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Partial Amino Acid Sequence and Crystal Structure of a Calcium-Independent Phospholipase A2 - Like Protein from the Venom of *Bothrops godmani*

M. V. Sousa[1], L. Morhy[1], R. K. Arni[2], R. J. Ward[2], J. M. Gutierrez[3]
[1] Centro Brasileiro de Sequenciamento de Proteínas, Dep. de Biologia Celular, UnB, Brasília-DF; [2] Departmento de Física, UNESP/IBILCE, São J. do Rio Preto-SP, Brazil; [3] Instituto Clodomiro Picado, San José, Costa Rica.

The substitution of Asp49 by Lys in pancreatic phospolipase A2 (PLA2) disrupts the ability of this protein in binding Ca^{2+}, resulting in a catalytically inactive enzyme. However, it has been shown that although naturally occuring Lys49 PLA2 variants do not hydrolyze phospholipids, they nevertheless disrupt the integrity of liposomes by a unique calcium independent process. In an attempt to understand the structural basis of this phenomena, we have purified, crystallized and solved the structure of a calcium independent Lys49 like PLA from the venom of *Bothrops godmani*. Sequencing of the N-terminal region and tryptic and chymotryptic fragments of the protein was carried out in an ABI 477A Automatic Sequencer. The partial sequence was instrumental in the structural refinement. The crystal structure indicated that the Nε group of Lys49 occupies the position of the calcium ion in catalytically active PLA2 molecules. Bgodmani PLA2 sequence confirmed the presence of Lys49 and showed good homology to other PLA2 sequences, as shown below by the CLUSTAL V multiple sequence alignment of their N-terminal regions.

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PA22_BOTAS      SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYKKLTGCNPK
PA2B_TRIFL      SLVQLWKMIFQETGKEAAKNYGLYGCNCGVGRRGKPKDATDSCCYVHKCCYKKVTGCDPK
PA2B_TRIMU      SLIELGKMIFQETGKNPVKNYGLYLCNCGVGNRGKPVDATDRCCFVHKCCYKKVTGCDPK
PA2H_AGKPI      SVLELGKMILQETGKNAITSYGSYGCNCGWGHRGQPKDATDRCCFVHKCCYKKLTDCNHK
BtxtxI          SLLELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYKKLTGCDPK
Batrox          SLVELGKMILQETGKNPLTSYGVYGCNCGVGSRHKPKDDTDRCCYVHKCCY---------
Bgodmani        SMYQLWKHILQETGKNAVPSYGLYGCNCGVGSRGKPKDATDRCCFVHKCC----------
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DETECTION OF CALCIUM-BINDING PROTEINS IN VENOM AND VENOM GLANDS OF SOUTH AMERICAN SNAKES.

Gonçalves[1], L.R.C., Nuñes-Burgos[2], G.B.; Furtado[3], M.F.D. and Nicolau[2], J.
Labs. de [1]Fisiopatologia e [3]Herpetlogia, Instituto Butantan, SP, and [2]Depto. Bioquimica, Instituto de Química, USP, SP.

Previously we have described (Int.J.Biochem., 25:1491, 1993) alterations in protein composition of *Bothrops jararaca* (Bj) venom and venom gland by isoproterenol (IPR). Then, we reported (XXIIIa SBBq, 1994, p.141) the presence of calcium-binding protein (CaBP), in both venom and venom gland of Bj, and that these CaBP were not altered either by IPR or secretory cycle phases. We extended our studies examining the presence of CaBP in venom glands and their secretions from three families of South American poisoning snakes. Venom glands from Viperidae (Bj and *Crotalus durissus terrificus*-Cdt) and Elapidae (*Micrurus coralinus*-Mc) snakes or Duvernoy's glands from Colubridae (*Phylodryas patagoniensis*-Pp and *Oxyrhopus* sp.-Oxy) snakes were obtained and homogenized, after the manual extraction of the venom. The samples from the glands and venoms were electrophoresed at 50V for 16 hrs on a 7.5-15% SDS-PAGE. After electrophoresis it was transfered to Zeta probe membranes for 3 hrs and the membrane treated with ^{45}Ca (Maruyama *et al.*, J. Biochem., 95:511, 1984) and developed for 72 hrs at -20°C. A CaBP of 12 kDa was detected in all glands studied. A CaBP of 17, and 30 kDa were detected in Bj, Cdt, Mc, and Pp. A Band of 18 kDa was detected in Bj, Cdt, and Mc. Two bands, one of 67 and other of 90 kDa were found only in Viperidae snakes. It was possible to detect CaBP (14, 16, 30, 42, and 65 kDa) in the venom sample only from Bj. The role of these CaBP on the gland and in venoms are under investigation in our laboratory.

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PURIFICATION AND CHARACTERIZATION OF SIII - 1 : A PROTEIN OF *Bothrops alternatus* VENOM THAT BINDS TO VON WILLEBRAND FACTOR.

V.R.C. Gimenez[1], S. Marangoni[1], B. Oliveira[1], G. de Nucci[2], S. Marcondes[2], E. Antunes[2] and J.C. Novello[1].

1 - Depto. Bioquímica - Inst. Biologia - CEP 13084-100 - UNICAMP.

2 - Depto. Farmacologia - FCM - CEP 13084-100 - UNICAMP.

Botrocetin is a platelet coaglutinin described in *Bothrops jararaca* venom, wich acts in the interaction between GP Ib-IX complex and von Willebrand factor (vWF) that lead to platelet agglutination (Read et al., 1978; Howard et al., 1984). A single chain and a two chain botrocetin were described in *B. jararaca* venom (Fujimura et al., 1991; Usami et al., 1993). The apparent molecular mass determined for the one chain botrocetin was 28kDa before and 32kDa after reduction of disulfide bonds. The aminoacid composition revealed a high content of acidic residues (30%) and the minimal concentration necessary to induce vWF binding to GP Ib was 1μg/ml. In this work we report the purification and characterization of a protein from *Bothrops alternatus* venom through procedures of gel filtration and reverse phase chromatography through a C_{18} column. The purified protein (SIII - 1) presents a single band (37.7kDa) in denaturing conditions (0.1M DTT) in 12.5% PAGE-SDS. Automatic microsequencing of aminoacid residues was performed in a 477 Applied Biosystems sequencer and the N - terminal sequence obtained I I/V SPPVCG present 100% homology to the one chain botrocetin. SIII - 1 binds to vWF and inhibit the ristocetin binding to vWF and unlike one chain botrocetin, SIII - 1 doesn't induced platelet aggregation. The present results indicate the existence of two funcionally distinct forms of structurally related proteins: botrocetin and SIII - 1. Further studies of SIII - 1 may help the understand the mechanism of complex formation with vWF and should clarify the structural features that make the complex a competent ligand for GP Ib-IX.

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Structural, biological and immunological alterations of crotoxin following irradiation in the presence of thiol groups.

E. P. Andriani, P. J. Spencer, N. Nascimento, Clissa, P.B. and J. R. Rogero
Coordenadoria de Bioengenharia-Supervisão de Radiobiologia IPEN/CNEN SP-11049-Pinheiros

Aqueous solutions irradiated with gamma rays leads to free radicals formation, mainly $OH^{\bullet}$ and hidrated electron. When irradiating proteins in solution, those reactive species react with the proteic molecule, resulting in structural alterations leading to partial or total inactivation even loss of function. These properties of ionizing radiation have been employed by us to atenuate toxins and produce immunogenic toxoids. In the present work, we investigated the influence of thiol compounds , well known $OH^{\bullet}$ scavengers, in structural, immunological, toxic and enzimatic alterations resulting from irradiation of crotoxin, the main toxic component of *Crotalus durissus terrificus* venom.

Methods: 2 mg/ml crotoxin solution in 150 mM NaCl, 50 mM DTT or 100 mM cysteine were irradiated with ^{60}Co γ rays to a 2000 Gy dose. Controls were native and irradiated without scavengers crotoxin as well as crotoxin incubated with the scavengers. All samples were dialyzed against water and structural alterations were investigated by HPLC size exclusion chromatography. Toxic and Phospholipase A_2 activities were determined by LD_{50} and indirect hemolysis respectively. Immunogenicity was assayed by immunizing mice and the obtained antibodies were tested by ELISA, *in vitro* and *in vivo* neutralization as well as chalenging the immunized animals with the native toxin.

Results: Incubation of the samples with the scavengers led to dissociation of the polypeptidic chains as a result of S-S bonds reduction. However, when irradiating these samples and removing the scavengers, a large amount of aggregates could be observed, suggesting reassociation via $RS^{\bullet}$ radicals. The aggregates amount was higher in those samples than in irradiated control. Toxicity was not reached although we assayed it till 27,8 LD_{50} of native crotoxin. Enzymatic activity decreased as a consequence of incubation with the scavenging substances and no further inactivation could be observed following irradiation. The antibodies titers obtained were similar for all samples, but when assaying neutralizing capacity, the antibodies raised against irradiated with scavengers crotoxin were much more protective.

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A POSSIBLE ROLE FOR BETA1 INTEGRIN ASSOCIATED LBP IN ADHESION TO LAMININ

M.C.Q.B.Elias*, S.S.Veiga, R.Chammas, C.L.S.Santos, R.Giorgi and R.R.Brentani.

Ludwig Institute For Cancer Research, São Paulo, Brazil.

Cell adhesion to the basement membrane is a key step of tumor cell invasion, and is mediated by specific cell surface receptors.

Cell adhesion assays showed that MEL-85, a human melanoma cell line, has a high capacity to adhere to laminin. By means of overlay assays, it was possible to identify a 100/110 kDa glycoprotein with laminin binding activity(LBP-laminin binding protein) (Veiga et al, SBBq 1995). However, the overlay assay does not show this LBP in HL-60 cells, a human leukemia cell line not able to adhere to laminin, although FACS analysis showed the presence of alpha6/beta1 integrin, a laminin receptor, on this cell's surface.

Besides this, F9 cells, a mouse embryonic carcinoma cell line, have similar characteristics to those of the embryo inner cell mass and under retinoic acid and cyclic AMP induction, they differentiate into parietal endoderm cells. This differentiation is followed by a decrease in cell adhesiveness to laminin and a lower expression of alpha6/beta1 integrin at the cell surface. Interestly, overlay assays showed that it was not possible to identify LBP in retinoic acid differentiated F9 cells, while in F9 stem cells LBP has an apparent molecular mass of 120 kDa.

We propose a crucial role for LBP in adhesion to laminin and are studying the possibility that this protein and laminin binding integrins act together in adhesion to laminin (preliminary studies with the MEL-85 cell line showed a physical association between LBP and beta1 laminin binding integrin, Veiga et al, SBBq 1995), since cells that have integrins, but not LBP, are not able to adhere to laminin, while cells that have LBP became unable to adhere to laminin when we utilize an anti-alpha6 integrin subunit monoclonal antibody in inhibition assays.

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THE ANTI-PRION PROTEIN AND PRION DISEASES

E. Granер*; A.F. Mercadante*; S.J. de Souza*; V.M.Neto**; R.R. Brentani*
* Instituto Ludwig de Pesquisa sobre o Câncer, 01509-010 - São Paulo, SP.
** Instituto de Biofisica Carlos Chagas Filho - UFRJ, RJ.

Prions are proteinaceous infectious particles involved in both animal and human diseases. The animal prion diseases are scrapie, transmissible mink encephalophaty, chronic wasting disease of muler deer and elk and bovine spongiform encephalophaty. In humans prions cause kuru, Creutzfeuldt-Jakob disease (CJD) and Gerstmann-Straussler-Scheinker syndrome (GSS). CJD and GSS are both transmissible and inherited diseases. The major component of the infectious particle is the scrapie prion protein (PrPsc), a posttranslationally modified isoform of the normal cellular prion (PrPc). PrPc is a plasma membrane glycoprotein expressed in the central nervous system with unknown function. The cellular mechanism of the conversion of PrPc to PrPsc remains unclear. Recently, the discovery of an ORF in the DNA strand complementary to the prion gene led to speculations concerning an anti-PrPc protein. This find is corroborated by the demonstration of the anti-PrPc message in several tissues.

In this work we are investigating the anti-PrPc expression in mouse nervous system. Mouse brain extracts were subjected to SDS-PAGE and transferred to nitrocellulose membranes. In Western blots two distinct bands were observed (60 and 85kDa) using a mouse serum produced with an anti-PrP peptide. In addition, the 60 kDa protein was eluted from a prion-sepharose column with a prion peptide, suggesting that prion and anti-PrP bind specifically by sense-antisense interactions. The anti-PrP protein was observed by immunofluorescence in primary culture of neurons and by immunoperoxidase technique in sections of mouse brain. Confocal immunofluorescence experiments demonstrated a plasma membrane localization for the anti-PrP protein. In conclusion, we have demonstrated the presence of the anti-PrP protein in mouse brain. We suggest that such molecule can play a role in prion diseases acting as a cell surface receptor for PrPsc.