The EMBO Journal vol.4 no.1 pp.45–49, 1985

Geometry of actin-membrane attachments in the smooth muscle cell: the localisations of vinculin and α-actinin

J.V.Small

Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstr. 11, A-5020 Salzburg, Austria

Communicated by J.V.Small

Antibodies to vinculin, a component of actin-membrane attachment sites, revealed by immunofluorescence microscopy a parallel co-axial array of continuous rib-like bands on the surface of isolated vertebrate smooth muscle cells. Images of extended and shortened cells showed that these ribs remain co-axially organised on contraction. Reference to earlier studies and labelling of thin sections indicates that the ribs correspond in position to the adhesion plaques previously described in electron microscope studies. Alpha-actinin showed a punctate distribution consistent with its presence in the cytoplasmic dense bodies, but did not show a constant association with the vinculin-containing ribs. It is suggested that α-actinin is an intracellular actin linker and not membrane associated, as earlier supposed, and that vinculin is, as deduced by others, a mediator of actin membrane attachment. The apparent co-association of these two proteins, noted previously, is concluded to arise from the inevitable geometrical apposition of peripheral and pre-terminal parts of the contractile machinery with the cell membrane.

Key word: actin attachment/α-actinin/contractile apparatus/smooth muscle/vinculin

Introduction

The three-dimensional organisation of the contractile apparatus of smooth muscle remains still a matter of debate and speculation (see reviews by Small and Sobieszek, 1980; Bagby, 1983). For example, while the existence of myosin in filament form in both relaxed and contracting living muscle appears to be settled (Lowy *et al.*, 1973; Shoenberg and Haselgrove, 1974; Somlyo *et al.*, 1981; Tsukita *et al.*, 1982) the precise structural interrelationships of the myofilaments has escaped definition.

Indications of some three-dimensional order within the smooth muscle cell have come from the demonstration of discrete, fibrillar and birefringent contractile units that become progressively oblique with respect to the cell axis, on shortening (Fay and Delise, 1973; Small, 1974; Fisher and Bagby, 1977). And the results of studies of thin and semi-thin sections in the electron microscope have been taken to suggest the existence of a sarcomere-like organisation, perhaps within these fibrils, involving an I-band type stucture centred around the cytoplasmic dense bodies (Heumann, 1977; Ashton *et al.*, 1975; Bond and Somlyo, 1982). However, these collected data have been so far insufficient to supply a clear picture of the spatial filament organisations in the cell.

To obtain further, independent structural information we, as well as others (Fay *et al.*, 1983; Bagby, 1980; Geiger *et al.*, 1981), have been applying immunocytochemical methods to isolated smooth muscle cells and sections of smooth muscle. In this report we describe the localisation of the protein vinculin, an established molecular marker of sites of actin-membrane attachment (Geiger, 1983) as well as of α-actinin, and discuss how their localisations put new constraints on models of the smooth muscle contractile apparatus.

Results and Discussion

In cross-sections of smooth muscle, labelling with antibodies to vinculin reveals a punctate localisation at the cell periphery (Geiger *et al.*, 1983; Drenckhahn and Mannherz, 1983; Figure 1b) that corresponds to the sites of the plasmalemma-associated adhesion plaques seen in the electron microscope (Geiger *et al.*, 1981). Labelling of cells, isolated from different smooth muscles, with vinculin antibodies shows that the punctate localisation in sections corresponds to the presence of longitudinal, mainly continuous, vinculin-containing ribs on the cell surface (Figure 2). Discontinuities in labelling were occasionally observed and showed themselves as merging of neighbouring bands (Figure 2e) or abrupt termination (Figure 2a). In spontaneously contracted (Figure 2e–g) as well as in extended cells (Figure 2e–d) the ribs showed a co-axial orientation, arguing against either a helicoidal arrangement of attachment sites (see e.g., hypothetical arrangement proposed earlier; Small, 1977) or a coiling of the cell as a whole on shortening (Fisher and Bagby, 1977).

In studies of smooth muscle using the freeze-etch procedure, longitudinal channels of smooth membrane, alternating with channels rich in vesicles have been clearly demonstrated (Devine *et al.*, 1971; Gabella and Blundell, 1978). The correspondence of the smooth membrane channels with the membrane-associated dense plaques (Devine *et al.*, 1971) is in full accord with the present data.

Since α-actinin as well as vinculin has been localised in the membrane plaques of smooth muscle (Schollmeyer *et al.*, 1976; Geiger *et al.*, 1981) it was relevant to recompare the localisation of these two proteins. Consistent with earlier studies on isolated amphibian smooth muscle cells (Fay *et al.*, 1983; Bagby, 1980) α-actinin antibodies revealed, in isolated mammalian cells, a fine, punctate label (Figure 3b, c). In ultrathin sections this label was, expectedly, more clear (Figures 1c and 3a) but showed one feature not completely consistent with previous reports. Thus, the ultrastructural immunocytochemical studies of Schollmeyer *et al.* (1976) and Geiger *et al.* (1981) were taken to imply the constant association of α-actinin with the dense plaque regions. Yet if this were so, cross-sections labelled with α-actinin antibodies should delineate the cell borders with a punctate label like that found for vinculin, which is not the case (Figure 1c). A continuous vinculin-like distribution for α-actinin was also not found at the surface of isolated cells (see also Bagby,

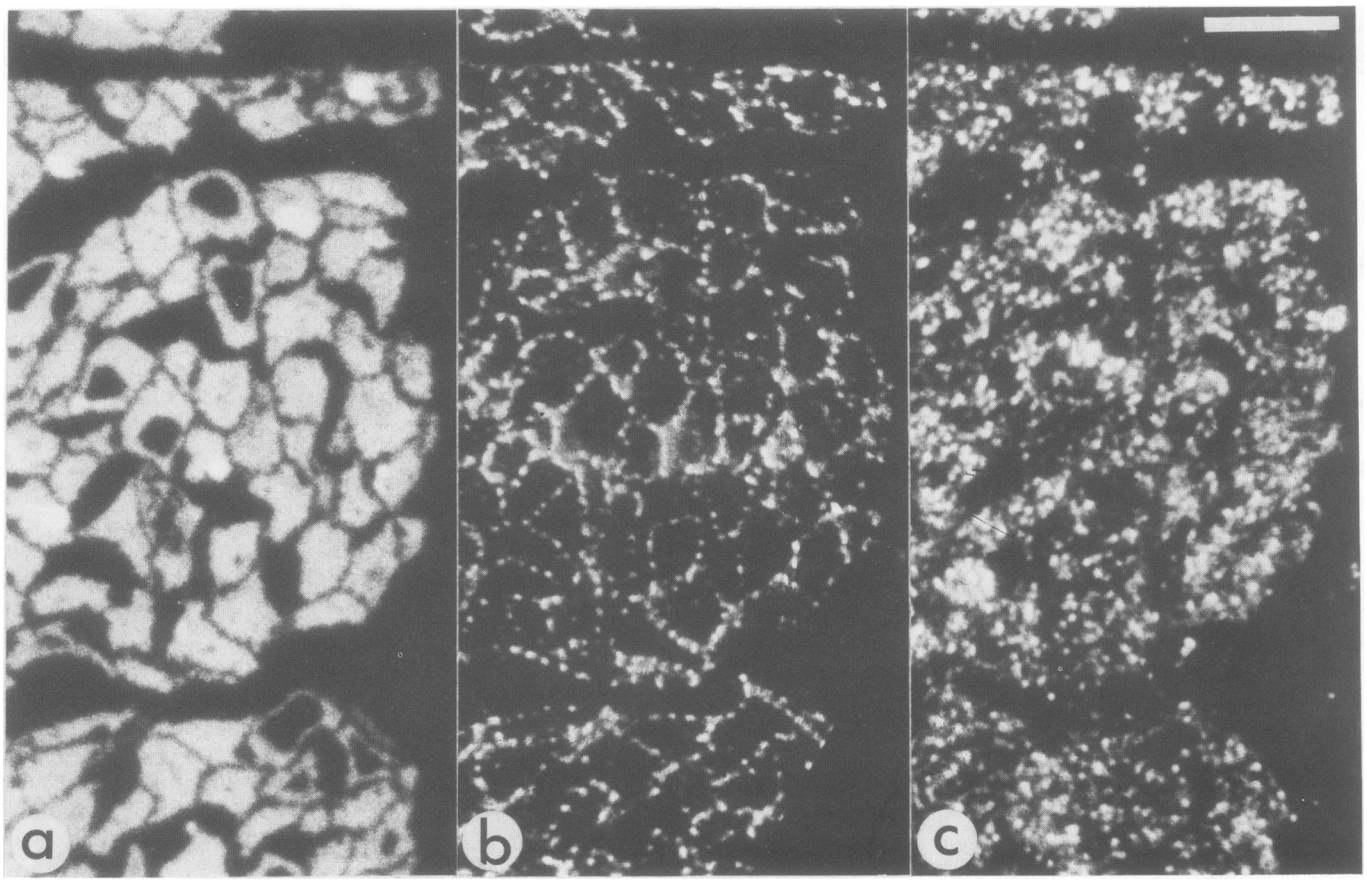

Fig. 1. Serial ultrathin cross-sections of polyvinyl alchol embedded guinea pig *taenia coli* muscle stained with antibodies to: **a**, actin; **b**, vinculin; **c**, α-actinin. Bar, 5 μm.

1980; Fay *et al.* 1983), although an apparent lining up of α-actinin sites, presumably beneath vinculin containing channels, was observed (Figure 3b, c). In the light of this striking difference between a linear surface label for vinculin and a punctate, cytoplasmic label for α-actinin one may question any direct association of these two proteins. Indeed, more recent studies of the focal contact regions of fibroblasts have indicated that the α-actinin localised there by immunofluorescence is not directly bound, like vinculin, to the contact sites (Burridge and McCullough, 1980; Avnur *et al.*, 1983). Likewise, we suggest that the noted association of α-actinin with the smooth muscle cell membrane does not actually relate to attachment but arises from the inevitable close proximity of the near to terminal regions of units of the contractile or cytoskeletal apparatus to the cell surface.

Recent studies of skeletal and cardiac muscle have documented the existence of transverse, vinculin-containing ribs on the sarcolemma (Pardo *et al.*, 1983a, 1983b). These ribs or 'costameres' do not, however, coincide with structures like the dense plaques of smooth muscle or adherens junctions of other cells (Geiger, 1983) and their relationship to these is therefore unclear. Notably, immunoblots of the vinculin monoclonal antibody used in this study (Figure 4) and raised using chicken gizzard vinculin (B.Geiger, personal communication) cross-reacted generally with mammalian smooth muscle but with neither cardiac (guinea pig) nor skeletal muscle (rabbit) homogenates. In smooth muscle the antibody reacted with both vinculin and a related, higher mol. wt. polypeptide, metavinculin (Feramisco *et al.*, 1982; D'Angelo Siliciano and Craig, 1982; B.Geiger, personal communication; Figure 4).

The continuous distribution of vinculin on the cell surface suggests a corresponding, continuous attachment of the actin filaments that anchor the contractile machinery. The apparent co-localisation, from cross and oblique sections, of an intermediate filament-associated protein, plectin (Wiche *et al.*, 1983) with the dense plaques would also suggest a potential for continuous association of intermediate filaments with these surface structures. Precisely how the contractile and cytoskeletal elements are accommodated together at these attachment sites remains to be established.

Materials and methods

Cells

Smooth muscle cells were isolated from three sources: the guinea pig taenia coli, guinea pig vas deferens and chicken expansor secundariorum. The isolation procedure was essential as described previously (Small, 1977) but with the omission of ATP and xylocaine from the collagenase digestion medium. For immunocytochemistry, freshly prepared cells were washed once *via* gentle centrifugation and then sedimented from suspension, at low speed in a table centrifuge, onto polylysine-coated 12 mm coverslips mounted in AMICON micropartition adaptors (type MPS-1) in place of the filter. This was found to be the most effective method to obtain cell monolayers that were resistant to

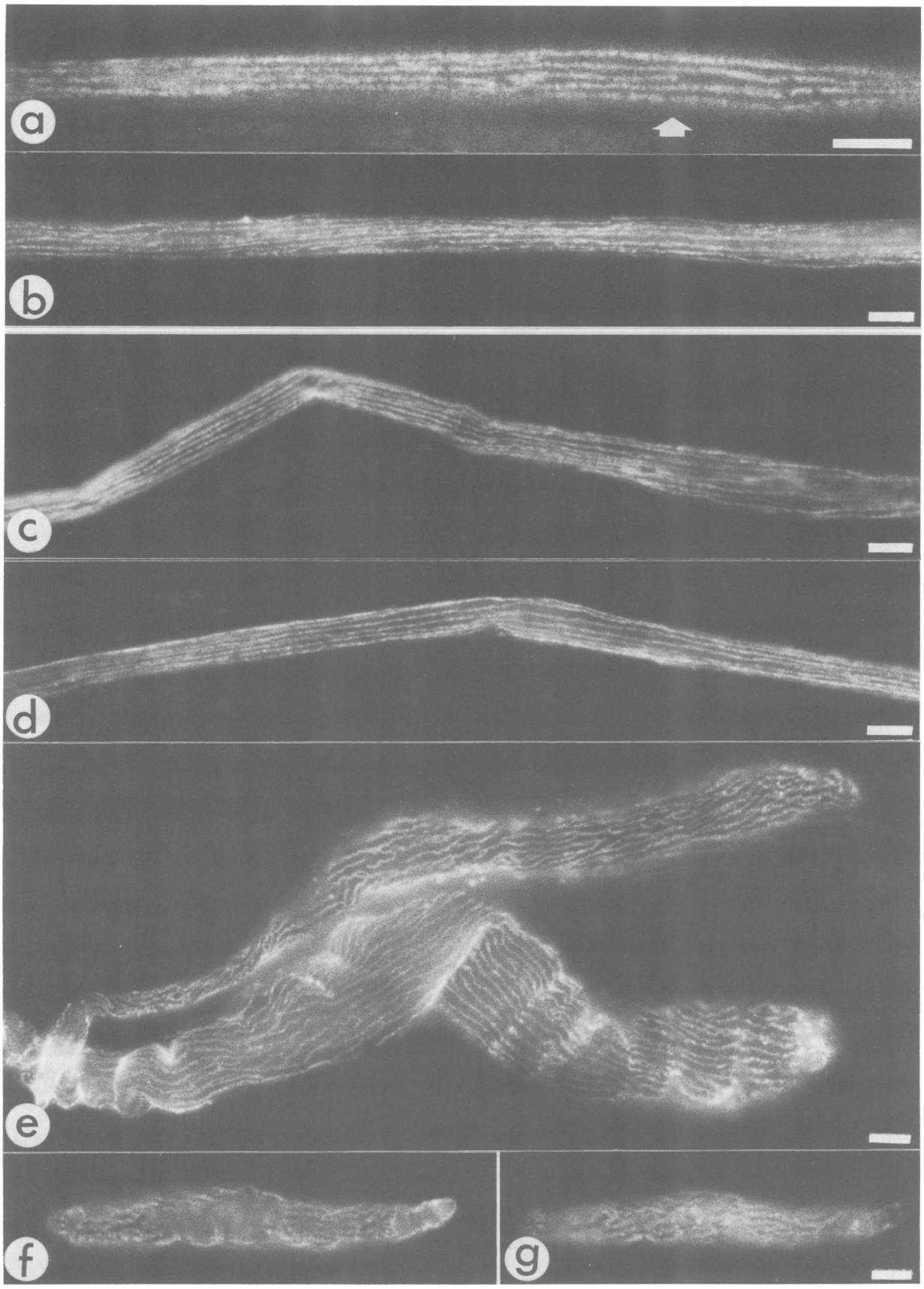

Fig. 2. Isolated smooth muscle cells labelled with the monoclonal vinculin antibody. **a**–**d**, Extended cells obtained from guinea pig *taenia coli*. Arrow in **a**, indicates termination of one of the surface ribs. **e**, Relatively short cells isolated from the chicken *expansor secundariorum*. **f** and **g**, Contracted cell isolated from the guinea pig *vas deferens* and photographed at two focal levels to show co-axial arrangement of surface ribs, as in the extended cells (**a**–**d**). Bars, 5 μm.

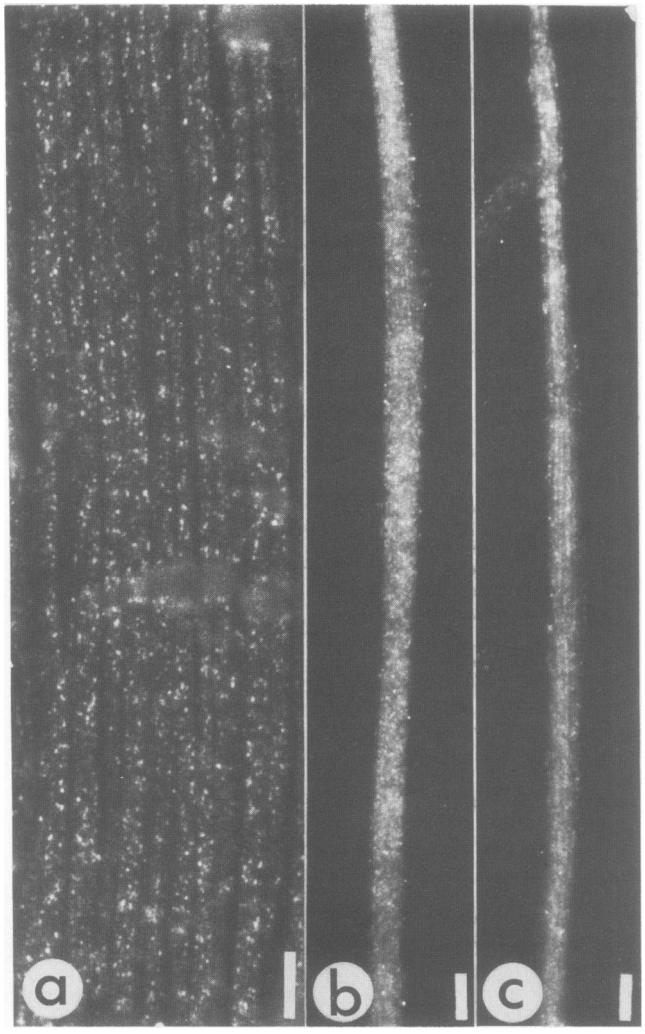

Fig. 3. a, Ultrathin longitudinal section of polyvinyl alcohol embedded guinea pig *taenia coli* muscle labelled with an α-actinin antibody. **b,** Isolated *taenia coli* cells labelled with α-actinin antibody. **c** is taken at a focal level close to the cell surface and shows lining up of α-actinin sites. Bars, 5 μm.

dissociation form the coverslips during the fixation and antibody procedure. For fixation, the cells were either extracted with Triton X-100 (0.5%), in 'solution 1' (Small, 1977) and then fixed in 3% paraformaldehyde in the same solution for 1 h or fixed directly in a mixture of 0.2% Triton X-100 and 3% paraformaldehyde for 1 h.

Immunocytochemistry

Monoclonal and polyclonal antibodies to vinculin were generously donated by Dr. B.Geiger (Weizmann Institute, Israel). Monospecific, polyclonal antibodies to α-actinin were raised against α-actinin purified from porcine stomach (Small and Sobieszek, 1983) in collaboration with Dr. J.De Mey (Janssen Pharmaceutica, Belgium). The α-actinin antibody used here was purified by affinity chromatography using porcine stomach α-actinin and reacted on immunoblots with pure α-actinin and a single co-migrating band in whole smooth muscle samples (unpublished data). The antibody staining procedure was as follows: after application of the first antibody (40 min in Tris-buffered saline, pH 7.6, supplemented with 1% normal goat serum) coverslips were washed by flotation (2 x 15 min) on the same buffer in multiwell dishes, on a rotatilt table. Application of the second antibody (rhodamine conjugated – a gift from Dr. B.Geiger) and the subsequent washes were carried out in the same manner prior to mounting in Gelvatol 20-30 (Monsanto Petrochemicals, USA). Ultrathin sections were made from paraformaldehyde-fixed smooth muscle embedded in polyvinylalcohol, as described elsewhere (Small, 1984, and in preparation). These were mounted on polylysine-coated 4 x 4 mm coverslips and stained as for the isolated cells. Observation and photography was carried out in a ZEISS photomicroscope III equipped with epifluorescence optics.

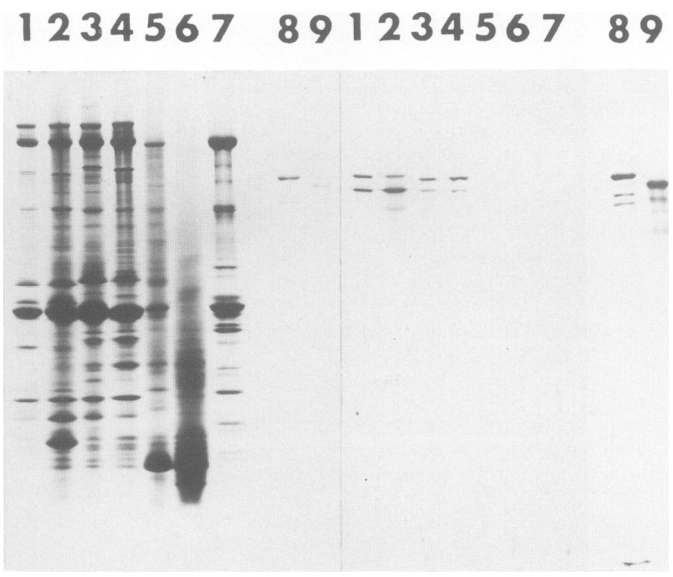

Fig. 4. SDS electrophoresis gel (left) and corresponding immunoblot with the vinculin monoclonal antibody (right). Immunoblotting was carried out essentially according to Towbin *et al.* (1979) and the antibody visualised using the immunogold-silver staining method of Moeremens *et al.* (1985). The samples were as follows: **1–5** whole muscle samples from respectively; **1**, chicken *expansor secundariorum;* **2**, chicken gizzard; **3**, porcine stomach; **4**, guinea pig *taenia coli*; **5**, guinea pig heart; **6**, guinea pig brain; **7**, rabbit psoas whole muscle; **8**, porcine stomach vinculin; **9**, turkey gizzard vinculin. Vinculin purified from porcine stomach runs predominantly as a polypeptide of ~150 000 daltons (D.Fürst, unpublished data), as for avian metavinculin (Feramisco *et al.*, 1982).

Acknowledgements

The author thanks Dr. B.Geiger for the gift of some of the antibodies used here and for useful discussion and criticism. Thanks also go to Dr. J.De Mey for raising the antibody to α-actinin, to Dr. H.Hinssen and A.Sobieszek for criticism of the manuscript and to Mr. D.Fürst for performing the immunoblot shown in Figure 4. The author thanks Miss M.Hattenberger and Mrs. H.Kunka for excellent technical assistance, Mr. G.Sulzer for photography and Mrs. G.McCoy for typing. This work was supported in part by a grant from the Austrian Science Research Council.

References

Ashton,F.T., Somlyo,A.V. and Somlyo,A.P. (1975) *J. Mol. Biol.*, **98**, 17-29.
Avnur,Z., Small,J.V. and Geiger,B. (1983) *J. Cell Biol.*, **96**, 1622-1630.
Bagby,R.M. (1980) *Histochemistry*, **69**, 113-130.
Bagby,R.M. (1983) in Stephens,N.L. (ed.), *Biochemistry of Smooth Muscle*, Vol. **I**, CRC Press, Florida, pp. 1-84.
Bond,M. and Somlyo,A.V. (1982) *J. Cell Biol.*, **95**, 403-413.
Burridge,K. and McCullough,L. (1980) *J. Supramol. Struct.*, **13**, 53-65.
D'Angelo Siliciano,J. and Craig,S.W. (1982) *Nature*, **300**, 533-535.
Devine,C.E., Simpson,F.O. and Bertaud,W.S. (1971) *J. Cell Sci.*, **8**, 427-443.
Drenckhahn,D. and Mannherz,H.G. (1983) *Eur. J. Cell Biol.*, **30**, 167-176.
Fay,F.S. and Delise,C.M. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 641-645.
Fay,F.S., Fujiwara,K., Rees,D.D. and Fogarty,K.E. (1983) *J. Cell Biol.*, **96**, 783-795.
Feramisco,J.R., Smart,J.E., Burridge,K., Helfman,D.M. and Thomas,P. (1982) *J. Biol. Chem.*, **257**, 11024-11031.
Fisher,B.A. and Bagby,R.M. (1977) *Am. J. Physiol.*, **232**, C5-C14.
Gabella,G. and Blundell,D. (1978) *Cell Tissue Res.*, **190**, 255.
Geiger,B. (1983) *Biochim. Biophys. Acta*, **737**, 305-341.
Geiger,B., Dutton,A.H, Tokuyasu,K.T. and Singer,S.J. (1981) *J. Cell Biol.*, **91**, 614-628.
Geiger,B., Schmidt,E. and Franke,W.W. (1983) *Differentiation*, **23**, 189-205.
Heumann,H.G. (1971) *Cytobiologie*, **3**, 259-281.
Lowy,J., Vibert,P.J., Haselgrove,J.C. and Poulsen,F.-R. (1973) *Phil. Trans. R. Soc. Lond. B*, **265**, 191-196.
Moermens,M., Daneels,G., Van Dijck,A., Langanger,G. and De Mey,J. (1985) *J. Immunol. Methods*, in press.
Pardo,J.V., D'Angelo Siliciano,J. and Craig,S.W. (1983a) *Proc. Natl. Acad.*

Sci. USA, **80**, 1008-1012.
Pardo,J.V., D'Angelo Siliciano,J. and Craig,S.W. (1983b) *J. Cell Biol.*, **97**, 1081-1088.
Schollmeyer,J.E., Furcht,L.T., Goll,D.E., Robson,R.M. and Stromer,M.H. (1976) in Goldman,R., Pollard,T. and Rosenbaum,J. (eds.), *Cell Motility*, Vol. **3**, Cold Spring Harbor Laboratory Press, NY, pp. 361-388.
Shoenberg,K. and Haselgrove,J.C. (1974) *Nature*, **249**, 152-153.
Small,J.V. (1974) *Nature*, **249**, 324-327.
Small,J.V. (1977) *J. Cell Sci.*, **24**, 327-349.
Small,J.V. (1984) in Csanády,Á., Röhlich,P. and Szabó,D. (eds.), *Proceedings of the Eighth European Congress on Electron Microscopy*, Vol. **3**, Budapest, pp. 1799-1800.
Small,J.V. and Sobieszek,A. (1980) *Int. Rev. Cytol.*, **64**, 241-306.
Small,J.V. and Sobieszek,A. (1983) in Stephens,N.L. (ed.), *The Biochemistry of Smooth Muscle*, CRC Press, Florida, pp. 85-140.
Somlyo,A.V., Butler,T.M., Bond,M. and Somlyo,A.P. (1981) *Nature*, **294**, 567-570.
Towbin,H., Straehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.
Tsukita,S., Tsukita,S., Usukura,J. and Ishikawa,H. (1982) *Eur. J. Cell Biol.*, **28**, 195-201.
Wiche,G., Krepler,R., Artlieb,U., Pytela,R. and Denk,H. (1983) *J. Cell Biol.*, **97**, 887-901.

Received on 16 October 1984; revised on 2 November 1984