

# Critical amino acid residues of AIP, a highly specific inhibitory peptide of calmodulin-dependent protein kinase II

Atsuhiko Ishida<sup>a</sup>, Yasushi Shigeri<sup>b</sup>, Yoshiro Tatsu<sup>b</sup>, Koichi Uegaki<sup>b</sup>, Isamu Kameshita<sup>a</sup>, Sachiko Okuno<sup>a</sup>, Takako Kitani<sup>a</sup>, Noboru Yumoto<sup>b</sup>, Hitoshi Fujisawa<sup>a,\*</sup>

<sup>a</sup>Department of Biochemistry, Asahikawa Medical College, Asahikawa 078-8510, Japan

<sup>b</sup>Department of Organic Materials, Osaka National Research Institute, AIST, MITI, Midorigaoka, Ikeda, Osaka 563-0026, Japan

Received 26 January 1998; revised version received 30 March 1998

**Abstract** The importance of the individual amino acid residues of AIP (KKALRRQEAVDAL), a highly specific inhibitor of calmodulin-dependent protein kinase II (CaMKII), was studied. Replacement of Arg<sup>6</sup>, Gln<sup>7</sup>, or Ala<sup>9</sup> by other amino acid residues produced a marked increase in the IC<sub>50</sub> value. Leu<sup>4</sup> and Val<sup>10</sup> were also sensitive to replacement, but some hydrophobic amino acids could substitute for these residues. Although replacement of Ala<sup>3</sup>, Glu<sup>8</sup>, Ala<sup>12</sup>, and Leu<sup>13</sup> by other residues produced no significant increase in the IC<sub>50</sub>, the substitution of Lys for Ala<sup>3</sup> decreased the IC<sub>50</sub>. An AIP analog (KKKLRRQEAFDAY), in which Ala<sup>3</sup> and Val<sup>10</sup> were replaced with Lys and Phe, respectively, showed an IC<sub>50</sub> value as low as 4 nM, suggesting that it is a useful tool for studying the physiological roles of CaMKII.

© 1998 Federation of European Biochemical Societies.

**Key words:** Structure-function relationship; Inhibitor; Synthetic peptide; Specificity; Calmodulin-dependent protein kinase II

## 1. Introduction

Calmodulin-dependent protein kinase II (CaMKII) is known to be a second-messenger-responsive multifunctional protein kinase, which plays important roles in controlling a variety of cellular functions in response to an increase in intracellular Ca<sup>2+</sup>, especially in the central nervous system [1–4]. For studying the physiological roles of CaMKII, a selective and potent inhibitor of the enzyme is a useful tool. We have recently demonstrated that a synthetic peptide, designated as AIP, a nonphosphorylatable analog of a peptide substrate, autocalmitide-2 [5], is a highly specific and potent inhibitor of CaMKII [6]. AIP is 50 and 500 times more potent than CaMK-(281–302 Ala<sup>286</sup>) and KN-93, respectively, which have been widely used as CaMKII-specific inhibitors, under the assay conditions used. The inhibition by AIP is not affected by Ca<sup>2+</sup>/calmodulin, unlike those by KN series inhibitors. The use of AIP has recently revealed the involvement of CaMKII in cyclic ADP-ribose-mediated intracellular Ca<sup>2+</sup> mobilization in pancreatic islets [7], Ca<sup>2+</sup>/calmodulin-dependent phosphorylation of AMPA type glutamate receptor subunits in the post-synaptic density [8], and phosphorylation of phosrestin I, a homolog of arrestin which is involved in light

inactivation of rhodopsin in *Drosophila* [9]. Similar peptides to AIP have also been used to examine the role of CaMKII in the induction and maintenance of long-term potentiation in the CA1 region of the rat hippocampal slice [10]. Thus, the development of a potent specific inhibitor of CaMKII will offer a strong means to explore its physiological function.

## 2. Materials and methods

### 2.1. Materials

ATP and 1,2-dioleoyl-rac-glycerol were purchased from Sigma. Phosphatidylserine was from Serdary Research Laboratories, Inc. [ $\gamma$ -<sup>32</sup>P]ATP (5000 Ci/mmol) was from Amersham International. Syntide-2 [11], a synthetic peptide substrate, was synthesized by American Peptide Company, Inc. AIP (KKALRRQEAVDAL) [6,12] and its analogs were synthesized by a Shimadzu PSSM-8 automated peptide synthesizer as described previously [13]. Fmoc-L amino acids used for the peptide synthesis were obtained from BACHEM and Shimadzu. All these peptides were purified by reversed-phase HPLC, and their molecular masses were confirmed by mass spectrometry on a time-of-flight mass spectrometer (Shimadzu/Kratos Kompact MALDI II) with matrix-assisted laser desorption/ionization as described [13]. Concentrations of the peptides containing Tyr were determined spectrophotometrically using an absorption coefficient ( $\epsilon_{278}$ ) of  $1.16 \times 10^3$  M<sup>-1</sup> cm<sup>-1</sup> [13]. Concentrations of the peptides without Tyr were determined by amino acid analysis.

### 2.2. Protein preparations

CaMKII was purified from rat cerebral cortex as described previously [14]. Catalytic subunit of cyclic AMP-dependent protein kinase (PKA) was prepared from bovine heart as described [15]. Protein kinase C (PKC) was purified from rat cerebral cortex essentially according to the method of Woodgett and Hunter [16]. Calmodulin-dependent protein kinase IV (CaMKIV) was prepared as described previously [17]. Calmodulin was purified from *Escherichia coli* transformed with expression vector pET11d carrying a cDNA encoding chicken brain calmodulin [6,14]. 'CaM kinase II-depleted' preparations of a rat brain extract, 'P-through' preparation, the flow-through fraction of a phosphocellulose column, and 'CaM-through' preparation, the flow-through fraction of a calmodulin-Sepharose column, were obtained as described previously [6].

### 2.3. Assay of protein kinases

The activities of CaMKII (0.31  $\mu$ g/ml), CaMKIV (3.46  $\mu$ g/ml), PKA (0.80  $\mu$ g/ml) and PKC (0.33  $\mu$ g/ml) were measured as described previously [6]. The activities in the presence of various concentrations of inhibitors were expressed as percentages of those determined in their absence. The IC<sub>50</sub> was defined as the concentration of the inhibitor which gave 50% inhibition.

### 2.4. Other analytical procedures

The concentrations of CaMKII and calmodulin were determined spectrophotometrically as described previously [14]. The concentration of CaMKIV was determined as described [17]. Other proteins were determined by the method of Lowry et al. [18], as modified by Peterson [19], with bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [20].

\*Corresponding author. Fax: +81 (166) 65-9416.  
E-mail: fujisawa@asahikawa-med.ac.jp

**Abbreviations:** CaMKII, calmodulin-dependent protein kinase II; CaMKIV, calmodulin-dependent protein kinase IV; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C

Table 1  
Ala scanning and Tyr scanning analysis of AIP

Position	Residue	Substitution	Analog AIP	IC <sub>50</sub> (nM) 32	IC <sub>50</sub> (analog)/IC <sub>50</sub> (AIP)
1	Lys	Ala	K1A	238	7.4
		Tyr	K1Y	391	12
2	Lys	Ala	K2A	162	5.1
		Tyr	K2Y	198	6.2
3	Ala	Tyr	A3Y	41	1.3
4	Leu	Ala	L4A	~12 000	~375
		Tyr	L4Y	718	22
5	Arg	Ala	R5A	397	12
		Tyr	R5Y	281	8.8
6	Arg	Ala	R6A	~30 000	~940
		Tyr	R6Y	~21 000	~660
7	Gln	Ala	Q7A	1500	47
		Tyr	Q7Y	3700	116
8	Glu	Ala	E8A	21	0.66
		Tyr	E8Y	24	0.75
9	Ala	Tyr	A9Y	727	23
10	Val	Ala	V10A	1800	56
		Tyr	V10Y	271	8.5
11	Asp	Ala	D11A	371	12
		Tyr	D11Y	116	3.6
12	Ala	Tyr	A12Y	22	0.69
13	Leu	Ala	L13A	23	0.72
		Tyr	L13Y	26	0.81

### 3. Results and discussion

To know which amino acids of AIP are important for inhibition of CaMKII, the inhibitory activities of AIP analogs in which each amino acid of AIP was replaced by Ala (Ala scanning) or Tyr (Tyr scanning) residue were examined as shown in Table 1. The IC<sub>50</sub> values were determined from the inhibition curves of the respective AIP analogs. Some typical inhibition curves of AIP and AIP analogs are given in Fig. 1. The substitution of Ala or Tyr for the residues Ala<sup>3</sup>, Glu<sup>8</sup>, Ala<sup>12</sup> or Leu<sup>13</sup> (the amino acids are numbered starting at the amino terminus) did not significantly increase the IC<sub>50</sub> value of the peptide, suggesting that the side chains of amino acid residues 3, 8, 12 and 13 were not essential for its inhibitory action. On the other hand, the replacement of Lys<sup>1</sup>, Lys<sup>2</sup>, Arg<sup>5</sup>, or Asp<sup>11</sup> by Ala or Tyr increased the IC<sub>50</sub> by 4- to 12-fold, and the replacement of Leu<sup>4</sup>, Arg<sup>6</sup>, Gln<sup>7</sup>, Ala<sup>9</sup>, or

Val<sup>10</sup> increased the IC<sub>50</sub> by more than 20-fold (except for V10Y), suggesting the importance of these amino acid residues.

For obtaining further information about contribution of each amino acid of AIP to the inhibitory action, the IC<sub>50</sub> values of a number of substituted AIP analogs were determined as shown in Table 2. For easy determination of the concentration of the AIP analogs, the C-terminal Leu<sup>13</sup> of AIP was replaced with Tyr in the analogs, since the substitution of Tyr for Leu<sup>13</sup> did not significantly affect the inhibitory action of AIP (Fig. 1). We examined whether or not Leu<sup>4</sup> could be replaced with another hydrophobic amino acid residue, but none of several residues tested could decrease the IC<sub>50</sub> value. Replacement of Arg<sup>6</sup> with another positively charged hydrophilic amino acid produced an increase in the IC<sub>50</sub>, but the extent of the increase was an order of magnitude

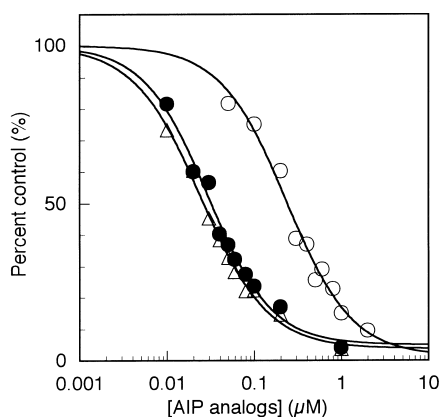


Fig. 1. Inhibition of CaMKII by AIP analogs. CaMKII activities were determined in the presence of the indicated concentrations of AIP (●), K1A-AIP (○) and L13Y-AIP (△), as described in Section 2.

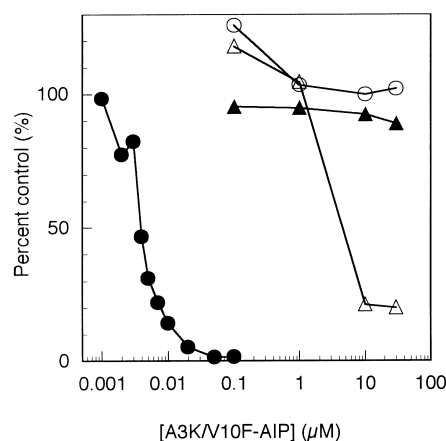


Fig. 2. Inhibition of several protein kinases by A3K/V10F-AIP. The activities of CaMKII (●), CaMKIV (○), PKA (▲) and PKC (△) were measured in the presence of the indicated concentrations of A3K/V10F-AIP as described in Section 2.

lower than that produced by the substitution of Ala or Tyr, suggesting the importance of a positively charged amino acid in this position of AIP. Although replacement of Arg<sup>5</sup> with Lys or His increased the IC<sub>50</sub>, the effects of the replacements were significantly smaller than those at the position 6. Replacement of Gln<sup>7</sup> with another amino acid, even Asn, produced a relatively great increase in the IC<sub>50</sub>, suggesting the importance of Gln in this position. Replacement of Ala<sup>9</sup> with other amino acids also produced a marked increase in the IC<sub>50</sub>. Since substitutions at the position 8 have no effect (Table 1), it is interesting that the adjacent positions 7 and 9 are highly susceptible to substitution. When substituted for Val<sup>10</sup>, some amino acids increased the IC<sub>50</sub>, but Phe and Ile decreased the IC<sub>50</sub> by 4-fold and 2-fold, respectively. Thus, the position 10 appears to be more tolerant for substitution than the position 4, though both of them absolutely require hydrophobic side chains.

Interestingly, the substitution of Lys for Ala<sup>3</sup> also decreased the IC<sub>50</sub> by 3- to 4-fold, and thus the substitutions of Phe for Val<sup>10</sup> and Lys for Ala<sup>3</sup> of AIP (A3K/V10F-AIP, Table 2) produced an almost 10-fold decrease in the IC<sub>50</sub>. The apparent IC<sub>50</sub> value of the A3K/V10F analog for CaMKII was as low as 4.1 nM, and this value is the lowest so far reported for CaMKII inhibitors. Since the apparent IC<sub>50</sub> value is comparable to the concentration of the enzyme used for the experiment, the actual IC<sub>50</sub> should be lower, because the IC<sub>50</sub> value largely depends on the concentration of the enzyme in such cases [21]. The selectivity of the A3K/V10F analog was examined with four representative second-messenger-responsive multifunctional protein kinases, PKA, PKC, CaMKIV, and

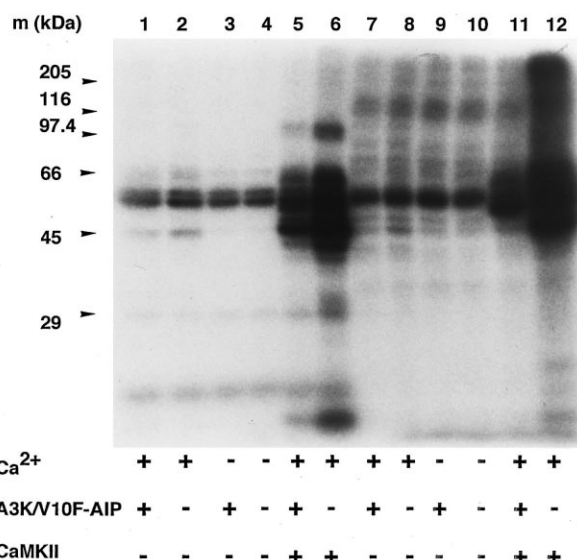


Fig. 3. Effect of A3K/V10F-AIP on the endogenous phosphorylation of the rat brain extract which was depleted of CaM kinase II. Two 'CaM kinase II-depleted' preparations of a rat brain extract, 'P-through' preparation (0.80 mg/ml, lanes 1–6) and 'CaM-through' preparation (0.26 mg/ml, lanes 7–12), were incubated at 30°C for 1 min in the mixture consisting of 40 mM HEPES-NaOH (pH 8.0), 5 mM magnesium acetate, 0.1 mM EGTA, 1 μM calmodulin, 0.01% Tween-20, and 50 μM [ $\gamma$ -<sup>32</sup>P]ATP, containing 1 mM CaCl<sub>2</sub> (lanes 1, 2, 5–8, 11, 12), 0.1 μM A3K/V10F-AIP (lanes 1, 3, 5, 7, 9, 11) or 0.96 μg/ml of purified CaM kinase II (lanes 5, 6, 11, 12). After incubation, 22.7 mM EDTA was added to stop the kinase reaction, and aliquots were subjected to SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel, followed by autoradiography.

Table 2  
IC<sub>50</sub> values of various substituted analogs of AIP

Position	Residue	Substitution	Analogues* AIP	IC <sub>50</sub> (nM) 32	IC <sub>50</sub> (analog)/IC <sub>50</sub> (AIP)
3	Ala	Lys	A3K	9.2	0.29
4	Leu	Phe	L4F	57	1.8
		Norleucine	L4Nle	66	2.1
		Ile	L4I	171	5.3
		Norvaline	L4Nva	247	7.7
		Met	L4M	363	11
		Val	L4V	418	13
		Gly	L4G	> 70 000	> 2200
5	Arg	Lys	R5K	66	2.1
		His	R5H	173	5.4
6	Arg	Orn	R6Orn	285	8.9
		Lys	R6K	338	11
		N <sup>G</sup> ,N <sup>G</sup> -dimethyl-arginine	R6dmR	960	30
		Citrulline	R6Cit	1200	38
		His	R6H	4300	134
7	Gln	Glu	Q7E	2600	81
		Asp	Q7D	5100	159
		Asn	Q7N	6800	213
		Orn	Q7Orn	8600	269
9	Ala	Gly	A9G	344	11
		Cys	A9C	354	11
		Val	A9V	938	29
		Ile	A9I	2800	88
		Leu	A9L	3700	116
10	Val	Phe	V10F	8.0	0.25
		Ile	V10I	16	0.50
		Leu	V10L	39	1.2
		Norvaline	V10Nva	60	1.9
		2-Aminobutylic acid	V10Abu	231	7.2
		Gly	V10G	5200	163
3, 10	Ala <sup>3</sup> /Val <sup>10</sup>	Lys <sup>3</sup> /Phe <sup>10</sup>	A3K/V10F	4.1	0.13

\*The C-terminal residue of the AIP analogs shown in this table except R5H was replaced with Tyr.

<b>AIP</b>				<b>K</b>	<b>K</b>	<b>A</b>	<b>L</b>	<b>R</b>	<b>R</b>	<b>Q</b>	<b>E</b>	<b>A</b>	<b>V</b>	<b>D</b>	<b>A</b>	<b>L</b>
residue number				1	2	3	4	5	6	7	8	9	10	11	12	13
substitution improving the potency				<i>K</i>									<i>F</i>			
<b>virtual optimal substrate</b>																
residue number																
				<b>K</b>	<b>R</b>	<b>Q</b>	<b>Q</b>	<b>S</b>	<b>F</b>	<b>D</b>	<b>L</b>	<b>F</b>				
				-4	-3	-2	-1	0	+1	+2	+3	+4				

Fig. 4. Alignment of amino acid sequences of AIP and a virtual optimal peptide substrate reported by Songyang et al. [23]. The amino acid residues of AIP which are essential for the potent inhibition of CaMKII are indicated by bold letters. The substitutions which markedly reduce the  $IC_{50}$  value are shown in italics below the residue numbers. In the sequence of the virtual optimal substrate, the residues which CaMKII prefers strongly are shown in bold letters. The amino acid residue which is phosphorylated by CaMKII is numbered 0, in the virtual optimal substrate.

CaMKII, as shown in Fig. 2. Among them, the activities of PKA and CaMKIV were not inhibited at all. The activity of PKC was inhibited at a concentration of the A3K/V10F analog as high as 10  $\mu$ M, but not significantly inhibited at a concentration (1  $\mu$ M) more than two orders of magnitude higher than the  $IC_{50}$  for CaMKII (4.1 nM). The selectivity of the analog was also examined with two 'CaM kinase II-depleted' preparations of a rat brain extract, 'P-through' and 'CaM-through' preparations, which were prepared by two distinct procedures as described in Section 2 (Fig. 3). The A3K/V10F analog (0.1  $\mu$ M) did not significantly affect the endogenous phosphorylation of both preparations (lanes 1–4 and 7–10), whereas phosphorylation of the two preparations caused by the addition of purified CaM kinase II was markedly inhibited by 0.1  $\mu$ M A3K/V10F-AIP (lanes 5, 6, 11, 12). These results indicate that 0.1  $\mu$ M of the analog had no effect on the activities of protein kinases other than CaM kinase II in the brain extract, whereas it markedly inhibited the phosphorylation by CaM kinase II. Thus, the A3K/V10F-AIP is a very potent and specific inhibitor, and it will provide a strong means for studying the physiological functions of CaMKII.

The question of whether the peptide inhibitor is susceptible to proteolytic degradation in cells is important. It has been reported that acetylation and amidation of N- and C-terminals, respectively, of an AIP analog greatly enhances its effectiveness in neuronal cells, probably owing to the increased resistivity to proteolysis [10]. Replacement of the Arg residue within the Arg cluster of a peptide inhibitor of PKA, PKI(6–24), with D-Arg is effective in increasing the in vivo half-life of the peptide [22]. Considering that Lys<sup>1</sup> (N-terminus), Arg<sup>5</sup>, and Leu<sup>13</sup> (C-terminus) of AIP can be replaced by other amino acid residues without leading to a great decrease in the inhibitory effect, such modifications or substitutions may improve resistivity to proteolytic degradation without significant loss of their inhibitory potencies.

Based on specificity analysis using an oriented degenerate peptide library, Songyang et al. [23] have recently reported that a virtual optimal substrate sequence for CaMKII is KRQQSFDLF, and that CaMKII prefers Arg at the –3 position, Gln at the –2 position and Phe at the +1 position in its substrate (Fig. 4). Since AIP is a nonphosphorylatable analog of autocalcine-2 (KKALRRQETVDAL), of which phosphorylation site is Thr<sup>9</sup>, AIP and the virtual optimal peptide substrate can be aligned as shown in Fig. 4. Thus, the optimal sequence of the peptide substrate suggested by them are in good agreement with that of the inhibitory peptide obtained in the present study. Since AIP inhibits CaMKII by binding to the substrate-binding site for autophosphorylation site, which is distinct from the binding site for the exogenous sub-

strate such as syntide-2 [6,12], the optimal sequence of CaMKII substrate reported by Songyang et al. appears to be not for the exogenous substrate but for the autophosphorylation site.

**Acknowledgements:** This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan, and by grants from the Byotai Taisha Research Foundation, the Mitsubishi Foundation, and the Uehara Memorial Foundation.

## References

- [1] Fujisawa, H. (1990) *BioEssays* 12, 27–29.
- [2] Braun, A.P. and Schulman, H. (1995) *Annu. Rev. Physiol.* 57, 417–445.
- [3] Soderling, T.R. (1996) *Biochim. Biophys. Acta* 1297, 131–138.
- [4] Lisman, J., Malenka, R.C., Nicoll, R.A. and Malinow, R. (1997) *Science* 276, 2001–2002.
- [5] Hanson, P.I., Kapiloff, M.S., Lou, L.L., Rosenfeld, M.G. and Schulman, H. (1989) *Neuron* 3, 59–70.
- [6] Ishida, A., Kameshita, I., Okuno, S., Kitani, T. and Fujisawa, H. (1995) *Biochem. Biophys. Res. Commun.* 212, 806–812.
- [7] Takasawa, S., Ishida, A., Nata, K., Nakagawa, K., Noguchi, N., Tohgo, A., Kato, I., Yonekura, H., Fujisawa, H. and Okamoto, H. (1995) *J. Biol. Chem.* 270, 30257–30259.
- [8] Hayashi, Y., Ishida, A., Katagiri, H., Mishina, M., Fujisawa, H., Manabe, T. and Takahashi, T. (1997) *Mol. Brain Res.* 46, 338–342.
- [9] Kahn, E.S. and Matsumoto, H. (1997) *J. Neurochem.* 68, 169–175.
- [10] Otmakhov, N., Griffith, L.C. and Lisman, J.E. (1997) *J. Neurosci.* 17, 5357–5365.
- [11] Hashimoto, Y. and Soderling, T.R. (1987) *Arch. Biochem. Biophys.* 252, 418–425.
- [12] Ishida, A. and Fujisawa, H. (1995) *J. Biol. Chem.* 270, 2163–2170.
- [13] Murase, S., Yumoto, N., Petukhov, M.G. and Yoshikawa, S. (1996) *J. Biochem.* 119, 37–41.
- [14] Ishida, A., Kitani, T., Okuno, S. and Fujisawa, H. (1994) *J. Biochem.* 115, 1075–1082.
- [15] Okuno, S. and Fujisawa, H. (1990) *Biochim. Biophys. Acta* 1038, 204–208.
- [16] Woodgett, J.R. and Hunter, T. (1987) *J. Biol. Chem.* 262, 4836–4843.
- [17] Kameshita, I. and Fujisawa, H. (1995) *J. Biochem.* 117, 85–90.
- [18] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275.
- [19] Peterson, G.L. (1977) *Anal. Biochem.* 83, 346–356.
- [20] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [21] Williams, J.W. and Morrison, J.F. (1979) *Methods Enzymol.* 63, 437–467.
- [22] Fernandez, A., Mery, J., Vandromme, M., Basset, M., Cavadore, J.-C. and Lamb, N.J.C. (1991) *Exp. Cell Res.* 195, 468–477.
- [23] Songyang, Z., Lu, K.P., Kwon, Y.T., Tsai, L.-H., Filhol, O., Cochet, C., Brickey, D.A., Soderling, T.R., Bartleson, C., Graves, D.J., DeMaggio, A.J., Hoekstra, M.F., Blenis, J., Hunter, T. and Cantley, L.C. (1996) *Mol. Cell. Biol.* 16, 6486–6493.