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Influence of the effector peptide of MARCKS-related protein on actin polymerization: a kinetic analysis

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Abstract

The members of the MARCKS protein family, MARCKS (an acronym for *m*yristoylated *a*lanine-*r*ich *C k*inase *s*ubstrate) and MARCKS-related protein (MRP), interact with membranes, protein kinase C, and calmodulin via their effector domain, a highly basic segment composed of 24–25 amino acid residues. This domain is also involved in the interaction between MARCKS/MRP and actin. In this article we show that a peptide corresponding to the effector domain of MRP, the effector peptide, strongly influences the dynamics of actin polymerization. Depending on the stoichiometric ratio of effector peptide to actin the peptide either accelerates or retards the actin polymerization process, which takes place in the presence of near-physiological salt concentrations. A model is developed in which this phenomenon is explained by two independent nucleation processes involving *free* actin monomers and *peptide-bound* actin monomers, respectively. As a control, a possible regulatory mechanism has been investigated: we show that calmodulin inhibits the actin polymerizing activity of the MRP effector peptide, thereby validating our model approach.

Keywords: Actin polymerization; MARCKS-related protein; Kinetics; Protein–protein interactions

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1. Introduction

Actin is a ubiquitous, 42-kDa globular protein which — due to its ability to polymerize into filaments (F-actin) — is one of the major constituents of the cytoskeleton. The actin cytoskeleton gives the cell its typical shape and maintains its flexibility to facilitate dynamic events such as cell crawling and cell shape changes (for reviews see [1–4]). These actin-based motility events are related to two properties of the actin cytoskeleton: (1) its rearrangement in different meshwork structures which can produce filopodia, lamellipodia, or 'stress fibers' [5]; and (2) its rapid turnover, which results from a dynamic combination of polymerization and depolymerization at the filament ends [6].

Both the change in the actin meshwork structure and the control of actin polymerization dynamics are governed by a large variety of actin-binding proteins (for reviews see [7–9]). Among the proteins that have been proposed to interact with actin, in particular to cross-link actin at the plasma membrane [10] are the two members of the myristoylated alanine-rich C kinase substrate (MARCKS) protein family: MARCKS and MARCKS-related protein (MRP). MARCKS is a ubiquitous protein with a molecular weight of 32 kDa [11,12], whereas MRP is a 20-kDa protein which is predominantly expressed in brain and reproductive tissues [13]. Both proteins are essential for brain development [14–16], and they colocalize with actin and actin-binding proteins during cellular events that require reorganization of the cytoskeleton [17,18]. At the molecular level MARCKS and MRP bind calmodulin (CaM) in a Ca^{2+}-dependent manner with very high affinity [19,20], are phosphorylated by protein kinase C (PKC) [21,22], and bind to acidic lipid membranes [23,24]. All of the aforementioned interactions are mediated by the so-called effector domain [11,25,26], a 24–25-amino acid residue long segment which is highly conserved in both proteins (approx. 96% homology). Hence, many properties of MARCKS and MRP are modeled in vitro by peptides representing the corresponding effector domains, the so-called effector peptides [10,25,26]. The MRP effector peptide is both highly basic and hydrophobic and has the following primary sequence:

KKKKK*F*S*F***KK**P*F***K***L*SG*L*S*F***KR**N**RK**

with the basic amino acid residues typed in bold letters and the hydrophobic residues in italics. It basically differs from the MARCKS effector peptide only in that it lacks one arginine after the five N-terminal lysine residues, and in the proline residue which is replaced by a serine in MARCKS.

Recently, we have documented that — besides its tendency to associate actin filaments laterally into bundles (for a more detailed review on the effect of polycations on actin see also [27,28]) — the effector peptide of MRP spontaneously, i.e. in the absence of salts, causes polymerization of actin monomers into filaments with high efficiency.[3] In the presence of 2 mM $MgCl_2$ and 50 mM KCl we have found that this effect on actin polymerization kinetics can be both accelerating as well as retarding, depending on the stoichiometric ratio of effector peptide to actin. In this report, we now analyze this puzzling result by developing a mathematical model which qualitatively explains the observed behavior. In addition, we present experimental data aimed at verifying our model by using CaM as an inhibitor of the interaction between the MRP effector peptide and actin.

2. Experimental procedures

2.1. Proteins and peptides

Actin was prepared from rabbit skeletal muscle according to the method of Spudich and Watt [29] as recently described [30] and stored at 4°C in G-buffer [2.5 mM imidazole (pH 7.4), 0.2 mM $CaCl_2$, 0.2 mM ATP] containing 0.001% NaN_3. For fluorescence measurements, actin was labeled at cysteine 374 with *N*-(1-pyrene)iodoacetamide (Molecular Probes Europe BV, Leiden, The Netherlands) according to the method of Kouyama and Mihashi [31]. The average extent of pyrene-labeling was $\approx 80\%$. Actin concentration

and its extent of labeling were determined as described by Cooper et al. [32]. The absorption measurements were performed on a Kontron UVIKON spectrophotometer (Kontron Instruments, Zürich, Switzerland).

A peptide corresponding to the effector domain of murine MRP (86–109), the MRP effector peptide, was obtained from AMS Biotechnology (Lugano, Switzerland). The amino acid composition as well as the concentration of the effector peptide was determined by quantitative amino acid analysis. CaM from bovine brain was purchased from Sigma (Div. of Fluka Chemie AG, Buchs, Switzerland). All other chemicals were purchased from Fluka (Fluka Chemie AG, Buchs, Switzerland) in the highest purity grade available.

2.2. Fluorescence spectroscopy

Actin polymerization was followed by observing the increase in fluorescence of the pyrenated actin moiety, according to Kouyama and Mihashi [31]. Unless indicated otherwise, pyrene-labeled actin was mixed with unlabeled actin to a final extent of 5%. A volume of 400 μl actin solution was added to 5×5-mm quartz glass cuvettes (Hellma Suprasil, Hellma, Müllheim, Germany), and the fluorescence was recorded at an angle of 90°. Excitation was at 365 nm with a bandwidth of 1.5 nm, and the fluorescence emission was measured at 407 nm with a bandwidth of 5 nm. To minimize scattering effects, a cut-off filter at 395 nm was used for the emitted light. The measurements were performed on a JASCO Spectrofluorometer FP-777 (Japan Spectroscopic Co., Ltd., Tokyo, Japan). In all experiments a temperature of 20°C was maintained. The data were evaluated with EasyPlot 3.0 (Spiral Software, Brookline, MA, USA, and Massachusetts Institute of Technology, Cambridge, MA, USA). Unless otherwise indicated, polymerization of actin was induced by addition of $MgCl_2$ and KCl to G-buffer to final concentrations of 2 mM and 50 mM, respectively (F-buffer). All polymerization experiments were performed with at least three different actin preparations to make sure that the observed results were not due to variations in the quality of single actin preparations.

2.3. Kinetic theory of actin polymerization

Wegner and co-workers [33–35] documented that the time course of actin polymerization can be qualitatively described as a nucleation/elongation process. In this scheme, the first step in filament formation is a nucleation step in which n monomers associate to form a nucleus. Nucleation is followed by a second step, elongation, i.e. the binding of monomers to actin filament ends with the association and dissociation rates of monomers to a filament end being independent from the filament length. By neglecting fragmentation of actin filaments, the polymerization problem can be reduced to a system which is described by two differential equations [36,37]:

$$\frac{dC}{dt} = (kc_1 - k')c_1^n \prod_{i=1}^{n-1} \frac{k_i}{k'_i} \quad (1)$$

and

$$\frac{dc_1}{dt} = -(kc_1 - k')C \quad (2)$$

In these equations C (in M) denotes the molar concentration of filaments, c_1 (in M) is the monomer concentration, n is the number of monomers building up a stable nucleus, k (in $M^{-1}\ s^{-1}$) and k' (in s^{-1}) are the association and dissociation constants for filament elongation (i.e. when the elongation steps are independent from filament length), and k_i (in $M^{-1}s^{-1}$) and k'_i (in s^{-1}) are the rate constants for the association and dissociation of nuclei of size i.

It should further be noted that both k and k' represent the sum of the rate constants at both ends of the actin filaments.

2.4. Modeling actin polymerization

Numerical calculations for the simulation of actin polymerization in the presence of the MRP effector peptide were performed with Mathematica 2.0 (Wolfram Research Inc., Champaign, IL, USA).

3. Results and discussion

We have recently documented that a peptide corresponding to the MRP effector domain spontaneously, i.e. in the absence of salts, causes actin to polymerize into filamentous bundles.[3] Furthermore, we found that, depending on the stoichiometry of the effector peptide to actin, this peptide can either accelerate or retard salt-induced actin polymerization. To better understand this surprising effect on a molecular basis, we assessed this problem quantitatively by titrating G-actin solutions with different amounts of MRP effector peptide in the presence of salt. A model which accounts for the unusual effector peptide concentration dependence is then developed.

3.1. Actin polymerization in the presence of salt and the MRP effector peptide

Actin polymerization was induced by addition of 2 mM $MgCl_2$ and 50 mM KCl to pyrene-labeled G-actin in the presence of MRP effector peptide, and the pyrene–actin fluorescence signal was monitored. Fig. 1a reveals the fluorescence signal of 4 μM actin at different concentrations of the MRP effector peptide. At equimolar concentrations of peptide and actin the polymerization velocity is markedly increased. Upon decreasing the concentration of peptide, however, the kinetics of polymerization is strongly decreased to a rate which is dramatically slower than observed with the actin control in the absence of peptide, until a minimum is reached, approximately at a 1:4 peptide/actin molar ratio. By further lowering the peptide/actin molar ratio (1:16) the polymerization velocity increases again until the control curve without peptide is reached. Note that the steady-state amount of F-actin does not change significantly as a function of peptide concentration (data not shown).

Increasing the KCl concentration from 50 to 100 mM results in the same qualitative behavior

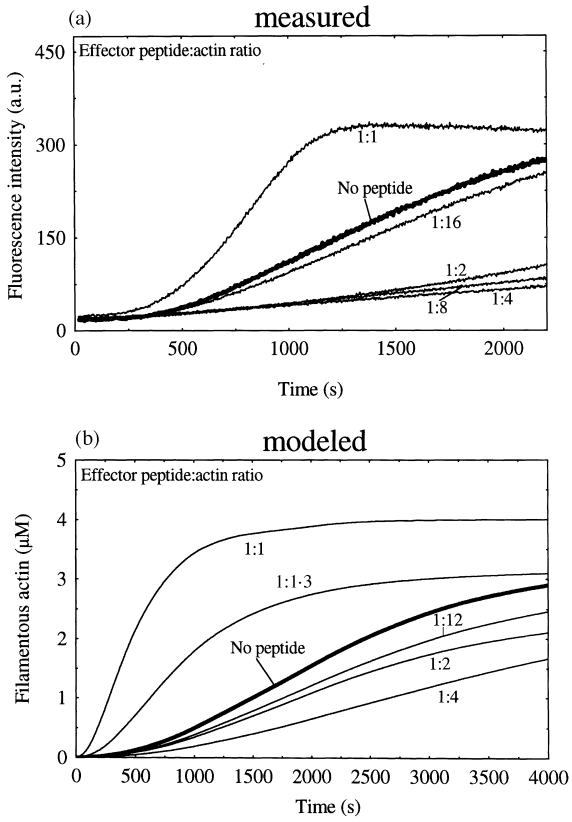

Fig. 1. Polymerization of actin by $MgCl_2$/KCl at different effector peptide concentrations: (a) 4 μM actin is polymerized by addition of 2 mM $MgCl_2$/50 mM KCl in the presence of different concentrations of MRP effector peptide; (b) Mathematical simulation of the actin polymerization process according to model II in Fig. 2. The polymerization of 4 μM actin in the presence of different concentrations of MRP effector peptide is modeled according to Eqs. (3)–(6) following an approach that was developed by Wegner et al. [33] and modified by Frieden [36]. The parameters used for the simulations were: $c_{total} = 4$ μM; $n = 3$; $k_m = 5 \times 10^5$ M^{-1} s^{-1}; $k'_m = 0.3$ s^{-1}; $k_p = 2 \times 10^6$ M^{-1} s^{-1}; $k'_p = 0$; $k_{pm} = 5 \times 10^4$ M^{-1} s^{-1}; $\prod_{i=1}^{2} \frac{k_{im}}{k'_{im}} = \prod_{i=1}^{2} \frac{k_{ip}}{k'_{ip}} = 1 \times 10^4$ M^{-1}.

of the effector peptide towards actin, i.e. low peptide concentrations inhibit actin polymerization whereas higher concentrations increase the rate of actin polymerization. However, the amount of peptide required to observe acceleration relative to polymerization of actin alone is increased by approximately one order of magnitude (data not shown).

[3] F. Wohnsland, A.A.P. Schmitz, M.O. Steinmetz, U. Aebi, G. Vergères, manuscript submitted.

In order to understand the observed phenomenon, we developed a model based on calculations performed by several other groups [33,35,36] and slightly modified to fit our needs.

We assume that upon addition of effector peptide to a G-actin solution the peptide molecules bind with high affinity to actin monomers at a 1:1 stoichiometry. In this complex, actin monomers become insensitive to nuclei formation by mono- and divalent cations. Instead, these 1:1 actin/peptide complexes rapidly self-associate independent of salt to yield F-actin nuclei. Hence, two independent nucleation reactions occur simultaneously (Fig. 2): (i) formation of F-actin nuclei by actin/peptide complexes as described above; and (ii) salt-induced formation of nuclei from uncomplexed actin monomers. For the elongation following actin nuclei formation, all steps are allowed, i.e. salt-independent, fast addition of complexed monomers to complexed nuclei; slow, salt-induced addition of uncomplexed actin monomers to uncomplexed nuclei; and slow, but non-negligible addition of uncomplexed monomers to complexed nuclei and vice versa.

According to this scheme, addition of the effector peptide at a 1:1 stoichiometry to actin will engage all actin molecules in 1:1 actin/peptide complexes which rapidly polymerize in a salt-independent manner, and hence the resulting polymerization kinetics is accelerated. Upon lowering the peptide concentration to a stoichiometry $< 1{:}1$, however, only a fraction of monomers have an effector peptide bound, and the effective concentrations of both 'monomer pools' are decreased. With the kinetic constants of the salt-induced and salt-independent reaction steps being different, this can well lead to a strong attenuation of actin polymerization due to the non-linear character of the polymerization reaction. This scenario can be readily modeled using the kinetic approach worked out by Wegner and co-workers [33,35] (see Section 2.3) by modifying the two differential equations [Eqs. (1) and (2)] in the following way:

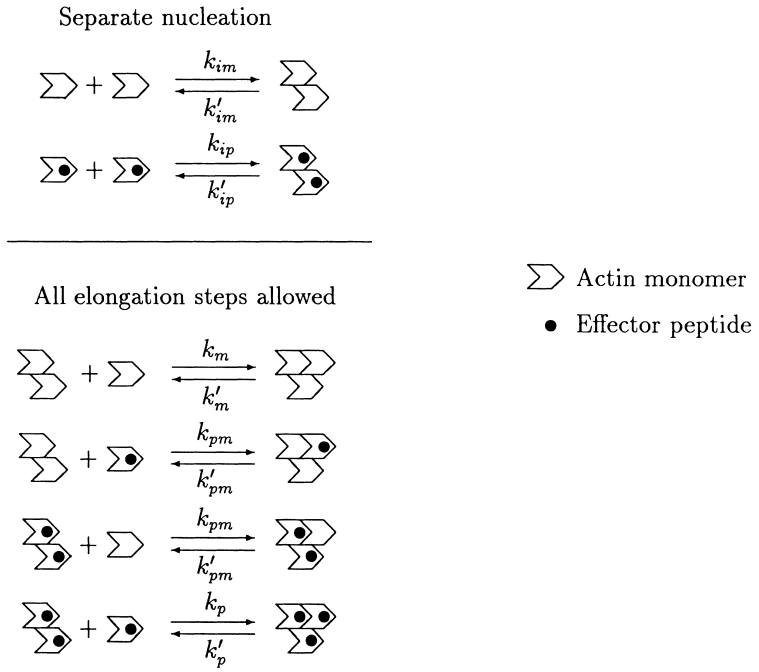

Fig. 2. Model for actin polymerization in the presence of the MRP effector peptide. Uncomplexed actin monomers and actin/peptide complexes can only form nuclei with their own kind. This leads to two completely independent nucleation processes. For elongation, all combinations are allowed. For each reaction there exists a different rate constant.

$$\frac{dC_m}{dt} = (k_m c_{1m} + k_{pm} c_{1p} - k'_m) c_{1m}^n \prod_{i=1}^{n-1} \frac{k_{im}}{k'_{im}} \quad (3)$$

$$\frac{dc_{1m}}{dt} = -(k_m c_{1m} - k'_m) C_m - k_{pm} c_{1m} C_p \quad (4)$$

$$\frac{dC_p}{dt} = (k_p c_{1p} + k_{pm} c_{1m} - k'_p) c_{1p}^n \prod_{i=1}^{n-1} \frac{k_{ip}}{k'_{ip}} \quad (5)$$

$$\frac{dc_{1p}}{dt} = -(k_p c_{1p} - k'_p) C_p - k_{pm} c_{1p} C_m \quad (6)$$

with C_m (in M) and C_p (in M) denoting the molar concentrations of filaments with an uncomplexed actin subunit or a peptide/actin subunit complex at their ends, c_{1m} (in M) and c_{1p} (in M) the molar concentrations of uncomplexed and complexed actin monomers, respectively, k_m (in M^{-1} s^{-1}) and k'_m (in s^{-1}) the association and dissociation constants of uncomplexed monomers to filament ends formed by uncomplexed actin, k_p (in M^{-1} s^{-1}) and k'_p (in s^{-1}) the association and dissociation constants of peptide/monomer complexes to complexed actin filament ends, k_{pm} (in M^{-1} s^{-1}) and k'_{pm} (in s^{-1}) the corresponding constants for association and dissociation of uncomplexed monomers to complexed ends and vice versa, and k_i (in M^{-1} s^{-1}) and k'_i (in s^{-1}) the association and dissociation constants describing nucleation from monomers (index m) or peptide/monomer complexes (index p). In the numerical calculations, the dissociation constants involving peptide/actin complexes (i.e. k'_{pm} and k'_p) were neglected since previous results indicate that these complexes are highly stabilized against depolymerization (data not shown).

As depicted in Fig. 1b, our model can clearly explain the concentration-dependent kinetic effect of the MRP effector peptide on actin polymerization (compare with Fig. 1a). Actin polymerization by the MRP effector peptide in the presence of 2 mM $MgCl_2$/50 mM KCl strongly accelerates the actin polymerization process at a 1:1 peptide/actin molar ratio. Upon decreasing the molar ratio down to 1:4, the polymerization process is increasingly retarded due to the presence of two pools of actin monomers (i.e. uncomplexed and complexed actin monomers). By further lowering the molar ratio, the polymerization reaction — now mainly induced by the mono- and divalent cations in the solution — slowly, but definitely, approaches the 'control' curve with no peptide in solution. Note that in this particular numerical example the parameters $\prod_{i=1}^{2} \frac{k_{im}}{k'_{im}}$, and $\prod_{i=1}^{2} \frac{k_{ip}}{k'_{ip}}$ describing the nucleation rates for free and complexed actin nuclei, respectively, are assumed to be equal. Only the elongation rate for complexed actin monomers, k_p, is increased by a factor of four vs. k_m, the elongation of uncomplexed actin monomers. Note also that at steady-state the same amount of actin is polymerized independently of the effector peptide concentration, i.e. all curves reach the same plateau value at t_∞ (not shown).

It should be emphasized that our model only *qualitatively* explains our results. In fact, the system is more complicated since this kinetic model assumes a simple nucleation elongation process whereas the actin polymerization induced by the MRP effector peptide seems to be accompanied — and influenced — by actin filament bundle formation as has been shown elsewhere[3].

That more effector peptide is required to enhance actin polymerization when increasing the KCl concentration from 50 to 100 mM suggests that the binding of the effector peptide to actin is, at least to some extent, governed by electrostatic interactions. However, the mechanism by which the complexation of actin monomers affects the actin polymerization reaction is independent of the salt concentration.

It is, of course, tempting to assume an even simpler mathematical model with two completely independent polymerization reactions of complexed and uncomplexed monomer pools instead of only the nucleation being completely independent. Recent findings, however, indicated that addition of $MgCl_2$/KCl to already existing filament bundles of complexed actin, which had been obtained by sub-stoichiometric amounts of MRP

effector peptide in a salt-free environment, induces a comparatively fast polymerization of the remaining free monomers towards the final plateau[3], with the filament bundles obviously acting as nuclei for elongation. Nevertheless, it should be noted that this rather simple model is also able to explain an attenuation of the kinetics when the stoichiometry peptide/actin is decreased (data not shown).

3.2. Calmodulin interferes with the actin-polymerizing activity of the MRP effector peptide

So far we have shown that the MRP effector peptide affects the actin polymerization kinetics in a concentration-dependent manner, and that this effect can be qualitatively described by a model in which two pools of actin monomers undergo independent nucleation. In order to test whether this model can be used to predict the effects of additional components in the system, the influence of CaM, a known reaction partner of MRP, on the interaction between actin and the MRP effector peptide was investigated. This is of particular interest in light of results from previous experiments which revealed that effector peptide, pre-incubated with CaM, loses its ability to cause spontaneous polymerization of actin in the absence of salts and that actin filaments polymerized only by MRP effector peptide can completely be depolymerized by CaM[3].

Typically, 4 μM actin was polymerized with 2 mM $MgCl_2$ and 50 mM KCl in the presence of 8 μM MRP effector peptide and an excess of CaM (Fig. 3, curve 3). Since CaM binds to the MRP effector peptide with nanomolar affinity, it should — at least partially — interfere with the peptide's polymerizing effect on actin.

In agreement with the data presented in Fig. 1a, 8 μM effector peptide increases the polymerization kinetics of a 4 μM G-actin solution in the presence of 2 mM $MgCl_2$/50 mM KCl (Fig. 3, curves 1 and 2). A fivefold excess of CaM over the effector peptide, however, strongly inhibits the polymerization process (Fig. 3, curve 3), whereas in the absence of MRP effector peptide CaM does not change the actin polymerization kinetics (Fig. 3, compare curves 1 and 4).

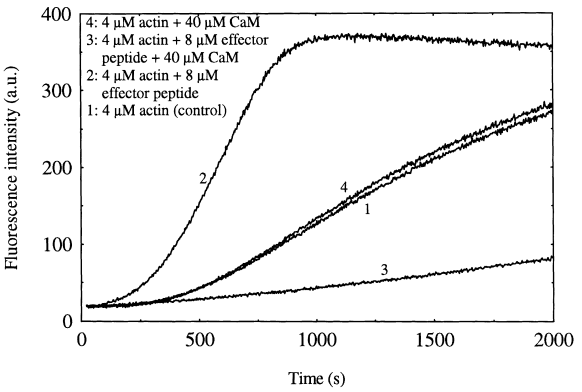

Fig. 3. Regulation of the influence of the MRP effector peptide on actin polymerization by CaM in the presence of salts: 4 μM actin is polymerized by parallel addition of 2 mM $MgCl_2$/50 mM KCl and 8 μM MRP effector peptide in the absence (curve 2) or presence (curve 3) of an excess of CaM (40 μM).

This effect is strongly reminiscent of the actin polymerization process at high ionic strength when monitored in the presence of decreasing amounts of MRP effector peptide (Fig. 1). The most simple model which can qualitatively explain this inhibition in polymerization kinetics is again the polymerization of two separate pools of actin monomers: one pool is composed of monomers that are associated with effector peptide molecules which can only form nuclei with other such complexes; a second pool contains free actin monomers as a consequence of the removal of the effector peptide molecules from actin by CaM. Alternatively, the second pool might consist of actin/effector peptide/CaM complexes which behave like free actin monomers.

In this context, it should be noted that, since phosphorylation of the MRP effector peptide by PKC is inhibited when CaM is associated with the effector peptide [26], we would expect that phosphorylation also affects the actin-polymerizing activity of the effector peptide; indeed, recent results indicate that phosphorylation of the effector peptide leads to inhibition of the actin polymerization kinetics in the presence of salts[3]. This is again readily explained in light of our observation that sub-stoichiometric peptide concentrations attenuate actin polymerization; incomplete phosphorylation might leave a small amount of un-

phosphorylated effector peptide which, in turn, yields the observed time course.

4. Conclusions

Taken together, we propose a model in which effector peptide molecules associate very fast with actin monomers, thus leading to a pool of complexed actin molecules that can only form nuclei with other such complexes (see Fig. 2). In this way, the resulting actin-polymerization kinetics can be either accelerated (i.e. with stoichiometric amounts of peptide) or retarded (i.e. with substoichiometric amounts of peptide), depending on the effector peptide/actin molar ratio. Furthermore, CaM can regulate this mechanism by inhibiting the interaction between the MRP effector peptide and actin.

5. Nomenclature

CaM:	calmodulin
F-actin:	filamentous actin
G-actin:	globular actin
MARCKS:	myristoylated alanine-rich C kinase substrate
MRP:	MARCKS-related protein
PKC:	protein kinase C

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