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Cyclolinopeptide A (CLA) mediates its immunosuppressive activity through cyclophilin-dependent calcineurin inactivation

Terry J. Gaymes[a], Marek Cebrat[b], Ignacy Z. Siemion[b], John E. Kay[a,*]

[a]*School of Biological Sciences, University of Sussex, Brighton BN1 9QG, UK*
[b]*Institute of Chemistry, University of Wroclaw, 50-383 Wroclaw, Poland*

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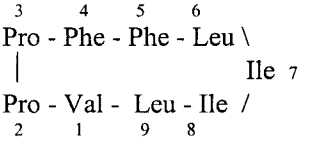
Abstract The immunosuppressive cyclic nonapeptide cyclolinopeptide A inhibits calcium-dependent, but not calcium-independent, activation of T lymphocytes comparably to the actions of cyclosporin A and FK506. The concentration required for complete inhibition, however, is 10 times higher than that of cyclosporin A. In addition, we demonstrate that calcineurin, a phosphatase which plays an important role in T lymphocyte signalling, is inhibited in vitro by cyclolinopeptide A by a mechanism dependent on the peptidyl-prolyl *cis-trans* isomerase (PPIase) cyclophilin A but not FKBP12. Direct binding of cyclolinopeptide A to cyclophilin A was confirmed using tryptophan fluorescence studies and PPIase assays. These results represent a third example of the production of a natural product that neutralises calcineurin by a mechanism dependent on the primary binding to a PPIase.

Key words: Cyclolinopeptide A; Cyclophilin A; Calcineurin; T lymphocyte activation; Peptidyl-prolyl *cis-trans* isomerase

1. Introduction

Cyclolinopeptide A (CLA) is a naturally occurring cyclic nonapeptide isolated from linseed oil [1] with the sequence:



It is one of a much larger group of cyclic oligopeptides reported to have immunosuppressive activities [2]. It was found that CLA inhibited primary and secondary humoral responses in CBA mice and that cellular immune responses were down-regulated in vivo suggesting that CLA was comparable to cyclosporin A (CsA) in its immunosuppressive activity [3]. CsA is an immunosuppressive hydrophobic undecapeptide derived from the fungus *Tolypocladium inflatum* [4] used clinically to prevent transplant rejection and to treat autoimmune disease. The structurally dissimilar macrolide FK506 derived from *Streptomyces tsukubaenis* [5] inhibits T lymphocyte activation at concentrations 2–3 orders of magnitude lower than CsA. However, both agents block calcium-dependent induction of T lymphocyte activation within the first few hours after mitogen stimulus, whilst neither drug affects early changes induced in the absence of calcium, most notably those that occur in response to the activation of protein kinase C by phorbol esters [6].

*Corresponding author. Fax: (44) (1273) 678433.
E-mail: j.e.kay@sussex.ac.uk

The presence of a pair of *cis*-linked proline residues suggests that CLA might resemble CsA or FK506 in targeting the cyclophilin or the FKBP12 family of peptidyl-prolyl *cis-trans* isomerases (PPIases). Once CsA and FK506 bind to their respective protein, PPIase activity is abolished [7]. Cyclophilins and FKBPs are sequence-unrelated families of proteins that accelerate in vitro protein folding and have been postulated to have chaperone and membrane trafficking properties in vivo [8]. CSA and FK506 become active once complexed with the PPIases, binding to calcineurin and inhibiting its phosphatase activity [9]. Calcineurin plays a key role in initiating T lymphocyte activation by dephosphorylating the nuclear factor of activated T cells (NF-AT) which in turn translocates from the cytoplasm to the nucleus, where it is required for the induction of transcription of mRNA for interleukin-2 and other cytokines [10].

In this communication, we show that CLA and its sulphonated analogue MC-172 mirror the action of CsA through their inhibition of T cell activation by binding to cyclophilin, inhibiting its PPIase activity and in turn creating a composite site for the interaction and inhibition of calcineurin.

2. Materials and methods

2.1. Drugs and proteins

For cell culture assays, cyclosporin A (Sandoz Ltd, Basle, Switzerland) was dissolved in ethanol/Tween 80 as described previously [11] and diluted to 100 μM in Eagle's minimal essential media (EMEM). The synthesis of the linear CLA precursor with the sequence Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe and the cyclic nonapeptide CLA was shortly described in [3]. The synthesis of the sulphonated analogue of CLA, MC-172, with the sequence c-(Val-Pro-Pro-Phe-Phe[SO_3Na]-Leu-Ile-Ile-Leu) is described in [12]. CLA, linear CLA and MC-172 were dissolved as for CsA. For all other assays, these peptides were dissolved solely in ethanol. FK506 (Fujisawa Pharmaceuticals Ltd., Osaka, Japan) and rapamycin (Wyeth-Ayerst Res. Labs., Princeton, NJ, USA) were dissolved in ethanol. Concanavalin A (Sigma Aldrich Co. Ltd., Poole, UK) was dissolved in EMEM and added to cultures to give a final concentration of 5 μg/ml. 12-*O*-Tetradecanoyl phorbol-13-acetate (TPA, Sigma) was dissolved in ethanol and added to cultures to give a final concentration of 10 nM. The calcineurin-specific peptide DLDVPIPGRFDRRVSVAAE was synthesised by Chris Kowalczyk, Interdisciplinary Research Centre, School of Biological Sciences, University of Sussex, Brighton, UK.

Cyclophilin A was purified from porcine kidney using gel filtration (FPLC, Superose-12, Pharmacia Biotech, Uppsala, Sweden) and cation exchange chromatography (Fast Flow Sepharose, Sigma) and suspended in 20 mM MES-NaOH pH 6.5. Human recombinant FKBP12 [13] was expressed in *Escherichia coli* strain XA90 and purified by gel filtration and cation exchange techniques before suspension in 20 mM MES-NaOH pH 6.0.

2.2. Preparation and activation of lymphocytes

Lymphocytes were prepared from heparinised porcine blood using Hypaque-Ficoll gradients and cultured at 2×10^6/ml in EMEM supplemented with 5% heat-inactivated foetal calf serum and 2 mM glutamine. Activation was assessed by determining the rate of incorpo-

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ration of [^{35}S]methionine (ICN Biomedicals Ltd., Thame, UK) into protein at 24 h and [^{3}H]thymidine (Amersham International, Amersham, UK) incorporation into DNA at 48 h. One microcurie of radioisotope was added to each of three replicate cultures and left for 4 h at 37°C before harvesting and incorporation was quantified by liquid scintillation counting.

Activated porcine lymphocyte extracts were prepared by stimulating 10 ml cultures of porcine lymphocytes (20×10^{6} cells) with 20 μg/ml concanavalin A for 30 min and then lysing the cells in 40 mM MOPS pH 7.2, 2 mM benzamidine, 0.5 mM PMSF, 125 mM NaCl, 7 mM β-mercaptoethanol, 10 μg/ml leupeptin, 0.5 mM EDTA and 0.5% NP-40. The total protein concentration of the cell extracts was determined by Bradford assay and adjusted to 10 mg/ml. Extracts were flash-frozen in liquid nitrogen and stored at −80°C.

2.3. PPIase assays and fluorescence spectroscopy

PPIase activity was determined as described by Fischer et al. [8] using 25 μM succinyl-AFPF.pNA as substrate. Fluorescence spectroscopy was based on the procedure described by Handschumacher et al. [14] where CsA binding increases the fluorescence intensity of the single tryptophan residue of cyclophilin A. In a typical assay, 1 μl volumes of CsA or CLA at a final concentration ranging from 10 nM to 10 μM were mixed with 300 nM porcine cyclophilin A in 20 mM MES-NaOH pH 6.5. Fluorescence was measured in a Perkin-Elmer LS3 spectrofluorimeter with an excitation wavelength set at 289 nm and the emission spectra recorded between 290 and 385 nm. Values shown are the peak fluorescence recorded at 336 nm.

2.4. Calcineurin assay

Phosphorylation of the peptide was performed as described by Blumenthal et al. [15]. 300 μM of the peptide DLDVPIPGRFDRRVS-VAAE was phosphorylated by 20 μg/ml cAMP-dependent protein kinase (Sigma) in a reaction mixture containing 20 mM MOPS pH 7.2, 2 mM Mg-acetate, 14 mM β-mercaptoethanol, 2 μM cAMP, 100 μM ATP and 300 μM [γ-^{32}P]ATP (200–400 cpm/pmol). The reaction was started by adding cAMP-dependent protein kinase and the mixture was incubated at 30°C for 30 min. Labelled peptide was separated from unincorporated ATP by passing the peptide down a 1 ml Dowex AG-1 X-8 (Sigma) anion-exchange column. Calcineurin dephosphorylation of the peptide was carried out according to the method of Katagiri et al. [16]. The reaction mixture was incubated at 30°C and consisted of 10 μM phosphopeptide (240 000 input cpm) in calcineurin buffer (50 mM MOPS pH 7.2 with 2 mM Mg-acetate, 2 mM $MnCl_2$, 1 μM calmodulin (Sigma), 14 mM β-mercaptoethanol, 100 μM $CaCl_2$, 2 nM okadaic acid, 0.5 μM calcineurin (Sigma) and 0.1 mg/ml bovine serum albumin). The reaction was started by addition of the phosphorylated peptide and the reaction mixture was incubated for 20 min. Where indicated incubations also contained 1 μM porcine kidney cyclophilin A, 1 μM human recombinant FKBP12, 50 nM FK506, 1 μM CsA, 10 μM CLA or 50 nM rapamycin. In some experiments calcineurin and the PPIases were omitted and replaced by activated porcine lymphocyte lysates. Dephosphorylation of the peptide was carried out in a reaction buffer containing 50 mM MOPS pH 7.5, 0.2 mg/ml bovine serum albumin, 3 μM calmodulin, 2 mM $CaCl_2$, 1 mM EGTA and 10 μM phosphorylated peptide (200 000 input cpm).

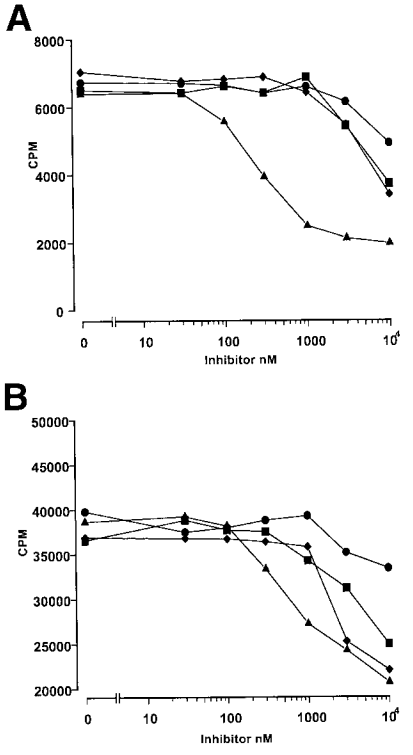

Fig. 1. Comparison of the effects of cyclolinopeptide A and cyclosporin A on porcine peripheral lymphocyte activation. Cells were activated using the lectin concanavalin A and incubated with cyclic cyclolinopeptide A (CLA) (■), linear CLA (●), MC-172 (◆) and CsA (▲). A: Protein synthesis was determined by monitoring the incorporation of [^{35}S]methionine into protein at 24 h. B: DNA synthesis was assessed by determining the incorporation of [^{3}H]thymidine into DNA at 48 h.

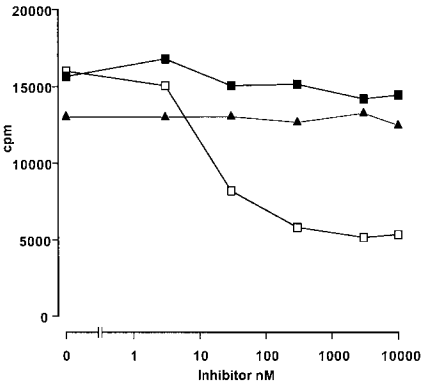

Fig. 2. Effect of cyclolinopeptide A, cyclosporin A and rapamycin on TPA-stimulated porcine lymphocytes. Lymphocytes were activated with 10 nM TPA and incubated with CLA (■), CsA (▲) and rapamycin (□). Activation was assessed by monitoring the incorporation of [^{35}S]methionine into protein at 24 h.

3. Results and discussion

Lectins such as concanavalin A crosslink the T lymphocyte receptor and activate the calcium-dependent pathway of activation. CsA and FK506 exhibit their immunosuppressive action through the inhibition of this pathway [6]. To determine whether cyclolinopeptide A could also inhibit this pathway by the same mechanism, we assessed its ability to inhibit porcine peripheral lymphocyte activation in comparison to CsA. Fig. 1 shows the action of CLA, linear CLA and MC-172 on protein and DNA synthesis through incorporation of labelled methionine and thymidine respectively. Both CLA and MC-172 were able to inhibit synthesis at concentrations above 3 μM, whilst linear CLA exhibited a lower level of inhibition. Thus, sulphonation of one of two Phe residues of CLA increased its solubility, but did not affect its inhibitory action. In comparison, CsA inhibits activation at concentrations above 300 nM.

T lymphocytes can also be activated to increase protein synthesis through a calcium-independent pathway by addition of phorbol esters such as TPA, a response which is insensitive to CsA [6]. Like CsA, CLA failed to inhibit protein synthesis in TPA-stimulated cells (Fig. 2).

It has been established that for CsA to inhibit lymphocyte activation, it needs to bind the PPIase cyclophilin. In addi-

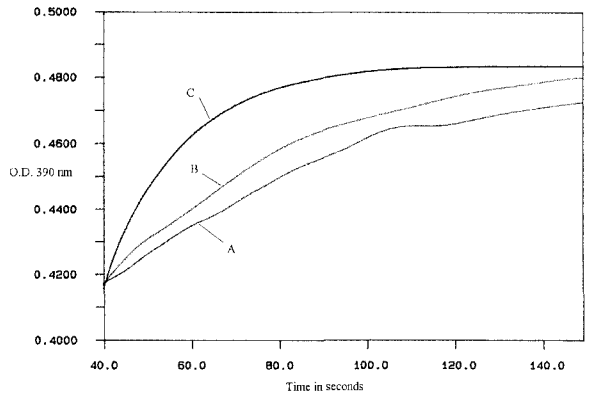

Fig. 3. Effect of MC-172 and cyclophilin A on the second phase of peptide hydrolysis in the α-chymotrypsin-coupled spectrophotometric assay. 25 μM of the peptide Succ-AFPF-pNa in HEPES buffer pH 7.5 was incubated with 50 nM porcine kidney cyclophilin A, 10 μM MC-172 or 1 μM CsA. Line A: 50 nM porcine kidney cyclophilin A+1 μM CsA; line B: 50 nM porcine kidney cyclophilin A+10 μM MC-172; line C: 50 nM porcine kidney cyclophilin A.

tion, the PPIase activity of cyclophilin is inhibited on binding CsA, though it has been shown using synthetic analogues of CsA that PPIase activity is not sufficient for the inhibition of T cell activation. CLA possesses a pair of proline bonds in the *cis* conformation which is analogous to the MLeu^{9}-MLeu^{10} *cis* linkage in CsA and would therefore be the likely site of interaction. We can exploit the inhibition of PPIase activity to determine whether cyclophilin A is the intracellular receptor for CLA. PPIase assays were carried out according to the method of Fischer et al. [8]. The assay relies on the fact that α-chymotrypsin can only hydrolyse the chromophore *p*-nitroaniline when the X-Pro bond is in the *trans* conformation. Approximately 15% of the bond is in *cis* at equilibrium and in the presence of excess α-chymotrypsin, hydrolysis of the *trans* peptide occurs in seconds (first stage) whilst the cleavage of the *cis* peptide is rate-limited by *cis-trans* isomerisation (second phase). PPIases such as cyclophilin catalyse the isomerisation. Fig. 3 depicts the second phase of α-chymotrypsin hydrolysis and shows that 1 μM CsA (line A) and 10 μM MC-172 (line B) were able to retard peptide hydrolysis by inhibiting the PPIase activity of cyclophilin A (line C). Using 10 μM CLA gave variable but lower inhibition, probably due to hydrolysis of the peptide itself by chymotrypsin and its poor solubility in the assay buffer. MC-172 and CLA and CsA did not inhibit the PPIase activity of FKBP12 (not shown).

It has been shown previously that there is a significant modification in the tryptophan fluorescence of cyclophilin when titrated with CsA [12]. This modification in fluorescence is due to cyclophilin A possessing a single tryptophan in the CSA binding pocket and ligation of CsA results in an enhancement of fluorescence [17]. This demonstrated a strong binding between CsA and cyclophilin. We demonstrated that the cyclolinopeptides were also able to increase fluorescence of porcine cyclophilin A (Fig. 4) by adding 1 μl volumes of CLA to give concentrations ranging from 10 nM to 10 μM. Fig. 4 is a Bjerrum plot of fractional fluorescence change against log concentration of CLA. Assuming a 1:1 stoichiometry of cyclophilin and peptide, a binding constant K_d can be estimated at a fractional fluorescence change of 0.5. If you assume 50% occupancy of cyclophilin sites, bound CLA equals unbound CLA, then the total concentration of CLA divided by 2 at a fluorescence change of 0.5 is the K_d. For CLA the calculated K_d is 125 nM (Fig. 4). Linear CLA and MC-172 showed a similar response with K_d values of 109 nM and 120 nM respectively (not shown). In comparison CsA gave a K_d of 30 nM, significantly lower than CLA and indicating a stronger affinity for cyclophilin A. Together the PPIase and tryptophan fluorescence studies show that CLA binds to and inhibits the PPIase activity of cyclophilin A.

To determine whether CLA was able to neutralise the phosphatase activity of calcineurin, phosphatase assays were performed. As expected CsA inhibits calcineurin phosphatase activity only if cyclophilin is present and FK506 inhibition is dependent on the presence of FKBP12 (Fig. 5). Rapamycin, which binds to FKBP12 to form a complex incapable of neutralising calcineurin [18], is ineffective. However, CLA was able to inhibit dephosphorylation in the presence of cyclophilin A, but not in the presence of FKBP12. In addition, CLA at concentrations of 5–10 μM was able to inhibit the calcineurin activity of activated lymphocyte extracts (Fig. 6). Thus CLA, which is structurally different from CsA, is nevertheless also able to bind cyclophilin and inhibit calcineurin phosphatase activity. Nature has evolved two dissimilar naturally based products which have similar antibiotic and immunosuppressive action dependent on binding to cyclophilin. FKBP12 cannot replace cyclophilin in either case.

A limitation on the use of CsA is its poor solubility. The more soluble sulphonated peptide MC-172 inhibited T lymphocyte activation equally effectively and thus this change in the conformation of the peptide did not affect its interaction with either cyclophilin or calcineurin. The sulphonyl group on residue Phe^{5} of MC-172 was added to the cyclic peptide to improve its solubility, and studies using several different sulphonated CLA analogues showed this was the most immunosuppressive [12]. Solubility of CLA can also be improved by alternative substitutions [19] but their effect of these modified cyclic peptides on interaction with cyclophilin and calcineurin remains to be determined.

It is remarkable that CLA is the third natural product which can target calcineurin via a PPIase. The structurally distinct immunosuppressant FK506 inhibits calcium-depend-

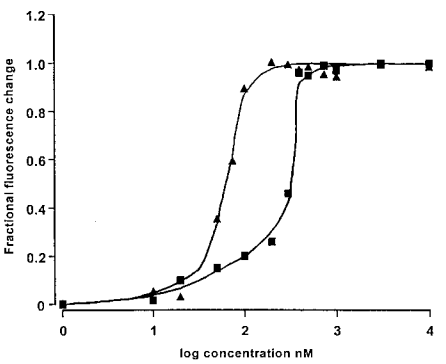

Fig. 4. Bjerrum plot assessing the binding of CLA and CsA to cyclophilin A through enhancement of tryptophan fluorescence. The excitation wavelength was set at 289 nm, emission wavelength set between 290 and 385 nm with values being recorded at peak fluorescence (336 nm). Cyclophilin concentration was held at 300 nM in MES-NaOH pH 6.5 whilst CLA (■) and CsA (▲) were added in increasing concentrations from 10 nM to 10 μM.

ent activation of T lymphocytes at a 1000 times lower concentration than CsA. In many respects its actions are similar to those of CsA, but it executes its activity through binding to FKBP12, rather than cyclophilin. CsA/cyclophilin and FK506/FKBP12 have quite different composite interfaces [20] and interact with calcineurin at slightly different sites. It would appear that the CLA/cyclophilin interface would share no obvious similarity with CsA/cyclophilin and FK506/FKBP12 interfaces, though that needs to be established. The concept that nature has promoted three distinct natural products which can promote interaction between a PPIase and calcineurin gives substance to the idea that there might be a physiological interaction between PPIases and calcineurin in the absence of exogenous ligand. It has been claimed on the basis of weak interactions using the yeast two-hybrid system that cyclophilin and FKBP12 can interact with calcineurin in the absence of CsA and FK506 respectively and that addition of the ligands only potentiates this interaction [21]. It remains to be determined whether CLA is able to bind to other cyclophilin isoforms with the same affinity and whether these complexes are able to target calcineurin with same effectiveness.

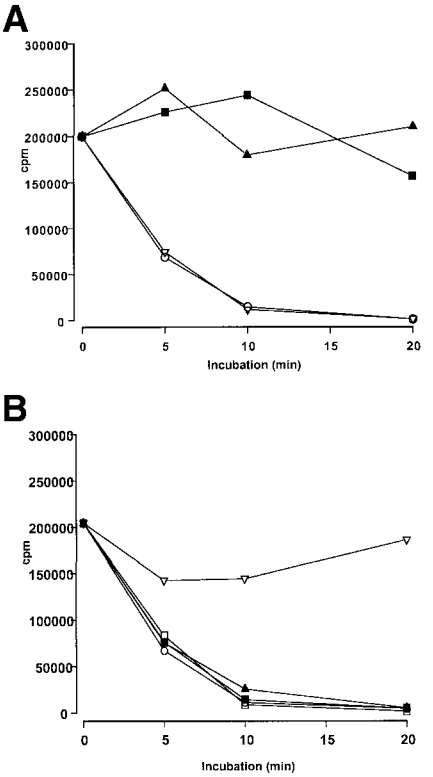

Fig. 5. Effects of PPIase and associated binding drugs on the dephosphorylation of the peptide DLDVPIPGRFDRRVSVAAE by calcineurin. The calcineurin-specific peptide was phosphorylated by cAMP-dependent protein kinase with [^{32}P]ATP. Labelled peptide was incubated in a reaction mixture containing (A) calcineurin, porcine cyclophilin A, CLA 10 μM (■), CsA 1 μM (▲), FK506 50 nM (▽) and an ethanol control (○); (B) calcineurin, human FKBP12, CLA 10 μM (■), CsA 1 μM (▲), FK506 50 nM (▽), rapamycin 50 nM (□) and an ethanol control (○). Dephosphorylation was assessed by removing 1 μl aliquots, spotting onto p81 paper and quantified by liquid scintillation counting.

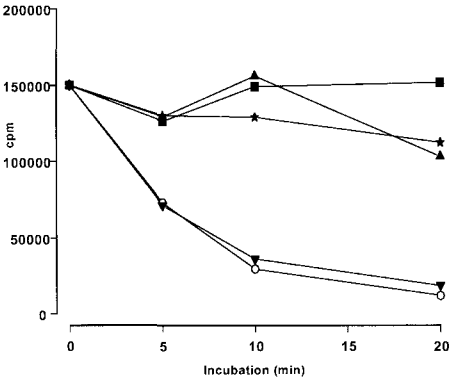

Fig. 6. Dephosphorylation of the calcineurin-specific peptide by activated porcine lymphocyte extracts and the effect of inhibitory peptides. Lymphocytes were activated by concanavalin A for 20 min and extracts were prepared as stated in Section 2. Phosphorylated peptide was incubated with extract diluted 1:250 in calcineurin buffer with the inhibitors CsA 1 μM (▲), CLA at 1 μM (▼), 5 μM (★) and 10 μM (■). An ethanol control was performed (○). Dephosphorylation was assessed by removing 1 μl aliquots, spotting onto p81 paper and quantified by liquid scintillation counting.

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References

[1] Kaufmann, H.P. and Tobschirbel, A. (1959) Chem. Ber. 92, 2805–2809.
[2] Wieczorek, Z., Siemion, I.Z., Zimecki, M., Bolewska-Pedyczak, E. and Wieland, T. (1993) Peptides 14, 1–5.
[3] Wieczorek, Z., Bengtsson, B., Trojnar, J. and Siemion, I.Z. (1991) Peptide Res. 4, 275–283.
[4] Borel, J.F. (1989) Pharmacol. Rev. 41, 259–371.
[5] Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H. and Imanaka, H. (1987) J. Antibiotics (Tokyo) 40, 1249–1255.
[6] Kay, J.E., Benzie, C.R., Goodier, M.R., Wick, C.J. and Doe, S.E.A. (1989) Immunology 67, 473.
[7] Harding, M.W., Galat, A., Uehling, D.E. and Schreiber L, S. (1989) Nature 341, 761–763.
[8] Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T. and Schmid X, F. (1989) Nature 337, 476–478.
[9] Liu, J., Albers, M.W., Wandless, T.J., Luan, S., Alberg, D.G., Belshaw, P.J., Cohen, P., Macintosh, C., Klee, C.B. and Schreiber, S.L. (1992) Biochemistry 31, 3896–3901.
[10] McCaffrey, P.G., Perrino, B.A., Sorderling, T.R. and Rao, A. (1993) J. Biol. Chem. 268, 3747–3752.
[11] Wiesinger, D. and Borel, J.F. (1979) Immunobiology 156, 454.
[12] Cebrat, M., Lisowski, M., Siemion, I.Z., Zimecki, M. and Wieczorek, Z. (1997) J. Peptide Res. (in press).
[13] Standaert, R.F., Galat, A., Verdine, G.L. and Schreiber, S.L. (1990) Nature 346, 671–674.
[14] Handschumacher, R.E., Harding, M.W., Rice, J., Drugge, R.J. and Speicher, D.W. (1984) Science 226, 544–546.
[15] Blumenthal, D.K., Takio, K., Hansen, R.S. and Krebs, E.G. (1986) J. Biol. Chem. 261, 8140–8145.
[16] Katagiri, C., Kawamura, T., Matsuzawa, S.-I., Mizuno, Y., Matsusama, S. and Kikuchi, K. (1993) J. Biochem. 114, 874–878.
[17] Gallo, P., Saviano, M., Rossi, F., Pavone, V., Pedone, C., Ragone, R., Stiuso, P. and Colonna, G. (1995) Biopolymers 36, 273–281.
[18] Dumont, F.J., Staruch, M.J., Koprak, S.L., Merino, M.R. and Sigal, N.H. (1990) Immunology 144, 251.
[19] Siemion, I.Z., Cebrat, M., Lisowski, M., Zimecki, M. and Wieczorek, Z. (1992) Arch. Immunol. Ther. Exp. 40, 257–261.
[20] Husi, H., Luyten, M.A. and Zurini, M.G.M. (1994) J. Biol. Chem. 269, 14199–14209.
[21] Cardenas, M.E., Hemenway, C., Scott Muir, R., Ye, R., Fiorentino, D. and Heitman, J. (1994) EMBO L. 13, 5944–5957.