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Participation of annexins in protein phosphorylation

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Abstract. Simultaneous discovery of members of the annexin family of calcium and phospholipid binding proteins by several groups is intimately linked to the possibility that these proteins may be controlled by phosphorylation. Indeed, annexin I and annexin II have been identified as major substrates for the tyrosine kinase activity associated with epidermal growth factor receptor (EGF-R) and for the retrovirus encoded protein tyrosine kinase $pp60^{v\text{-}src}$. Both annexins are also in vitro and/or in situ substrates for platelet derived growth factor (PDGF), insulin and hepatocyte growth factor/scatter factor (HGF/SF) receptor tyrosine kinases. In addition, to serve as substrates for tyrosine protein kinases some annexins are cellular targets for serine/threonine protein kinases such as protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA). Although the role of annexin phosphorylation has not been studied in detail, it is thought to influence their vesicle aggregation and phospholipid binding properties. Some annexins are also potent inhibitors of various serine/threonine and tyrosine kinases. The physiological functions of the annexins have still not been clearly defined. Therefore the identification of the ability of these proteins to undergo phosphorylation may be helpful in assigning them a precise biological role.
Key words. Annexins; protein kinases; phosphorylation; phospholipids; calcium.

Introduction

Phosphorylation is a reversible post-translational covalent modification used by prokaryotic and eukaryotic cells to control the properties of a wide variety of proteins including enzymes, receptors, ion channels and regulatory or structural proteins. This mechanism is controlled by the activation of intracellular protein kinases, leading to the phosphorylation of intracellular target proteins which relay the effects of various agonists on cellular processes such as proliferation and differentiation. Depending on the amino acid(s) phosphorylated, these proteins are classified as serine/threonine kinases, tyrosine kinases and dual specificity protein kinases (serine/threonine and tyrosine).

The effects of protein kinases are counterbalanced by protein phosphatases with the following specificities: serine/threonine phosphatases, tyrosine phosphatases and dual specificity phosphatases (serine/threonine and tyrosine).

Phosphoproteins can be characterized according to the protein kinase involved, the residues phosphorylated and the cellular and subcellular distribution.

The discovery of members of the annexin family of proteins is closely related to studies intended to identify cellular substrates of various protein kinases.

Physiological functions of annexins

Numerous physiological functions have been attributed to annexins including regulation of membrane traffic during exocytosis and endocytosis, mediation of cytoskeletal-membrane interactions, mitogenic signal transduction, transmembrane ion channel activity, anti-inflammatory properties, inhibition of blood coagulation and inhibition of phospholipase A_2. It is still a matter of debate as to whether these two latter functions are the result of a calcium-dependent sequestration of phospholipids or a direct effect of the annexins acting via protein-protein interactions.

During the development of these studies, numerous investigations attempting to unravel the physiological role of these proteins led to the identification of post-translational modifications such as phosphorylation.

Annexins are targets of protein tyrosine kinases

Reversible protein tyrosine phosphorylation is an essential regulatory mechanism that governs the control of fundamental cellular signalling events involved in growth, proliferation, differentiation and transformation. Tyrosine protein kinases are enzymes that catalyse these reactions. The effects of various effectors such as growth factors (EGF, PDGF) and hormones (insulin) are transduced by a class of transmembrane receptors with intrinsic tyrosine kinase activity (R-PTK). The tyrosine-specific phosphorylation function of these R-PTKs is indispensable for the activation of signalling pathways that promote cellular responses. Products of retrovirus-encoded oncogenes define another class of cytosolic nonreceptor tyrosine kinases, the prototype being $pp60^{v\text{-}src}$.

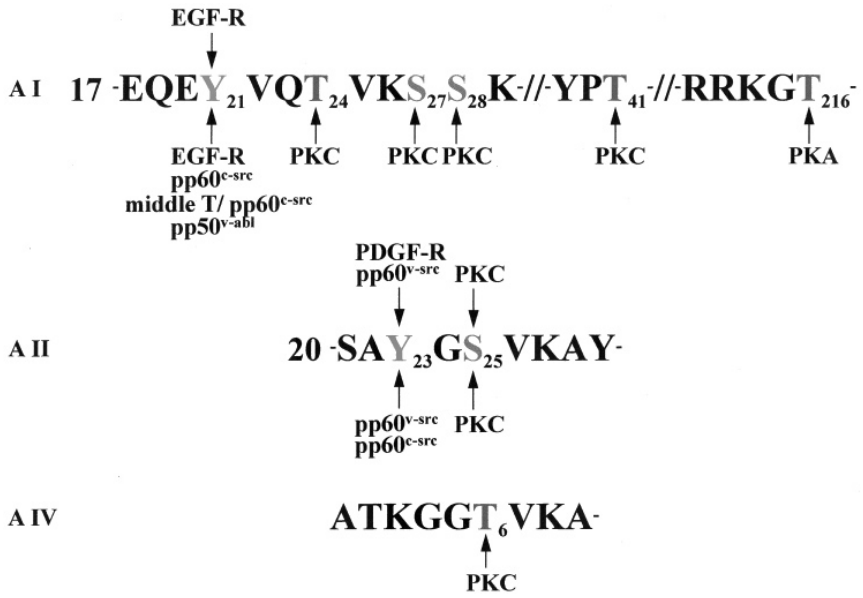

Figure 1. Sites of human annexins I, II and IV phosphorylation by protein serine/threonine kinases and tyrosine kinases. The different protein kinases involved are shown above the sequences when the sites of phosphorylation have been identified in situ and below when they have been obtained in vitro. Amino acids are represented by the single letter code.

Pioneering studies devoted to the identification of substrates of the EGF receptor kinase and $pp60^{v\text{-}src}$ led to the discovery of annexin I [1–3] and annexin II [4–6]. A unique tyrosine residue phosphorylated by the EGF receptor kinase was identified in the N-terminal domain of annexin I at position Tyr-21 [7] (fig. 1). The EGF receptor-dependent phosphorylation was shown to occur when cells were treated with EGF [8, 9], and when the reaction was developed in vitro using purified proteins [7, 10]. Tyr-21 was also characterized as the major site of annexin I phosphorylation by recombinant $pp60^{c\text{-}src}$, polyoma middle T/$pp60^{c\text{-}src}$ and $pp50^{v\text{-}abl}$ [11].

More recently a role has been attributed to annexin I in the processing of the EGF receptor. A form of annexin I seems to be associated in a Ca^{2+}-independent manner with internal vesicles involved in the endocytotic degradation of the receptor [12]. Only the tyrosine phosphorylated form is released from the membrane, showing that it may influence the binding of annexin I to membranes.

In addition to EGF, other growth factors are involved in the phosphorylation of annexin I, including HGF/SF [13]. This growth factor was shown in vivo to induce phosphorylation on tyrosine of annexin I and the translocation of annexin I to the membrane fraction where it associated with the receptor. This interaction was independent of the phosphorylation state of the receptor. In this respect, a functional link between HGF/SF-stimulated cell proliferation and the phosphorylation of annexin I has been suggested.

The stimulation of a seven-transmembrane G-protein coupled receptor (which lacks an intrinsic tyrosine kinase activity) provides another example of tyrosine phosphorylation [14]. In mesangial cells, the mitogenic agent angiotensin II induced a sustained tyrosine phosphorylation of annexin I. However, the tyrosine kinase responsible for this effect has not been identified.

Tyrosine phosphorylation of annexin II was shown to occur both in vivo [15, 16], and in vitro [17]. Sequencing of peptides from bovine annexin II phosphorylated in vitro with $pp60^{v\text{-}src}$ has shown that Tyr-23 is the major site [18] (fig. 1). Activation of the PDGF receptor also increased tyrosine phosphorylation of annexin II [19] at position Tyr-23 [20]. Annexin II has been identified as a major cellular substrate for the constitutive protein tyrosine kinase activity of bovine articular chondrocytes [21]. Annexins I and II are also in vitro and in vivo substrates for the insulin receptor tyrosine kinase [22].

Additional information concerning the possible role of annexin II in insulin signal transduction has appeared recently [23]. The authors demonstrate that insulin induces the tyrosine phosphorylation of annexin II in vivo and that this event could be part of the internalization and sorting mechanism of the insulin receptor. Those results could be related to the tyrosine phosphorylation of annexin I by an active EGF receptor kinase [12], required for the correct sorting and recycling of the receptor [24].

Although these results show that annexins I and II are substrates for both receptor tyrosine protein kinases and oncogene encoded protein kinases, the precise consequences of these post-translational modifications in the transduction of mitogenic signals have still to be determined.

Annexins are substrates of serine/threonine kinases

Intracellular signalling pathways include systems in which specific kinases, for example PKC and PKA, are activated following the generation of second messengers.

PKC serine/threonine kinases, a family of at least 12 isoenzymes, have been subdivided, on the basis of different primary structures and enzymatic properties, into Ca^{2+}-dependent or conventional PKCs (cPKC) and Ca^{2+}-independent or novel and atypical PKCs (n + aPKC) [25]. Although PKC and annexins have some features in common, such as binding of calcium and phospholipids and association with cytoskeletal elements, there are no structural similarities between them. Activation of PKC by growth factors, hormones or phorbol esters leads to the phosphorylation of many endogenous proteins which are involved in the control of cell proliferation, differentiation and motility [26].

Annexin I [11, 27] and annexin II [28, 29] are among the substrates phosphorylated by PKC. The major sites of human annexin I phosphorylation by PKC are Ser-27, located six residues from the tyrosine kinase phosphorylation site, and Thr-41 [11] (fig. 1). In addition, phosphorylation of Ser-27, Ser-28 and Thr-24 have also been identified [27]. Annexin II is a target for PKC both in vivo and in vitro. Ser-25 has been shown to represent the major phosphorylation site [28]. Its seems that Ser-25 and Tyr-23 phosphorylation events are mutually exclusive since both sites have not been identified on the same molecule. Another annexin, namely annexin IV, has also been phosphorylated in vitro by PKC on Thr-6 [30] (fig. 1).

These PKC-dependent sites are located in a consensus sequence motif (Ser/Thr-Val-Arg/lys). Interestingly, other annexins which possess the putative PKC substrate motif (Thr-Val/Ile-Arg/lys) are also potential substrates for PKC. These include annexins III, VII, VIII and X. Annexins V and VI, which both lack this consensus sequence, have not been described as PKC substrates.

To investigate which PKC isoforms were involved in the phosphorylation of annexins in vitro studies have been performed. Thus we recently showed that annexin I [31] and annexin II [32] are substrates only for cPKCs (Ca^{2+} and phospholipid-dependent) and not for (n + a)PKCs. In addition to PKC-dependent phosphorylation, annexin I is also an in vitro substrate for PKA. The unique site identified was Thr-216 located near the beginning of the third repeated sequence of the conserved domain [11]. The sequence preceding Thr-216 (Arg-Arg-Lys-Gly) is positively charged, as expected for a protein kinase A phosphorylation site. This is, until now, the only phosphorylation site that has been identified outside the N-terminal domain of an annexin. However the physiological meaning of this phosphorylation has not yet been investigated.

Effect of phosphorylation on the properties of annexins

Previous studies suggested that tyrosine and serine phosphorylations of annexin-like proteins could abolish their ability to inhibit the activity of PLA_2 [33]. However, it still remains to be established whether this inhibitory activity is the result of a sequestration of phospholipids or a direct protein-protein interaction.

The tyrosine phosphorylation of annexins I and II has previously been shown to influence their lipid binding characteristics. Thus phosphorylation of annexin I by the EGFR reduced the Ca^{2+} requirement of phospholipid vesicle binding [34, 35], whereas phosphorylation of annexin II by $p60^{v\text{-}src}$ decreased binding of the protein to phospholipid vesicles at low Ca^{2+} concentrations [36]. Tyrosine phosphorylation has also been reported to promote proteolytic degradation of annexin I [37].

Phosphorylation of annexin I and of the annexin II tetramer (A-II-t:two A-II subunits and two p11 subunits [38–40]) by PKC did not inhibit their phospholipid binding properties, but in vitro it inhibited their membrane aggregating properties as well as the association of annexin II with p11 [41, 42]. Phosphorylation also increased the calcium requirement of annexin I to promote aggregation of chromaffin granules [43].

Recently, the aggregative and binding properties of the phosphorylated and unphosphorylated forms of annexin II toward chromaffin granules have been compared [44]. The authors show that the phosphorylation of annexin II decreased the affinity of the binding to chromaffin granules without affecting the maximum binding capacity. Phosphorylation of annexin II was followed by dissociation of the light chains (p11 subunits) from the heterotetramer. Interestingly, when annexin II, bound to chromaffin granules, was phosphorylated by PKC in the presence of phorbol ester (TPA), fusion of granules was observed. These results suggest that PKC may play an important role in the regulation of annexin II during Ca^{2+}-dependent exocytosis, since both are involved in stimulus secretion coupling [45].

Another example is provided by the phosphorylation of annexin XI in rat embryonic fibroblasts transformed by Rous sarcoma virus oncogene (v-*src*). Compared to nontransformed cells, phosphorylation of annexin XI was increased on both serine and threonine residues by an as yet unidentified kinase [46]. This phosphorylation was shown to abolish the phosphatidylserine vesicle-binding ability of annexin XI even in the presence of high concentrations of calcium. It also altered the subcellular distribution of the protein. These results show that phosphorylation can affect the properties of annexins in different manners but represents a critical event that modifies their biological character.

Annexins are inhibitors of serine/threonine and tyrosine kinases

In addition to serving as substrates of various protein kinases, some annexins also have the capacity to inhibit these enzymes. Thus, annexins V [47] and VI [48] are potent inhibitors of PKC in vitro. Although the inhibition seems specific for this kinase (EGF receptor kinase and PKA were not affected by annexin V), there is no consensus concerning the mechanism of action. In some cases annexin V was shown to inhibit PKC directly [47, 48], whereas others maintain a substrate depletion model [49]. The main difficulty in elucidating a conclusive mechanism is that all these proteins bind calcium and phospholipids. Nevertheless, among the PKC isoforms tested, only cPKC-dependent phosphorylation of annexin I was affected by annexin V [31].

Another recent study also provides evidence that annexin IV specifically inhibited Ca^{2+}/calmodulin-dependent protein kinase II (CaM KII) activated chloride current [50]. However, the authors show that in vitro annexin IV did not inhibit CaM KII activity and did not act as a substrate for this kinase. These results suggest that the inhibitory effect of annexin IV is not the result of a direct interaction with CaM KII, but rather acts by preventing CaM KII-ion channel interaction and subsequent activation.

An additional example is provided by the inhibitory effect of annexin I on the insulin receptor protein tyrosine kinase [51]. This inhibition was specific (annexin V had no effect), occurred in the absence of calcium and phospholipids and seemed to involve a direct interaction between annexin I and the insulin receptor. Although, this observation needs to be confirmed in vivo, it opens new perspectives on the mechanisms of action of the annexins.

Conclusion

During the last 15 years, a substantial amount of data has accumulated which deals with the phosphorylation of members of the annexin family of calcium and phospholipid binding proteins. However, those results are not always satisfactory because most studies were obtained in vitro. A clear picture of the biological properties of these phosphoproteins needs an investigation in vivo. Such experiments are usually complicated by the presence of phosphatases that counterbalance the effects of specific kinases. One approach could be to generate a mutated form of an annexin that could affect a known phosphorylation site. For example a selected tyrosine residue could be changed to a glutamic acid residue in order to introduce a stable negative charge that could mimic the presence of a phosphate in this location. These mutations could provide a tool to understand better the translocation of annexins and/or their interactions with other proteins, and to modulate the mechanism of signal transduction.

Identification of protein kinases and protein phosphatases and the study of their accessibility to substrates such as annexins will be of great importance to the better understanding of the physiological role of these proteins. No doubt these experiments are already 'en route'.

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