Disulphide cross-linking of smooth-muscle and non-muscle caldesmon to the C-terminus of actin in reconstituted and native thin filaments

Philip GRACEFFA*, Leonard P. ADAM† and William LEHMAN‡

*Department of Muscle Research, Boston Biomedical Research Institute, 20 Staniford St., Boston, MA 02114, †Department of Medicine, Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN 46202, and ‡Department of Physiology, Boston University School of Medicine, Boston, MA 02118, U.S.A.

It was reported that chicken gizzard smooth-muscle caldesmon Cys-580 can be disulphide-cross-linked to the C-terminal penultimate residue (Cys-374) of actin, indicating that these residues are close in the protein complex [Graceffa, P. and Jancso, A. (1991) *J. Biol. Chem.* **266**, 20305–20310]. Since the possibility that the cross-link involves a cysteine residue other than actin Cys-374 was not absolutely excluded, more direct evidence was sought for the identity of the cysteine residues involved in the cross-link. We show here that caldesmon could not be disulphide-cross-linked to actin which had Cys-374 removed by carboxypeptidase A digestion, providing direct support for the participation of actin Cys-374 in the cross-link to caldesmon. In order to assign the caldesmon cysteine residue involved in the cross-link, use was made of caldesmon from porcine stomach muscle, which is shown to contain one cysteine residue close to, or at, position 580, in contrast with chicken gizzard caldesmon, which has an

additional cysteine residue at position 153. The porcine stomach caldesmon also formed a disulphide-cross-link to actin, further supporting the original conclusion that Cys-580 of the chicken gizzard caldesmon had been cross-linked to actin. Disulphide-cross-linking with similar yield was also observed in native chicken gizzard muscle thin filaments, indicating that the interaction between actin and the C-terminal domain of caldesmon is the same in native and reconstituted thin filaments. The much smaller non-muscle isoform of caldesmon, from rabbit liver, could be similarly cross-linked to actin, consistent with the sequence similarity between the C-terminal domain of muscle and non-muscle caldesmon. The ability to cross-link caldesmon Cys-580 to actin Cys-374 suggests the possibility that the Cys-580 region of caldesmon and the C-terminus of actin form part of the actin–caldesmon binding interface.

INTRODUCTION

The actin-binding protein caldesmon is thought to be involved in the thin-filament regulation of smooth-muscle contraction (for reviews, see [1,2]). Such a function might involve caldesmon's ability to inhibit the actin-activated myosin ATPase activity. It is at present undecided whether this inhibition is due to a reduction in myosin binding to actin [3,4] or to a slowing of the rate-limiting step in the ATPase kinetic pathway [5]. Since actin has binding sites for caldesmon in both its N-terminal [6–8] and C-terminal [9] regions, regions which also contain myosin-binding sites (see [9] for reference), the inhibition could be due to caldesmon directly competing with myosin for binding sites on actin or to caldesmon indirectly affecting the actin–myosin interaction and thus the actomyosin ATPase rate.

Chicken gizzard caldesmon contains a single 756-amino-acid polypeptide chain formed into a thin elongated molecule [1,2]. The C-terminal portion contains the actin-binding region and the ATPase-inhibiting function, the N-terminal portion contains a myosin-binding site, and the central portion is thought to be a long α -helical spacer rod [1,2,10]. The molecule contains two cysteine residues [11], one at position 153 in the N-terminal domain and the other at position 580 in the C-terminal domain [12,13]. Two cDNA clones have been isolated for chicken gizzard caldesmon. One represents a 756-residue protein [12] and the other a 771-residue protein [13], the latter corresponding to a 15-residue peptide insertion into the central region of the former. For convenience we will refer to the amino-acid sequence of the smaller protein. The non-muscle isoform of caldesmon lacks the central region [1,2] and, consequently, is a shorter molecule [14].

The direct interaction of caldesmon with the C-terminus of

actin was concluded from evidence which indicated that Cys-580 in caldesmon's C-terminal region could be disulphide cross-linked to actin Cys-374, the C-terminal penultimate residue, in reconstituted thin filaments [9]. The involvement of actin Cys-374 was based on the use of actin which had been modified with reagents which had been reported to modify specifically Cys-374. However, studies of Liu et al. [15] suggested that other cysteine residues of actin (total of five cysteine residues) might also be modified with reagents which had previously been reported to react specifically with Cys-374. Furthermore, in recent work on the interaction of caldesmon with actin labelled at Cys-374 with fluorescent probes, it was concluded that although caldesmon affects the probe, it does not directly make contact or interact with the Cys-374 region of actin [16]. These two studies raise the possibility that caldesmon could have been disulphide-cross-linked to a cysteine other than Cys-374 on actin. We therefore sought further evidence for deciding which cysteine residues of actin and caldesmon are involved in the cross-link between them. Since the previous cross-linking study was conducted with reconstituted actin thin filaments, we also wished to investigate whether a similar cross-link occurs in native thin filaments.

EXPERIMENTAL

Caldesmon from chicken gizzard muscle [9], porcine stomach muscle [17] and rabbit liver [14] was prepared as previously described. Chicken gizzard native thin filaments were prepared as described by Marston and Lehman [18], except that the last dialysis was performed in the absence of dithiothreitol and there was an additional dialysis against cross-linking buffer (see below). Actin was prepared from rabbit skeletal muscle as described by

Spudich and Watt [19]. SDS/PAGE was performed either as described by Laemmli [20], under non-reducing conditions unless otherwise stated, or as described by Porzio and Pearson [21] in the presence of dithiothreitol. The Cu^{2+} (*o*-phenanthroline)₂-catalysed oxidation of caldesmon and the determination of caldesmon binding to actin were carried out as reported [9].

Caldesmon was disulphide-cross-linked to actin via disulphide exchange by treatment with 5,5'-dithiobis-(2-nitrobenzoic acid) (NbS_2), as detailed previously [9]. Essentially, a solution of caldesmon and actin or of native thin filaments, in 40 mM NaCl/5 mM Mops/2 mM MgCl_2 /0.1 mM EGTA (pH 7.5)/0.01% NaN_3 , plus the proteolytic inhibitors phenylmethanesulphonyl fluoride (0.25 mM), benzamidine (0.75 mM), and leupeptin (1 $\mu\text{g}/\text{ml}$) (cross-linking buffer), was incubated with 50–100 μM NbS_2 for 15–30 min at room temperature; any further reaction was stopped by addition of excess *N*-ethylmaleimide, which blocks all free thiol groups. Protein concentrations during cross-linking were as follows: actin, 10–14 μM , plus muscle caldesmon, 0.75 μM , or liver caldesmon, 0.3 μM ; native thin filaments, 1 mg/ml.

Carboxypeptidase A-digested actin was prepared essentially as described by Field and Bowen [22]. G-actin, in 2 mM Mops/0.2 mM CaCl_2 /0.2 mM ATP, pH 7.5, was incubated for 3 h at 25 °C with carboxypeptidase A (Worthington) at an actin/carboxypeptidase molar ratio of 25:1. The digested G-actin was converted into F-actin by adding NaCl to 40 mM, MgCl_2 to 2 mM and NaN_3 to 0.01% and stored on ice. Control actin was similarly treated in the absence of carboxypeptidase A. At various times during the digestion, aliquots were taken for analysis. Acetic acid was added to the aliquots in order to stop the reaction and precipitate actin and, after centrifugation, the supernatant was analysed for amino acid content on a Beckman 118/119CL analyser.

The cysteine content of porcine stomach caldesmon was determined with NbS_2 as described by Ellman [23], as we have done previously for gizzard caldesmon [11]. The caldesmon was first reduced with 5–10 mM dithiothreitol and then dialysed exhaustively against 20 mM NaCl/10 mM Mops/1 mM EDTA, pH 7.5. The concentration of the stomach caldesmon was determined by the Lowry assay [24], using gizzard caldesmon as a standard, the concentration of which was determined from A_{280} [11]. NbS_2 (2 mM) was added to the reduced caldesmon denatured in 4 M guanidinium chloride, and the concentration of NbS^- released, equivalent to the thiol content, was calculated from A_{412} by using an absorption coefficient of $13600 \text{ cm}^{-1} \cdot \text{M}^{-1}$ [23].

Caldesmon was chemically cleaved at cysteine with 2-nitro-5-thiocyanatobenzoic acid (NTCB; Sigma) as described by Jacobson et al. [25]. Caldesmon was incubated at 25 °C for 3 h in a solution containing 1 mM NTCB, 1 mM EDTA, 20 mM Tris, pH 9.0 and 50 mM NaCl. After incubation, the reaction mixture was diluted 1:1 with gel-electrophoresis sample buffer and subjected to SDS/PAGE for the separation of caldesmon fragments. In some instances caldesmon was first maximally phosphorylated with protein kinase C in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, as described by Adam et al. [26] and then cleaved with NTCB.

RESULTS

Porcine stomach caldesmon

Determination of the cysteine content of porcine stomach caldesmon by the NbS_2 method (see the Experimental section) resulted in a value of 0.92 ± 0.05 cysteine/molecule. This is in contrast with two cysteine residues/molecule for chicken gizzard caldesmon, which can form an intramolecular disulphide cross-

link and intermolecular disulphide-cross-linked dimers and oligomers upon oxidation catalysed by Cu^{2+} (*o*-phenanthroline)₂ ([9,27]; Figure 1, lanes A and B). Consistent with one cysteine residue for porcine stomach caldesmon, only disulphide-cross-linked dimers were formed by treatment with Cu^{2+} (*o*-phenanthroline)₂ (Figure 1, lanes C and D).

Chemical cleavage of chicken gizzard caldesmon at cysteine residues results in a fragmentation pattern consistent with two cysteine residues [28–30]. As expected from the one cysteine residue in porcine stomach caldesmon, only two fragments resulted from chemical cleavage by NTCB (Figure 2). One fragment migrated with an apparent molecular mass of 110 kDa (caldesmon and its fragments migrate anomalously during SDS/PAGE [11]), termed 'caldesmon heavy subfragment' (or CHS), and the other, called 'caldesmon light subfragment' (or CLS), migrated with an apparent molecular mass of 24 kDa. NTCB cleavage of $[\text{P}^{32}]\text{phosphorylated}$ porcine stomach caldesmon results in all of the $[\text{P}^{32}]\text{phosphate}$ being contained in the 24 kDa fragment (Figure 2). Since the C-terminal portion of porcine stomach caldesmon contains all three protein kinase C phosphorylation sites, at serine-726, -600 and -587 (positions based on the chicken gizzard caldesmon sequence; [31]), then the 24 kDa fragment is the C-terminal portion of the molecule and the single cysteine residue must be at a position lower than 587. Furthermore, since chemical cleavage at cysteine-580 of chicken gizzard caldesmon also results in a 24 ± 1 kDa C-terminal fragment [29,30], then the single cysteine residue of porcine stomach caldesmon must be close to, or at, position 580.

Of the several muscle and non-muscle caldesmons which have been sequenced, all have a cysteine at a position which is equivalent to Cys-580 of chicken gizzard muscle caldesmon [2,12,13,32–36]. The single cysteine residue of porcine stomach caldesmon presents a convenient site for specific labelling of the actin-binding C-terminal region of the molecule by thiol-group-directed spectroscopic and cross-linking probes. Other caldesmons that contain a single cysteine residue, located in the C-terminal region, include those from duck gizzard muscle [37], human fibroblast [34] and human aorta muscle [36].

Porcine stomach caldesmon could be disulphide-cross-linked to actin by treating the caldesmon-actin complex with NbS_2 , as described in the Experimental section. This treatment resulted in

Figure 1 Cu^{2+} (*o*-phenanthroline)₂-catalysed oxidation of muscle caldesmon from chicken gizzard (cCaD) and porcine stomach (pCaD)

SDS/6.7% PAGE [20] of: lane A, cCaD; lane B, oxidized cCaD; lane C, pCaD; lane D, oxidized pCaD. M, intramolecular disulphide-cross-linked monomer; D, intermolecular disulphide-cross-linked dimer; bands above D, intermolecular disulphide-cross-linked oligomers (according to Lynch et al. [27]). pCaD migrates slightly slower than cCaD during SDS/PAGE [50].

Figure 2 NTCB cleavage of porcine stomach caldesmon phosphorylated by protein kinase C

SDS/7.5%-PAGE [21]: lanes A and B are from a Coomassie Blue-stained gel, and lane C is an autoradiogram. Lane A, M_r markers; lane B: NTCB-digested caldesmon; lane C: caldesmon phosphorylated by protein kinase C in the presence of [γ - 32 P]ATP and digested with NTCB. CAD, caldesmon; CHS, caldesmon heavy subfragment; CLS, caldesmon light subfragment.

the generation of a single band on SDS/PAGE, migrating slower than caldesmon, with a concomitant reduction in the intensity of the caldesmon band (Figure 3, lanes A and B). The new band co-migrated with the electrophoretic band resulting from the same treatment of a solution of chicken gizzard caldesmon and actin (Figure 3, lanes C and D). This band had previously been shown to be due to a disulphide cross-link between chicken gizzard caldesmon and actin [9]. Since the stomach caldesmon contains a single cysteine at, or close to, position 580, these results give further evidence that the cross-link between the gizzard caldesmon and actin must involve Cys-580 and not Cys-153, confirming the original conclusion [9].

Carboxypeptidase A-digested actin

Actin was treated for 3 h with carboxypeptidase A under conditions [22] where it has been shown that the C-terminal residue, phenylalanine-375, was completely released in 20 min of incubation and about 35% of the penultimate residue, Cys-374, was released in 40 min [38]. The amino acid analysis of our carboxypeptidase-digested actin showed a similar rapid release of phenylalanine without any further increase after 30 min. Since we incubated for much longer than 40 min, presumably much more than 35% of Cys-374 was removed from actin, although cysteine could not be quantified, owing to side reactions. After 3 h of digestion we also found that about 20% of lysine-373, and essentially none of arginine-372, was released. This is to be expected, since carboxypeptidase A digests lysine only slowly and arginine essentially not at all [39]. Thus the digestion of actin did not go beyond the three C-terminal residues, but for most molecules did not go beyond the first two residues.

In order to test for the ability of digested actin to form a disulphide cross-link to caldesmon, a solution of chicken gizzard

Figure 3 Disulphide cross-linking of muscle caldesmon (CaD) to actin by NbS_2 treatment

SDS/6.7%-PAGE [20] of: lane A, porcine stomach CaD (pCaD); lane B, NbS_2 -treated (pCaD + actin); lane C, chicken gizzard CaD (cCaD); lane D, NbS_2 -treated (cCaD + actin); lane E, NbS_2 -treated (cCaD + carboxypeptidase-digested actin); lane F, native thin filaments from chicken gizzard muscle; lane G, NbS_2 -treated native thin filaments. X, CaD-actin disulphide-cross-linked product. In lanes F and G, one of the tropomyosin bands co-migrates with actin and the other ran off the gel. Lanes A-E and F-G are from two separate gel runs.

caldesmon plus digested or control actin was treated with NbS_2 and subjected to SDS/PAGE (Figure 3, lanes C-E). (Although carboxypeptidase A was present during this treatment, caldesmon would not be expected to be digested, since its C-terminal residue is proline which is resistant to carboxypeptidase A digestion [39].) Caldesmon in the presence of control actin showed an intense band due to a caldesmon-actin disulphide-cross-linked product, whereas in the presence of the digested actin only a faint cross-link band could be discerned. Under the conditions of the cross-linking experiment essentially all of the caldesmon was bound to the digested actin, and thus the lack of cross-linking is not due to a lack of binding. Therefore, although we cannot absolutely exclude the possibility that the removal of Cys-374-Phe-375 indirectly reduces the cross-linking yield between an actin cysteine residue other than Cys-374 and caldesmon, these results provide strong evidence that the disulphide cross-link between caldesmon and actin involves actin Cys-374.

Native chicken gizzard thin filaments

In all of the above experiments and in the previous study [9] it was shown that caldesmon could be easily disulphide-cross-linked to actin ($\pm$ tropomyosin), in reconstituted actin thin filaments [9]. We show in Figure 3 (lanes F and G) that the same cross-link can be easily and efficiently formed by NbS_2 treatment of native thin filaments, prepared without the dissociation of the component proteins of actin, tropomyosin and caldesmon. A small fraction of the caldesmon was cross-linked to actin even before NbS_2 treatment (Figure 3, lane F). The 'cross-linked' band, present before NbS_2 treatment, was not filamin or myosin, two trace contaminants of native thin filaments [18], since it migrates faster than these proteins when run on the same gel (results not shown). The band indeed corresponded to a disulphide cross-linked between caldesmon and actin, since it co-migrated with the cross-linked product and did not appear if the sample was run under reducing conditions, i.e. in the presence of dithiothreitol (results not shown). Thus the interaction between actin and the C-terminal domain of caldesmon appears to be the same in reconstituted and native thin filaments.

Figure 4 Disulphide-cross-linking of liver caldesmon (LCaD) to actin by NbS_2 treatment

(a) SDS/6.7%-PAGE [20] of: lane A, LCaD; lane B, NbS_2 -treated (LCaD + actin); lane C, NbS_2 -treated [chicken gizzard caldesmon (cCaD) + actin], used here to show the position of the cCaD band as an approximate M_r marker. X_L , cross-linked product between LCaD + actin. (b) SDS/10%-PAGE [20] of band X_L cut out rerun in the presence of dithiothreitol.

Non-muscle rabbit liver caldesmon

Non-muscle caldesmon lacks the central region of muscle caldesmon, but it shows considerable sequence similarity to the rest of the muscle isoform [1,2]. Thus we would expect the interaction site between the C-terminal actin-binding domain of non-muscle caldesmon and actin to be equivalent to that for the muscle protein. This, indeed, appears to be the case, since rabbit liver caldesmon can be disulphide-cross-linked to actin by treatment with NbS_2 (Figure 4), as is the case for muscle caldesmon. The evidence for this is that NbS_2 treatment results in a new SDS/PAGE band which: (1) is formed at the expense of the caldesmon band; (2) re-runs as actin plus caldesmon in the presence of dithiothreitol; and (3) has an apparent M_r slightly less than that of muscle caldesmon (140 000), consistent with the sum of the apparent M_r values for liver caldesmon (77 000) [14] and actin (43 000) (Figure 4).

DISCUSSION

In this work we have provided further evidence to support the original conclusion [9] that Cys-580, in caldesmon's actin-binding C-terminal domain, can be disulphide-cross-linked with high yield to Cys-374, the C-terminal penultimate residue of actin. In principle there are at least three possible interpretations of this finding: (i) the two cysteine residues are located close to each other in the binding site and they and/or their adjacent residues contribute to the strength of the interaction; (ii) the two cysteine residues are close to each other in the complex, but neither themselves nor their adjacent amino-acid residues contribute to the interaction; (iii) the two cysteine residues are on average far from each other and come close (and become cross-linked) only due to a significant flexibility of the relevant protein segment(s) [40]. Although none of these possibilities can be rigorously ruled out at present, we believe that it is unlikely that the cross-linking results from large-scale flexibility, since the regions of interest on the two proteins exhibit only slow, small-scale, movements as judged by the low mobility of n.m.r. [41,41a] and spin [42] labels attached to Cys-374 of F-actin and by the high polarization of a fluorescence probe attached to Cys-580 of caldesmon bound to actin ([43]; D. Szczesna, P. Graceffa, C.-L. A. Wang and S. S. Lehrer, unpublished work). The inability of large-scale flexibility to account for cross-linking is also consistent with the fact that

high cross-linking yields were achieved both by allowing NbS_2 -modified actin to react with caldesmon and by adding NbS_2 to the actin-caldesmon complex [9]. Large-scale flexibility could expose both cysteine residues to solvent, with the result that both cysteine residues would be modified by added NbS_2 , resulting in low cross-linking yields [9]. Thus our observations are explained more by proximity than by flexibility. Further studies are needed to delineate the interaction interface between the two proteins and to evaluate the role of the two cysteine regions in the interaction.

In contrast with our conclusion of proximity, Crosbie et al. [16] have concluded that caldesmon indirectly affects the conformation of the C-terminus of actin, without directly interacting or coming in contact with it, from studies of the interaction of caldesmon with actin labelled at Cys-374 with fluorescence probes. First of all, they found that a label on Cys-374 of actin weakens the binding between caldesmon and actin, which is consistent with either a proximity between actin Cys-374 and caldesmon or with an indirect conformational effect of caldesmon on the Cys-374 region of actin. They also observed that the fluorescence of labels attached to Cys-374 of actin became less intense and more exposed to solvent upon interaction with caldesmon, and reasoned that an indirect conformational change was responsible. However, although binding close to a label often shields the label from solvent with a resulting increase in fluorescence, close contact between two proteins at the label site might, alternatively, displace the label and expose it to solvent. Thus the proximity between Cys-580 of caldesmon and Cys-374 of actin can be reconciled with the fluorescence data of Crosbie et al. [16]. Furthermore, each caldesmon molecule most likely binds to more than one actin monomer, since the actin-binding region of caldesmon extends over a larger part of its C-terminal domain [1,2,32,44] and the actin/caldesmon binding stoichiometry is much greater than 1 [1,2]. Thus it is possible that the Cys-580 region of caldesmon could be binding close to Cys-374 in the C-terminus of one actin monomer, at the same time that another region of the same caldesmon molecule could be indirectly affecting the conformation of the C-terminus of another actin monomer, consistent with the conclusions of Crosbie et al. [16].

Finally, Crosbie et al. [16] reported that the removal of the last three C-terminal residues of actin had no effect on the strength of binding between actin and caldesmon, which they interpreted as an indication of there being no direct interaction between these residues and caldesmon. On the other hand, this could also be consistent with direct contact, since proximity between protein segments does not require that they provide a significant contribution to the binding strength.

However, in contrast with the finding of Crosbie et al. [16], Makuch et al. [45] and Kolakowski et al. [46] have presented strong evidence that the removal of the last three C-terminal residues of actin, especially Lys-373 [46], weakens its interaction with caldesmon. These results could be due either to a direct interaction between these residues of actin and caldesmon, as proposed by Kolakowski et al. [46], or to an indirect effect of the removal of these residues on the actin-caldesmon interaction. The latter explanation must be considered, since it has been shown that the removal of the last three C-terminal residues (especially Lys-373) of actin decreases the actin intermonomer interaction, resulting in decreased stability and greater flexibility of the actin filament [47–49]. The former explanation, if correct, together with our ability to cross-link actin Cys-374 to caldesmon Cys-580, would strongly suggest that an interaction of the Cys-580 region of caldesmon with the C-terminus of actin forms part of the actin-caldesmon binding interface.

We thank Ms. Elizabeth Gowell and Dr. Paul Leavis for the preparation of carboxypeptidase A-digested actin, Ms. Anna Wong and Dr. Renne Lu for amino acid analysis, and Drs. Zenon Grabarek and John Gergely for a critical reading of the manuscript before its submission. This work was supported by National Institutes of Health grants AR-30917 to P.G., HL-36153 to W.L., and a grant from the American Heart Association, Indiana Affiliate, to L.P.A.

REFERENCES

- 1 Sobue, K. and Sellers, J. R. (1991) *J. Biol. Chem.* **266**, 12115–12118
- 2 Marston, S. B. and Redwood, C. S. (1991) *Biochem. J.* **279**, 1–16
- 3 Horiuchi, K. Y. and Chacko, S. (1989) *Biochemistry* **28**, 9111–9116
- 4 Velaz, L., Ingraham, R. H. and Chalovich, J. M. (1990) *J. Biol. Chem.* **265**, 2929–2934
- 5 Marston, S. B. and Redwood, C. S. (1992) *J. Biol. Chem.* **267**, 16796–16800
- 6 Bartegi, A., Fattoum, A. and Kassab, R. (1990) *J. Biol. Chem.* **265**, 2231–2237
- 7 Levine, B. A., Moir, A. J. G., Audemard, E., Mornet, D., Patchell, V. B. and Perry, S. V. (1990) *Eur. J. Biochem.* **193**, 687–696
- 8 Adams, S., DasGupta, G., Chalovich, J. M. and Reisler, E. (1990) *J. Biol. Chem.* **265**, 19652–19657
- 9 Graceffa, P. and Jancso, A. (1991) *J. Biol. Chem.* **266**, 20305–20310
- 10 Wang, C.-L. A., Chalovich, J. M., Graceffa, P., Lu, R. C., Mabuchi, K. and Stafford, W. F. (1991) *J. Biol. Chem.* **266**, 13958–13963
- 11 Graceffa, P., Wang, C.-L. A. and Stafford, W. F. (1988) *J. Biol. Chem.* **263**, 14196–14202
- 12 Bryan, J., Imai, M., Lee, R., Moore, P., Cook, R. G. and Lin, W.-G. (1989) *J. Biol. Chem.* **264**, 13873–13879
- 13 Hayashi, K., Kanda, K., Kimizuka, F., Kato, I. and Sobue, K. (1989) *Biochem. Biophys. Res. Commun.* **164**, 503–511.
- 14 Stafford, W. F., Jancso, A. and Graceffa, P. (1990) *Arch. Biochem. Biophys.* **281**, 66–69
- 15 Liu, D. F., Wang, D. and Stracher, A. (1990) *Biochem. J.* **266**, 453–459
- 16 Crosbie, R., Adams, S., Chalovich, J. M. and Reisler, E. (1991) *J. Biol. Chem.* **266**, 20001–20006
- 17 Lash, J. A., Sellers, J. R. and Hathaway, D. R. (1986) *J. Biol. Chem.* **261**, 16155–16160
- 18 Marston, S. B. and Lehman, W. (1985) *Biochem. J.* **231**, 517–522
- 19 Spudich, J. A. and Watt, S. (1971) *J. Biol. Chem.* **246**, 4866–4871
- 20 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 21 Porzio, M. A. and Pearson, A. M. (1977) *Biochim. Biophys. Acta* **490**, 27–34
- 22 Field, V. L. S. and Bowen, W. J. (1968) *Arch. Biochem. Biophys.* **127**, 59–64
- 23 Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- 24 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 25 Jacobson, G. R., Schaffer, M. H., Stark, G. R. and Vanaman, T. C. (1973) *J. Biol. Chem.* **248**, 6583–6591
- 26 Adam, L. P., Haeberle, J. R. and Hathaway, D. R. (1989) *J. Biol. Chem.* **264**, 7698–7703
- 27 Lynch, W. P., Riseman, V. M. and Bretscher, A. (1987) *J. Biol. Chem.* **262**, 7429–7437
- 28 Sutherland, C. and Walsh, M. P. (1989) *J. Biol. Chem.* **264**, 578–583
- 29 Riseman, V. M., Lynch, W. P., Nefsky, B. and Bretscher, A. (1989) *J. Biol. Chem.* **264**, 2869–2875
- 30 Katayama, E. (1989) *J. Biochem. (Tokyo)* **106**, 988–993
- 31 Adam, L. P., Gapinski, C. J. and Hathaway, D. R. (1992) *FEBS Lett.* **302**, 223–226
- 32 Hayashi, K., Fujio, Y., Kato, K. and Sobue, K. (1991) *J. Biol. Chem.* **266**, 355–361
- 33 Yamashiro, S., Yamakita, Y., Hosoya, H. and Matsumura, F. (1991) *Nature* **349**, 169–162
- 34 Novy, R. E., Lin, J. L.-C. and Lin, J. J.-C. (1991) *J. Biol. Chem.* **266**, 16917–16924
- 35 Bryan, J. and Lee, R. (1991) *J. Muscle Res. Cell Motil.* **12**, 372–375
- 36 Humphrey, M. B., Herrera-Sosa, H., Gonzalez, G., Lee, R. and Bryan, J. (1992) *Gene* **112**, 197–204
- 37 Vorotnikov, A. V. and Gusev, N. B. (1991) *Biochem. J.* **273**, 161–163
- 38 Drabikowski, W., Lehrer, S., Nagy, B. and Gergely, J. (1977) *Arch. Biochem. Biophys.* **181**, 359–361
- 39 Ambler, R. P. (1972) *Methods Enzymol.* **35**, 143–154
- 40 Chantler, P. D., Tao, T. and Stafford, W. R. (1991) *Biophys. J.* **59**, 1242–1250
- 41 Barden, J. A., Phillips, L., Cornell, B. A. and dos Remedios, C. G. (1989) *Biochemistry* **28**, 5895–5901
- 41a Braver, M. and Sykes, B. D. (1986) *Biochemistry* **25**, 2187–2191
- 42 Thomas, D. D., Seidel, J. C. and Gergely, J. (1979) *J. Mol. Biol.* **132**, 257–273
- 43 Szczesna, D., Graceffa, P., Wang, C.-L. A. and Lehrer, S. S. (1992) *Biophys. J.* **61**, A154 (abstr.)
- 44 Wang, C.-L. A., Wang, L.-W. C., Xu, S., Lu, R. C., Saavedra-Alanis, V. and Bryan, J. (1991) *J. Biol. Chem.* **266**, 9166–9172
- 45 Makuch, R., Kolakowski, J. and Dabrowska, R. (1992) *FEBS Lett.* **297**, 237–240
- 46 Kolakowski, J., Makuch, R. and Dabrowska, R. (1992) *FEBS Lett.* **309**, 65–67
- 47 Johannes, F.-J. and Gallwitz, D. (1991) *EMBO J.* **10**, 3951–3958
- 48 O'Donoghue, S. I., Miki, M. and dos Remedios, C. G. (1992) *Arch. Biochem. Biophys.* **293**, 110–116
- 49 Mossakowska, M., Moraczewska, J., Khaitlina, S. and Strzelecka-Golaszewska, H. (1993) *Biochem. J.* **289**, 897–902
- 50 Furst, D. O., Cross, R. A., De Mey, J. and Small, J. V. (1986) *EMBO J.* **5**, 251–257