POSTER SESSION

B

Ca^{2+}-BINDING PROTEINS

LIPID MODIFICATIONS OF PLANT CALCINEURIN-B LIKE PROTEINS REGULATE THE LOCALIZATION AT DIFFERENT CELLULAR MEMBRANES

BATISTIC O. & KUDLA J.

Institut für Botanik, Universität Münster, Schlossplatz 4, D-48149 Münster, Germany.

Arabidopsis thaliana calcineurin B–like proteins (CBLs) interact specifically with a group of CBL-interacting protein kinases (CIPKs). CBL/CIPK complexes phosphorylate target proteins at different cellular membranes.

Here, we report that lipid modifications are required for localization of these Ca^{2+}-sensor proteins at the plasma membrane and vacuolar membrane. Myristoylation of AtCBL1 first targets the Ca^{2+}-sensor protein to the endoplasmic reticulum. Second, S-acylation of AtCBL1 is crucial for endoplasmic reticulum-to-plasma membrane trafficking via a targeting pathway that is insensitive to brefeldin A. Moreover, the lipid modification status of the calcium sensor moiety determines the cellular localization of preassembled CBL/CIPK complexes. Additionally, a related Ca^{2+}-sensor protein, AtCBL2, is potentially palmitoylated at three cysteine-residues and thereby targeted to the vacuolar membrane.

Keywords: calcineurin-B like proteins, lipid modification, Arabidopsis

USE OF NMR TO CHARACTERIZE THE INTERACTION OF S100 PROTEINS WITH SMALL LIGANDS AND PHYSIOLOGICAL PARTNERS

BORSI V. & LUCHINAT C.

CERM, Magnetic Resonance Center, Polo Scientifico University of Florence, via Sacconi 6, I-50019 Sesto Fiorentino, Italy.

The S100 family is a highly conserved group of EF-hand proteins involved in a variety of cellular processes, such as cell cycle regulation, cell growth, cell differentiation, and motility. These proteins have gained a growing interest due to their deregulated expression in cardiomyopathies, neurodegenerative and inflammatory disorders, and certain cancers.

Several members of the family (S100A2, S100A5, S100A7, S100A9, S100A10, S100A13, S100A16, S100B, S100P) have been expressed with high yield in ^{15}N enriched media. The structure of S100A13 in solution has been already solved, while for S100A5 and S100A16 the structural analysis is currently in progress.

In order to elucidate the different molecular mechanisms and the pathological roles of S100s, the interaction with selected protein partners and with a library of small molecules has been investigated by NMR.

The interaction of S100B with p53 and of S100P with the V-domain of RAGE have been monitored, and in the former case small molecules that hamper the interaction have been identified.

Keywords: S100 proteins, NMR

CALCIUM FINGERPRINTS INDUCED BY CALMODULIN INTERACTORS IN EUKARYOTIC CELLS

DAGHER R.[1], BRIERE C.[2], FEVE M.[1], ZENIOU M.[1], PIGAULT C.[1], RANJEVA R.[2], KILHOFFER M.C.[1] & HAIECH J.[1]

[1] UMR CNRS 7175, University Strasbourg I, F-67401 Illkirch, France.
[2] UMR CNRS-UPS 5546, BP42617 Auzeville, F-31326 Castanet-Tolosan, France.

Calcium (Ca^{2+}) is one of the prominent elements in cell physiology. Its transient intracellular concentration change leads to cell response. This activity is sustained by a whole set of proteins constituting a Ca^{2+}-signaling toolkit involved in Ca^{2+}-signal generation, deciphering and arrest, with spatial and temporal dynamics to control the function of specific cell types. Among the intracellular proteins involved in Ca^{2+}-signaling deciphering, calmodulin (CaM) plays a pivotal role both by its ubiquity in eukaryotic cells and the variety of proteins it interacts with.

As CaM is ubiquitously expressed in eukaryotic cells, we addressed the question of whether Ca^{2+}-signaling pathways might be modified by various molecules known to interact with CaM and able to disrupt or modify its interaction with target proteins. Considering the central role of CaM in the Ca^{2+} network, one can assume that, inside the cell, these molecules might disrupt Ca^{2+} homeostasis, even in resting cells. The resulting Ca^{2+} response will vary depending on which interactions between CaM and target proteins are altered by the molecules and on the Ca^{2+} toolkit expressed in a given cell.

The effect of six classical CaM interactors (W5, W7, W12, W13, bifonazole and calmidazolium) was studied on Ca^{2+} signaling in tobacco cells (BY-2) and tumor-initiating cells isolated from human glioblastoma (TGNM01) using aequorin technology and the fluorescent Ca^{2+}-sensitive Indo-1 dye, respectively. Various Ca^{2+} fingerprints were obtained depending both on the CaM interactor used and the cell type investigated. This observation corroborates the idea that a differential interaction between the antagonists and CaM results in a differential inhibition of CaM-dependent proteins involved in Ca^{2+}-signal regulation. In addition, the distinct Ca^{2+} fingerprints in tobacco and human tumor initiating glioblastoma cells induced by a given CaM interactor highlights the specificity of the Ca^{2+} signalosome in eukaryotic cells.

Keywords: Ca^{2+} fingerprint, calmodulin, calmodulin antagonists

NEW INSIGHTS INTO THE FUNCTIONAL MECHANISM OF ANNEXINS

DASTVAN R.[1] & AMININASAB M.[2]

[1] Department of Biotechnology, University of Tehran, P.O. Box 14155-6455 & National Research Centre for Genetic Engineering and Biotechnology, Tehran, Iran.
[2] Department of Cell and Molecular Biology, University Of Tehran, P.O. Box 14155-6455, Tehran, Iran.

In this study, two distinct, interchangeable patterns of tension alteration in coupled plasma membrane monolayers have been proposed that are involved in various physiological processes and diseases such as cystic fibrosis and MDR which could solve the formerly proposed conundrum of dual activity of some released agents including membrane intercalating chemoattractants.

In addition, a new model has been developed for the functional mechanism of annexins, by highlighting the importance of their interaction with biological membranes, in line with their role in membrane–cortical cytoskeleton/extracellular matrix coupling and modulating the activity of transport proteins, all of which cause the modulation of membrane tension. The promising novel predictions of the model may shed light on the annexins' related puzzles including their opposing roles in the tumor progression and metastasis, and externalization of annexins. A novel secretion based model has also been proposed for the externalization of annexins in response to various autocrine/paracrine motility and growth factors that may possibly resolve the previous ambiguities in the secretion mechanism of annexins. Phosphorylation of the N-terminal domain of membrane-bound annexins by various activated kinases, by improvement of their ability to bind and penetrate into membranes and therefore local lateral pressure increase of cytosolic leaflet, promotes endocytosis of membrane-attached annexins and this in cooperation with pH alterations and membrane curvature changes triggers refolding and externalization of them via secretion and this unprecedented model provides oriented translocation and extrusion of annexins.

Findings may pave the way to understand wide variety of cell membrane originated phenomena such as multidrug-resistance, metastasis, cell proliferation, apoptosis and inflammation.

Keywords: annexins

CBL/CIPK REGULATION OF A PLANT K^+ CHANNEL

ECKERT C., HELD K., PASCAUD F., THIBAUD J.-B. & KUDLA J.

Institut für Botanik, Universität Münster, Schlossplatz 4, D-48149 Münster,Germany.
Biochimie et Physiologie Moléculaire des Plantes, F-34060 Montpellier, France.

Ca^{2+} ions play an important role in signalling processes in all living cells. Ca^{2+}-binding proteins are involved in sensing and relaying primary signals to downstream signalling and adaptation responses. Calcineurin B-like proteins (CBLs) represent a novel group of Ca^{2+}-sensor proteins likely to function in deciphering Ca^{2+} signals in plants. CBLs interact with a group of serine-threonine kinases designated as CBL-interacting protein kinases (CIPKs). In Arabidopsis, 10 CBL-type Ca^{2+}-sensor proteins form an interaction network with 25 CIPKs. Preferential formation of distinct CBL-CIPK complexes and differential subcellular localization of these complexes are mechanisms likely to contribute to specificity in signal-response coupling (Batistic and Kudla, 2004). The CBL-CIPK network has been shown to be involved in adaptation reactions to abiotic stresses like osmotic stress, as well as in the regulation of ion homeostasis in plants. In the latter regard it has recently been shown that the shaker-type K^+ channel AKT1 is activated by phosphorylation through the kinase CIPK23 that is complexed with either the Ca^{2+} sensor CBL1 or the Ca^{2+} sensor CBL9 (Xu et al., 2006).

Here we will present a second example of K^+ channel regulation by specific CBL/CIPK complexes. We identified a potential CBL-CIPK-K^+ channel combination using combinatorial yeast two-hybrid analysis. These interactions and the subcellular localization of these complexes were further verified by BiFC analyses in planta. In contrast to AKT1 the K^+ channel investigated in this study is already active in Xenopus oocytes in the absence of additionally expressed proteins. However, two-electrode voltage-clamp analyses in oocytes revealed a dramatic enhancement of K^+ currents of the K^+ channel after co-expression of both a specific CBL and a defined CIPK.

These finding suggest a novel mode of K^+ channel regulation by a specific CBL/CIPK complex.

Batistic & Kudla (2004) Planta 219, 915-924.
Xu et al. (2006) Cell 125, 1347-1360.

Keywords: K^+ channel, calcineurin B-like proteins, CIPK

INSIGHTS INTO S100-RAGE SIGNALING BY STRUCTURES OF S100B AND RAGE LIGAND-BINDING DOMAIN

KOCH M., DIEZ J., DATTILO M., CHAZIN W.J. & FRITZ G.

Department of Biology, University of Konstanz, Germany
Departments of Biochemistry and Chemistry, Center for Structural Biology, Vanderbilt University, Nashville, Tennessee, USA.

With 21 members in humans, S100 proteins are the largest subgroup within the family of EF-hand calcium-binding proteins. Most S100 proteins form homo- and heterodimers under physiological conditions. Larger non-covalent multimers are reported for S100A8/A9, S100A12, and we recently identified and structurally characterized tetra-, hexa-, and octameric species of S100B (Ostendorp et al., 2007). Several S100 proteins are secreted to the extracellular space where they exert neurotrophic, proinflammatory or antimicrobial functions. Prominent examples are S100B and S100A12, which act via the receptor for advanced glycation end products (RAGE).

We investigate the interactions between RAGE and S100 proteins by in vitro binding studies and X-ray crystallography. The X-ray structure of human Ca^{2+}-loaded S100B revealed that S100B can assemble into large multimers, which were confirmed in human brain and by recombinant expression in E. coli. Such multimers exhibit decreased dissociation from RAGE resulting most likely in a sustained activation of the receptor signal cascade. Besides Ca^{2+}, S100B and S100A12 bind Zn^{2+} with high affinity in a conserved Zn^{2+}-binding motif. Zn^{2+}-binding increases the affinity of dimeric S100B and S100A12 towards RAGE. We determined the structure of Ca^{2+}-Zn^{2+}-S100B at different pH values showing that Zn^{2+} binding induces a more open conformation and conformational changes in C-terminus which are important for target protein interaction.

To characterize the counterpart of S100-RAGE interaction we determined the X-ray structure of RAGE ligand-binding domain at 1.85 Å resolution. The arrangement of the molecules in the crystal suggests a pre-assembly of the receptor during activation which is supported by our biochemical studies.

Ostendorp et al. (2007) EMBO J., 26, 3868-3878.

Keywords: S100B, Zinc, RAGE, X-ray structural analysis

CELL-TYPE SPECIFIC RAGE SIGNALING PROMOTES TUMOR DEVELOPMENT BY SUSTAINING INFLAMMATION

GEBHARDT C.*, RIEHL A., NÉMETH J., FURSTENBERGER G., MUELLER-DECKER K., ARNOLD B., ENK A., NAWROTH P.P., BIERHAUS A., HESS J. & ANGEL P.

German Cancer Research Center (DKFZ), INF 240, D-69120 Heidelberg, Germany.
University Hospital Heidelberg, Vosstrasse 2, D-69115 Heidelberg, Germany.

A broad range of experimental and clinical evidence has highlighted the central role of chronic inflammation in promoting tumor development. However, the mechanisms that sustain a tumor-promoting microenvironment remain largely elusive.

We demonstrate that mice deficient for the receptor for advanced glycation end-products (RAGE) are resistant to DMBA/TPA-induced skin carcinogenesis. Furthermore, RAGE-deficient mice showed severely reduced inflammatory response to treatment with TPA accompanied by impaired infiltration with neutrophils, macrophages and mast cells and impaired upregulation of pro-inflammatory genes, such as Ptgs2, MIPs, S100A8 and S100A9 using confocal microscopy and gene expression analysis. S100A8 and S100A9 encoding strong chemoattractants for myeloid cells were induced upon TPA treatment of mouse back skin and overexpressed in advanced stages of mouse and human skin tumors using RQ-PCR and tissue microarrays. Since S100 proteins represent activating ligands of RAGE and ligand expression is RAGE dependent, we propose the existence of an S100/RAGE-driven feed-forward-loop in chronic inflammation and tumor formation. Finally, TPA-induced dermal infiltration and epidermal hyperplasia was restored in wild type-bone marrow-chimeric RAGE-deficient mice and revealed that RAGE expression on immune cells, but not keratinocytes, is essential for a sustained inflammatory microenvironment and effective tumor promotion.

In conclusion, we demonstrate that RAGE signaling drives the strength and maintenance of an inflammatory reaction during tumor promotion and provide direct genetic evidence for a novel cell type-specific role for RAGE in linking chronic inflammation and cancer.

* E-mail: christoffer.gebhardt@med.uni-heidelberg.de

Keywords: RAGE, inflammation, tumor development

ANNEXIN A6-INDUCED INHIBITION OF cPLA2 IS LINKED TO CAVEOLIN-1 EXPORT FROM THE GOLGI

GREWAL T.[1], CUBELLS L.[2], SVILÀ DE MUGA S.[2] & ENRICH C.[2]

[1] Faculty of Pharmacy, University of Sydney, Sydney, NSW 2006, Australia.
[2] Departament de Biologia Cellular, Idibaps, Facultat de Medicina, Universitat de Barcelona, E-08036 Barcelona, Spain.

The molecular mechanisms regulating the exit of caveolin from the Golgi complex are not fully understood. Cholesterol and sphingolipid availability affect Golgi vesiculation events and involve the activity of phospholipase A2 (cPLA2).

We recently demonstrated that high expression levels of annexin A6 (AnxA6) perturb the intracellular distribution of cellular cholesterol, thereby inhibiting caveolin export from the Golgi complex (Cubells et al., 2007). In the present study, we show that in CHO cells overexpressing AnxA6, sequestration of cholesterol in late endosomes, leading to reduced amounts of cholesterol in the Golgi, inhibits cPLA2 activity and its association with the Golgi complex. This correlates with the blockage of caveolin export from the Golgi in cells treated with MAFP, a Ca^{2+}-dependent cPLA2 inhibitor. AnxA6-mediated downregulation of cPLA2 activity was overcome upon addition of exogenous cholesterol or transfection with siRNA targeting AnxA6.

These findings indicate that AnxA6 interferes with caveolin transport through the inhibition of cPLA2.

Cubells et al. (2007) Traffic 8, 1568-1589.

Keywords: annexin A6, caveolin, phospholipase A2

IN VITRO Ca^{2+}-BINDING ANALYSIS OF CALCINEURIN B-LIKE PROTEINS AND Ca^{2+} REGULATION OF CBL-CIPK INTERACTION

GROTE J. & KUDLA J.

Universität Münster, Institut für Botanik und Botanischer Garten, Schlossplatz 4, D-48149 Münster, Germany.

Networks of Ca^{2+}-sensing proteins and their interplay with kinases contribute to deciphering temporal and spatial changes in calcium concentrations. They are able to transduce calcium signals and to mediate accordant cell responses. Recently, such a protein signaling network comprising of 10 Calcineurin B-like (CBL) calcium sensor proteins and 25 CBL-interacting protein kinsases (CIPK) has been identified in Arabidopsis (Batistic et al., 2004). CBL proteins harbor four EF-hands for potential calcium binding and interact with their target kinases via a CBL-binding domain (NAF-domain) located in the C-terminal regulatory domain of CIPKs.

Although general calcium binding to CBL proteins has been demonstrated in vitro previously (Kudla et al., 1999; Sanchez-Barrena et al., 2005), a detailed characterization of the Ca^{2+}-binding properties of any CBL protein is still lacking and it is currently not understood how changes in calcium concentration affect CBL-CIPK interaction.

Here we present our approaches to characterize the Ca^{2+}-binding abilities of selected CBL proteins and to investigate the Ca^{2+}-dependence of CBL-CIPK interaction. Spectrofluorimetric techniques in combination with calcium titrations are applied to examine calcium affinities. Ca^{2+} binding to CBLs are measured by changes of intrinsic fluorescence due to binding-induced conformational changes or by monitoring a Ca^{2+}-binding chromophoric chelator as a competitor during calcium titration. For the investigation of CBL-CIPK complex formation in dependence of calcium concentration we use surface plasmon resonance (SPR, Biacore 3000). SPR is a label-free method that allows quantitative examination of dissociation constants.

The combination of both techniques will provide first insights into how calcium signals are channeled by the CBL-CIPK network.

Batistic et al. (2004) Planta, 219, 915-924.
Kudla et al. (1999) Proc. Natl. Acad. Sci. USA 96, 4718-4723.
Sanchez-Barrena et al. (2005) J. Mol. Biol. 345, 1253-1264.

Keywords: surface plasmon resonance, calcineurin B-like protein, CIPK, Arabidopsis

DIRECT AND Ca^{2+}-DEPENDENT INTERACTION OF S100P WITH IQGAP1

HEIL A.[1], KOLTZSCHER M.[1], AUSTERMANN J.[1], PÖTER M.[1], BAUDIER J.[2] & GERKE V.[1]

[1] *Institute of Medical Biochemistry, Center for Molecular Biology of Inflammation, University of Münster, Münster, Germany.*
[2] *Institut de Recherche en Technologies et Sciences pour le Vivant, Commissariat à l'Energie Atomique, Grenoble, France.*

S100P is an EF-hand Ca^{2+}-binding protein initially identified in placenta that interacts with the cortical actin regulator ezrin.

Here we identified the multi domain protein IQGAP1 as another specific interaction partner of S100P. By association with numerous binding partners IQGAP1 is involved in different cellular activities such as modulation of the actin cytoskeleton, microtubule dynamics, cell-cell-adhesion, gene transcription and signaling pathways. To further investigate the S100P-IQGAP1 interaction and to map the respective binding motives we performed affinity chromatography approaches with mutant forms of the two proteins. Our results indicate that the CHD and the IQ domains of IQGAP1 as well as amino acids 21-25 of S100P are crucially involved in the interaction that occurs in a direct and Ca^{2+}-dependent manner. Our analyses also reveal that the monomeric mutant of S100P, $S100P^{F15A}$ is capable of binding towards IQGAP1 Ca^{2+}-dependently.

Therefore, the mechanism underlying S100P-IQGAP1 binding is different from that mediating the S100P-ezrin interaction, which is strictly dimer-specific and occurs via the C-terminus of S100P.

Keywords: S100P, IQGAP1

IDENTIFICATION OF CRITICAL RESIDUE IN ALIX FOR ALG-2 BINDING

INUZUKA T.[1], SUZUKI H.[1], KAWASAKI M.[2], KAKIUCHI T.[1], SHIBATA H.[1], WAKATSUKI S.[2] & MAKI M.[1]

[1] Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Japan.
[2] Structural Biology Research Center, Kek, Tsukuba, Japan.

Alix (also known as AIP1) was originally identified as an interacting protein of ALG-2, which is a Ca^{2+}-binding protein with five repetitive EF-hand motifs called penta-EF-hand (PEF) domain. ALG-2 is known to associate various proteins such as TSG101 (a component of endosomal sorting complex required for transport-Ⅰ (ESCRT-Ⅰ)), phospholipid scramblase 3 (PLSCR3), a COPⅡ component Sec31A, annexin A7 and annexin A11.

We previously demonstrated that a short segment containing the characteristic four-tandem PxY repeat region in the C-terminal proline-rich domain of Alix is crucial for direct interaction with ALG-2, and we named this region ABS (ALG-2 binding site). From the X-ray crystallographic analysis of the complex between ALG-2 and a 16-residue peptide of AlixABS (corresponding to 799-814; 1-QGPPYPTYPGYPGYSQ-16), it was revealed that the ABS peptide is held in two hydrophobic pockets in ALG-2 (Pocket1, Pocket2).

In this study, we attempted to identify which residues in the AlixABS peptide are critical for binding to ALG-2. We constructed E. coli expression plasmid GST-AlixABS-His which contained two flexible GGS linkers between N-terminal GST and AlixABS and between AlixABS and C-terminal 8xHis, and various alanine-substituted mutants. Direct binding of GST-AlixABS-His with ALG-2 was analyzed by an overlay assay using biotin labeled ALG-2 as a probe. The residues of 3-PPYP-6 captured in Pocket1 were found to be more important for ALG-2 binding than those of 11-YP-12 held in Pocket 2. Although the residues of 7-TYPG-10 do not interact with ALG-2, those of 8-YP-9 contribute to efficient binding, probably by stabilization of the peptide structure by intramolecular interaction. Indeed, GST fusion protein of GPPYPGGGGYPGG poorly bound to ALG-2.

Keywords: ALG-2, Alix

TYROSINE PHOSPHORYLATION OF ANNEXIN A2 REGULATES RHO-MEDIATED ACTIN REARRANGEMENT AND CELL ADHESION

KONIETZKO V., LUDWIG C., RESCHER U. & GERKE V.

Institute of Medical Biochemistry, Centre for Molecular Biology of Inflammation, Münster, Germany.

Dynamic remodelling of the membrane-associated actin cytoskeleton plays an important role in organizing cell adhesion and motility. Annexin A2, a Ca^{2+}-dependent membrane and F-actin binding protein, is involved in the regulation of membrane-cytoskeleton contacts and has been linked to certain sites of actin rearrangement. In BHK cells, which stably overexpress the human insulin receptor, annexin A2 is one of the most prominent tyrosine-phosphorylated proteins that are seen upon insulin stimulation.

The increase in annexin A2 tyrosine phosphorylation is accompanied by massive rearrangements of the actin cytoskeleton, with cells showing peripheral actin accumulations and a subsequent detachment. These morphological changes are inhibited by annexin A2 depletion and require Rho/Rock signaling downstream of annexin A2. In contrast, expression of a phospho-mimicking annexin A2 mutant, anx2-Y23E, is sufficient to drive peripheral actin accumulations and the resulting loss in cell adhesion in the absence of insulin stimulation.

The results indicate a critical role of tyrosine-phosphorylated annexin A2 in the regulation of Rho/ROCK-dependent actin-mediated changes in cell morphology and adhesion.

Keywords: actin, annexin A2, tyrosine phosphorylation, cell adhesion

IN SILICO STUDY OF PUTATIVE CALCIUM-BINDING DOMAINS IN HUMAN BK AND BEST1 CHANNELS

KRANJC A., MENINI A., CARLONI P. & ANSELMI C.

International School for Advanced Studies (SISSA/ISAS), Via Beirut 2-4, I-34014, Trieste, Italy.

Human big conductance calcium- and voltage-gated potassium channels (McCobb et al., 1995) (hBK) play an important role in the repolarization of the membranes and are putative drug targets for cardiovascular, respiratory and urological diseases. Human Bestrophin1 (Sun et al., 2002) (hBest1) are suggested to function as chlorine channels, having calcium sensitivity and are associated with age-related macular degeneration, particularly with Best vitelliform macular dystrophy.

The crystal structures for both channels are unknown. However they share the known calcium-binding motif pattern DDD-x-[D,E]. We have used bioinformatics and molecular simulation approaches to construct models of putative calcium-binding domains in hBK and hBest1 channels. Models could help to rationalize the available experimental data and to plan new experiments in order to understand the physiological role of these classes of channels. The search of this calcium-binding motif in the Swiss-Prot database by means of Prosite produced a high number of results. However, only a few of them correspond to proteins, which are actually known to bind calcium. Among them just the human Thrombospondin-1 (Kvansakul et al., 2004) (TSP-1) has the three-dimensional structure available. Therefore the human TSP-1 calcium-binding domain was used as a template for the modeling of the putative calcium-binding domains of hBK and hBest1.

Molecular dynamics simulations were then carried out on both models to evaluate the thermodynamic stability and to study the dynamical properties of both calcium-binding domains.

Kvansakul et al. (2004) EMBO J. 23, 1223-1233.
McCobb et al. (1995) Am. J. Physiol. 269, H767-777.
Sun et al. (2002) Proc. Natl. Acad. Sci. USA 99, 4008-4013.

Keywords: Ca^{2+}-gated channels, Ca^{2+}-binding domain, homology modelling

SYNAPTIC PROTEINS IN Ca^{2+}-DEPENDENT EXOCYTOSIS FROM CHROMAFFIN CELLS

LUKYANETZ E.A., SADOVIY A.V., POCHINYUK O.M. & ZAIKA O.L.

Bogomoletz Institute of Physiology &
International Center for Molecular Physiology, Bogomoletz Str. 4, Kiev, Ukraine

Ca^{2+}-dependent exocytosis of synaptic vesicles is the main processes in synaptic transmission in neurons and neuroendocrine cells.

We studied the role of synaptic proteins such as synaptotagmin (STG), Munc-13, Munc-18 and CAPS on Ca^{2+}-dependent kinetic properties of secretory events in the rat chromaffin cells using microfluorescent and amperometric methods and specific antibodies anti-STG, anti-Munc-13-1, anti-Munc-13-3, anti-Munc-18 and anti-CAPS. Simultaneously, fluorescent dextran-FITC was injected into a cell for the control of antibody entering into the cell. Exocytosis was induced by activating of the potential-dependent Ca^{2+}-channels by activation of voltage-operated Ca^{2+}-channels with KCl solution or increasing of intracellular Ca^{2+} concentration via activating of acetylcholine receptors.

The injection of antibodies resulted in the reduction of frequency of appearance of secretory peaks registered by carbon fiber electrode. Most substantially this was observed under anti-STG treatment. Influence of anti-Munc-18 resulted in deceleration of kinetic characteristics of secretory peaks, possible due to narrowing of fusion pore. Using of anti-Munc-18 revealed two types of cells which differed by the value of reduction of the frequency of secretory events. In the first group of tested cells, there was greater reduction of probability of appearance of secretory peaks (by ~71%) after injection of anti-Munc-18. While in the second group, there was the far less this value (~12%). We observed enough substantial decline of frequency of secretory appearances during introduction of specific antibodies to Munc-13, and the value of the reduction depended on the kind of stimulus. Anti-Munc-13-1 was more effective in suppression of secretory peaks than anti-Munc-13-3. Anti-CAPS antibodies induced also reduced appearance of secretory spikes.

We concluded that explored synaptic proteins participate in the secretory processes of chromaffin cells, but play their specific role.

Keywords: exocytosis, synaptic proteins, chromaffin cells

LOOKING FOR Ca^{2+}-BUFFERING PROTEINS IN THE NITROGEN-FIXING BACTERIUM MESORHIZOBIUM LOTI

MOSCATIELLO R.[1], ROVERI A.[2], MARIN O.[2], DAMIANI E.[3], MARIANI P.[1] & NAVAZIO L.[1]

1 Dipartimento di Biologia, Università di Padova, Via U. Bassi 58/B, I-35131 Padova, Italy.
[2] Dipartimento di Chimica Biologica, V.le G. Colombo 3, I-35131 Padova, Italy.
[3] Dipartimento di Scienze Biomediche Sperimentali, V.le G. Colombo 3, I-35131 Padova, Italy.

The involvement of a Ca^{2+}-binding protein in the regulation of intracellular free Ca^{2+} concentration has been recently demonstrated in the cyanobacterium Anabaena during heterocyst differentiation (Zhao et al., 2005; Shi et al., 2006).

Within a project aimed at unraveling the participation of Ca^{2+} ions in rhizobia during the early stages of their symbiotic interactions with legumes, we checked in the nitrogen-fixing bacterium Mesorhizobium loti the possible occurrence of Ca^{2+}-buffering proteins as a component of the bacterial Ca^{2+} homeostat.

As no sequence homologous to that encoding the Ca^{2+}-binding protein of the cyanobacterium Anabaena was found in the M. loti genome database, a biochemical approach was carried out. Evidence was provided for a 20 kDa soluble protein which was found to share with eukaryotic calsequestrin and calreticulin several biochemical features: i) purification by an ammonium sulphate precipitation procedure followed by anion-exchange chromatography; ii) an acidic isoelectric point (pI 4.2); iii) Stains All blue staining on SDS-PAGE; iiii) Ca^{2+}-dependent mobility shift.

To confirm the Ca^{2+}-binding ability and elucidate the identity of the putative rhizobial Ca^{2+}-binding protein, ^{45}Ca-overlay assays and MALDI-TOF/TOF mass analysis are in progress on the protein purified to homogeneity. Further work will be aimed at investigating the regulation of gene expression and/or protein level during the transition from free-living rhizobia into symbiotic bacteroids.

Shi et al. (2006) Proc. Natl. Acad. Sci. USA 103, 11334-11339.
Zhao et al. (2005) Proc. Natl. Acad. Sci. USA 102, 5744-5748.

Keywords: Ca^{2+}-binding proteins, bacteria, rhizobium

THE DUAL ROLES OF ANNEXIN A2 AS A mRNA-BINDING PROTEIN

RADDUM A., ERIKSEN I., HOLLÅS H., STRAND E., FLATMARK T. & VEDELER A.

Dept. of Biomedicine, University of Bergen, Jonas Lies Vei 91, N-5009 Bergen, Norway.

The cellular transformation process, which may eventually lead towards cancer development, is the result of an accumulation of several changes involving complex interactions and post-translational modifications. The expression of both the multi-functional protein Annexin A2 (AnxA2) and the proto-oncogene c-Myc are modulated in several cancers. Furthermore, both AnxA2 and c-myc mRNA are constituents of the same mRNP complexes associated with the cytoskeleton. Untranslated regions (UTRs) of mRNAs, together with specific proteins, regulate mRNA transport, translation and decay. We have previously shown that AnxA2 binds to a specific sub-population of mRNAs (Vedeler & Hollås, 2000) including its cognate mRNA (Hollås et al., 2006) via helices C-D in its domain IV (Aukrust et al., 2007).

The mRNA-binding assays in the present study confirm the finding that AnxA2 binds to the localisation signal in the c-myc 3'-UTR (Mickleburgh et al., 2005) and in addition shows that AnxA2 interacts with the c-myc 5'-UTR, suggesting that it regulates both the transport and subsequent translation of c-myc mRNA.

In order to study the effect of AnxA2 on the translation of c-myc mRNA, several chimeric transcripts were constructed using a seamless cloning strategy at the DNA level. The chimeras contain various combinations of regions coding for the c-myc and β2-microglobulin UTRs (the latter as negative controls) or no UTR, with the coding region of Renilla Luciferase (hRLuc) as a reporter protein. PCR products of the chimeric cDNAs included a T7 promoter site at their 5'-end to enable T7-driven transcription and subsequent translation in an in vitro system based on rabbit reticulocyte lysate.

The results strongly suggest that AnxA2 elicits a specific inhibitory effect on the translation of the chimeric mRNAs containing one or both UTRs of c-myc mRNA. This may imply that AnxA2 is not only involved in the transport of specific mRNAs, but also in their silencing during transport in order to initiate synthesis of their cognate proteins at specific cellular compartments.

Aukrust et al. (2007) J. Mol. Biol. 368, 1367-1378.
Hollås et al. (2006) Biochim. Biophys. Acta 1763, 1325-1334.
Mickleburgh et al. (2005) FEBS J. 272, 413-421.
Vedeler & Hollås (2000) Biochem. J. 348, 565-572.

Keywords: annexin A2, mRNA, c-myc

EXPRESSION OF ZEBRAFISH SPECIFIC NEURONAL CALCIUM SENSOR PROTEINS COINCIDES WITH THE ONSET OF VISUAL FUNCTION IN THE LARVAL RETINA

RAETSCHO N., SCHOLTEN A. & KOCH K.W.

Biochemistry Group, Dept. of Biology & Environm. Sciences, University of Oldenburg, D-26111 Oldenburg, Germany.

Among the family of neuronal calcium sensor proteins (NCS) is a group of small acidic proteins called guanylate cyclase-activating proteins (GCAPs). These proteins are predominantly expressed in photoreceptor cells of the vertebrate retina and function as regulators of membrane bound guanylate cyclases (GCs) via calcium dependent feedback loops. Adult zebrafishes express a variety of six zGCAP isoforms, whereas four of these are cone specific (Imanishi et al., 2004).

Establishing of visual function in the zebrafish larvae takes place between 3 and 5 days post fertilization (dpf) and can be attributed to cones only.

Therefore, we analyzed the expression profiles of the cone specific zGCAPs during these period of retina maturation. We detected the onset of expression for zGCAP3, zGCAP4 and zGCAP7 around 3 dpf, and found dynamic changes in expression profiles between 3 and 4 dpf. Only zGCAP5 transcripts appeared in later larval stages.

Furthermore, zGCAPs probably target to membrane bound GCs. We here report about the cloning of three sensory GCs (zGC1-3) and investigated their spatial-temporal expression pattern in the adult and larval zebrafish retina. Expression of zGC3 was restricted to cones in the adult retina, whereas zGC1 and zGC2 are expressed in rod nuclei as well as in UV-sensitive cones. Larval expression of all zGCs could be detected around 3 dpf, but only zGC3 showed dynamic changes in its expression profile and a spatial overlap with zGCAP transcripts. To test for functional zGCs we measured cGMP synthesis in larval eye preparations and revealed high endogenous guanylate cyclase activities.

The precisely controlled coexpression of zGCAPs and zGCs and corresponding guanylate cyclase activities indicate a crucial role of cGMP homeostasis in larval zebrafish cone phototransduction.

Imanishi et al. (2004) J Mol Evol. 59, 204-217.

Keywords: neuronal Ca^{2+} sensors, guanylate cyclase-activating proteins, retina, zebrafish, phototransduction

IMPLICATION OF S100A8/A9 IN THE Ca^{2+}-DEPENDENT NOX2 ACTIVATION IN NEUTROPHIL-LIKE HL-60 CELLS

SCHENTEN V., BRÉCHARD S., MELCHIOR C., PLANÇON S. & TSCHIRHART E.J.

Life Sciences Research Unit, University of Luxemburg, 162a, Avenue de la Faïencerie, L-1511 Luxemburg, Luxemburg.

The neutrophil NADPH oxidase NOX2, via reactive oxygen species production, participates to host defense but its dysfunction can contribute to the inflammatory process. Some studies have provided evidence for a role of Ca^{2+}-binding proteins S100A8 and S100A9 in NOX2 activation. Most of these data have been obtained in cell-free systems and the exact regulatory mechanism remains unclear.

The objective of this study is to characterize the role of S100A8/A9 in the Ca^{2+}-dependent regulation of NOX2 activity in differentiated neutrophil-like HL-60 cells (≠HL-60). fMLF-induced H_2O_2 production was reduced by half in cells co-transfected with S100A8 and S100A9 siRNAs, suggesting that both proteins are required for NOX2 activity.

Further, we characterized the role of intracellular Ca^{2+} ($[Ca^{2+}]_i$) variations on the S100A8/A9-regulated NOX2 activity. H_2O_2 production and $[Ca^{2+}]_i$ measurements showed that Ca^{2+}-independent NOX2 activity induced by PMA was enhanced by ionomycin, a Ca^{2+} ionophore. This amplification was abolished by S100A8/A9 knock-down. To determine Ca^{2+} conditions necessary for an optimal S100A8/A9-regulated NOX2 activity, we measured H_2O_2 production in S100A8/A9 siRNAs transfected ≠HL-60 subjected to increasing concentrations of BAPTA, an intracellular Ca^{2+} chelator. The resulting Ca^{2+}-sensitivity curve indicated that low BAPTA concentrations (10 nM) were sufficient to inhibit H_2O_2 production by half in S100A8/A9 siRNA-transfected cells whereas higher concentrations were necessary to obtain a similar inhibition in control cells. Confocal microscopy experiments on fixed ≠HL-60 revealed a translocation of S100A8/A9 to the plasma membrane after stimulation by fMLF which was inhibited by BAPTA. This indicates that intracellular Ca^{2+} is necessary for the recruitment of these proteins at the membrane.

Our results show that Ca^{2+} and S100A8/A9 are involved in the regulation of NOX2 activity and that the S100 complex is partly responsible for the Ca^{2+} sensitivity of NOX2.

Keywords: S100A8/A9, NADPH oxidase, neutrophils

FUNCTION OF CacyBP/SIP-TUBULIN INTERACTION IN NEURONAL CELLS

SCHNEIDER G.[1], NIEZNANSKI K.[1], KILANCZYK E.[1], BIEGANOWSKI P.[1], MIETELSKA A.[1], NIEWIADOMSKA G.[1], KUZNICKI J.[1,2] & FILIPEK A.[1]

[1] Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland.
[2] International Institute of Molecular and Cell Biology, 4 Trojdena Street, 02-109 Warsaw, Poland.

CacyBP/SIP [calcyclin (S100A6) binding protein and Siah-1 interacting protein] was identified in Ehrlich ascities tumor cells and later in other cells and tissues. CacyBP/SIP interacts with several targets such as proteins from the S100 family, Siah-1 and Skp1. The role of CacyBP/SIP is unclear; it has been suggested however, that it might be involved in protein ubiquitination and in cell differentiation.

In this work we show that CacyBP/SIP is up-regulated during differentiation of mouse neuroblastoma NB2a cells. Since the process of differentiation is accompanied by an extensive rearrangement of microtubules, we examined whether CacyBP/SIP interacts with tubulin. Using chemical cross-linking, immunoprecipitation and affinity chromatography we found that CacyBP/SIP binds tubulin. Immunofluorescence studies showed that CacyBP/SIP and tubulin co-localized in NB2a cells, particularly in cellular processes formed during cell differentiation. Co-sedimentaion assays revealed that CacyBP/SIP does not have any effect on tubulin polymerization and disruption of microtubules, however the light scattering measurements and electron microscopy studies showed that CacyBP/SIP, but not its homologue, Sgt1, forms globular tubulin assemblies. Although CacyBP/SIP was discovered as an S100A6-binding partner, S100A6 has no effect on CacyBP/SIP-tubulin interaction under the in vitro conditions. In this work we also showed that CacyBP/SIP changes its localization, similarly to the tau protein, in brain neurons of aged rats. These observations confirmed that CacyBP/SIP is a novel partner of tubulin and that the interaction between these two proteins might be of physiological importance.

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Keywords: Calcyclin-binding protein, tubulin, neurons

Ca^{2+}-SENSOR PROPERTIES OF CONE SPECIFIC GUANYLATE CYCLASE-ACTIVATING PROTEINS IN ZEBRAFISH

SCHOLTEN A., BEHNEN P., KEHL A., RAETSCHO N. & KOCH K.W.

Biochemistry Group, Dept. of Biology & Environm. Sciences, University of Oldenburg, D-26111 Oldenburg, Germany.

Guanylate cyclase-activating proteins (GCAPs) serve as neuronal Ca^{2+}-sensor proteins in vertebrate rod and cone photoreceptor cells. There they participate in the Ca^{2+}-dependent feedback mechanisms of light adaptation. Unresolved issues of these feedback routes include the molecular details enabling cones to operate over a much larger light intensity range than rods. Interestingly, the Ca^{2+} homeostasis in both celltypes, rods and cones, appears to be more complex than originally thought. Different GCAP isoforms seem to be designed to respond to these subtle differences in cytoplasmic [Ca^{2+}]. By this means they provide a regulatory strategy for fine-tuning of vision over the whole range of light intensities. A large variety of GCAPs was discovered in zebrafish (Imanishi et al., 2004), a teleost fish with tetrachromatic vision.

In the present study we cloned the genes of the cone specific GCAP isoforms from zebrafish (zGCAP3, zGCAP4, zGCAP5, zGCAP7), expressed them in E. coli, and investigated the biochemical properties of the purified proteins. As an example here we show that heterologous zGCAP4 exhibits properties suitable for its role as a Ca^{2+} sensor like Ca^{2+}-binding, Ca^{2+}-induced conformational changes, and regulation of membrane-bound guanylate cyclases, which occurs in the physiological range of cytoplasmic [Ca^{2+}] in zebrafish cones.

A comparison of the biochemical properties between the different zebrafish specific GCAPs and mammalian isoforms, GCAP1 and GCAP2, points to their function as Ca^{2+} sensors in a complex network of Ca^{2+}-dependent regulation of phototransduction and light adaptation.

Imanishi et al., (2004) J. Mol. Evol. 59, 2204-217.

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Keywords: neuronal Ca^{2+} sensors, guanylate cyclase-activating proteins, retina, zebrafish, phototransduction

RAPID DOWNREGULATION OF THE Ca^{2+} SIGNAL AFTER EXOCYTOSIS IN PARAMECIUM CELLS: ESSENTIAL ROLE OF A CENTRIN-RICH FILAMENTOUS CORTICAL NETWORK

SEHRING I.M.[1], KLOTZ, C.[2], BEISSON J.[2] & PLATTNER H.[1]

[1] Department of Biology, University of Konstanz, P.O. Box 5560, D-78457 Konstanz, Germany.
[2] Centre de Génétique Moléculaire, CNRS, F-91190 Gif-sur-Yvette & Université Paris-Sud, F-91405 Orsay & Université Pierre et Marie Curie, Paris 6, F-75005 Paris, France.

We analysed in Paramecium tetraurelia cells the role of the infraciliary lattice (ICL), a cytoskeletal network containing numerous centrin isoforms tightly bound to large binding proteins, in the re-establishment of Ca^{2+} homeostasis following exocytosis stimulation.

The wild type (wt) strain d4-2 has been compared with the mutant cell line ΔPtCenBP1 which is devoid of an ICL ("ΔPtCenBP1" cells). Exocytosis is known to involve the mobilisation of cortical Ca^{2+} stores and a superimposed Ca^{2+} influx and was analysed using Fura Red ratio imaging. No difference in the intitial signal generation was found between wt and ΔPtCenBP1 cells. In contrast, decay time was greatly increased in ΔPtCenBP1 cells particularly when stimulated, e.g., in presence of 1 mM extracellular Ca^{2+}, $[Ca^{2+}]_o$. Apparent halftimes of f/f_0 decrease were 8.5 s in wt and ~125 s in ΔPtCenBP1 cells, requiring ~30 s and ~180 s, respectively, to re-establish intracellular $[Ca^{2+}]$ homeostasis. Lowering $[Ca^{2+}]_o$ to 0.1 and 0.01 mM, caused an acceleration of intracellular $[Ca^{2+}]$ decay to t½ = 33 s and 28 s, respectively, in ΔPtCenBP1 cells as compared to 8.1 and 5.6 for wt type cells.

We conclude that, in Paramecium cells, the ICL is the most efficient endogenous Ca^{2+}-buffering system allowing the rapid downregulation of Ca^{2+} signals after exocytosis stimulation.

Keywords: centrin, exocytosis, Paramecium, Ca^{2+} signalling

ELUCIDATION OF THE Ca^{2+}-DEPENDENT INTERACTION MECHANISM BETWEEN ALG-2 AND ALIX BY X-RAY CRYSTALLOGRAPHIC ANALYSIS

SUZUKI H., KAWASAKI M., INUZUKA T., KAKIUCHI T., SHIBATA H., WAKATSUKI S. & MAKI M.

Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University and
Structural Biology Research Center, Photon Factory, Institute of Materials Structure Science, Kek, Japan.

ALG-2 is a member of the penta-EF-hand (PEF) protein family and interacts with various intracellular proteins such as Alix, TSG101, Annexin A7, Annexin A11, phospholipid scramblase 3 and Sec31A in a Ca^{2+}-dependent manner.

Previously we reported that ALG-2 recognizes four tandem PxY repeat sequence in proline-rich region of Alix.

In the present study, we performed X-ray structural analysis to elucidate the interaction mechanism between ALG-2 and Alix. We solved the ALG-2 structures of Ca^{2+}-free, Ca^{2+}-bound form and Zn^{2+}-bound form as well as the structure of the ALG-2/Alix peptide complex in Zn^{2+}-bound form. In the Ca^{2+}bound form, Ca^{2+} ions were found at EF1, EF3 and EF5, whereas in the Zn^{2+}-bound and ALG-2/Alix peptide complex form, Zn^{2+} ions are coordinated at EF1 and EF3, but not at EF5. Comparison of the structure of Ca^{2+}-free form with those of the Ca^{2+}-bound and Zn^{2+}-bound form reveals that Ca^{2+} ions induce conformational change at EF3, but not at EF1 and Zn^{2+} ions similarly induce the conformational change. In the ALG-2/Alix complex form, the Alix peptide was found to bind ALG-2 in two different patterns, in which both Alix peptides were captured in two hydrophobic pockets. We investigated critical residues in ALG-2 for interaction with Alix by GST-pulldown assay. Substitutions of residues in Pocket 1 of ALG-2 (Y124, R125, D169 and Y180) resulted in loss of binding abilities. The binding abilities were lost by mutation of Y91 and W95 present in Pocket 2.

The Ca^{2+}-dependent binding of ALG-2 to Alix peptide is explained as follows: i) change in geometry of helices E3 and F3 causes detachment of Y124 side chain from L158, ii) configuration change of R125 side chain, iii) opening of Pocket 1, iv) entry of the peptide, v) half closure of Pocket 1 by the R125 side chain and completing the peptide trapping. R125 is a key element of the open-close mechanism of Pocket 1 and acts as a switch driven by Ca^{2+}/EF3 conformational change.

Keywords: X-ray structural analysis, ALG-2, Alix, zinc

MEMBERS OF THE S100 FAMILY BIND p53 IN TWO DISTINCT WAYS

VAN DIECK J., FERNANDEZ-FERNANDEZ M.R. & FERSHT A.

Centre for Protein Engineering, Medical Research Council, Cambridge, CB2 0QH, UK.

The homotetrameric transcription factor p53 acts as a tumour suppressor and is often referred to as the “guardian of the cell” due to its crucial roles in apoptosis, DNA-damage repair and cell cycle arrest. The level of active p53 is carefully regulated within the cell by posttranslational modifications and through interactions with other proteins.

The S100-protein family consists of 24 members of small acidic proteins that remain soluble in a saturated ammonium sulphate solution and possess EF-hand Ca^{2+}-binding domains. They have been implicated in diverse biological processes including cell proliferation, differentiation and cell signalling. Furthermore, many S100 proteins are overexpressed in solid cancer tumours and have also been associated with the promotion of metastasis. Nevertheless, the exact molecular function of S100 proteins is not fully understood. P53 has been identified as a binding target and the interaction might be relevant for the oncogenic potential of S100 proteins.

Here we characterize the binding of six different members of the S100-family to p53 by fluorescence anisotropy and analytical ultracentrifugation. All analysed S100 proteins show a common binding to the tetramerization domain (325-355) of p53 whereas only a subset of the S100 proteins were able to bind to its negative regulatory domain (367-393). We also show that the binding is dependent on the oligomeric state of p53. Consequently S100 proteins might be involved in the regulatory network of p53 by influencing its ability to form active tetramers.

Keywords: S100 proteins, p53, tumor development

TOLL-LIKE RECEPTOR 4 AND S100A8/S100A9

VOGL T., VAN ZOELEN, M.A.D., EHRHARDT, C., WOLF M., FOELL D. & ROTH J.

Institute of Immunology, University of Münster, Roentgenstr. 21, D-48149 Münster, Germany.

Damage Associated Molecular Pattern (DAMP) proteins are known as important pro-inflammatory factors of the immune system. Recognition of DAMPs involves the multiligand Receptor for Advanced Glycation End products (RAGE) and Toll-like receptors (TLRs) in sensing not only Pathogen Associated Molecular Patterns (PAMPs) but also endogenous proteins released during cellular stress. Members of this fast growing family of DAMP proteins are phagocyte-specific S100 proteins (S100A8, S100A9, S100A12), which promote inflammatory processes. S100A8, S100A9, and S100A12 are released at high concentrations at local sites of inflammation by activated neutrophils and monocytes. They exhibit pro-inflammatory effects in vitro at concentrations found during inflammation in vivo. While S100A12 binds to RAGE, the pro-inflammatory effects of S100A8 and S100A9 depend upon interaction with TLR4 (Vogl et al., 2007). It is therefore important to differentiate between specific functions of different S100 proteins rather than subsuming them with the term “S100/calgranulins”.

Vogl et al. (2007) Nature Medicine 13, 1042-1049

Keywords: S100A8/A9, Toll-like receptor 4

BIOCHEMICAL CHARACTERISATION OF THE IN VIVO RELEVANT S-NITROSYLATED S100A1 AND S100B PROTEINS

BAJOR M., ZARĘBA M., POZNAŃSKI J., ZHUKOVA L. & WYSŁOUCH-CIESZYŃSKA A.

Institute of Biochemistry and Biophysics, PAS, Pawińskiego 5a, 02-106 Warszawa, Poland.

The transfer of a nitric oxide group to cysteine sulfhydryls on proteins, named S-nitrosylation, is a ubiquitous post-translational protein modification (Hess et al., 2005). It represents a form of physiological redox modulation in diverse tissues, including such where S100A1 and S100B calcium binding proteins are expressed i.e. brain, muscle and heart. Strong alteration of protein S-nitrosylation is observed in various human pathologies such as cancer, inflammation, heart disease and different forms of neurodegeneration (Stamler et al., 2008; Nakamura and Lipton, 2008; Saraiva and Hare, 2006). A hallmark of many of these pathologies is an increased expression of S100A1 and S100B proteins and their extracellular receptor RAGE (receptor for advanced glycation endproducts).

Here we document for the first time that S-nitrosylation is an in vivo post translational modification of S100B protein in C6 glioma cells. We present detailed biochemical characterization of S-nitrosylated S100A1 and S100B proteins showing differential effects on ion binding and interaction with the RAGE receptor.

Hess et al. (2005) Nat. Rev. Mol. Cell Biol. 2, 150-166.
Nakamura & Lipton (2008) Antioxid. Red. Sign. 10, 87-101.
Saraiva & Hare (2006) Curr. Opin. Cardiol. 21, 221-228.
Stamler et al. (2008) Cell 133, 33-35.

Keywords: S100A1, S100B, RAGE, S-nitrosylation

ROLE OF THE ANNEXIN A2 N-TERMINAL DOMAIN DURING Ca^{2+}- AND H^+-INDUCED MEMBRANE BRIDGING

ZIBOUCHE M.[1,2], ILLIEN F.[1,2], VINCENT M.[3,4], GALLAY J.[3,4] & AYALA-SANMARTIN J.[1,2]

[1] INSERM, UMRS893, CdR Saint Antoine, F-75012 Paris, France.
[2] Université Pierre et Marie Curie, CHU Saint Antoine, F-75012 Paris, France.
[3] CNRS UMR8619 IBBMC, F-91405 Orsay, France.
[4] Université Paris-Sud, F-91405 Orsay, France.

Annexins belong to a family of Ca^{2+}- and phospholipid-binding proteins involved in many cellular regulatory processes. They have two domains: a conserved core, containing the Ca^{2+}-binding sites, and a variable N-terminal segment, containing sites for interactions with other protein partners. A wealth of data exists on the structure and dynamics of the core, but little is known about the physicochemical properties of the N-terminal segment.

We present experimental data concerning the structure and dynamics of annexin A2 (AnxA2) in various conditions (free protein and when bridging phospholipid membranes), obtained with a fluorescence-based technique. We specifically labeled the reactive Cys8 residue in the N-terminus with the polarity-sensitive probe acrylodan or the excimer-forming probe pyrene-maleimide. We found that this segment of the molecule was flexible, with acrylodan largely exposed to the solvent. Ca^{2+} and H^+ had no effect on polarity. We studied the interaction with membranes in a calcium-dependent manner and in mild acidic condition in the absence of calcium. Our results show differences in the microenvironment of the acrylodan in AnxA2 when the protein associates to membranes.

In brief, the N-terminal tail does not to interact with the membrane phospholipids in the presence of Ca^{2+}, but interact strongly (it is probably inserted or "snorkelling" in the bilayer) at acidic pH. We present a model for the conformational changes of AnxA2 N-terminal domain in bridged membranes in the presence of Ca^{2+} and in mild acidic conditions.

Keywords: annexin A2, pH, membrane bridges