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FEBS Letters 338 (1994) 58–62

FEBS 13561

FEBS LETTERS

Characterisation of the F-actin binding domains of villin: classification of F-actin binding proteins into two groups according to their binding sites on actin

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Received 2 December 1993

Abstract

The F-actin binding properties of chicken villin, its headpiece and domains 2–3 (V2–3) have been analysed to identify sites involved in bundle formation. Headpiece and V2–3 bind actin with K_d values of ~7 μM and ~0.3 μM, respectively, at low ionic strength. V2–3 binding, like that of villin, is weakened with increasing salt concentration; headpiece binding is not. Competition experiments show that headpiece and V2–3 bind to different sites on actin, forming the two cross-linking sites of villin. Headpiece does not compete with the F-actin binding domains of gelsolin or α-actinin, but it dissociates actin depolymerizing factor. We suggest that the F-actin binding domains of actin severing, crosslinking and capping proteins can be organized into two classes.

Key words: Villin; Gelsolin; Actin depolymerizing factor; Actin-binding protein

1. Introduction

The actin bundles in the intestinal brush border microvilli contain two actin crosslinking proteins, villin and fimbrin. Villin (M_r 92,300), originally isolated from brush border microvilli of chicken intestine [1] was characterized as an actin-bundling protein that is converted to an actin-severing protein by calcium ions. This dual activity was explained in part by villin's homology with the family of F-actin severing proteins that includes gelsolin, severin and fragmin. This family is characterized by a set of three homologous sequence repeats in severin and fragmin [2,3] and, as a result of gene duplication, villin core and gelsolin consist of six such segments here termed V1–6 and G1–6 respectively [4–6]. However, villin is unique in that it crosslinks actin filaments in low calcium concentrations, an activity requiring the C-terminal headpiece. This 76-residue F-actin binding domain is absent from other severing proteins, but has recently been identified in dematin [7]. Proteolytic removal of headpiece abolished crosslinking, but not the severing activity of the core fragment (V1–6) [8]. These observations demonstrated that one crosslinking site is located in headpiece.

There is evidence to suggest that the second crosslinking site is located in the core domain near the N-terminus. Villin N-terminal fragment 44T (equivalent to V1–3), severs F-actin in a calcium sensitive manner and must therefore bind to the filaments [9]. An F-actin binding site has been identified between V1 and V2 by actin footprinting/peptide mapping [10]. In gelsolin, this site, which participates in severing, lies at the N-terminus of the F-actin binding region (G2) [11].

We have characterised the F-actin binding properties of V2–3 and headpiece and compared them in competition assays with actin binding domains of other filament binding proteins. Headpiece does not compete with V2–3, G2 or the actin binding domain of α-actinin for binding to actin. A-actinin belongs to a family of crosslinking proteins that includes fimbrin, spectrin, dystrophin and filamin, characterized by a 250-residue F-actin binding domain [12]. Fimbrin contains a tandem pair of this domain [13]. Although the actin binding site for fimbrin has not been characterised, the related domain of α-actinin binds actin filaments in a calcium independent bmanner with a K_d of 4 μM and competes with G2 for the same site [11]. Headpiece displaces actin depolymerizing factor (ADF) from actin filaments. ADF (M_r 18,505) is a small actin severing protein, unrelated to gelsolin or villin, originally isolated from chick brain

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Abbreviations: ADF, actin depolymerizing factor from human brain; V2–3, segments 2 and 3 of chicken villin; G2, segment 2 of human gelsolin.

SSDI 0014-5793(93)E1478-5

[14]. It binds G-actin at pH values above 7.5, but associates with filament subunits below pH 7.2 [15, 16]. Thus ADF and headpiece bind to the same region of actin.

Our results indicate that villin crosslinks actin filaments through both headpiece and V2–3 and suggests that F-actin binding proteins can be classified into two distinct groups, based on their actin binding sites.

2. Materials and methods

2.1. Construction and expression of chicken villin headpiece and V2–3

Chicken villin headpiece (residues 751–825) was expressed from a construct engineered into the T7 based expression vector pAED 4 (Doering and Matsudaira, in preparation). V2–3, the actin binding site of the villin core (residues 125–389) was constructed in the following manner: the chicken villin 44T pAED4 construct was digested with *Nde*I and *Hin*dIII. The unique *Hin*dIII site is contained in the codons for amino acid residues 157–159 of the sequence. The missing N-terminal portion of V2 corresponding to residues 125–159 was assembled synthetically from two sense oligonucleotides and the two nonsense. The final construct was confirmed by restriction digests and sequencing.

The headpiece and V2–3 pAED4 expression constructs were transformed into the *E. coli* strain Bl21(DE3) for expression. Two liter of medium (2XTY) were inoculated with transformed Bl21(DE3), grown overnight at 37°C, and harvested without induction. Cell pellets were frozen and the soluble fraction or inclusion bodies prepared as described previously [17]. The soluble fraction, containing villin headpiece, was dialysed against 3 liter of 10 mM Tris-HCl pH 8.0, 0.2 mM EGTA, 0.5 mM DTT, 1 mM sodium azide (BufferA) loaded into a 50-ml syringe and passed through a Product IV DE52 column (BPS Separations Ltd., Spennymore, County Durham, DL16 6YL, UK), which bound the majority of the *E. coli* proteins. The flow-through fraction containing headpiece was concentrated on an Amicon membrane, filtered through an 0.45 μm membrane (Millipore) and chromatographed on a S200 Sephacryl column, yielding 35 mg of pure protein/l. Protein concentration was determined from absorbance, using a calculated value A_{280} = 1.0 cm^{-1} = 175.7 μM, based on the presence of a single tryptophan residue.

The yield of V2–3 from inclusion bodies was improved by adding lysozyme (6 mg) to the resuspended cell pellet from a 2 liter culture. Inclusion bodies were solubilised in 8 M urea in Buffer A, clarified by centrifugation, and filtered through a 0.45 μm Millipore membrane. The filtrate was diluted to 6 M urea and loaded onto a 10 × 2.5 cm Whatman DE52 column equilibrated in Buffer A containing 6 M urea. V2–3 eluted in the flow through fraction which was concentrated and then dialysed against Buffer A. The protein concentration was calculated from tyrosine and tryptophan content – A_{280} = 1.0 cm^{-1} = 35.9 μM.

V2–3 showed a tendency to aggregate when it was concentrated under conditions of the assays. Self-association during preparation was prevented by purifying and concentrating in 6 M urea. Following removal of the urea, V2–3 could be used at concentrations up to 40 μM. Headpiece was fully soluble in all conditions to >300 μM.

2.2. Preparation of other proteins

The F-actin binding domains of chicken smooth muscle α-actinin (residues 1–269) and human gelsolin segment 2 (G2) were prepared as described previously [11]. Human actin depolymerising factor (ADF) was purified according to Hawkins et al. [15]. All proteins were dialysed into Buffer A for storage. Actin was prepared from rabbit skeletal muscle as described previously and stored as F-actin at 100 μM in F-buffer (10mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 100 mM NaCl, 0.1 mM ATP, 0.2 mM dithiothreitol, 3 mM NaN_3 and either 0.1 mM $CaCl_2$ or 0.2 mM EGTA) [18]. Concentration was measured using an A_{290} = 1.0 cm^{-1} = 38 μM.

2.3. Actin sedimentation assays

Sedimentation experiments were routinely performed in F-actin buffer, in the absence of calcium. Prior to the assays, all proteins except actin were clarified at 100,000 rev/min (av 390,000 × *g*) in a Beckman TL100 centrifuge for 30 min. Actin (5–22.5 μM) was mixed with F-buffer (for 100 μl final volume) before adding the binding protein. The mixture was left on ice for 20 min then centrifuged for 20 min at 75,000 rev/min (av 220,000 × *g*). Supernatants were carefully removed and 20 μl of SDS gel buffer was added to both supernatant and pellet. In addition, 96 μl of F-buffer was added to the pellets to equalize volumes of supernatants and pellets for SDS-PAGE analysis.

Gels were densitometered on a Molecular Dynamics gel densitometer and results corrected to take account of trapping of supernatant protein in the small actin pellets. Using bovine serum albumin as a control, we found that 2–3% of the supernatant protein was trapped. Controls in which the binding protein was centrifuged on its own showed that an additional correction of 2.2% was required to take account of supernatant protein left on the walls of the centrifuge tubes. (Based on previous observations, washing the tubes would be expected to give significant losses). Dissociation constants were calculated by non-linear least squares analysis using Enzfitter (Biosoft, Cambridge) on an IBM PC or Kaleidagraph (Synergy Software, 2457 Perkiomen Avenue, Reading, PA 19606, USA) on an Apple Macintosh.

3. Results

3.1. Effects of ionic conditions binding of V2–3 and headpiece to F-actin

We previously reported poor recovery of F-actin in pellets when low concentrations (<20 μM) were sedimented at 100,000 × *g* [19]. Because the maximum concentration of V2–3 was limited to 40 μM, it was necessary to work at much lower actin concentrations to achieve saturation. Using the TL 100 ultracentrifuge at 220,000 × *g* we found that over 90% of F-actin could be sedimented at concentrations as low as 2.5 μM.

At low ionic strength V2–3 binds tightly to F-actin at a 1:1 stoichiometry to actin subunits (Fig. 1). Binding was progressively weaker as the ionic strength was increased (Fig. 2). A similar decrease was observed with intact villin (Fig. 2). Binding of V2–3 to actin was unaffected in the pH range 6.0 to 8.9, whereas whole villin binds about 50% tighter at pH 6.5 than at 7.5 (data not shown). Because of this salt dependence and the 40 μM concentration limit on V2–3 (see section 2), it was not possible to demonstrate saturation binding at ionic strength values approaching physiological. Nevertheless,

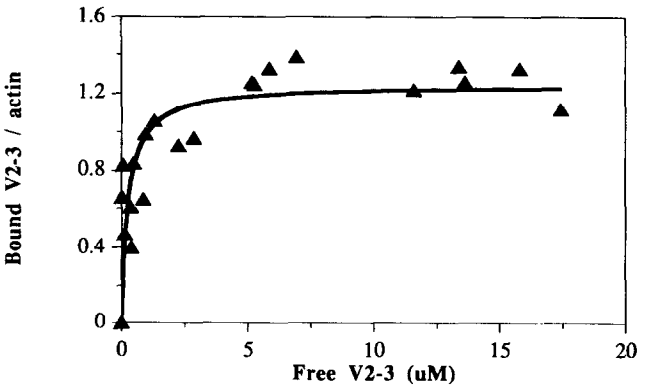

Fig. 1. Binding of V2–3 to F-actin. Sedimentation of V2–3 with 5 μM actin in 5 mM salt (closed triangles). Curve shows non-linear least squares fit with K_d = 0.26 μM.

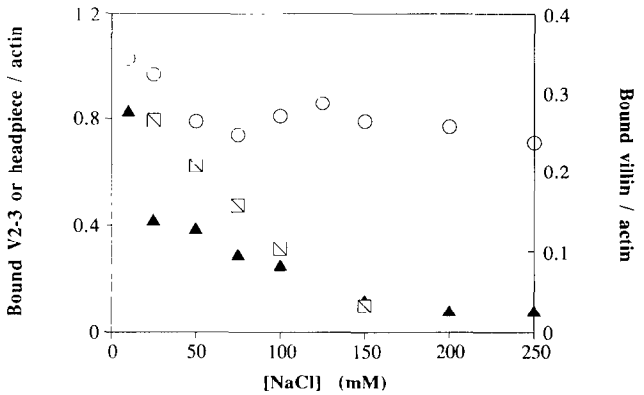

Fig. 2. Effect of ionic strength on the binding of V2–3, villin and headpiece to F-actin. 14 μM V2 3 (filled triangles) was centrifuged with 7.5 μM F-actin in EGTA-containing F-buffer with NaCl concentration varied between 5 and 250 mM. Similar experiments are shown with crossed squares for the binding of 5 μM intact villin to 5 μM F-actin at pH 6.5, while open circles show results using 43 μM headpiece and 22.5 μM actin.

K_d values, estimated from binding assays at fixed protein concentrations in 25 and 100 mM salt, were 8 and 40 μM in EGTA, and 2 and 20 μM in 1 mM $CaCl_2$, respectively.

Initially we found considerable variation in the level of binding of headpiece. This was due to protein loss during fixation in methanol-acetic acid solutions. To eliminate these losses, gels were fixed in 0.5% glutaraldehyde for 15–20 min before staining with Coomassie blue. As a result the stain uptake per μg of headpiece was found to be identical to that of all the other proteins (data not shown).

Binding of headpiece to actin was not significantly reduced at higher ionic strength values (Fig. 2). Fig. 3 shows that headpiece binds actin at a 1 : 1 molar ratio. Non-linear least squares analysis of the binding curve gave a K_d value of 7.2 μM. Headpiece binding was unaffected by the presence of 750 μM free calcium or pH in the range of 6.0 to 8.9.

3.2. *Headpiece has different binding sites on actin from V2–3 and G2*

We investigated whether headpiece would compete for F-actin binding sites with V2–3 or G2, the F-actin binding domain of gelsolin. Fig. 4a shows that prebound V2–3 is not displaced when actin is saturated with headpiece. The estimated K_d for headpiece was 4.6 μM, a value similar to that obtained for headpiece alone. In similar experiments, no displacement of G2 was observed when the filaments were saturated with headpiece (Fig. 4b). Non-linear least squares fitting gave a K_d value of 12 μM.

It has been previously shown that the F-actin binding domain of α-actinin competes with that of gelsolin for sites on F-actin [11]. Table 1 shows that the binding of headpiece was not affected by saturating amounts of α-actinin. At the highest concentrations of the two proteins, the total ratio of bound headpiece plus α-actinin to actin was 1.75:1.

3.3. *Human ADF competes with headpiece for F-actin binding sites*

ADF binds F-actin at pH values < 7.2 [15,16], but only to monomeric actin at higher values. Competition experiments (Fig. 5) showed that about 80% of the bound ADF is displaced when 30 μM headpiece is added to 10 μM actin presaturated with ADF.

4. Discussion

Villin crosslinks actin filaments in the absence of calcium. One binding site is located in the headpiece and we have shown here that the second site is located in V2–3. Our value for the K_d for headpiece (~7 μM) agrees well with binding data of headpiece prepared by proteolysis, but for which a K_d was not determined [8] and is of similar magnitude to that of G2–3 or the actin binding domain of α-actinin [11].

V2–3 binds F-actin very tightly at low ionic strength, but unlike headpiece, K_d increases considerably with ionic strength. Our value in 5 mM salt was 0.26 μM, but this increased to about 40 μM at 100 mM salt. By contrast, the F-actin binding domain of gelsolin, which is highly homologous to that of villin, has a 4 μM K_d value under these conditions [11]. Similar experiments with villin also showed a marked reduction in binding at physiological ionic strength (Fig. 2). The extent of binding by villin is consistent with earlier experiments showing that bundle formation was maximal at a 4:10 villin/actin ratio [20]. The affinity of villin, which co-operatively crosslinks filaments, for actin cannot be calculated from these experiments, but based on analogy with chicken gizzard α-actinin, it is probably much higher than that of V2–3.

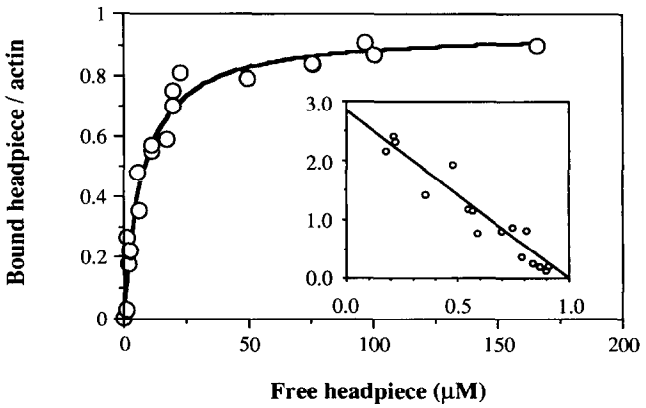

Fig. 3. Binding of headpiece to F-actin. Sedimentation of headpiece with 22.5 μM F-actin at pH 8.0. Curve shows non-linear least squares fit with K_d = 7.2 μM. Inset shows a Scatchard plot of the same data with bound headpiece/actin on the abscissa and bound/free on the ordinate. Saturation of binding is at 1 : 1 and K_d = 2.9 μM.

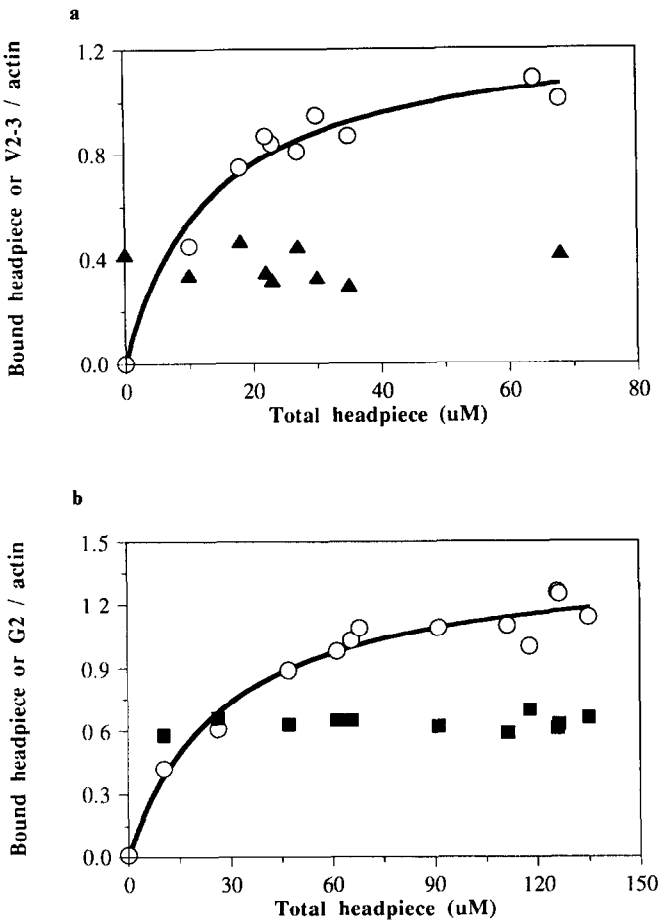

Fig. 4. (a) Binding of headpiece and V2–3 to F-actin. Sedimentation of 13 μM V2–3 (closed triangles) plus increasing concentrations of headpiece (open circles) with 10 μM actin in F-buffer containing 32 mM NaCl. The curve shows non-linear least squares fit with K_d = 4.6 μM. (b) Binding of headpiece and G2 to F-actin. Sedimentation of 32 μM G2 (closed squares) plus increasing concentrations of headpiece (open circles) with 18 μM actin in F-buffer containing 120 mM NaCl. The curve shows non-linear least squares fit with K_d = 11.6 μM.

α-actinin showed 50% saturation at 0.4 μM compared to a K_d for the isolated actin binding domain of 4.3 μM [11,21].

Although V2–3 binds F-actin in the absence of calcium, previous studies failed to detect F-actin binding in EGTA for villin core or the N-terminal proteolytic fragment 44T [8,9]. This could be due to inaccessibility of the F-actin binding sites when covalently linked to adjacent monomer binding domains, or to very low affinity in the larger fragments. Finidori et al. have recently shown that a villin deletion mutant containing V1–3 and headpiece, but lacking V4–6, induces growth of microvilli and F-actin redistribution in a manner similar to intact villin when transfected into fibroblast like CV1 cells [22]. This supports the low affinity hypothesis because cross-linking will stabilise multiple weak interactions. In a similar manner, video fluorescence microscopy has shown that gelsolin attached to glass coverslips binds F-actin in EGTA [23], although no binding could be detected in biochemical assays [24,25].

Based on the high degree of homology between villin and gelsolin, it is likely that V2–3 and G2 bind to the same site on actin. The competition experiments in Fig. 4 support this hypothesis. Taken with earlier experiments [11], these results suggest that the F-actin binding site of villin core overlaps that of gelsolin and α-actinin. Hence it is likely that the binding site for fimbrin, which shows about 50% sequence similarity with α-actinin [13], will overlap the same region.

Our competition experiments suggest that ADF and headpiece bind to the same region of actin (although competitive displacement could result from steric hindrance at adjacent overlapping sites). Deletion of the C-terminal seven residues of headpiece (KKEKGLF) abolishes the bundling activity of villin in vivo [26]. These authors also showed that a synthetic peptide corresponding to the 22 C-terminal residues of headpiece induces polymerization of actin, which suggests that the cluster of basic residues (KKEK) at the extreme C-terminus of villin forms part of the binding site. Cofilin, which is 71% identical to ADF (also known as destrin) [27], also contains a cluster of basic residues in the sequence DAIKKKL that is also present in ADF. A 7-residue peptide of this sequence binds F-actin and inhibits the binding of cofilin to filaments [28]. It will be interesting to find out whether either of these peptides affects the binding of the unrelated protein to filaments.

Gelsolin, villin, α-actinin and ADF contain F-actin binding domains that are characteristic of several major families of actin binding proteins. Calculations of K_d values here and elsewhere [29,30] for F-actin binding proteins all fall into the low micromolar range. Based on our experiments, these F-actin recognition domains can be classified into two groups. Group I consists of villin V2–3, gelsolin G2, α-actinin and, on the basis of sequence homology, dystrophin, filamin, fimbrin and other members of the spectrin family. Interchangeability of two of these proteins has been demonstrated in a chimaeric protein containing G1 of gelsolin and the α-

Table 1
Competition assays between α-actinin and headpiece for F-actin

Mols headpiece added per mol actin	Mols headpiece bound per mol actin	Mols α-actinin bound per mol actin	Mols α-actinin added per mol actin
3.0	0.60	0.0	0.0
2.5	0.70	0.66	1.7
3.0	0.55	1.25	4.0
3.4	0.78	0.97	2.6
0.0	0.0	1.14	3.6

Binding of approximately 3-fold molar excess of headpiece to 22.5 μM F-actin in competition with 0–90 μM α-actinin in EGTA containing F-buffer at pH 8.0.

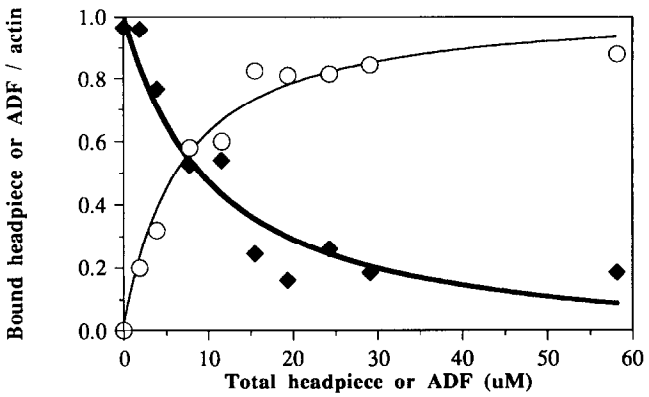

Fig. 5. Binding of headpiece and ADF to F-actin. Sedimentation of 60 μM ADF (closed diamonds) plus increasing concentrations of headpeice (open circles) with 9.5 μM actin in F-buffer at pH 6.5 containing 32 mM NaCl and Imidazole-HCl pH 6.5 in place of Tris-HCl.

actinin actin binding domain, which retains actin severing activity [11]. Group II domains consists of ADF, cofilin, villin headpiece, and dematin [7]. These groupings illustrate that there may be a limited number of potential binding sites on actin filaments.

Acknowledgements: We thank Sutherland Maciver for his kind gift of human ADF, Don Doering and Tomoko Nakayama for their headpiece and V2–3 constructs. The research was supported in part by NIH DK35306 to P.M.

References

[1] Bretscher, A. and Weber, L. (1980) Cell 20, 839–847.
[2] Ampe, C. and Vandekerckhove, J. (1987) EMBO J. 6, 4149–4157.
[3] André, E., Lottspeich, F., Schleicher, M. and Noegel, A. (1988) J. Biol. Chem. 263, 722–728.
[4] Arpin, M., Pringault, E., Finidori, J., Garcia, A., Jeltsch, J., Vandekerckhove, J. and Louvard, D. (1988) J. Cell Biol. 107, 1759–1766.
[5] Bazari, W.L., Matsudaira, P., Wallek, M., Smeal, T., R. Jakes and Ahmed, Y. (1988) Proc. Natl. Acad. Sci. USA 85, 4986–4990.
[6] Way, M. and Weeds, A.G. (1988) J. Mol. Biol. 203, 1127–1133.
[7] Rana, A.P., Ruff, P., Maalouf, G.J., Speicher, D.W. and Chishti, A.H. (1993) Proc. Natl. Acad. Sci. USA 90, 6651–6655.
[8] Glenney, J.R., Geisler, N., Kaulfus, P. and Weber, K. (1981) J. Biol. Chem. 256, 8156–8161.
[9] Matsudaira, P., Jakes, R. and Walker, J.E. (1985) Nature 315, 248–250.
[10] deArruda, M.V., Bazari, H., Wallek, M. and Matsudaira, P. (1992) J. Biol. Chem. 267, 13079–13085.
[11] Way, M., Pope, B. and Weeds, A.G. (1992) J. Cell Biol. 119, 835–842.
[12] Matsudaira, P.T. (1991) Trends Biochem. Sci. 16, 87–92.
[13] deArruda, M.V., Watson, S., Lin, C.-S., Levitt, J. and Matsudaira, P. (1990) J. Cell Biol. 111, 1069–1079.
[14] Bamburg, J.R., Harris, H.E. and Weeds, A.G. (1993) FEBS Lett. 121, 178–182.
[15] Hawkins, M., Pope, B., Maciver, S.K. and Weeds, A.G. (1993) Biochemistry 32, 9985–9993.
[16] Hayden, S.M., Miller, P.S., Brauweiler, A. and Bamburg, J.R. (1993) Biochemistry 32, 9994–10004.
[17] Way, M., Pope, B. and Weeds, A.G. (1992) J. Cell Biol. 116, 1135–1143.
[18] Taylor, R.S. and Weeds, A.G. (1976) Biochem. J. 159, 301–315.
[19] Pope, B. and Weeds, A.G. (1986) Eur. J. Biochem. 161, 85–93.
[20] Matsudaira, P.T. and Burgess, D.R. (1982) J. Cell Biol. 92, 648–656.
[21] Meyer, R.K. and Aebi, U. (1990) J. Cell Biol. 110, 2013–2024.
[22] Finidori, J., Friederich, E., Kwiatkowski, D.J. and Louvard, D. (1992) J. Cell Biol. 116, 1145--1155.
[23] Bearer, E.L. (1991) J.Cell Biol. 115, 1629-1638.
[24] Way, M., Gooch, J., Pope, B. and Weeds, A.G. (1989) J. Cell Biol. 109, 593–605.
[25] Yin, H.L. (1988) Bioessays 7, 176–179.
[26] Friederich, E., Vancompernolle, K., Huet, C., Goethals, M., Finidori, J., Vandekerckhove, J. and Louvard, D. (1992) Cell 70, 81–92.
[27] Moriyama, K., Nishida, E., Yonezawa, N., Sakai, H., Matsumoto, S., Iida, K. and Yahara, I. (1990) J. Biol. Chem. 265, 5768–5773.
[28] Yonezawa, N., Nishida, E., Ohta, M., M. Seki, Kumagai, H. and Sakai, H. (1989) Eur. J. Biochem. 183, 235–238.
[29] Ohanian, V., Wolfe, L.C., John, K.M., Pinder, J.C., Lux, S.E. and Gratzer, W.B. (1984) Biochemistry 23, 4416–4420.
[30] Way, M., Pope, B., Cross, R.A., Kendrick-Jones, J. and Weeds, A.G. (1992) FEBS Lett. 301, 243–245.