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Generation and functional characterization of a BCL10 inhibitory peptide that represses NF- κ B activation

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Running title: A BCL10 inhibitory peptide represses NF- κ B activation

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Abstract

The molecular complex containing BCL10 and CARMA proteins has been recently identified as key component in the signalling transduction pathways that regulate activation of NF- κ B transcription factor in lymphoid and non-lymphoid cells. Assembly of complexes containing BCL10 and CARMA proteins relies on homophilic interactions established between the CARD domains of these proteins. In order to identify BCL10 inhibitory peptides, we have established a method of assaying peptides derived from the CARD of BCL10 in a binding competition assays of CARD-CARD self-association. By this procedure, a short peptide corresponding to the amino acidic residues 91-98 of BCL10 has been selected as an effective inhibitor of the protein self-association. When tested in cell assays for its capacity to block NF- κ B activation, this peptide represses activation of NF- κ B mediated by BCL10, CARMA3 and PMA/ionomycin stimulation. Collectively, these results indicate that the region 91-98 of BCL10 is involved in BCL10 self-association and also participates in the interaction with external partners. We also show that blocking of the CARD domain of BCL10 may potentially be used for the treatment of pathologic conditions associated with inappropriate NF- κ B activation.

Introduction

Over the past few years, a number of adaptor molecules that contain the caspase recruitment domain (CARD) have been identified. The CARD was originally described as a protein-binding motif that interacts with caspase through a CARD-CARD homophylic interaction [1]. However, the CARD has also been found in many adaptor proteins that do not interact with caspases, but mediate the assembly of proteins involved in apoptosis and/or the control of the activation state of NF- κ B transcription factor. Thus, it is now well established that CARD-containing proteins play important roles in the assembly and activation of apoptotic and inflammatory complexes, which are mediated by homotypic interactions between CARD domains [2].

BCL10 is a CARD-containing protein that was initially identified as a target of the chromosomal translocation t(1;14)(p22;q32), which is found in certain aggressive cases of MALT B cell lymphomas [3, 4]. The resulting overexpression of BCL10 leads to constitutive activation of NF- κ B, which eventually promotes cell proliferation and tumorigenesis. Several groups independently identified BCL10 as a regulator of apoptosis and as an activator of NF- κ B [5, 6-10]. Altogether, these studies show that an intact CARD domain is required for the NF- κ B-inducing activity of BCL10, since the point mutations L41Q and G79R introduced in conserved residues of the CARD of BCL10 result in an inactive form of the protein [8, 10]. BCL10 knockout mice are immunodeficient because lymphocytes derived from these mice are unable to activate NF- κ B, either following antigen receptor stimulation or phorbol-12-myristate-13-acetate (PMA)- and ionomycin treatment [11]. In addition, BCL10-deficient mice also exhibit impaired neuronal-tube closure, thus implicating BCL10 not only in lymphocyte activation but also in neural development [11]. BCL10 interacts with other CARD-containing cytoplasmic molecules, via homophylic interaction mediated by its CARD domain. These include RIP2 (12), CARMA1 (also known as CARD11 or Bimp2) [13-15], CARMA2 (also known as CARD14 or Bimp3) [13], CARMA3 (also known as CARD10 or Bimp1) [16, 17] and CARD9 [18]. The three CARMA proteins share similar structural motifs, with an N-terminal CARD domain, followed by a coiled-coil (CC) domain, a PDZ domain, a SH3 domain and a C-terminal guanylate kinase-like (GUK) domain [13-17]. They display distinct expression profiles, being CARMA1 expressed in hematopoietic cells, CARMA2 in the placenta, and CARMA3 in all non-hematopoietic cells [13-17]. Functionally, CARMA proteins are supposed to function as molecular scaffolds that assist recruitment and assembly of signal transduction molecules. For example, in the TCR signaling pathway, phosphorylated CARMA1 recruits BCL10, MALT1 and TRAF6 [18-20]. MALT1 and TRAF6 function as E3 ligases to further induce K63-linked polyubiquitination of NEMO, leading to activation of the IKK complex [21-24]. Similarly to deficiency of BCL10, genetic disruption of CARMA1 results in severe immunodeficiency, due to lymphocyte failure to activate NF- κ B following antigen receptor-induced activation [14, 18-20, 24-26]. CARMA proteins interact with BCL10 via their CARD domain, as a CARD-domain mutant of CARMA1 (L39R) shows no binding to BCL10 [18]. Whereas BCL10 translocation is associated to MALT B cell lymphomas, missense mutations in the coiled coil domain of CARMA1 are frequently detected in diffuse large B cell lymphomas [35].

The complex of proteins that comprises CARMA3, BCL10 and MALT1 appears to play an important role in cells outside of the immune system. In fact, recent studies indicate that CARMA3 and BCL10 are implicated in the signal transduction pathways elicited by G protein-coupled receptors, a large family of cell surface receptors that regulate cell migration, differentiation, proliferation and survival [27-29]. Specifically, it appears that NF- κ B activation induced by G protein-coupled receptors utilizes a pathway dependent on the molecular complex containing CARMA3 and BCL10.

Altogether, these data indicate that BCL10 represents a valid molecular target for the treatment of pathologic conditions associated with inappropriate NF- κ B activation, ranging from the MALT B cell lymphomas with t(1;14)(p22;q32), to the inflammatory disorders involving G protein-coupled receptors, such as chronic liver injury, atherosclerosis and cardiac hypertrophy [30]. In this study,

we report the identification of a short BCL10 fragment able to block CARD self-association and prevent activation of the transcription factor NF- κ B promoted by BCL10. We also identify residues involved in this activity and thus potentially involved in CARD oligomerization and formation of large protein complexes that mediate activities of BCL10 and of its interacting partners.

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Results

Preparation and characterization of BCL10-CARD

BCL10-CARD was expressed as a Cys29Ser-Cys57Ser isosteric double mutant of the wild-type protein domain (Fig. 1) to prevent oligomerization by covalent cysteine crosslinking. The polypeptide used in this study also contained an N-terminal linker bearing the poly-histidine tag utilized for protein affinity purification. The sequence numbering adopted here is based on that of the native protein domain, ignoring for a matter of clarity the N-terminal fusion tag. The protein was successfully expressed in the BL21(DE3) *E. Coli*. The protein recovered after the affinity chromatography and gel filtration purification steps was more than 90% pure and was utilized in all the subsequent studies without any further manipulation. The polypeptide was characterized by LC-MS analysis confirming purity and the exact molecular weight (not shown). Protein sequence was further assessed by analysis of tryptic peptides during the protein fragmentation procedure. A preliminary study on protein solubility and stability showed that the protein was essentially insoluble for pH values between 5 and 8, therefore all binding experiments were performed in 50 mM AcNa buffer at pH 4.5. Under these conditions, the polypeptide adopted an all-alpha conformation as expected for a CARD domain and indeed the far UV-CD spectrum showed a minimum at 208 nm, a shoulder at 222 nm and a positive band at 196 nm (not shown).

Identification of BCL10-CARD regions involved in self-association

In order to determine protein regions involved in CARD self-association, peptide fragments generated by trypsin treatment of BCL10-CARD and successively RP-HPLC fractionated, were used as inhibitors of protein self-association in a competitive ELISA (see below). For this purpose, a preliminary dose-dependent binding assay was carried out, setting up the optimal conditions for the inhibition test. In Fig. 2A the plot of curves relative to the binding assay at different protein concentrations is reported. As shown, at protein coatings of 0.12 and 0.30 μM , the binding is saturated for concentrations of soluble protein higher than about 1.5 μM , suggesting that several BCL10-CARD molecule can bind to one coated polypeptide. K_D value was obtained by averaging the K_{Ds} of the three curves derived by non-linear regression fitting of data (GraphPad Prism, vers. 4.0) and was estimated to be $1.30 \pm 0.16 \mu\text{M}$. The binding competition test was performed by coating the protein at a fixed concentration, 0.12 μM , and using the biotinylated variant at 0.80 μM . Competitors were used at a nominal 5-fold excess over the soluble protein. Peptide fragments concentration was deduced by assuming an initial quantitative protein cleavage by trypsin and a subsequent recovery of 50% by RP-HPLC fractionation.

The LC-MS analysis of fractions showed that protein fragments were distributed as described in Table I. As can be seen, fractions F1 and F2 did not contain peptides, whereas fractions F3-F8 and fraction F10 contained more than one fragment while the fraction F9 was constituted only by the L68-K77 fragment. In spite of prolonged reaction times and of high temperature (37 °C), many fragments contained uncleaved lysines and arginines peptide bonds, suggesting the presence of poorly accessible cleavage sites. Several other fragments, such as the N-terminal M1-K17 bearing the linker tetrapeptide GSEF (See fig. 1) and the peptides H38-K45 and T59-K67 were only poorly detected and thus not further considered. For the assay purposes, the lyophilized fractions were reconstituted in water, properly diluted with buffer and subsequently assayed as competitors in the BCL10-CARD self-association test. Screening results are summarized in Table I (last column on the right hand side). As expected, fractions F1 and F2 in which no peptides were detected, did not provide binding reduction. Among the remaining fractions, only F8, containing the peptides BCL10 [91-98] (sequence: TQNFLIQK, abundance 63%) and the homolog BCL10 [89-98] (sequence: EKTQNFLIQK, abundance 37%), produced a relevant effect of binding reduction (about 50%). The sequence EKTQNFLIQK, that compared to the most abundant 91-98 fragment only contained two additional residues at the N-terminus, was not considered for further investigations, while the

peptide BCL10 [91-98] (peptide I, Table II) was readily re-prepared by chemical synthesis as amidated C-terminal and acetylated N-terminal variant. To also investigate the influence of the structured flanking region of the selected peptide on CARD self-association, we designed and synthesized two additional BCL10 peptides containing the 91-98 stretch at the C-terminus. These peptides, termed Peptide II and Peptide III, contained residues 78-98 and 68-98, respectively (see Table I and Table II). A model for the BCL10-CARD was retrieved from the ModBase server of the University of California at San Francisco (<http://modbase.compbio.ucsf.edu/>) and visualized by the WebLab ViewerPro software, vers. 3.7 (Molecular Simulations Inc.). This model was used to localize the putative structure of the selected peptide within the CARD framework. It was obtained by using as template the x-ray crystallographic structure of the apoptotic protease activating factor 1 CARD (pdb code: 1cy5, (31)), and, despite the very low sequence identity (17%), has a very low E-value (2×10^{-8}) and a model score of 0.99. On examination of this putative model, it can be seen that the 91-98 region is inside the modelled sequence and virtually corresponds to the domain 6th helix (partially distorted); the sequence 78-98 instead, covers the 5th and the 6th helix, and the 68-98 fragment corresponds to the protein region including helices 5 and 6, the loop between helix 5 and helix 4 and part of the helix 4 (see also Fig. 1). The synthetic peptides I-III purified to homogeneity by RP-HPLC and characterized by LC-MS, were utilized in a dose-response binding competition assay under the same conditions reported previously for the screening of protein fragments (coating 0.12 μM ; biotinylated protein 0.80 μM). The synthetic peptides were utilized at increasing concentrations between 0.03 and 15 μM and results are reported in Fig. 2B. Data show that the shorter BCL10 [91-98] strongly reduces protein self-association in a dose-dependent fashion, whereas, unexpectedly, the longer polypeptides II and III, although containing this active sequence, only slightly interfered with the interaction. In particular, Peptide I inhibited the protein association by 90% at the highest dose of 15 μM and exhibited an IC_{50} of 70 nM. At variance, Peptides II and III exhibited an efficacy between 30% and 40% only, with no further effects at higher concentrations (see Fig. 2B). This result was suggestive of a tendency of peptides to self-aggregate; thereby, to further investigate this issue, we carried out SEC experiments in order to determine the apparent molecular weight of the synthetic peptides. Repeated analyses of Peptide I and Peptide II onto the SEC column at increasing concentrations (see Table III) demonstrated that, while the 91-98 peptide appeared invariably monomeric, the 78-98 variant eluted according to a dimer even at the lowest concentration of 5 μM . This property, together with the incapability to efficiently suppress the CARD BCL10 self association at concentrations higher than about 1 μM , suggests that the presence of residues 78-90 may favour the formation of peptide oligomers wherein amino acids 91-98, required for binding to the CARD, are not accessible. Next we explored single residues within the 91-98 peptide involved in protein-protein interactions, and, to this purpose, we designed and synthesized four mutated variants of Peptide I (see again Table II) where adjacent residue pairs were substituted by alanines. These molecules, named as reported in Table II as Peptides IV, V, VI and VII were again tested in a dose-response assay along with the parent BCL10 [91-98] and results are summarized in Fig. 3. As shown, Peptides I, V, VI and VII still reduced the BCL10-CARD self-association, whereas Peptide IV, with Thr91 and Gln92 changed to Alanine, was almost ineffective even at the highest dose (15 μM). Notably, Peptide VI, having residues Leu95 and Ile96 changed to Alanines, exhibited only a reduced efficacy, being able to lessen the interaction to 50% only at the highest concentration. Also the Peptide V, with substitutions on residues N93 and F94, had an efficacy ten times lower (about 1 μM) than Peptides I and VII. Altogether, these data suggest that residues 91-98 of BCL10 can have Thr91, Gln92, Leu95 and Ile96 may represent key contact points between protein monomers.

In vivo effect of the BCL10 peptide

To further confirm the efficacy of the selected peptide to block self-association between CARDS of BCL10 and possibly the association between BCL10 and other protein partners, we planned cellular experiments using Tat-conjugated peptide variants to allow cell entry [32]. Peptides were also

labeled with fluoresceine to confirm and to quantify peptide entry. As a first step, we assessed whether the new fluorescent Tat-conjugated Peptide VIII containing the active sequence TQNFLIQK, was still capable of disrupting the CARD BCL10 self-association. We therefore prepared HEK293 cells co-transfected with plasmids encoding for two tagged versions of BCL10 (1-127) bearing the HA and the FLAG epitopes. Then lysates from transfected cells were immunoprecipitated with anti-FLAG antibodies either in the absence or presence of increasing concentrations of Peptide VIII and the co-precipitating HA-tagged BCL10 (1-127) was detected by immunoblot assay. Peptide VIII repressed self-association of BCL10 (1-127) in a dose dependent manner and at 100 μ M the binding was totally abolished (Fig. 4A). Peptide VIII, but not the control Peptide IX, also repressed association of BCL10 with the CARD domain of CARMA3 (Fig. 4B), although at higher concentration (Fig. 4B). We also assessed the effect of peptide VIII on the preformed complex containing BCL10 and Malt1. This analysis was conducted on endogenous proteins, rather than overexpressed molecules. As shown in Fig. 4C, association of BCL10 with endogenous Malt1 was unaffected by the presence of Peptide VIII (Fig. 4C).

To assess peptide entry, HEK293 cells were treated for 5 hrs at room temperature with different concentrations of FITC and Tat-conjugated Peptides VIII and Peptide IX (used as a control). Samples were then fixed and analyzed by fluorescence microscopy. Under these conditions, both peptides appeared uniformly distributed in HEK cells at the concentration of 200 μ M (Fig. 5A). Cytofluorimetry experiments also confirmed peptides entry in the cells (Fig. 5B). In addition, the peptides were not toxic for the cells at the concentration and time periods used for the study (Fig. 5C).

To analyze the effect of Peptide VIII in an *in vivo* assay, we transiently transfected HEK293 cells with an expression plasmid encoding the full length BCL10, along with a luciferase reporter plasmid for NF- κ B. 16 hrs later, cells were treated with both Peptide VIII and Peptide IX (used as a control) for 5 hrs and luciferase activity was determined. The results of these experiments, shown in Fig. 6A, indicated that Peptide VIII repressed, in a dose dependent manner, the activation of NF- κ B promoted by BCL10 overexpression with a maximum efficacy of about 50% at the dose of 50 μ M. In the same experiment, treatment of cells with the control Peptide IX had no effect on NF- κ B activity. In addition, Peptide VIII had no effect on NF- κ B activation elicited by expression of p65, indicating that its inhibitory effect is exerted at an upstream level (Fig. 6B).

BCL10 and CARMA3 have been implicated in lysophosphatidic acid (LPA)-induced NF- κ B activation in mouse embryonic fibroblasts (MEFs) [27-29]. Since PKCs activity is required for LPA-induced NF- κ B activation, we tested whether Peptide VIII inhibited activation of NF- κ B following PKCs activation. For this purpose, an NF- κ B-luciferase reporter plasmid was transfected into HEK293 cells, and cells were then left untreated or stimulated with ionomycin, a calcium ionophore, plus PMA, a surrogate of 1,2-diacylglycerol, the natural activator of conventional and novel PKCs. In agreement with the previous results, treatment of cells with Peptide VIII, but not with the control Peptide IX, repressed the activation of NF- κ B following PKCs activation in a time and dose dependent manner (Fig. 7).

Discussion

Originally identified as a protein–protein interaction motif present in signalling molecules involved in the activation of apoptotic and pro-inflammatory caspases, CARD motifs also function in caspase-unrelated signalling pathways, by mediating CARD–CARD association between CARD-containing binding partners [1, 2]. BCL10 was initially identified in a subset of MALT B cell lymphomas with t(1;14)(p22;q32) (3,4). This 233 amino acids protein is ubiquitously expressed and contains an amino-terminal CARD domain. Importantly, BCL10^{-/-} lymphocytes show absence of NF-κB activation following antigen receptor stimulation or PMA/ionomycin-induced cell activation [9] which results in severe immunodeficiency. A subfamily of membrane-associated guanylate kinase (MAGUK) proteins bind to and cooperate with BCL10 in promoting activation of NF-κB [13-17]. These proteins, which include CARMA-1, -2 and -3, function as molecular scaffolds and contain a PSD-95/Dlg/ZO-1 homologous (PDZ) domain, a Src-homology (SH3) domain and a guanylate kinase (GUK)-like domain [13-17]. Association of these proteins with BCL10 is mediated by a N-terminal CARD, which interacts specifically with the CARD of BCL10, and this interaction is necessary for their function. Accordingly, the experimental hypothesis here addressed is that the disruption of the pattern of interactions involving the CARD of BCL10 should down-regulate BCL10-dependent NF-κB activation.

Given these premises, knowledge of regions involved in protein-protein interactions becomes of utmost importance for the design of BCL-10-targeted NF-κB inhibitors. Hence, by means of a procedure described in a previous paper [33], we have fragmented the CARD of BCL10 with trypsin and the peptides obtained following HPLC fractionation, have been used as competitors in a CARD-CARD interaction assay. By this method, we have isolated a fragment corresponding to residues 91-98 of the protein (Peptide I) that strongly abolishes protein self-association both in vitro and in a BCL-10-overexpressing cell line. Noticeably, Peptide I has an IC₅₀ of about 70 nM, although when it is fused to the basic region of the TAT protein (Peptide VIII) it is much less effective, and shows an IC₅₀ of only about 5 μM (see Fig. 4). This result can be imputed to both a reduced affinity of the Tat-conjugated peptide for the protein domain and to a higher stability of the complexes formed by BCL10 in the presence of other proteins. This hypothesis is confirmed by the cellular assays, where a concentration of about 50 μM is needed to block NF-κB activation by about 70% (see Fig. 6 and Fig. 7AB). Importantly, the loss of activity of Peptide VIII is not due to peptide degradation within the cell medium, as it appeared highly stable when in contact with serum for over 20 hrs (data not shown). Also Peptides II and III, designed to account for adjacent CARD helices and containing at the C-terminus the wild type sequence 91-98, resulted poorly effective in reducing the BCL10-CARD self-association in vitro. This behavior can be explained by the propensity of the longer polypeptides to self-aggregation and by a consequent inaccessibility of contact residues even at low concentrations. This hypothesis is supported by the observation that the 78-98 segment is dimeric even at 5 μM and we expect a similar dimeric structure also for the 68-98 peptide. Indeed, the oligomeric organization of these peptides reflects the CARD propensity to self-associate and corroborates the view that recognition is mediated by the C-terminal helix of the globular domain [36]. However the possibility that other residues within the 78-90 stretch mediates the self-recognition or that the longer peptides likely adopt a conformation which is closer to that within the full length CARD, can not be ruled out. Our biochemical data also provide the evidence that the side chains of Thr91, Gln92, Leu95 and Ile96 are implicated in the pattern of interactions involving the CARD self-recognition of BCL10 and the incapability of the ThrGln91-92AlaAla synthetic mutant to activate NF-κB even after PMA/ionomycin stimulation, strengthens this view and suggests a very critical role for these amino acids in mediating CARD-CARD contacts. Notably, residues 91, 92, 95 and 96, given their alternate distribution along the sequence, could be part of an extended surface on a side of the putative helix 6.

It is well known that BCL10 forms with CARMA1 and MALT1 a large protein complex known as CBM (CARMA1-BCL10-MALT1), which is the ultimate effector of BCL10 activity [36]. Several

studies have reported direct interactions between the CARDS of BCL10 and CARMA1 and between the Ig-like domain of MALT1 and residues 107-119 of BCL10. Very recently, it has been found that residues from the putative helix 5 of the BCL10 CARD (D80 and E84) mediate the binding to the death domain (DD) of MALT1 and that they are essential for the downstream NF- κ B activation; furthermore it has been demonstrated that mutations within the 6th helix (Q92, L95, I96) preclude this binding by seemingly altering the CARD structural stability [37]. These findings essentially agree with our data as they fit with a model of multiple interactions between CARDS and between CARD and the MALT1 DD, whereby helix 5 mediates the recognition with MALT1 and residues from the adjacent helix 6 are involved in CARD self association. Importantly, data also suggest that residues 91-92 of BCL10 might have a role for the recognition of CARMA3 and therefore for the G-coupled receptors mediated NF- κ B activation, although a higher peptide concentration is required to observe this effect. Compounds targeting this BCL10 region could downregulate activation of the transcription factor via multiple pathways, thus resulting much more effective in suppressing cell growth. NF- κ B is an obvious pharmacological target for treatments aiming at blocking the inflammatory response in instances where this process becomes chronic or dysregulated, and a variety of anti-inflammatory agents inhibit the NF- κ B pathway. However, a major difficulty in the design of these drugs is that it may not be feasible to block the NF- κ B pathway for prolonged periods, since NF- κ B plays an important role in the maintenance of host defense responses. Recently, it has been shown that pathway-specific NF- κ B inhibition could be obtained by targeting transcriptional regulators of NF- κ B [40]. In this context, our report offers the proof of concept that blocking the CARD of BCL10, and possibly the association with other interacting partners, is an effective way to selectively reduce NF- κ B activation and can be a valuable therapeutic approach for the treatment of specific pathologic conditions associated with inappropriate NF- κ B activation.

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Materials and Methods

Reagents: N-protected Fmoc-amino acid derivatives and coupling reagents for peptide synthesis were from Inbios (Pozzuoli, Napoli). Peptides were prepared by standard solid phase synthesis as C-terminally amidated and N-terminally acetylated derivatives following Fmoc chemistry protocols [34]. Reagents for peptide synthesis, solvents and other reagents were all from Sigma-Aldrich (Milano, Italy) and LabScan (Stillorgan, Dublin, Ireland). Anti-BCL10 and anti CARMA3 antibodies have been generated in our laboratory [2,9,10]. All plasmids and expression vectors used in this study were generated by standard procedures and confirmed by sequencing [39].

Expression and biotinylation of BCL10-CARD domain The doubly mutated Cys29Ser-Cys57Ser N-terminal region of BCL10, comprising residues 1-107, named BCL10-CARD, was recombinantly expressed in E.coli as a His6-tagged fusion protein. Aliquots of the recombinant BCL10-CARD were biotinylated using the EZ Link NHS-LC-biotin reagent (Pierce) according to manufacturer's instructions. Biotinylated samples were dialyzed against 50 mM sodium acetate buffer pH 4.0 and protein biotinylation was confirmed by LC-MS analysis ascertaining the MW increase by 340 amu due to the biotin moiety.

BCL10-CARD self-association and competition ELISA assays.

To identify protein regions involved in self-association, the procedure described in Tornatore et al. [33] was adopted. To monitor BCL10-CARD self-association, we set up an ELISA-like assay where the protein was adsorbed on the plate surface at different concentrations, whereas the biotinylated variant was utilized to detect the binding. Adsorption was performed in a 96-well plate (Falcon) in sodium acetate 50 mM pH 4.0 (AcNa buffer) at protein concentrations of 0.12, 0.31 and 0.62 μM overnight at 4°C (100 μL /well). A detailed procedure for these assays are reported in the Supplementary Methods. Binding competition assays were carried out by coating the unlabelled BCL10-CARD at a fixed concentration of 0.12 μM and using a fixed concentration of 0.80 μM soluble biotinylated protein (7:1 mol/mol ratio, pre-saturation condition). Peptides contained within the 11 HPLC fractions obtained by trypsin digestion were used at a nominal concentration of 8.0 μM (10:1 ratio with the biotinylated protein).

Cell culture and luciferase assay

HEK293 cells and MEFs were grown in complete DMEM supplemented with 10% fetal calf serum (FCS) and 100 $\mu\text{g}/\text{mL}$ penicillin /streptomycin, and maintained at 37°C with 5% CO_2 . For plasmid transfection, HEK293 cells were transfected in 100 mm Petri dishes by calcium phosphate precipitation. Immunoblot analysis and luciferase tests were performed as previously described [38].

Immunoblot analysis and co-precipitation

Cell lysates were made in lysis buffer (150 mM NaCl, 20 mM Hepes, pH 7.4, 1% Triton X-100, 10% glycerol, and a mixture of protease inhibitors). Proteins were resolved by 10% SDS-PAGE, and gels were transferred to nitrocellulose membrane. Primary antibody incubations were carried out overnight at 4°C in PBS + 0.05% Tween 20 + 2.5% dry nonfat milk. Secondary antibody incubations were carried out for 1 h at room temperature in blocking buffer. Reacting bands were detected using the ECL system (Amersham Biosciences). For co-immunoprecipitation experiments, cells were lysed in lysis buffer and immunocomplexes were bound to protein A/G, resolved by SDS-PAGE and analyzed by immunoblot assay.

Table I

<i>Fraction</i>	BCL10-CARD domain fragments	Relative distribution of fragments within fractions (%)	Relative composition of the fraction (%)	Extent of inhibition of self-association (%)
F1	//	//	//	//
F2	//	//	//	//
F3	I46-R49	100	24	20
	I32-R36	100	41	
	E50-R58	100	35	
F4	K45-R49	100	7	0
	L111-K115	100	44	
	V26-K31	100	56	
F5	G78-R88	100	100	16
	G78-R87	100	8	
F6	H37-R42	100	0.7	10
	T91-K105	100	0.3	
	I99-K105	93	88	
	D19-R25	22	3	
	K18-R25	100	19	
F7	D19-R25	88	83	7
	N108-K115	100	15	
	E89-K98	32	2	
F8	E89-K98	68	37	45
	T91-K98	100	63	
F9	L68-K77	100	100	10
F10	E89-K115	100	87	12
	I99-K105	7	10	
	I46-R58	100	3	

Table II

	PEPTIDE	SEQUENCE
I	Bcl10[91-98]	TQNFLIQK
II	Bcl10[78-98]	GLDTLVESIRREKTQNFLIQK
III	Bcl10[68-98]	LLDYLQENPKGLDTLVESIRREKTQNFLIQK
IV	Bcl10[91-98]T91A,Q92A	AANFLIQK
V	Bcl10[91-98]N93A,F94A	TQAALIQK
VI	Bcl10[91-98]L95A,I96A	TQNFAAQK
VII	Bcl10[91-98]Q97A,K98A	TQNFLIAA
VIII	Tat-Bcl10[91-98]	<i>Fluor</i> - β A-GRKKRRQRRRPPQ- β A- β A-TQNFLIQK
IX	Tat-Bcl10[91-98]T91A,Q92A	<i>Fluor</i> - β A-GRKKRRQRRRPPQ- β A- β A-AANFLIQK

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Table III

Conc (μM)	Peptide I MW_{exp} (Da) ($\text{MW}_{\text{th}}= 1032$ Da)	Peptide II MW_{exp} (Da) ($\text{MW}_{\text{th}}= 2746$ Da)
400	1314	5802
100	1322	5470
50	1309	5352
10	1294	4981
5	1245	5045

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Figure legends

Fig. 1. Amino acidic sequence of the BCL-10 CARD protein used in this study. The two mutated cysteines are boxed and the linker (without numbering) is in bold. Residues 91-98 are in bold and italicized. Amino acids enclosed in the CARD domain are all italicized; helices 1 to 6, according to the model retrieved from the server <http://modbase.compbio.ucsf.edu/>, are underlined.

Fig. 2. *A)* Dose-dependent binding curves of biotinylated BCL10-CARD to coated BCL10-CARD at different concentrations (0.12, 0.30 and 0.60 μM). Data fitting and analysis (GraphPrism, vers. 4.0) allowed the determination of a self-association K_D of about 3.0 μM . *B)* Dose-dependent competitive ELISA assay with the synthetic Peptide I-III corresponding to fragments 91-98, 78-98 and 68-98 of BCL10, respectively. For this assay, the coating solution was fixed at 0.12 μM ; the concentration of the soluble biotinylated protein was kept at 0.80 μM .

Fig. 3. Dose-dependent competitive ELISA assay with the synthetic Peptides I, IV, V, VI, VII. Peptides IV-VII are doubly-mutated synthetic variants of the most active 91-98 fragment.

Fig. 4. *A)* In vitro inhibition of BCL10 CARD (region 1-127) self-association in lysates of BCL10-overexpressing cells. Two differentially tagged protein constructs were utilized in this assays. 50% binding inhibition was roughly achieved by a concentration of Peptide VIII of about 5 μM . *B)* Inhibition of BCL10/CARMA3 CARD association. HEK 293 cells were transfected with BCL10-CARD or CARMA3-CARD and 24hrs later cell lysates were pre-treated for 1 hour with PBS or with the indicated concentration of Peptide VIII or Peptide IX. Samples were pooled and immunoprecipitated with anti-FLAG beads overnight. Immunocomplexes were then washed, resolved by SDS-PAGE and analyzed by western blot. *C)* Association of BCL10 with Malt1 is unaffected by Peptide VIII. HEK 293 cells were treated with indicated concentration of Peptide VIII and Peptide IX or left untreated. Cells were left untreated or stimulated with PMA and ionomycin for 40 min. to induce NF- κ B activation and then lysed. Samples were immunoprecipitated with α -BCL10 beads overnight, washed, resolved by SDS-PAGE and analyzed by western blot.

Fig. 5. *A)* Assessment of peptide entry in 293HEK cells. Cells were incubated with peptide solutions at different concentrations (100-300 μM) and subsequently analyzed by fluorescence microscopy. A picture of the untreated cells is also reported in the middle. *B)* Cellular uptake of Peptide VIII and Peptide IX. HEK 293 cells were incubated for 5 hours in presence of FITC-conjugated Tat-Peptide VIII and Tat-Peptide IX at indicated concentration. Samples were then harvested, washed and analyzed by FACS. *C)* Cell viability in presence of Peptide VIII or Peptide IX. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) dye method was used to assess cell viability. 3×10^3 viable HEK293 cells were plated in 96-well plates untreated or treated with 100 μM of Peptide VIII or Peptide IX. MTT assay was performed in triplicate at the indicated times. Absorbance was examined at 590 nm by use of a microplate reader. Data are mean \pm s.d. for at least three separate determinations.

Fig. 6. Selective inhibition of BCL10-induced NF- κ B activation by TAT-Peptide VIII. HEK293 cells were transiently co-transfected with an expression vector encoding for *(A)* BCL10 and *(B)* p65, together with an NF- κ B-luciferase and β -galactosidase reporter vectors. 16 hrs after transfection, cell were treated for 5 hrs with TAT- Peptide VIII and TAT-ctrl (Peptide IX), at the indicated concentrations. Data shown represents relative luciferase activity normalized on β -galactosidase activity and is representative of six independent experiments done in triplicate.

Fig. 7. TAT-91-98 BCL10 inhibits PKC-mediated NF- κ B activation. **A)** HEK293 cells were transfected with an NF- κ B-luciferase reporter plasmid. 24 hrs later, cells were left untreated or treated with PMA (40 ng/mL) plus ionomycin (1 μ M) for 5 hrs, together with Peptide VIII or Peptide IX at the indicated doses, and luciferase activity was measured. Data shown are representative of six independent experiments. **B)** HEK293 cells were transfected with an NF- κ B-luciferase reporter plasmid. 24 hrs later, cells were left untreated or treated with PMA (40 ng/ml) plus ionomycin (1 μ M) for 5 hr. Before cell lysates were made, Peptide VIII (50 μ M) or Peptide IX, 50 μ M were added to the cell culture for the indicated time periods. Data shown are representative of five independent experiments.

Table I Tryptic BCL10-CARD domain peptide fragments as identified by LC-MS/MS analysis. Relative distributions of fragments in column 3 were calculated by comparing area integration of extracted ion peaks from a given fragment taken from the different fractions. The relative composition within each fraction was derived by comparing area integrations of extracted ion peaks of all the fraction components (See also reference 33).

Table II Peptides utilized in this study. Single letter codes are reported. β A stands for the non natural amino acid β -alanine that was used as a spacer between the TAT and the CARD sequences. *Peptides VIII and IX described in the experiment of Fig. 6, were also fluoresceinated at their N-terminus with Fluoresceine- β Ala.

Table III Characterisation of the oligomeric state of Peptide I and Peptide II. Gel-filtration analysis of synthetic peptides on Biosep/SEC/S 2000 buffer phosphate 50 mM, pH=6.8. Gel-filtration analyses were performed at five different concentrations between 400 and 5 μ M (each run was repeated at least twice). A calibration curve was used to determine the MWs. Calibrants were: Trypsinogen, 23.0 kDa; Myoglobin, 17.0 kDa; Cytochrome C, 12.4 kDa; Angiotensin II, 1.0 kDa; an unrelated synthetic peptide I, 3.5 kDa; an unrelated synthetic peptide II, 1.67 kDa.

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Figure 1

MGSSHHHHHH SGLVPRGSH MASMTGGQQM GRGSEF

10 20 30 40 50 60
MEPTAPSLTE EDLTEVKKDA LENLRVYLQE KIIAERHFDH LRAKKILSRE DTEEISQRTS

70 80 90 100 107
SRKRAGKLLD YLQENPKGLD TLVESIRREK TQNFLIQKIT DEVLKLR

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Figure 2A

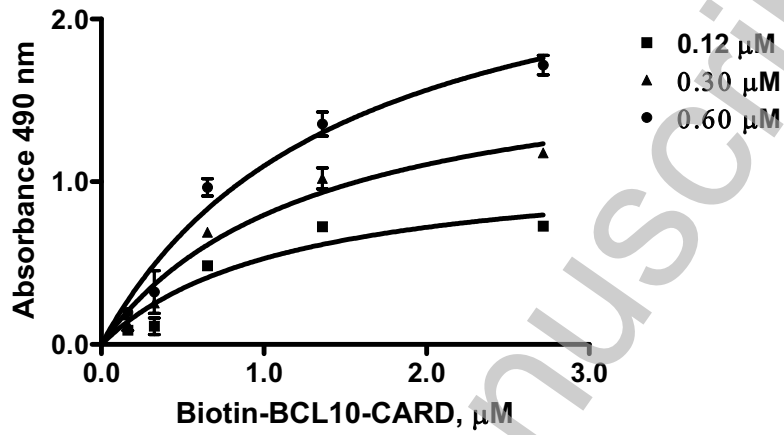


Figure 2B

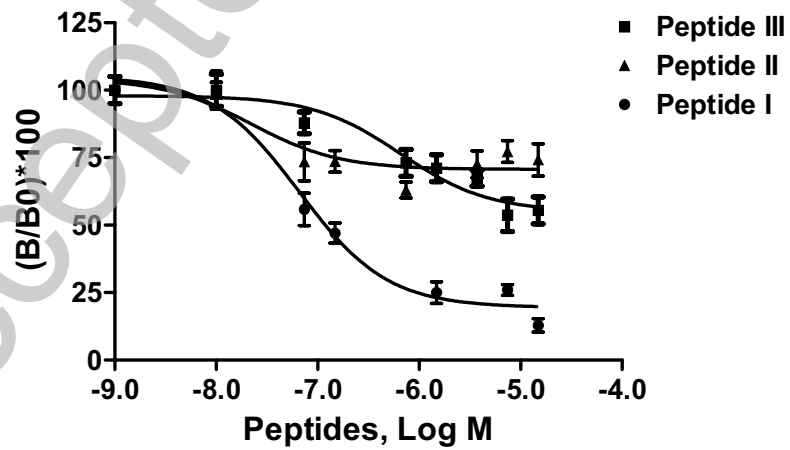


Figure 3

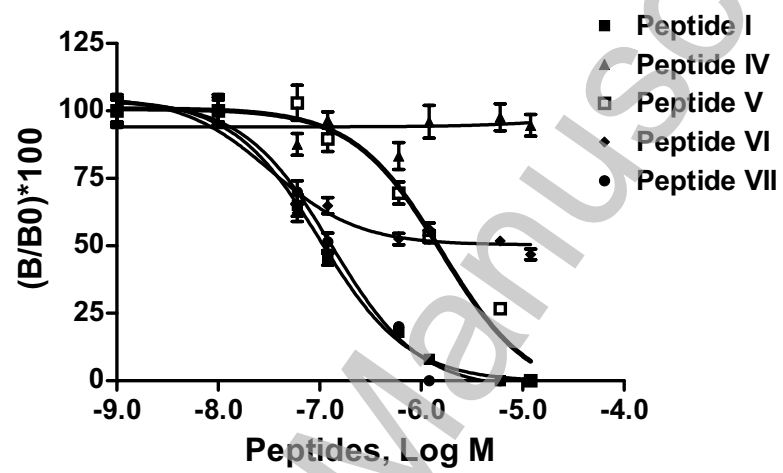


Figure 4

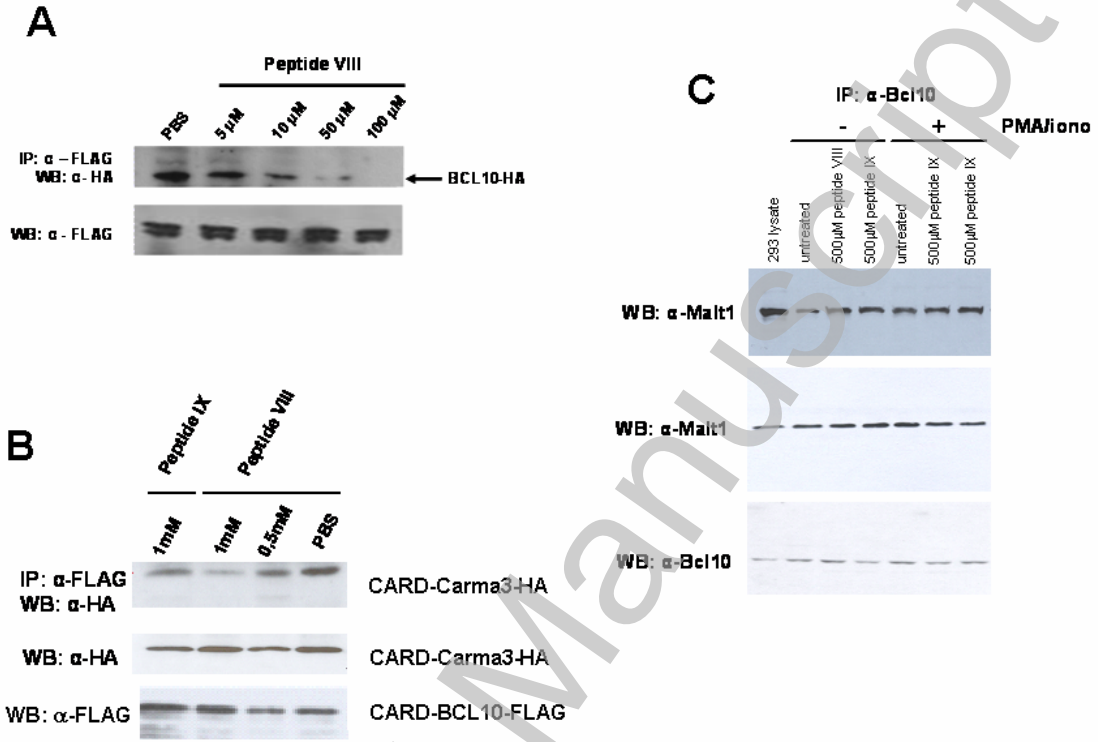


Figure 5A

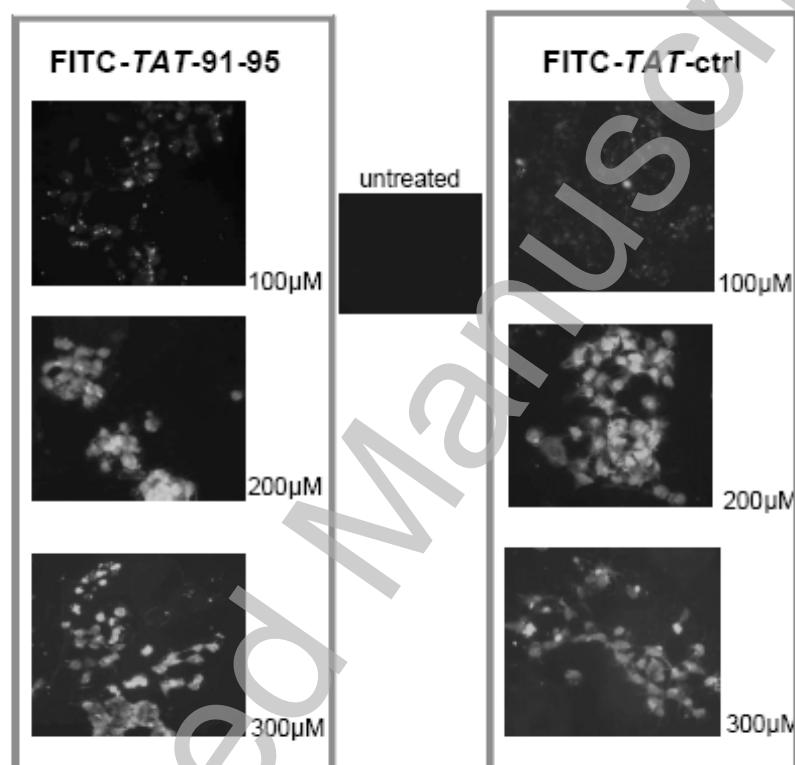


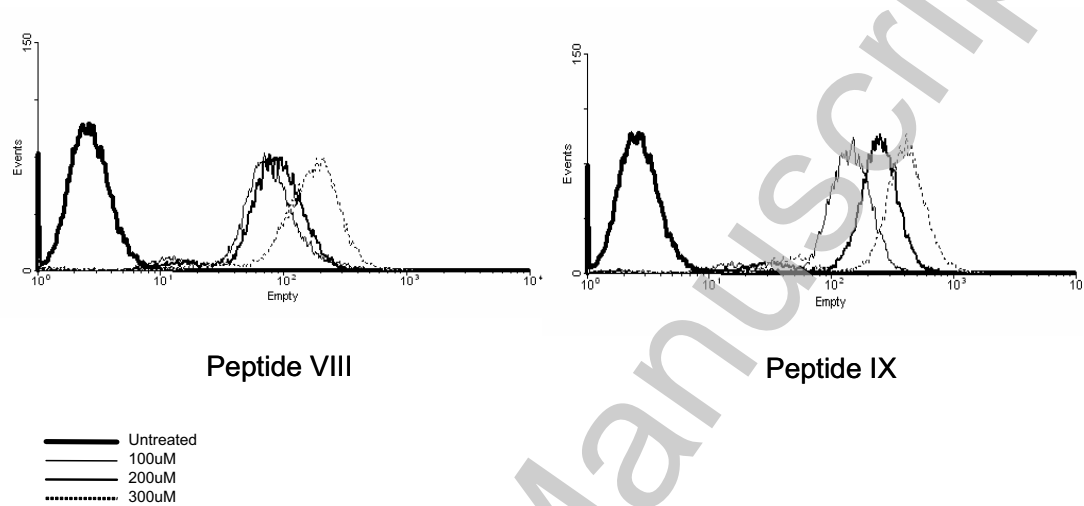
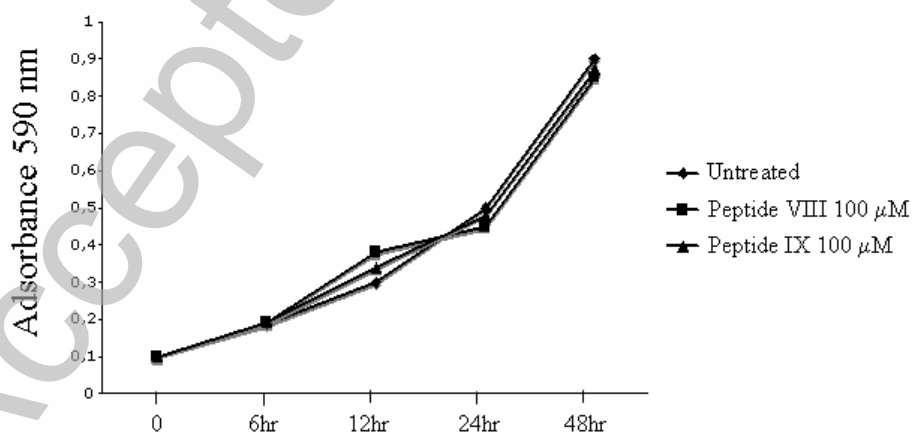
Figure 5B**Figure 5C**

Figure 6 A

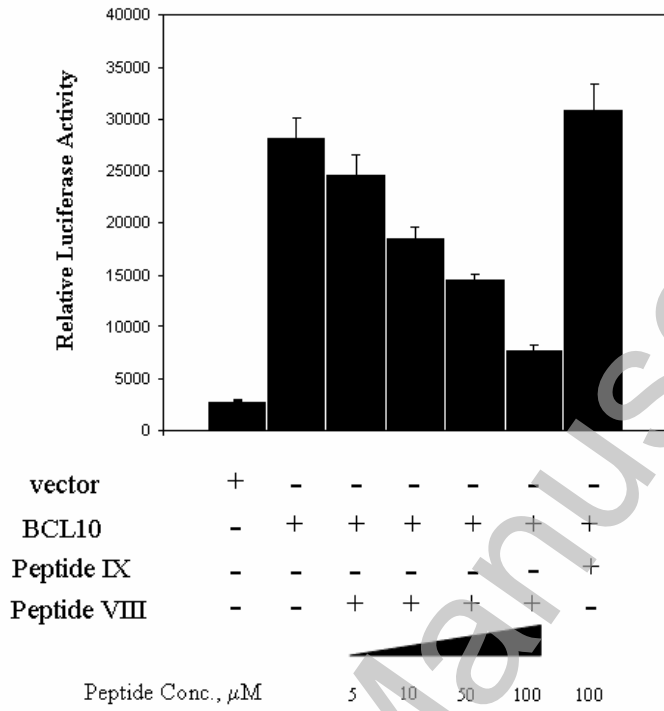
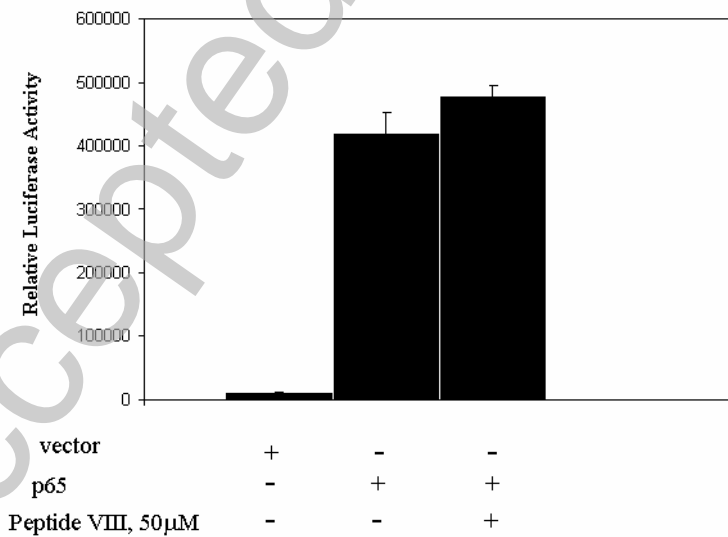


Figure 6 B



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Figure 7 A

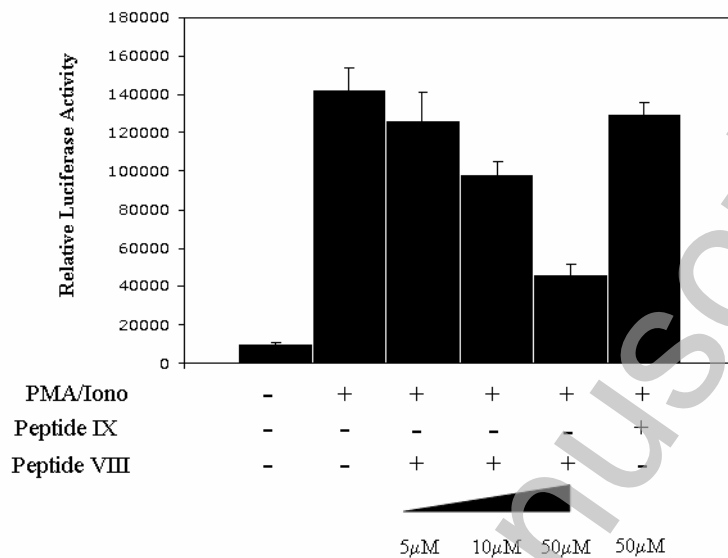
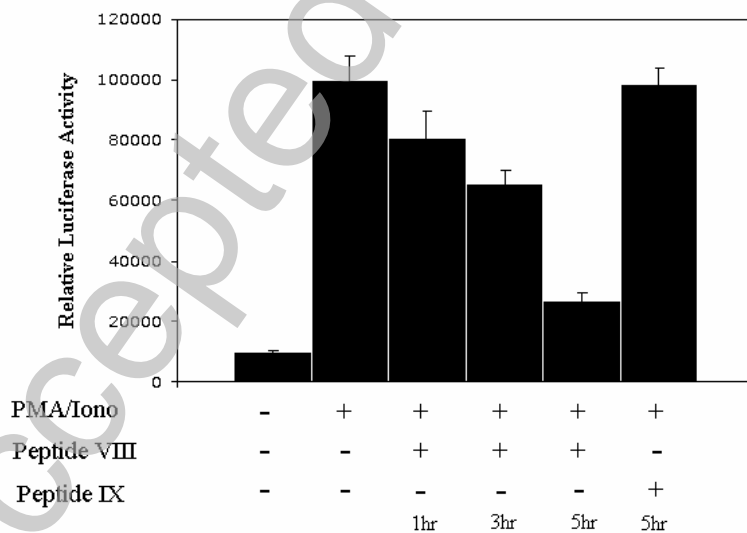


Figure 7 B



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