Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 11, pp. 1147-1150, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00184-0

A Medicinal Chemistry Evaluation of the Autoinhibitory Domain of Calcineurin

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Abstract : *Truncation of, and substitutions in, the 25 amino acid autoinhibitory element of the phosphatase calcineurin indicate that most of the segment is required for inhibition. The peptide does not, therefore, represent a convenient starting point for small molecule drug development.*

Calcineurin (CaN) is a Ca^{2+} and calmodulin (CaM) dependent protein phosphatase that has a key role in the regulation of expression of a broad range of genes whose transcription is stimulated by Ca^{2+}.[1] It is now well established that CaN has an important role in the biochemical events associated with the action of the immunosuppressive agents cyclosporin A and FK-506. Much evidence has accumulated indicating that these agents bind to endogenous proteins, designated immunophilins, and that the active drug species are the ligand/protein complexes so-formed; CsA/CyP and FK-506/FKBP respectively.[2,3] Both of these complexes are thought to exert their effect by binding to and inhibiting CaN.[3] Such a scenario raises the issue of whether it would be possible to directly inhibit the phosphatase activity of CaN (i.e. without immunophilin intervention) and whether there are any advantages to be gained in doing so.[4]

CaN is composed of two subunits CaN A (61 kDa) and CaN B (19 kDa). CaN A comprises four distinct domains, denoted by function as catalytic, CaN B binding, CaM binding and autoinhibitory.[5,6] Soderling and co-workers have further delimited the autoinhibitory element.[7] By assessing CaN inhibition of fragments of CaN A retrieved from limited proteolysis they were able to define a 25-mer peptide (sequence ITSFEEAKGLDRINERMPPRRDAMP) that appeared to house most of the inhibitory activity. This peptide inhibited CaN with an IC_{50} of 10 μM.[7] Chart 1 shows the autoinhibitory regions from a number of eukaryotes.[8] The amino acid sequence is fairly conserved across these species. Residues 4, 7, 11, 14, 15, 18 & 19 are absolutely conserved and only conservative substitutions appear in positions 1, 3, 8, 13, 16 & 17. The motif -RRXS/T- is present in many of CaN's preferred substrates and consequently the presence of -RRDA- at the C-terminal end of the peptide has prompted speculation that the autoinhibitory domain is a substrate mimic.[7,9] We were interested in determining whether the inhibitory activity could be further localized to a shorter sequence within the 25-mer which could provide a starting point for the design of a small molecule inhibitor.[10]

Chart 1 : Phylogenetic Comparison of Autoinhibitory Domains

(hum-human, mur-murine, bov-bovine)

	5							10							15							20					25
bov1,huml,mur1,rat1	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	A	M	P
hum2,rat2	I	C	S	F	E	E	A	R	G	L	D	R	I	N	E	R	M	P	P	R	K	D	A	V	Q
hum3	I	R	S	F	E	E	A	R	G	L	D	R	I	N	E	R	M	P	P	R	K	D	S	I	H
mur3	I	R	S	F	E	E	A	R	G	L	D	A	V	N	E	R	M	P	P	R	K	E	A	S	
drosophila	I	H	S	F	A	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	P	L	L	M
neurospora	I	S	S	F	E	D	A	R	K	V	D	L	Q	N	E	R	L	P	P	S	H	D	E	V	V
yeast1	L	S	T	F	E	K	A	R	K	E	D	L	I	N	E	K	L	P	P	S	L	S	E	V	E
yeast2	L	S	T	F	E	R	A	R	K	H	D	L	I	N	E	K	L	P	P	S	L	D	E	L	K

*Chart 2 : IC_{50}'s for Selected Analogs**

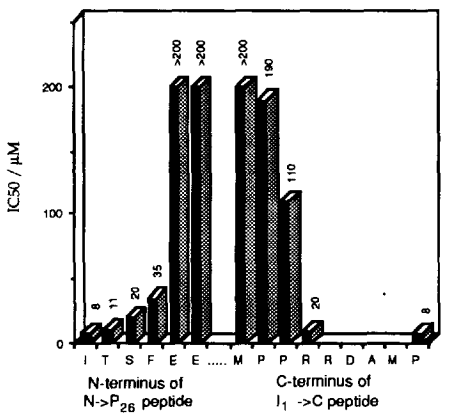
		5							10							15							20					25		IC_{50} (μM) †
1.	mam1	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	A	M	P-NH_2			8
2.	yeast2	L	S	T	F	E	R	A	R	K	H	D	L	I	N	E	K	L	P	P	S	L	D	E	L	K-NH_2			35
C-Truncation																													
3.	L-751,262	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R-NH_2								20
4.	L-748,265	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P-NH_2									110
5.	L-751,232	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P-NH_2										190
6.	L-750,347	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M-NH_2											>200
7.	L-751,676	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	**A**-NH_2								140
N-Truncation																													
8.	L-754,007		T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	A	M	P-NH_2			11
9.	L-754,872			S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	A	M	P-NH_2			20
10.	L-751,675				F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	A	M	P-NH_2			35
11.	L-750,258					E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	A	M	P-NH_2			>200
12.	L-750,258						E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	A	M	P-NH_2			>200
Substitutions																													
13.	L-750,257	I	T	S	**A**	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	A	M	P-NH_2			>200
14.	L-750,346	I	T	S	F	**A**	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	**A**	M	P-NH_2			21
15.	L-763,914	I	T	S	F	E	**A**	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	A	M	P-NH_2			7
16.	L-763,915	I	T	S	F	E	E	A	K	G	L	**A**	R	I	N	E	R	M	P	P	R	R	D	A	M	P-NH_2			>200
17.	L-749,291	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	**A**	P	R	R	D	A	M	P-NH_2			>200
18.	L-749,317	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	**A**	R	R	D	A	M	P-NH_2			40
19.	L-749,759	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	**A**	R	D	A	M	P-NH_2			85
20.	L-749,760	I	T	S	F	E	E	A	K	G	L	D	R	I	N	E	R	M	P	P	R	R	D	**E**	M	P-NH_2			9

* Peptides were synthesized using an ABI 431A peptide synthesizer, employing Fmoc chemistry on Rink amide resin. They were synthesized as amides to circumvent diketopiperazine formation induced by proline at the C-terminus. Resin cleavage conditions were 95%TFA/5%thioanisole/5h/RT. The resin was filtered and the solvent removed. The peptide was precipitated in ether, filtered and dried in a dessicator. Purification was by C18 RP-HPLC using a 15% acetonitrile/0.1% TFA to 50% acetonitrile/0.1%TFA gradient over 40 minutes. The peptides were checked for purity by analytical RP-HPLC and validated by mass spectroscopy and amino acid analysis.

† IC_{50}'s refer to inhibition of the dephosphorylation of phospho-RII peptide using a modified assay for phosphate detection (Anner, B.; Moosmayer, M. *Anal. Biochem.* **1975**, *65*, 305). The reaction mixture contained 50μM phospho-RII peptide in 50mM HEPES buffer (pH 7.0), 1mM $MnCl_2$, 0.1mM $CaCl_2$, and 30nM calmodulin in a volume of 195μL. Reactions were initiated with 5μL of CaN (12nM; rat gene 1δ isoform purified from baculovirus-infected insect cells (Parsons, J. N.; Wiederrecht, G. J.; Salowe, S.; Burbaum, J. J.; Rokosz, L. L.; Kincaid, R. L.; O'Keefe, S. J., *J. Biol. Chem.* **1994**, *269*, 19610)) and terminated after 10min at room temperature with 25μL of a dye reagent composed of 0.04% Malachite green, 16mM Na_2EDTA, 1% polyvinyl alcohol. The absorbance at 600nm was read 30min after the subsequent addition, with vigorous mixing, of 25μL 0.1 M Na_2MoO_4 in 5N H_2SO_4.

Chart 2 shows a selection of the peptides synthesized with their corresponding IC_{50} values. The native peptide (entry 1) has an IC_{50} of 8 μM, in agreement with the reported value.[7] The value for the analogous peptide derived from a yeast CaN A sequence[8] (entry 2) is 35μM. Deletion of five amino acids from the C-terminus (entry 3) has little effect on the inhibitory potency. Thereafter stepwise truncations are detrimental (entries 4-6). A five fold increase in IC_{50} with the loss of Arg_{20} culminates in complete loss of inhibitory activity at the erasure of Pro_{18}. This behaviour can be correlated with the phylogenetic comparison (Chart 1). Residues 22-25 (and arguably also 21) at the C-terminus appear somewhat heterogeneous and hence, perhaps, predictably unimportant, while residues 18-20 (-PPR-) are more highly conserved. The importance of the nature of the 'cut-off residue' (R_{20}) is clear from the poor performance of the Ala substituted truncate (entry 7). That these five residues can be dispensed with does not invalidate the concept of substrate analogy, but certainly implies that, if true, the segment at the catalytic active site makes no significant binding contribution.[11,12] Stepwise paring from the N-terminus (entries 8-12) reveals an unmistakable frontier at Phe_4. That this is the first absolutely conserved residue reached (Chart 1) again illustrates the significance of the phylogenetic comparison. A graphical summary of this data highlights this activity profile:

Effect of C- and N-Terminal Truncations



These truncation limits were already suggesting that the autoinhibitory peptide (only a micromolar inhibitor in its own right) would be a poor candidate as a lead for small molecule inhibitor design. Peptidomimetic approaches for anything larger than a hexa-peptide are probably impractical. Furthermore, the scatter of conserved and conservatively substituted residues (see above) suggested that elements throughout the peptide might be critical components. To gauge this we chose to prepare a limited number of Ala substituted analogs (entries 13-19). We suspected that, for our purposes, a complete 'Ala scan' would not be necessary. All inhibitory activity was lost by substituting the absolutely conserved Phe at position 4 (the N-terminal truncation limit), the absolutely conserved Asp at position 11, and the absolutely conserved Pro at position 18 (entries 13,16,17).[10] Several other Ala substitutions generated interesting results. Substitution is accepted at position 5 (entry 14), which displays a Glu in all but drosophila, where Ala itself is naturally present. $Glu_6 \Rightarrow$Ala is also acceptable (entry 15). This is the only site where both an anionic and cationic residues appear. The rather moderate effect of the $Pro_{19} \Rightarrow$Ala substitution was somewhat surprising, given the conserved nature of that residue (entry 18). At Arg_{20} (the C-terminal truncation limit) substitution generated a peptide with an IC_{50} in the

same range as the 1-19 truncation (entries 19 & 4), presumably reflecting the lack of that side chain without misorienting the remainder of C-terminus. One rather different substitution was examined. If the -RRDA- sequence *does* lie where the -RRXS/T(OPO_3^-)- motif would ordinarily be placed, it might be possible to mimic the anionic phosphate with a Glu and in doing so introduce a new and tenacious interaction for the autoinhibitor.[13] The data (entry 20) proffered no encouragement in this regard.[14]

It appears that a substantial portion of the peptide is required for expression of inhibitory activity and that key interactions (whether intermolecular or self-structural) are provided by residues throughout.[15] While this may have functional implications for the autoinhibitory domain, it does not present an appealing proposition to the medicinal chemist. A search for small molecule inhibitors might more reasonably seek other origins.

Acknowledgment. We thank Amy Bernick for mass spectral analysis, David Kolodin for amino acid composition analysis and John Hannah for helpful discussions.

References and Notes.

1. Klee, C. B.; Draetta G. F.; Hubbard, M. J. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1988**, *61*, 149.
2. CsA=cyclosporin A, CyP=cyclophilin, FKBP=FK-506 binding protein.
3. a) Liu, J.; Farmer, J. D.; Lane, W. S.; Friedman, J.; Weissman I.; Schreiber, S. L. *Cell* **1991**, *66*, 807. b) O'Keefe, S. J.; Tamura, J; Kincaid, R. L.; Tocci, M. J.; O'Neill, E. A. *Nature* **1992**, *357*, 692. c) Fruman, D. A.; Klee, C. B.; Bierer, B. E.; Burakoff, S. J. *Proc. Natl. Acad. Sc. U.S.A.* **1992**, *89*, 3686. d) Rosen, M. K.; Schreiber S. L. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 384.
4. In our laboratories, this approach was first proposed by J. Burbaum, M. Goulet and W. H. Parsons.
5. Hubbard, M. J.; Klee C.B. *Biochemistry* **1989**, *28*, 1868.
6. For an interesting review of protein kinase and phosphatase autoinhibitory domains see Soderling, T. R. *Biotechnol. Appl. Biochem.* **1993**, *18*, 185.
7. Hashimoto, Y.; Perrino, B. A.; Soderling, S. R.*J. Biol. Chem.* **1990**, *265*, 1924.
8. See Guerini, D.; Montell, C.; Klee, C.B.*J.Biol.Chem.* **1992**, *267*, 22542 and references therein.
9. This is probably a consequence of kinase selectivity, since -RRXS/T- is a consensus sequence for these enzymes, but this need not invalidate the proposition. Of perhaps more concern were Soderling's initial findings suggesting that the peptide is not competitive with substrate[7] (recently reiterated[10]), although other recent findings suggest that it is.[12]
10. During manuscript preparation a report appeared that described the effect of two Ala substitutions (including L-763,915, entry 16) and two double Ala substitutions in this 25-mer - Perrino, B.A.; Ng, L. Y.; Soderling, T. R. *J. Biol. Chem.* **1995**, *270*, 340.
11. There may be analogy to the protein kinase situation (Knigton, D. R.; Zheng, J.; Ten Eyck, L. F.; Xuong, N.; Taylor, S. S; Sowadski, J. M. *Science* **1991**, *253*, 407).
12. This corroborates recent biochemical indications - Parsons, J. N.; Wiederrecht, G. J.; Salowe, S.; Burbaum, J. J.; Rokosz, L. L.; Kincaid, R. L.; O'Keefe, S. J., *J. Biol. Chem.* **1994**, *269*, 19610.
13. Metal ions may play a key role at the active site (Martin, B. L.; Graves, D. J. *Biochim. Biophys. Acta* **1994**, *1206*, 136); we would be seeking either a direct or an indirect M^+ interaction.
14. Phosphorylated RII peptide (DLDVPIPGRFDRRVS(OPO_3^{2-})VAAE) is a substrate for CaN. We were unable to generate a peptide capable of inhibiting CaN by substituting the phospho-serine with either alanine (DLDVPIPGRFDRRVAVAAE) or glutamic acid (DLDVPIPGRFDRRVEVAAE).
15. This has recently been demonstrated to be the case for CaN *substrates* (Donella-Deana, A.; Krinks, M. H.; Ruzenne, M.; Klee, C.; Pinna L.A. *Eur. J. Biochem.* **1994**, *219*, 109).

(*Received in USA* 20 *March* 1995; *accepted* 20 *April* 1995)