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Review

Calnexin phosphorylation: Linking cytoplasmic signalling to endoplasmic reticulum lumenal functions

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ABSTRACT

Calnexin is an abundant integral membrane phosphoprotein of the endoplasmic reticulum (ER) of eukaryotic cells. The role of the luminal domain as an N-glycoprotein specific lectin has been well-established. Cytosolic C-terminal domain phosphorylation of calnexin has recently been elucidated in glycoprotein folding and quality control. Signalling of the presence of unfolded proteins from the lumen of the ER is mediated by the three ER membrane sensor proteins Ire1, ATF6 and PERK. The observation that the C-terminus of calnexin is differentially phosphorylated when glycoproteins are misfolded initiated our search for functional roles of calnexin phosphorylation. Recent studies have defined a role for phosphorylation at a proline-directed kinase site (Ser563) in ER protein quality control, while phosphorylation at a casein kinase 2 site (Ser534, Ser544) may be linked to transport functions. There are also four other abundant integral membrane phosphoproteins in the ER, and these may be components of other signalling pathways that link and coordinate other ER functions with the rest of the cell.

Contents

1. Introduction

Calnexin was originally uncovered as a prominent integral membrane protein of the endoplasmic reticulum (ER) which was *in vitro* phosphorylated by ER-associated kinases [1]. This property, as well as the observation that the phosphorylated protein was the antigen for an ER-specific polyclonal antibody which recognized an unknown protein of the same molecular weight, enabled its purification, partial protein sequencing, and full-length sequencing of its cloned cDNA [1]. The identity of calnexin with a previously reported molecular chaperone of the ER (p88, IP90) predicted a role in protein folding [2,3]. The raising of mono-specific polyclonal antibodies to calnexin then enabled its functional significance to be elucidated as the first example of an ER molecular chaperone sens-

Abbreviations: ER, Endoplasmic reticulum; Ire1, Inositol-requiring protein 1; ATF6, Activating transcription factor 6; PERK, PRKR-like endoplasmic reticulum kinase; CK2, Casein kinase 2; ERK1, Extracellular signal-regulated kinase 1; MAPK, Mitogen-activated protein kinase; PKC, Protein kinase C; PACS-2, Phosphofurin acidic cluster sorting protein 2; TMX-4, Thioredoxin-related transmembrane protein 4; ERAD, Endoplasmic reticulum-associated degradation; UPR, Unfolded protein response; EDEM, Endoplasmic reticulum degradation-enhancing mannosidase-like protein; OS-9, Osteosarcoma 9; MEK1, Mitogen-activated protein kinase/ERK kinase 1; AAT, Alpha-1-antitrypsin; TRAP, Translocon-associated protein.

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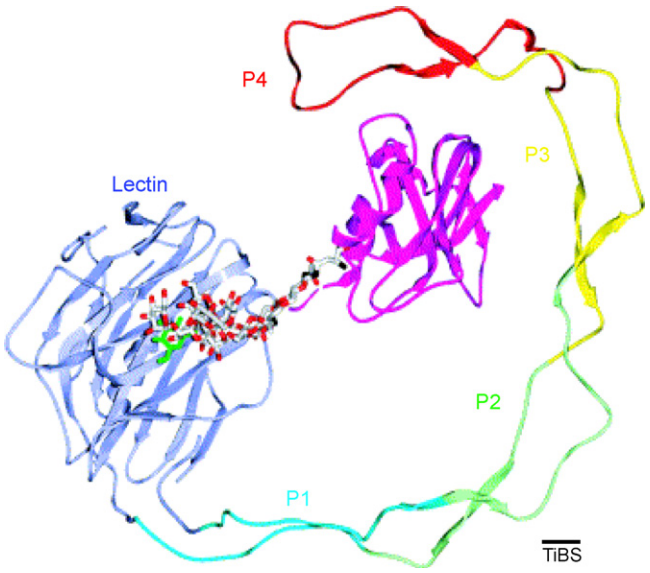

Fig. 1. 3D model of the interaction between calnexin and the client glycoprotein, ribonuclease b. The lumenal structure of calnexin is made up of two domains: the lectin domain (blue) and the P-domain. The P-domain is comprised of four repeats (labelled P1-P4) of anti-parallel interactions between two different proline-rich sequences. While the binding of the glycoprotein to calnexin is through the lectin domain, an interaction involving the P-domain is also likely. The P-domain also interacts with the protein-folding enzyme ERp57. The client glycoprotein is indicated in magenta. Taken from Schrag et al. [28].

ing the protein-folding status of client glycoproteins through their sugar complement [4–6].

The lumenal domain of calnexin has been established as a Glc1Man9-specific lectin [5,7,8] with a long arm (P-domain) potentially encompassing and restricting the dissociation of client glycoproteins (Fig. 1). Immediate upon sequencing the cDNA of calnexin, it was recognized as a paralogue to calreticulin [1]. Calreticulin is a soluble ER lumenal protein and is also a Glc1Man9-specific lectin that recognizes the protein-folding status of client glycoproteins via their glycans [9].

A third calnexin homologue has been found, and this is the male germ cell-specific protein calmegin (calnexin-t) [10,11]. Calmegin has a similar lumenal, transmembrane, and cytosolic domain to that of calnexin, except that its cytosolic domain is slightly longer [10,11].

Phosphorylation of calnexin (and calmegin [12]) occurs on its cytosolic domain [13] (Fig. 2). The significance of this phosphorylation has given us insight into new mechanisms involving ER microdomains, productive protein folding, and quality control.

2. Identification of the phosphorylation sites of calnexin

It has been previously demonstrated that the casein kinase CK2 is an ER-associated kinase responsible for the phosphorylation of calnexin [14,15]. Subsequently, calnexin phosphorylation *in vivo* was analyzed in the protein purified from HepG2 cells and MDCK cells [13]. By phospho-amino-acid analysis as well as by nano-electrospray mass spectrometry employing a selective scan specific mode for phosphorylated peptides, three phosphorylation sites were identified (Fig. 2) [13].

Several kinases have been shown to participate in calnexin phosphorylation. Among them CK2 was found to be responsible for the phosphorylation of Ser534 and Ser544 on human calnexin [13,14]. In addition, ERK1 MAPK was also found to phosphorylate Ser563 both *in vitro* and *in vivo* [16,17]. Finally, indirect evidence showed that PKC may be involved in calnexin phosphorylation through the regulation of ER calcium concentrations [18].

High-throughput approaches to identify and to characterize phosphoproteins have also confirmed that calnexin is phosphorylated *in vivo*, with confirmation of the phosphorylation sites that had been previously identified [19,20] (Fig. 3A).

As shown in Fig. 3B, numerous kinases have been predicted to phosphorylate calnexin on its cytosolic domain as deduced from a NetworKIN analysis of calnexin [21,22]. Thus far, only ERK1 and CK2 have been found to phosphorylate calnexin *in vitro* and *in vivo* [13,14,16,17,23]. Remarkably, although the NetworKIN analysis may reveal novel paths to investigate experimentally, ERK1 appears to be missing from this analysis (Fig. 3B). Because ERK1 was found to be a relevant calnexin kinase both *in vitro* and *in vivo* [13,14,16,17,23], we may conclude that predictive tools provide only partial information.

3. Functional roles of calnexin phosphorylation

Calnexin phosphorylation was first shown as relevant by the specific recruitment of calnexin to the translocons that are bound to ribosomes [16]. This was deduced through an *in vitro* approach using dog pancreatic microsomes and an *in vivo* approach carried out in Rat-2 cells [16]. A further study has uncovered a role for calnexin phosphorylation in ER quality control [17] (see below). For both phenomena, regulation is via phosphorylation on Ser563. At Ser534/544, CK2 phosphorylation has been shown to disrupt the association of calnexin with the cytosolically associated PACS-2 protein, leading to a re-distribution of calnexin from peripheral ER to juxtanuclear ER [23]. Similar potential sites of phosphorylation and PACS-2 association have been found for the transmembrane protein-folding enzyme TMX-4 [24]. This suggests a broader involvement of phosphorylation of cytosolic domains of ER membrane proteins with PACS-2. However, neither phosphorylation of TMX-4 nor PACS-2 association has yet been demonstrated. Calmegin has also been shown to be a phosphoprotein *in vivo* [12], with as yet no established function.

3.1. Role of calnexin phosphorylation in ER quality control

The ER quality control machinery enables the retention of misfolded proteins within the ER lumen [25]. While correctly

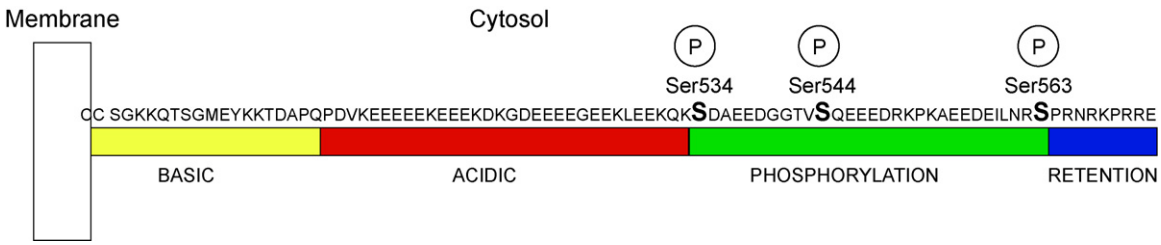

Fig. 2. Schematic representation of calnexin cytosolic domain. Four sub-domains were identified in the cytosolic domain of calnexin. A juxta-membrane basic domain (yellow) is followed by an acidic domain (red), the phosphorylation region (green), and a potential ER retrieval motif (blue) [24]. Amino acid numbering starts after signal sequence cleavage.