-Butxs (a group of structurally unique molecules found only in the venoms of *Bungarus* snakes) are covalent heterodimers of a GIA sPLA₂ (A subunit) and a dendrotoxin-like B subunit. In spite of many attempts, no other sPLA₂ has been demonstrated to bind any type of K⁺ channel to date, so it seems clear that the specificity of β-Butxs for certain K⁺ channels is conferred by the dendrotoxin-like subunit and not by the sPLA₂ subunit. Strictly speaking, K⁺ channels in fact are not sPLA₂-binding molecules.

2. 7. Neuronal-type sPLA₂ Receptor (N-type sPLA₂R)

It is more than twenty years since a neuronal-type sPLA₂ receptor (N-type sPLA₂R) was detected in rat brain presynaptic membranes using ¹²⁵I-OS₂³⁹ but its identity is still not known. It consists of membrane proteins of 18–24, 36–51 and 85 kDa. The N-type sPLA₂ receptor is a neuronal receptor for most of the presynaptically neurotoxic sPLA₂s. Its receptor-binding affinity and the lethality of neurotoxic sPLA₂s correlate strongly, so this receptor remains the most likely candidate for the sPLA₂-neurotoxicity-specific receptor on nerve cells.³⁹

3. Intracellular sPLA₂-binding Proteins

3. 1. CaM

A protein of 16 kDa was purified by Atx-affinity chromatography from a porcine cerebral cortex membra-

ne extract and shown to be a calmodulin (CaM).⁴⁰ It also binds some other venom sPLA₂s of GII and GIII, *i.e.* agkistrodotoxin, crotoxin and bee venom sPLA₂. Recently, its association with human GV and mouse GX sPLA₂s has been reported.⁴¹

CaM is a cytosolic protein and as long as the translocation of extracellular sPLA₂s into the cytosol of mammalian cells was undisputedly demonstrated^{42–44} it was questionable as to whether the interaction between CaM and sPLA₂s can occur *in vivo* at all. CaM is a highly conserved Ca²⁺-binding protein implicated in regulation of many cellular processes.⁴⁵ *In vitro* experiments demonstrated that CaM stabilizes sPLA₂s in reducing, cytosol-like conditions and substantially increases their enzymatic activity.^{41,46} Importantly, these findings suggest a new mechanism of modulating the enzymatic activity of GV and GX sPLA₂s in certain phospholipase activity-dependent processes in mammalian cells. Further, stabilization of sPLA₂s and stimulation of their enzymatic activity in specific cellular compartments offer a possible interpretation of the neuropathological effects produced by some sPLA₂s.

CaM is structurally similar to two other sPLA₂-binding proteins, taipoxin-associated calcium-binding protein of 49 kDa (TCBP-49)⁴⁷ and crocalbin.⁴⁸ All these proteins are EF hand Ca²⁺-binding proteins.⁴⁹

3. 2. 14-3-3 Proteins

Two other highly conserved cytosolic proteins, γ and 14-3-3 isoforms, were detected and identified as sPLA₂-binding proteins using Atx-affinity chromatography. The specific interaction between Atx and 14-3-3p was confirmed by SPR.⁵⁰ Atx also interacts with a similar affinity with the yeast 14-3-3p homologues, Bmh1 and Bmh2.⁵¹ Binding to 14-3-3p is not limited to Atx – human GIIA sPLA₂,⁵² β-Butx, taipoxin and the bee venom GIII sPLA₂ were also found to interact (Mattiazzi, unpublished).

14-3-3p are acidic proteins of 28–33 kDa that spontaneously form biologically active homo- or heterodimers. Seven isoforms of 14-3-3p exist in mammals. Atx was suggested to interact with 14-3-3p through the non-canonical KEESEK amino acid sequence at its C-terminus.^{50,53}

14-3-3p are widespread in eukaryotic cells but are mainly localized in the brain. They are involved in several, usually isoform-specific, processes including intracellular trafficking/targeting, signal transduction, cell cycle regulation, Ca²⁺-regulated exocytosis, regulation of phosphorylation, cytoskeletal structure organization, transcription and apoptosis.^{54–57} They have also been linked to cancer⁵⁸ and several neurological disorders such as Creutzfeldt–Jakob disease,⁵⁹ Parkinson's⁶⁰ and Alzheimer's disease.⁶¹

AtxA, heterologously expressed in yeast cytosol, inhibits cell cycle arrest specifically in the G₂ phase, which is known to be regulated by 14-3-3p. This effect is probably not related directly to neurotoxicity, but suggests a

possible mechanism in mammalian cells by which an endogenous sPLA₂ induces one of two opposite effects, proliferation or apoptosis.⁵¹ 14-3-3p bind to membranes.^{62,63} Connected with the process of sPLA₂ neurotoxicity, membrane bound 14-3-3p could act as an adaptor to localize the toxic sPLA₂ at active zones on the presynaptic membrane. In support of this suggestion, several 14-3-3p isoforms have been detected associated with the synaptic junction and synaptic vesicle membranes.⁶²

3. 3. v-Src Kinase

In yeast two-hybrid screen, one of the v-Src kinase-interacting proteins in Rous sarcoma virus (RSV)-transformed hamster cells was suggested to be a GIIA sPLA₂ molecule with a C-terminal 8-amino acid residue extension (srPLA₂). The srPLA₂ was found to be Tyr-phosphorylated. Using *in vitro* and *in vivo* methods a direct interaction between the v-Src oncoprotein and the srPLA₂ was demonstrated. It was suggested that the interaction occurs at the PM, however it remains a question as to how the v-Src at the cytoplasmic face of the PM meets the precursor of the srPLA₂ in the process of maturation before its secretion from the cell.⁶⁴

v-Src is an RSV non-receptor tyrosine kinase, an Src family kinase lacking the regulatory C-terminal phosphorylation site (Tyr-527). For this reason it is constitutively active, unlike the normal c-Src kinase which is only activated when required, and found to be responsible for the formation of cancer. Possibly connected to the observed interaction between v-Src and srPLA₂ (with Tyr-phosphorylation of the latter), it was noted that RSV-transformed hamster cells were secreting PgE. PgE inactivates the cytotoxic activity of natural killer lymphocytes and macrophages thus aiding the infected cell to avoid the body defence system. Whether or not the c-Src kinase also possesses affinity for sPLA₂s is not known.

3. 4. A Taipoxin-associated 49 kDa Calcium Binding Protein (TCBP-49)

Taipoxin is a complex trimeric sPLA₂ neurotoxin from the venom of the taipan snake (*Oxyuranus s. scutellatus*). Using taipoxin-affinity chromatography, a Ca²⁺-binding protein of 49 kDa (TCBP-49), with similarity to reticulocalbin, was detected in the lumen of the endoplasmic reticulum (ER). TCBP-49 was shown to reside in reticular organelles of neurons and glia, but its mRNA is also present in other tissues, such as liver, muscle, heart, kidney, and testis.⁴⁷ So far, taipoxin is the only sPLA₂ known to associate with TCBP-49. Besides taipoxin, TCBP-49 also binds neuronal pentraxins, NP1, NP2 and NPR.⁶⁵

TCBP-49 contains six EF-hand Ca²⁺-binding motifs and the C-terminal HDEL, the ER-retaining signal. Currently, TCBP-49 is known as ERC-55, past names being E6BP, VAF1 and reticulocalbin-2.⁶⁶ Several splicing va-

riants of ERC-55 exist, including the cytosolic ERC-55-C.⁶⁷

The EF-hand structural motif is present in various Ca²⁺-binding proteins. These proteins are involved in buffering Ca²⁺ in the cytosol, in signal transduction, Ca²⁺-dependent processes in the secretory pathway, chaperone activity and in muscle contraction. Specifically, TCBP-49 is a member of the CREC family of EF-hand Ca²⁺-binding proteins. CREC proteins were first thought to be localised exclusively in the secretory pathway of mammalian cells, but later some have been detected in the cytosol of cells as well as on their surface. Several pathological conditions, for example malignant transformation of cells, neuromuscular and cardiovascular diseases, have been related to dysfunction of CREC proteins.^{49,66}

It was proposed that TCBP-49 is involved in retro-transport of the neurotoxic taipoxin from the cell surface and in its activation inside the neuronal cell.^{65,68} This novel cell import pathway would include NPR, NP1 and NP2. However, the participation of TCBP-49 in the sPLA₂-neurotoxicity has not been confirmed.

3. 5. Crocalbin

Crotoxin is a dimeric sPLA₂ neurotoxin from the venom of the South American rattlesnake (*Crotalus durissus terrificus*). Crocalbin, another sPLA₂-binding protein in the lumen of the ER, homologous to TCBP-49, was discovered and isolated from porcine brain using crotoxin-affinity chromatography.⁶⁹ In competition studies, Atx did not inhibit the interaction of ¹²⁵I-crotoxin with crocalbin,⁷⁰ so crotoxin remains currently its only confirmed sPLA₂ ligand.

In its structure, crocalbin contains six EF-hand Ca²⁺-binding motifs and a C-terminal HDEF sequence for retention in either the ER or the Golgi apparatus.⁷¹ As TCBP-49, it belongs to the CREC family of proteins and is now referred to as calumenin-2, a splicing variant of calumenin.⁶⁶

The involvement of crocalbin in sPLA₂-neurotoxicity has not been proven. As suggested for TCBP-49, it might be implicated in the retro-transport of the toxic sPLA₂ into a target cell. However, the function of crocalbin may not be strictly confined to the ER or the Golgi apparatus. The HDEF C-terminal sequence is not an effective retention signal, as is confirmed by the finding that human calumenin is distributed throughout the secretory pathway and is secreted from the cell.⁷² Therefore, it may be that crocalbin and crotoxin also interact extracellularly.⁶⁶

3. 6. Protein Disulphide Isomerase (PDI)

One of the proteins from the porcine cerebral cortex retained on an AtxC-affinity column was PDI.⁷³ The specificity of the interaction between the neurotoxic GIIA sPLA₂ and PDI was confirmed by the SPR technique using a chip-immobilized AtxC and a solution of pure porcine brain PDI. Additionally, PDI was affinity-labelled with sulfo-SBED-AtxC, a photo-reactive derivative of AtxC.⁷⁴

Using this technique and different combinations of recombinant domains of yeast PDI,⁷⁵ the binding site of Atx in PDI was located (Kovačič et al., unpublished).

PDI is the founding member of a family of 20 related mammalian proteins. Members of the PDI family vary in length and domain arrangement, but share the common structural feature of having at least one domain with a thioredoxin-like structural fold, $\beta\alpha\beta\alpha\beta\alpha\beta\alpha$, either catalytic or non-catalytic.⁷⁶

PDI is an enzyme present abundantly in the lumen of the ER. It catalyzes the formation and breaking of disulfide bonds within proteins, thereby assisting proper protein folding, redox signalling, antigen presentation and HIV infection for example. It was suggested that the interaction of Atx with PDI represents a step in the mechanism to deliver the sPLA₂ neurotoxin from outside the cell into the cytosol.⁷³

3. 7. Mitochondrial sPLA₂ Receptor

An sPLA₂ receptor in mitochondria (R25) has been known for more than ten years. It was detected by high affinity for ¹²⁵I-Atx and characterized as a 25 kDa integral membrane protein⁷⁷ in porcine mitochondria.⁷⁸ Interestingly, only AtxA and its mutants have been found to be able to bind to R25 so far, although this observation is based only on ¹²⁵I-Atx binding competition and not on direct sPLA₂-binding assays.

4. Secreted sPLA₂-binding Proteins

4. 1. sPLA₂ Inhibitors (PLI) from Serum

It has been known for a long time that snakes are resistant to envenomation by their own venoms. sPLA₂s are major toxic components of a large part of *Viperidae*, *Hydrophiidae* and *Elapidae* snake venoms. Responsible for such innate immunity were found to be protein inhibitors of venom sPLA₂s not belonging to the Ig family of proteins, circulating in the blood of these animals. That the neutralization of pharmacological activities of toxic sPLA₂s is the primary function of PLIs is consistent with the fact that PLIs from the serum of a particular snake bind toxic sPLA₂s from the venom of that snake with the highest affinity. The snake venom-poisoning-resistance of some animals feeding on snakes, such as opossum, mongoose and hedgehog, has been linked to similar proteins in their blood. Below is a basic description of the three different structural classes of PLI described to date. Further details can be found in several comprehensive reviews about snake venom PLIs.⁷⁹⁻⁸¹

4. 1. 1. α -PLI

An α -type PLI was the first PLI described.⁸² α -Type PLIs have been found in viperid snakes, in snakes of the

Crotalinae and *Viperinae* subfamilies, but apparently not in *Elapidae* and the *Hydrophiidae* snakes.

Typically, type- α PLIs are globular glycoproteins composed of 20–25 kDa subunits. Three to six subunits, identical or differing slightly in sequence, form non-covalently associated oligomers of 75–120 kDa. According to their three-dimensional structure, α -PLIs belong to the C-type lectin superfamily. The sPLA₂-binding element in α -PLIs probably resides in the C-type CRD-like domain, the most conserved portion of α -PLI molecules, comprising 60-70% of the sequence of each subunit.⁸³ Binding of sPLA₂s by α -PLIs is Ca²⁺-independent, as in the case of M-type sPLA₂R and in contrast to the pulmonary SP-A. Type- α PLIs bind specifically GII, but not GI or GIII, sPLA₂s.⁸⁴

The proteins containing CRD-like domains, presented in sections 2.1. and 4.5., are structural homologues of α -PLI.

4. 1. 2. β -PLI

The first type- β PLI was purified from the venom of the Chinese snake *Agkistrodon blomhoffii siniticus*.⁸⁵ Each subunit of β -PLI binds one molecule of basic GII sPLA₂. β -PLIs have not so far been found in blood of elapid or hydrophid snake species.

Structural characterization revealed that this type of PLI consists of three identical or, in the case of some other β -PLIs, highly homologous glycosylated subunits of about 50 kDa (without the sugar moiety 35 kDa). The β -PLI sequence is unique showing no significant homology to the then known α -PLIs. It however exhibits 33% identity to the human leucine-rich α_2 -glycoprotein. It contains 9 leucine-rich repeats (LRRs), each of 24 amino acid residues, encompassing over two-thirds of the molecule.⁸⁶ In addition, characterized as the motif involved in diverse protein-protein interactions, LRRs in β -PLI could be responsible for the specific binding to basic sPLA₂s.

LRR motifs have been found in proteins with a wide array of biological functions, such as hormone receptors, toll-like receptors, enzymes, enzyme inhibitors, cell adhesion proteins, and ribosome binding proteins. Thousands of protein sequences containing LRR repeats have been detected. About 90 three-dimensional structures of LRR proteins and domains reveal a considerable degree of structural variability, accounting for the large functional versatility of this protein superfamily.⁸⁷

4. 1. 3. γ -PLI

γ -Type PLIs were first purified in 1994.^{88,89} They are glycoproteins with molecular masses ranging from 90 to 140 kDa and are oligomeric, incorporating at least three non-covalently associated subunits. This type of PLI is distributed more widely than α - and β -PLIs. It has been found in sera of both venomous and non-venomous *Elapidae*, *Hydrophiidae*, *Viperidae*, *Boidae* and *Colubridae*

snakes. Likewise, PLIs of the γ -type display broader sPLA₂ specificity than α - and β -PLIs, being able to bind GI, GII and also GIII sPLA₂s.

The inhibitors in this group possess a pattern of cysteine residues constituting two internal repeats of the characteristic three-finger shaped motifs. They therefore belong to the three-finger superfamily of proteins.

The three-finger scaffold is characteristic of an extensive group of snake venom toxins.⁹⁰ Mammalian members of this family include the urokinase-type plasminogen activator receptor, several membrane proteins of the Ly-6 family, including Ly-6A/E, Ly6C, thymocyte B cell antigen (ThB) and the complement regulatory protein CD59, and a bone-specific membrane protein RoBo-1.

4. 2. DM64, Inhibitor of sPLA₂ Myotoxic Activity

An anti-myotoxic protein was isolated from the serum of Southern opossum (*Didelphis marsupialis*). The protein of 64 kDa (DM64) was shown to be composed of five Ig-like domains. In serum, DM64 exists as a dimer. It forms a non-covalent complex with snake venom-derived myotoxic sPLA₂ molecules, either Asp49 or Lys49 sPLA₂s from *Bothrops asper* venom, and neutralizes their myotoxicity. Associated with Asp49 sPLA₂, DM64 did not inhibit its enzymatic activity.⁹¹

DM64 is a member of the Ig superfamily. The sPLA₂-binding receptors VEGFR-1/Flt-1 and VEGFR-2/KDR, described in section (see 2.4.), also consist of Ig-like domain tandem repeats.³⁴ An important observation is that the mode of interaction of sPLA₂s with the Ig-like domain-containing binding proteins is such that it does not affect their phospholipolytic activity. That would imply that the function of this type of sPLA₂-binding proteins is not inhibition of phospholipase activity.

4. 3. Activated Blood Coagulation Factor X (FXa)

The non-enzymatic inhibition mechanism of the prothrombinase complex was first demonstrated for CM-IV, a GI sPLA₂ from the *Naja nigricollis* venom.⁹² Using human GIIA sPLA₂ the sPLA₂-binding molecule was identified as FXa. GIIA sPLA₂ binds to the FVa-binding site on FXa, in this way preventing the generation of prothrombinase complex and its physiological activity.⁹³ According to the computer-generated model of the complex between AtxA and FXa, the sPLA₂-binding site in FXa includes both the light and the heavy chain, but not the proteinase catalytic site.⁹⁴

FXa consists of a light and a heavy chain linked by a single disulfide bond. At the N-terminal of the light chain is the Gla domain, rich in γ -carboxyglutamic acid, which is followed by a short stack of hydrophobic residues and two epidermal growth factor-like domains. The catalyti-

cally active proteinase domain is located in the heavy chain of FXa and is homologous to serine proteinases of the chymotrypsin family.

FXa is an important element of the haemostatic system. It associates with FVa to form a prothrombinase complex on the surface of the tissue factor-bearing cells. In the complex, FXa then activates prothrombin to thrombin in the blood coagulation cascade.⁹⁵

4. 4. Neuronal Pentraxins (NP)

A soluble, 47 kDa extracellular taipoxin-binding protein was discovered in rat brain membrane extract, using taipoxin affinity-chromatography. The sequence of this protein revealed homology with the acute phase proteins serum amyloid P protein and C-reactive protein of the pentraxin family.⁹⁶ Based on the homology of this protein to the pentraxins and its brain distribution, the taipoxin-binding protein was termed neuronal pentraxin (NP1). Subsequently, an isoform of NP1 (NP2/Narp) was found that also binds taipoxin.⁶⁸ Binding of taipoxin to both NPs was Ca²⁺-dependent. No other sPLA₂s have been reported to bind NPs. NP1 and NP2 were shown to oligomerize (pentamer) and form very stable hetero-oligomers with the NPR on the surface of neurons. In this way NPs are retained on the cell surface.

The neuronal expression of NPs and their homology to serum amyloid P protein and C-reactive protein suggest that they may be involved in synaptic uptake, and could play an important role in synaptic remodelling. NP–NPR complexes formed on the surface of neuronal cells can bind taipoxin and, in this way, localize taipoxin to synapses. They are also able to bind TCBP-49.⁶⁸ As TCBP-49 is a protein in the lumen of the ER, it may be that all these proteins are elements of a novel neuron uptake pathway for the neurotoxic sPLA₂. Recently, the first intracellular (cytosolic) NP isoforms were found.⁹⁷ These proteins are encoded by an extended NP gene that links the pentraxin domain with an N-terminal chromo (chromatin organization modifier) domain. For this reason, they were named neuronal pentraxin with chromo domain (NPCD). NPCDs probably have specific functions in neuronal differentiation, possibly including axon growth and/or guidance. Their interaction with an sPLA₂ has not been reported.

4. 5. Surfactant Protein A (SP-A)

Clinical studies demonstrated that ARDS, caused by a marked increase in lyso-PC content, correlated with destruction of SP-A in pulmonary surfactant. This observation suggested an important role for SP-A in the control of the GIIA sPLA₂ hydrolytic activity towards surfactant phospholipids. By inhibiting sPLA₂ activity, SP-A supposedly plays a protective role by maintaining surfactant integrity during lung injury. As shown in an SPR experiment, SP-A binds directly to GIIA sPLA₂.⁹⁸

SP-A is an innate immune system collectin which possesses a C-terminal CRD. In this respect it belongs to the same structural group of sPLA₂-binding proteins as the M-type sPLA₂R and α -PLIs. Like the M-type sPLA₂R and α -PLIs, SP-A inhibits the phospholipolytic activity of sPLA₂s. Besides GIIA sPLA₂, SP-A was reported to be an endogenous inhibitor of GX sPLA₂, but it fails to inhibit GV sPLA₂ which also effectively hydrolyzes surfactant phospholipids *in vitro*.⁹⁹

4. Conclusions

The survey of sPLA₂-binding proteins shows that sPLA₂s are capable of interacting with structurally very diverse molecules (Table 1). In addition to differences in enzymatic specificity, this further explains the plethora of physiological functions as well as pathological situations in which these molecules take part.

LRR, three-finger fold and thioredoxin-like motifs have been detected, each in a single sPLA₂ receptor type. According to its characteristics, LRR is also expected to assume the sPLA₂-binding structure (Figure). Protein-binding sites are positioned on different sides of an sPLA₂ molecule, as concluded from the fact that while some interactors inhibit the phospholipase activity (CRD and CRD-like domain-containing interactors, LRR and three-finger interactors), others do not (Ig domain-containing interactors, vimentin and integrins) or even augment the activity (EF-hand Ca²⁺-binding CaM). sPLA₂-binding molecules are thus implicated in negative (soluble M-type sPLA₂R, SP-A, PLIs) and probably also positive (CaM, maybe Src) regulation of the enzymatic function of sPLA₂s. Their role as signalling transducers (the M-type sPLA₂R, integrins) or as molecules that assist in specific cellular localization of sPLA₂s (NPR/NPs, 14-3-3p) has been revealed.

Our understanding of the identity and function of the sPLA₂-binding molecules is still very limited and

Table 1. sPLA₂-binding molecules and some of their properties.

Structural characteristic	Representative(s)	Protein type	Effect on PLA ₂ activity	Ca ²⁺ -dependence of sPLA ₂ binding
CRD-like domain	M-type sPLA ₂ R	membrane	inhibition	no
	SP-A	soluble	inhibition	yes
	α -PLI	soluble	inhibition	no
Leucine-rich repeat	β -PLI	soluble	inhibition	n. d.
Three-finger motif	γ -PLI	soluble	inhibition	no
EF-hand Ca ²⁺ -binding motif	CaM	soluble	augmentation	yes
	Crocalbin	soluble	n. d.	yes
	TCBP-49	soluble	n. d.	yes
Ig-like domain	VEGFR-1/Flt-1	membrane	no effect	n. d.
	VEGFR-2/KDR	membrane	no effect	n. d.
	DM64	soluble	no effect	n. d.
Thioredoxin-like fold	PDI	soluble	no effect*	no*
Negatively charged carbohydrates	HSPG	membrane	no inhibition	n. d.
Other or undefined	Integrins	membrane	n.d.	n.d.
	K ⁺ -channel	membrane	n.d.	n. a.
	N-type sPLA ₂ R	membrane	n. d.	yes
	R25	membrane	n. d.	yes
	14-3-3 proteins	soluble	no effect*	n.d.

n.d., not determined; n.a., not applicable; * Lidija Kovačič, personal communication

It is evident that some well-defined structural elements occur repeatedly in sPLA₂ receptors. These are the CRD and CRD-like folds (the M-type sPLA₂R, SP-A and α -PLIs), the EF-hand Ca²⁺-binding motif (CaM, crocalbin and TCBP-49), and the Ig-like domain (VEGFR-1/Flt-1, VEGFR-2/KDR and DM64). Direct implication in the sPLA₂ binding has been demonstrated experimentally for the CRD and CRD-like motifs. The Ig-like domain probably harbours an sPLA₂ binding-site but experimental proof is still lacking (Figure 1).

many questions still remain to be answered. For example, what is the physiological function of catalytically inactive mammalian sPLA₂-XIIA homolog sPLA₂-XIIIB, or whether presynaptically toxic sPLA₂s exert their neuromuscular blocking effect by affecting neurons extra- or intracellularly, are questions that will be solved much more easily when the nature of the respective receptor proteins on target cells is known.

As the number of protein three-dimensional structures grows rapidly, definition of general sPLA₂-binding

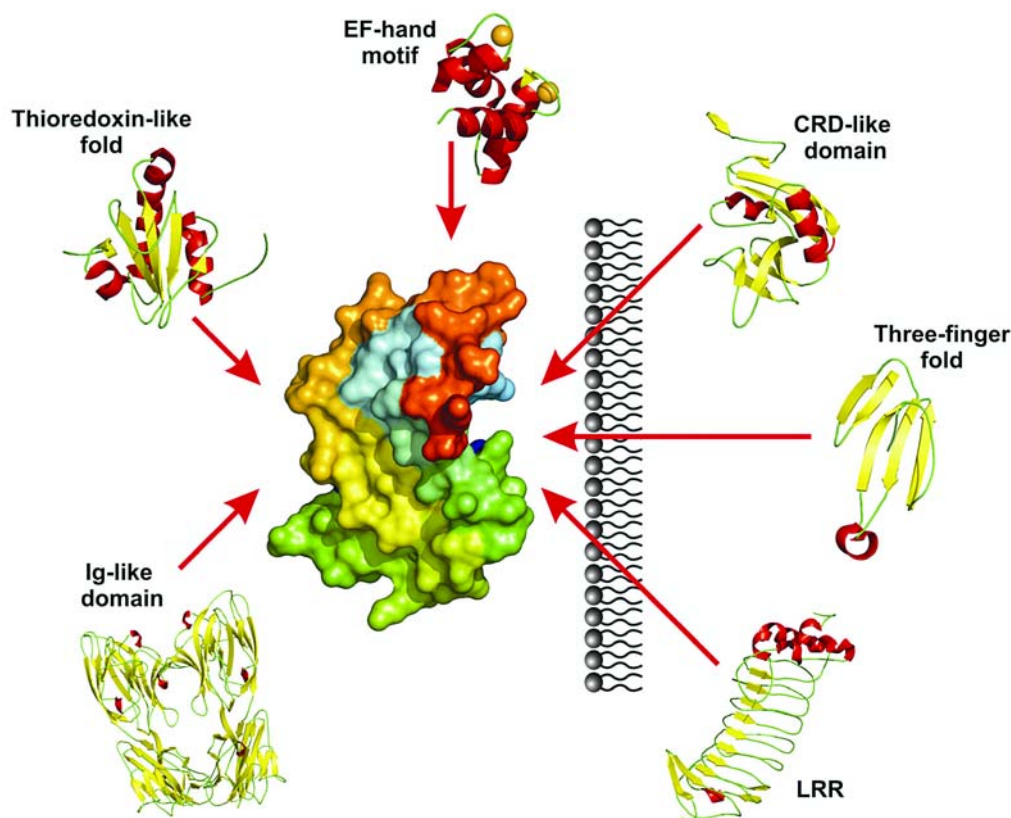


Figure 1. sPLA₂-binding molecules contain some well defined structural motifs. A CRD-like domain has been experimentally demonstrated as the sPLA₂-interacting structure. An Ig-like domain and a LRR are also likely involved directly in sPLA₂ binding. Deduced from structural and/or phospholipase activity-interfering studies, protein-binding regions on an sPLA₂ molecule are located either on the interfacial binding site (IBS) or outside this area. As symbolically shown on the picture, in the case of sPLA₂-binding molecules containing a CRD-like domain, a three-finger fold or a LRR, access of substrate phospholipids into the enzyme active site is physically prevented resulting in the inhibition of the enzymatic activity. If an sPLA₂-binding molecule interacts with an sPLA₂s outside its IBS, the phospholipase activity of the latter is not affected or it is even augmented.

motifs appears as a promising approach to advance our understanding of the mechanisms of the pathophysiological actions of sPLA₂ molecules.

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6. References

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Povzetek

Sekrecijske fosfolipaze A₂ (sPLA₂) predstavljajo tako po fiziološki kot po patološki plati zelo pomembno družino encimov. Večino učinkov sPLA₂ lahko pojasnimo z njihovo fosfatidilglicerol *sn*-2 hidrolazno aktivnostjo. Od trenutka, ko so bile odkrite tudi farmakološko aktivne molekule sPLA₂ brez encimske aktivnosti, najprej v kačjih strupih, kasneje pa tudi pri človeku, postaja očitno, da je v številnih primerih učinke teh beljakovin potrebno obravnavati kot rezultat prepleta njihove vezave na specifične receptorje (vloga liganda) in encimske aktivnosti. Število odkritih sPLA₂-vezavnih molekul narašča in z razvojem vedno bolj občutljivih biokemijskih tehnik lahko pričakujemo nova odkritja. V preglednem članku podajava pregled vseh trenutno poznanih sPLA₂-vezavnih proteinov. Strukturna raznolikost teh molekul izpostavlja sPLA₂ kot ligande širokega interakcijskega spektra, v skladu z njihovim več funkcijskim značajem. Mehanišična analiza različnih aktivnosti sPLA₂ so danes vznemirljiva in obetavna področja znanosti. Opis interaktoma sPLA₂ je zagotovo eden od ključnih elementov teh raziskav.