High affinity calmodulin target sequence in the signalling molecule PI 3-kinase

Roland Fischer, Juanitta Julsgart, Martin W. Berchtold*

Institute of Molecular Cell Biology, University of Copenhagen, Oster Farimagsgade 2A, 1353 Copenhagen K, Denmark

Received 12 February 1998

Abstract In this study we report that phosphatidylinositol 3-kinase (PI 3-kinase), a lipid kinase which participates in downstream signalling events of heterotrimeric G protein-coupled receptors and receptor tyrosine kinases, contains a high affinity binding site for calmodulin (CaM). The putative CaM-binding peptide derived from the p110γ isoform interacts with CaM in a calcium-dependent way. Using gel shift analysis and fluorescence spectrophotometry we discovered that the peptide forms a high affinity complex with CaM. Titration experiments using dansylated CaM gave an affinity constant of 5 nM. Furthermore, a sequence comparison among different PI 3-kinase isoforms revealed that the sequence which can bind CaM is highly conserved within different PI 3-kinase isoforms. These results indicate a novel mechanism for regulating PI 3-kinase and provide a new direct link between Ca^{2+} and phospholipid signalling pathways.

Key words: Calcium; Calmodulin; Phosphatidylinositol 3-kinase; Signal transduction

1. Introduction

Calcium and its principal receptor calmodulin (CaM) are important signalling molecules. Ca^{2+}/CaM is involved in multiple processes such as mitogenesis, cell growth, differentiation and apoptosis [1]. However, molecular mechanisms of Ca^{2+}/CaM-regulated growth and death pathways are poorly understood. We and others have recently described that members of a novel subgroup of ras-related GTP-binding proteins bind CaM with high affinity and in a Ca^{2+}-dependent manner [2,3]. We have extended our efforts to identify signalling molecules which are CaM targets. In this study we show evidence that phosphatidylinositol 3-kinase (PI 3-kinase) is a putative CaM target.

PI 3-kinase is a lipid kinase capable of phosphorylating phosphoinositides at the 3′ position. PI 3-kinases are activated upon stimulation of cells with growth factors and agonists which transmit their signals through either tyrosine kinase receptors or heterotrimeric G proteins. Several variants of PI 3-kinases have been cloned and characterised [4,5]. Heterodimeric PI 3-Kα and PI 3-Kβ, consisting of p110 catalytic subunits and different p85 adaptor molecules, are regulated by receptors with tyrosine kinase activity. Another PI 3-kinase isotype, termed PI 3-K p110γ, can be activated in vitro by both α and βγ subunits of heterotrimeric G proteins but does not interact with p85 [6–8]. The role of PI 3-kinase family members in growth- and survival-related signalling has only been discovered recently. Interestingly, mutations in a PI 3-kinase gene of *Caenorhabditis elegans* cause a threefold increase in the lifespan [9]. A retrovirus-encoded PI 3-kinase was found to cause haemangiosarcomas in chickens and to transform fibroblasts [10] Various downstream effector targets of PI 3-kinase activity have been identified, including the serine-threonine kinase PKB/Akt and GRP1 [11–13]. PKB/Akt participates in the activation of the p70 ribosomal protein S6 kinase ($p70^{S6K}$). Moreover, PKB/Akt provides a survival signal that protects cells from apoptosis induced by various stress signals in several cell types [14–16].

To understand the cross-talk between the Ca^{2+}/CaM and the ras or other growth- or death-signalling pathways in more detail we are specifically looking for potential CaM-binding motifs in molecules which participate in important signal transduction pathways. In this study we show evidence that PI 3-kinase is a target for Ca^{2+}/CaM.

2. Materials and methods

2.1. Materials

The peptide L-A-R-F-L-L-K-R-G-L-R-N-K-R-I-G-H-F-L-F-W-F-L-R-S corresponding to residues 677–700 of the reported PI 3-kinase p110γ sequence [6] was purchased from Anawa, Switzerland. The peptide was 70% pure as measured by mass spectrometry. The peptide was purified by reverse-phase HPLC on a Sephasil C8 column (4.6/100 mm). Buffer A was 0.1% TFA in water, buffer B was 0.1% TFA in 50% acetonitrile. The peptide was eluted using a linear gradient from 100% buffer A to 100% buffer B. The purity of the peptide was confirmed by amino acid analysis and mass spectrometry. Dansylcalmodulin was purchased from Sigma.

2.2. Fluorescence measurements

Fluorescence measurements were performed with a Shimadzu fluorescence spectrophotometer RF-5301PC as described by Fischer et al. [2]. The concentration of the peptide was determined by amino acid analysis. The affinity constant of the PI 3-kinase peptide was calculated according to the method of Stinson and Holbrook [17].

2.3. Gel electrophoresis in the presence of urea

Gel shift analyses of PI 3-kinase peptide/CaM complexes were performed according to [18].

3. Results and discussion

The amino acid sequences of several recently discovered signal transduction molecules were compared with those of known high affinity CaM-binding proteins. We have searched for amino acid sequences which match the described structural features of known CaM-binding motifs [19,20]. We discovered that the lipid kinase PI 3-kinase (p110γ) contains a putative sequence which matches the described features of typical CaM-binding peptides. The CaM-binding motif generally has a hydrophobic/basic amino acid composition with a propensity to form an amphiphilic helix and frequently con-

*Corresponding author. Fax: (45) 33 93 52 20.
E-mail: mabe@biobase.dk

PII S0014-5793(98)00225-7

p110γ	677	A	R	F	**L**	L	K	R	**G**	L	R	**N**	K	R	I	G	H	**F**	L	F	W	F	L	R	S	700
p110α	650	V	R	F	**L**	L	K	K	**A**	L	T	**N**	Q	R	I	G	H	**F**	F	F	W	H	L	K	S	673
p110β	657	S	R	F	**L**	L	E	R	**A**	L	G	**N**	R	R	I	G	Q	**F**	L	F	W	H	L	R	S	680
p110δ	630	T	K	F	**L**	L	D	R	**A**	L	A	**N**	R	K	I	G	H	**F**	L	F	W	H	L	R	S	653
Kir/Gem	267	R	R	F	**W**	G	K	I	**V**	A	K	**N**	N	K	N	M	A	**F**	K	L	K	S	K	S	C	290
Rad	240	K	R	F	**L**	G	R	I	**V**	A	R	**N**	S	R	K	M	A	**F**	R	A	K	S	K	S	C	263
Capump	1101	R	R	G	**Q**	I	L	W	**F**	R	G	**L**	N	R	I	Q	T	**Q**	I	R	V	V	N	A	F	1124
sm MLCK	473	R	R	K	**W**	Q	K	T	**G**	H	A	**V**	R	A	I	G	R	**L**	S	S	M	A	M	I	S	496

Fig. 1. Alignment of the putative CaM-binding sequences in PI 3-kinase isoforms with known CaM-binding proteins. The putative CaM-binding sequences of PI 3-kinases are aligned with the known CaM-binding domains of Kir/Gem, Rad, the Ca^{2+}-pumping ATPase and smooth muscle myosin light chain kinase. The locations of conserved hydrophobic residues are shown in bold.

tains an aromatic residue at the N-terminus of the domain [20]. In addition, two aromatic and/or long chain aliphatic residues are often separated by 12 amino acid residues. Fig. 1 shows the putative CaM-binding region of PI 3-kinase p110γ and the highly related sequences in the α, β α and δ isoforms and for comparison the recently described ras-related Kir/Gem [2] and the well characterised CaM-binding domains of the Ca^{2+}-pumping ATPase and smooth muscle myosin light chain kinase.

We have synthesised a peptide corresponding to amino acid residues 677–700 of the reported PI 3-kinase p110γ sequence (Fig. 1). We first tested whether the putative CaM-binding domain of PI 3-kinase is able to bind CaM by the band shift assay in urea gels. Non-denaturing gel electrophoresis in the presence of 4 M urea revealed that the PI 3-kinase peptide bound to CaM and induced a shift of the complex in the presence of 0.1 mM $CaCl_2$ (Fig. 2A). Since two shifted bands with different mobilities were found, it is possible that the CaM/peptide complex has two different conformations. At a 1:1 peptide/CaM ratio CaM was almost quantitatively found in a shifted complex. This indicates a peptide/CaM interaction with a 1:1 stoichiometry. Addition of 2 mM EGTA completely abolished complex formation indicating that the interaction of PI 3-kinase with CaM was Ca^{2+}-dependent (Fig. 2B).

The interaction of PI 3-kinase with CaM was also studied using fluorescence spectrophotometry. As shown in Fig. 3, titration of dansylcalmodulin with the PI 3-kinase peptide saturated in the nanomolar concentration range in the presence of 0.1 mM $CaCl_2$. The maximum in the emission spectrum of the binding peptide underwent a shift to a lower wavelength and an increase in intensity upon complex formation with dansylcalmodulin. No increase in intensity and shift of the spectrum was observed in control experiments (peptide alone plus calcium, dansylcalmodulin plus calcium, peptide and dansylcalmodulin in the presence of EGTA, data not shown).

The PI 3-kinase peptide bound CaM with an affinity constant of 5 nM as determined by titration experiments with dansylcalmodulin (Fig. 4). The plot of relative fluorescence versus the molar ratio of PI 3-kinase to dansylcalmodulin is shown. This is well in the range of known high affinity CaM-binding proteins such as the Ca^{2+}-pumping ATPase or smMLCK. Both proteins bind CaM with affinity constants in the nanomolar range.

Recently it has become clear that PI 3-kinases form a large

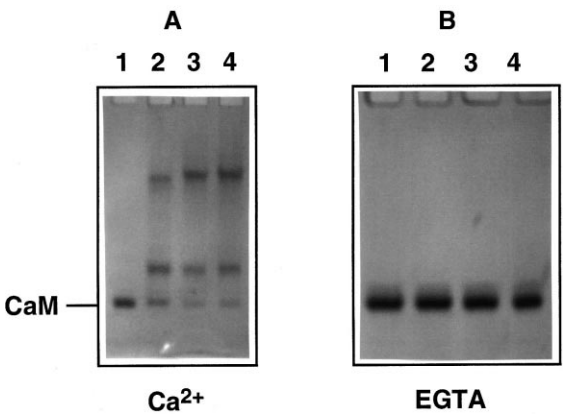

Fig. 2. Urea gel shift analysis. Gel shift analyses of PI 3-kinase peptide with CaM were performed according to [15]. Slab gels of 12% acrylamide, 4 M urea, 0.375 M Tris-HCl, pH 8.8 and 0.1 mM $CaCl_2$ (A) or 2 mM EGTA (B) were run at a constant current of 25 mA. All lanes contain 200 pmol CaM. Lane 1, CaM alone; lane 2, plus 100 pmol of peptide; lane 3, plus 200 pmol of peptide; lane 4, plus 400 pmol of peptide.

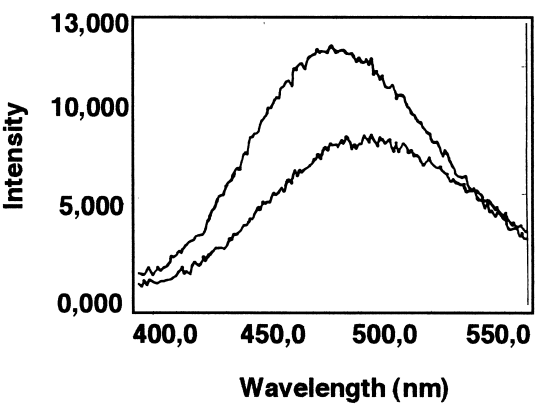

Fig. 3. Direct interaction of CaM with the PI 3-kinase p110γ peptide. Emission spectrum of dansylcalmodulin after complex formation with the PI 3-kinase peptide. Dansylcalmodulin (50 nM) was treated with 30 nM peptide in the presence of 100 μM calcium in 20 mM HEPES, pH 7.2, 130 mM KCl. Excitation was performed at 340 nm. Binding of PI 3-kinase peptide to dansylcalmodulin results in fluorescence enhancement and a blue shift.

Table 1
Comparison of the full length and CaM-binding sequences of α, β, γ and δ isoforms of PI 3-kinases

	p110α		p110β		p110δ	
	CaM peptide	Protein	CaM peptide	Protein	CaM peptide	Protein
p110γ	70 (78)	36 (47)	71 (75)	36 (48)	70 (83)	36 (48)
p110α			70 (78)	43 (53)	65 (83)	42 (52)
p110β					70 (86)	59 (67)

Values are given in % identity (values for % similarity are given in parentheses).

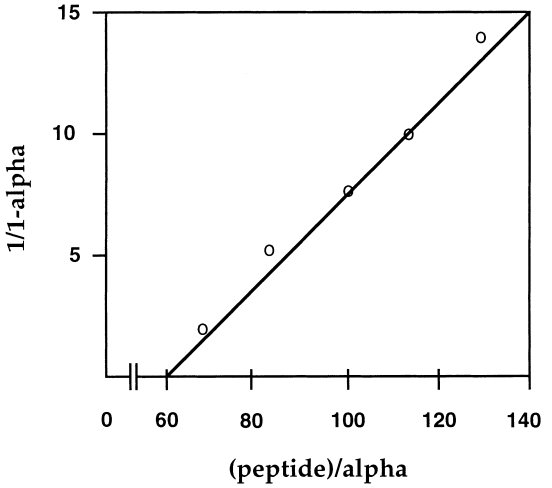

Fig. 4. Determination of the affinity constant for the interaction between CaM and the PI 3-kinase p110γ peptide. Dansylcalmodulin (140 nM) was titrated with the PI 3-kinase peptide in the presence of 100 μM Ca^{2+}. The data points were recorded, and the fractional degree of saturation of dansylcalmodulin was calculated. The plot of $1/(1-\alpha)$ against the free concentration (nanomolar) of the peptide divided by α results in a straight line if a 1:1 complex is formed and the titration end point is correctly estimated. The reciprocal of the slope gives the affinity constant. The affinity constant was calculated according to [17]. The calculated K_d is 5 nM.

family of important signalling enzymes which act downstream of either heterotrimeric G protein-coupled receptors or tyrosine kinase receptors. Interestingly, a comparison of the different PI 3-kinase isoforms revealed that the putative CaM-binding domain of p110γ is highly conserved in α and β isoforms of PI 3-kinases (see Fig. 1). Whereas α, β and δ isoforms have an average identity/similarity of 42%/53%, it is 69%/80% in the putative CaM-binding domain (Table 1). That the putative CaM-binding sequence is highly conserved within the different isoforms suggests that CaM might regulate or modulate the lipid kinase activity of all PI 3-kinase family members. The finding that the used peptide derived from p110γ is able to bind CaM with high affinity and in a Ca^{2+}-dependent manner suggests that α, β and δ isoforms of PI 3-kinases also are targets for Ca^{2+}/CaM. Evidence that PI 3-kinases are indeed targets for Ca^{2+}/CaM has recently been reported by Jojal et al. [21]. These authors describe that CaM binding to p85 activates PI 3-kinase. Interestingly, they mapped the CaM-binding domain within the Src homology 2 domain (SH2) of the regulatory subunit p85 using a CaM-Sepharose 'pull down' assay, though without showing the precise CaM-binding sequence. The putative CaM-binding peptide in our study, however, has been derived from the catalytic subunit of p110γ. The sequence lies in the so-called PIK or homology region 2 (HR2) domain which is located N-terminal of the kinase core domain [22]. Taken together these results indicate that additional mechanisms of regulation of PI 3-kinases exist and that PI 3-kinases interconnect with Ca^{2+}/CaM signalling pathways within cells.

References

[1] Means, A.R. (1994) FEBS Lett. 347, 1–4.
[2] Fischer, R., Wei, Y., Anagli, J. and Berchtold, M.W. (1996) J. Biol. Chem. 271, 25067–25070.
[3] Wes, P.D., Yu, M. and Montell, C. (1996) EMBO J. 15, 5839–5848.
[4] Vanhaesebroeck, B., Leevers, S.J., Panayotou, G. and Waterfield, M.D. (1997) Trends Biochem. Sci. 22, 267–272.
[5] Toker, A. and Cantley, L.C. (1997) Nature 387, 673–676.
[6] Stoyanov, B., Volinia, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R., Nurnberg, B., Gierschik, P., Seedorf, K., Hsuan, J.J., Waterfield, M.D. and Wetzker, R. (1995) Science 264, 690–693.
[7] Lopez-Iiasaca, M., Crespo, P., Pellici, P.G., Gutkind, J.S. and Wetzker, R. (1997) Science 275, 394–397.
[8] Stephens, L.R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A.S., Thelen, M., Cadwallader, K., Tempst, P. and Hawkins, P.T. (1997) Cell 89, 105–114.
[9] Morris, J.Z., Tissenbaum, H.A. and Ruvkun, G. (1996) Nature 382, 536–539.
[10] Chang, H.W., Aoki, M., Fruman, D., Auger, K.R., Bellacosa, A., Tsichlis, P.N., Cantley, L.C., Roberts, T.M. and Vogt, P.K. (1997) Science 276, 1848–1850.
[11] Franke, T.F., Kaplan, D.R., Cantley, L.C. and Toker, A. (1997) Science 275, 665–668.
[12] Stokoe, D., Stephens, L.R., Copeland, T., Gaffney, P.R., Reese, C.B., Painter, G.F., Holmes, A.B., McCormick, F. and Hawkins, P.T. (1997) Science 277, 567–570.
[13] Klarlund, J.K., Guilherme, A., Holik, J.J., Virbasius, J.V., Chawla, A. and Czech, M.P. (1997) Science 275, 1927–1930.
[14] Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J. and Evan, G. (1997) Nature 385, 544–548.
[15] Franke, T.F., Kaplan, D.R. and Cantley, L.C. (1997) Cell 88, 435–437.
[16] Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R. and Greenberg, M.E. (1997) Science 275, 661–665.
[17] Stinson, R.A. and Holbrook, J.J. (1973) Biochem. J. 131, 719–728.
[18] Erickson-Viitanen, S. and DeGrado, W.F. (1987) Methods Enzymol. 139, 455–478.
[19] O'Neil, K.T. and DeGrado, W.F. (1990) Trends Biochem. Sci. 15, 59–64.
[20] James, P., Vorherr, T. and Carafoli, E. (1995) Trends Biochem. Sci. 20, 38–42.
[21] Joyal, J.L., Burks, D.J., Pons, S., Matter, W.F., Vlahos, C.J., White, M.F. and Sacks, D.B. (1997) J. Biol. Chem. 272, 28183–28186.
[22] Vanhaesebroeck, B., Welham, M.J., Kotani, K., Stein, R., Warne, P.H., Zvelebil, M.J., Higashi, K., Volinia, S., Downward, J. and Waterfield, M. (1997) Proc. Natl. Acad. Sci. USA 94, 4330–4335.