The EMBO Journal Vol.2 No.10 pp.1701 – 1705, 1983

The p36 substrate of tyrosine-specific protein kinases co-localizes with non-erythrocyte α-spectrin antigen, p230, in surface lamina of cultured fibroblasts

V.-P. Lehto*, I. Virtanen, Raili Paasivuo[1], R. Ralston[2] and K. Alitalo[2]

Department of Pathology, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki 29, [1]Labsystems Research Laboratories, Labsystems Oy, Helsinki, Finland, and [2]Department of Microbiology and Immunology, University of California, San Francisco, CA 94143, USA

Communicated by A. Vaheri
Received on 6 June 1983; revised on 19 July 1983

Biochemical and immunofluorescence studies have demonstrated that p36, a major substrate for the tyrosine-specific protein kinases induced by several sarcoma viruses and epidermal growth factor, is associated with plasma membranes and detergent-resistant cytoskeletal structures of cultured cells. We have used here polyclonal antisera and monoclonal antibodies in indirect immunofluorescence microscopy to study the subcellular location of p36 and the p230, which is a subplasmalemmal polypeptide showing immunologic cross-reactivity with erythrocyte α-spectrin. Both p36 and p230 showed a diffuse distribution in fixed and permeabilized cells and were localized in a surface lamina-like network in Triton-extracted cells. In double-staining experiments, an extensive co-distribution between these proteins was seen in detergent-treated cultured fibroblasts. These results, together with our previous work, suggest that the p36 protein is an integral part of the detergent-resistant proteinaceous network at the cytoplasmic face of the plasma membrane.

Key words: cytoskeleton/phosphorylation/transformation

Introduction

Cells transformed by retroviruses have been a favoured model system to study the molecular mechanisms of malignant transformation because they harbor genetic loci, the viral oncogenes, whose activity can initiate and maintain the transformed phenotype (Bishop, 1983). In particular, studies with Rous sarcoma virus-transformed cells have revealed an oncogene-encoded, 60 000 dalton phosphoprotein (pp60$^{v\text{-}src}$), possessing a capacity for tyrosine-specific phosphorylation, a rare modification in normal cells (Collett and Erikson, 1978; Levinson *et al.*, 1978, 1980; Erikson *et al.*, 1979; Maness *et al.*, 1979; Collett *et al.*, 1980; Hunter and Sefton, 1980; Gilmer and Erikson, 1981; McGrath and Levinson, 1982). Among the identified substrates of the pp60$^{v\text{-}src}$ (see Sefton *et al.*, 1982) undergoing tyrosine phosphorylation, a protein of mol. wt. 36 000 daltons has been the subject of an intensive study. It represents ~0.3% of the total cellular protein (Erikson and Erikson, 1980; Radke *et al.*, 1980; Cooper and Hunter, 1983), and has been shown to be phosphorylated to the same tyrosine residue both in transformed cells and by purified pp60$^{v\text{-}src}$ *in vitro* (Radke and Martin, 1979; Erikson and Erikson, 1980). It is also phosphorylated at tyrosine in normal, epidermal growth factor (EGF) receptor-positive cells, upon exposure to EGF (Cooper and Hunter, 1981; Hunter and Cooper, 1981; Ghosh-Dastidar and Fox, 1983). In the latter case, the kinase responsible for p36 phosphorylation has not been identified, but it should be noted that the 160 000 mol. wt. EGF-receptor protein possesses tyrosine kinase activity (Cohen *et al.*, 1982) and an as yet incompletely known association exists between pp60$^{v\text{-}src}$ and EGF-receptor activity (Chinkers and Cohen, 1981; Kudlow *et al.*, 1981; Buhrow *et al.*, 1982; Cohen *et al.*, 1982).

The role of p36 in normal cells and its contribution to the pleiotropic alterations observed in transformed cells has remained unelucidated. One way to approach this problem has been to determine the subcellular distribution of p36 by using, for instance, cell fractionation. These studies have indicated that p36 is associated with plasma membranes (Cooper and Hunter, 1982; Amini and Kaji, 1983) or with detergent-resistant cytoskeletal structures (Cheng and Chen, 1981; Cooper and Hunter, 1982). Recently, results of studies with specific antisera and immunofluorescence microscopy have also suggested that p36 is closely associated with the plasma membrane in untransformed cultured cells (Courtneidge *et*

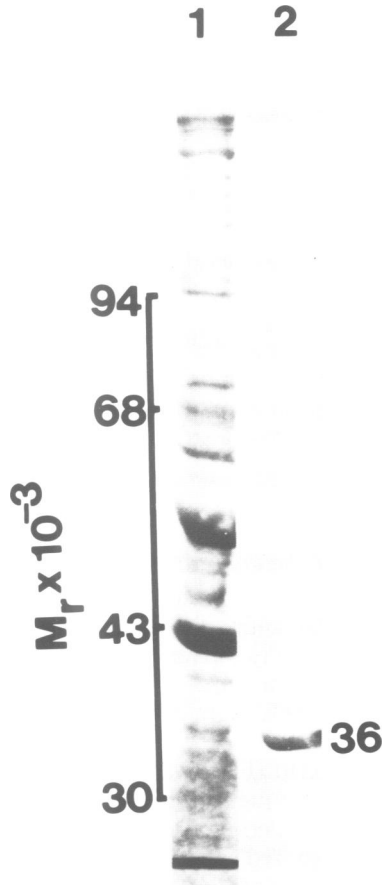

Fig. 1. Immunoblot of electrophoretically separated polypeptides of cultured human fibroblasts using anti-p36 antibodies. (1) Amido black staining. (2) Immunoblot with anti-p36 antibodies.

*To whom reprint requests should be sent.

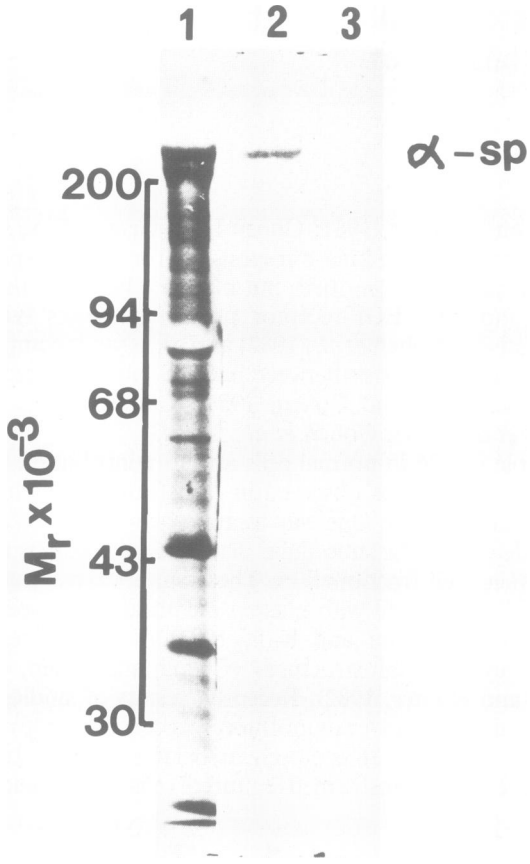

Fig. 2. Immunoblot of electrophoretically separated polypeptides of ghost preparations of human red blood cells. (1) Amido black staining. (2) Immunoblot with monoclonal anti-p-230 antibodies. (3) Immunoblot with monoclonal anti-p-230 antibodies preabsorbed with α-spectrin. α-sp denotes α-spectrin.

al., 1983; Nigg *et al.* 1983).

In this study we have used immunofluorescence microscopy to compare the subcellular location of p36 with that of p230, a recently described subplasmalemmal, lattice-forming protein which is immunologically cross-reactive with α-spectrin of human red blood cells (Lehto and Virtanen, 1983). The results show an extensive co-distribution between these proteins and suggest a structural or functional association between p36 and p230.

Results

Characterization of the antisera

The specificity of the anti-p36 antiserum and the monoclonal anti-p230 antibodies is demonstrated in Figures 1 and 2; electrophoretically separated polypeptides of cultured fibroblasts or ghost preparations (Steck *et al.*, 1970) of human red blood cells were transferred to nitrocellulose sheets which were then exposed to rabbit anti-p36 (Figure 1), or to mouse anti-p230 (Figure 2), followed by horseradish peroxidase (POX)-conjugated mouse anti-rabbit IgG or POX-conjugated goat anti-mouse IgG, respectively. The anti-p36 antibodies recognized specifically a single polypeptide of mol. wt. 36 000 daltons while the monoclonal anti-p230 antibodies recognized a single polypeptide of 230 000 daltons corresponding to α-spectrin (Marchesi, 1983). The α-spectrin reactivity of the rabbit anti-p230 antiserum has been demonstrated elsewhere (Lehto and Virtanen, 1983).

Immunofluorescence (IF) findings

No staining was seen either with anti-p36 or anti-p230 antibodies in IF microscopy of paraformaldehyde-fixed cells, suggesting that neither of the corresponding antigens is exposed at the cell surface (Figure 3a). On the other hand, for the most part homogeneous, membrane-associated staining was seen with both antisera in paraformaldehyde-Nonidet P-40 (NP-40)-fixed cells (Figure 3b and c). There was also some cytoplasmic staining discernible with both antisera as shown by focusing at different levels of the monolayer. In addition, a dense, ventrally located plaque-like formation could often be visualized with anti-p230 antibodies (Figure 3c). Both the anti-p36 and the anti-p230 staining reactions could be inhibited by preabsorption of the antisera with the corresponding purified antigens (Figure 3d and e). In cytoskeletal preparations of cultured fibroblasts, p36 was seen to be localized to an extensive, lace-like network which distinctly coincided with the externally disposed detergent-resistant cell surface glycoproteins that could be visualized with tetramethyl rhodamine isothiocyanate-conjugated wheat germ agglutinin (TRITC-WGA) (Figure 4a and b). Notably, this close co-distribution is probably not due to a collapse of Triton-non-extractable material onto other detergent-resistant structures in a non-specific manner since, for instance, myosin, a detergent-resistant cytoskeletal component which is closely associated with the cell surface (Zigmond *et al.*, 1979), showed a completely different distribution in these same preparations (Figure 4c). In double staining, a close co-distribution was seen with p36 and p230; they were both localized to a lattice, similar to that decorated by TRITC-WGA, and could be seen both at the ventral and dorsal surfaces of the cytoskeletal preparations (Figure 5a – d).

Discussion

Many studies have sought to determine the subcellular location of p36 in order to find topological associations which could elucidate the function of this major tyrosine-specific kinase substrate in both normal and transformed cells. It has become evident from cell fractionation, morphologic and immunochemical studies that p36, or at least a fraction of it, is closely associated with plasma membranes (Cooper and Hunter, 1982; Amini and Kaji, 1983; Courtneidge *et al.*, 1983; Nigg *et al.*, 1983), with detergent-resistant cytoskeletal structures (Cheng and Chen, 1981; Cooper and Hunter, 1982) or, according to one study, with ribonucleoprotein particles (Arrigo *et al.*, 1983). However, distinctly specific interactions, such as that of vinculin, another important tyrosine kinase substrate, and focal adhesion sites (Sefton *et al.*, 1981), have not been found between p36 and other membrane or cytoskeletal components. On the other hand, the suggestion that cytosolic malic dehydrogenase activity is associated with p36 (Rubsamen *et al.*, 1982), has recently been challenged (Cooper *et al.*, 1983; Courtneidge *et al.*, 1983).

The results presented here show that in cultured fibroblasts p36 co-distributes with p230, a subplasmalemmal, cytoskeleton-associated protein, which is closely associated with the cytoplasmic face of the plasma membrane (Lehto and Virtanen, 1983). It also remains in the detergent-resistant surface lamina, which consists of both detergent-resistant cytoplasmalemmal and cell surface proteins (Ben Ze'ev *et al.*, 1979; Mescher *et al.*, 1981) and which can be visualized with fluorochrome-conjugated WGA (Lehto *et al.*, 1983). This co-

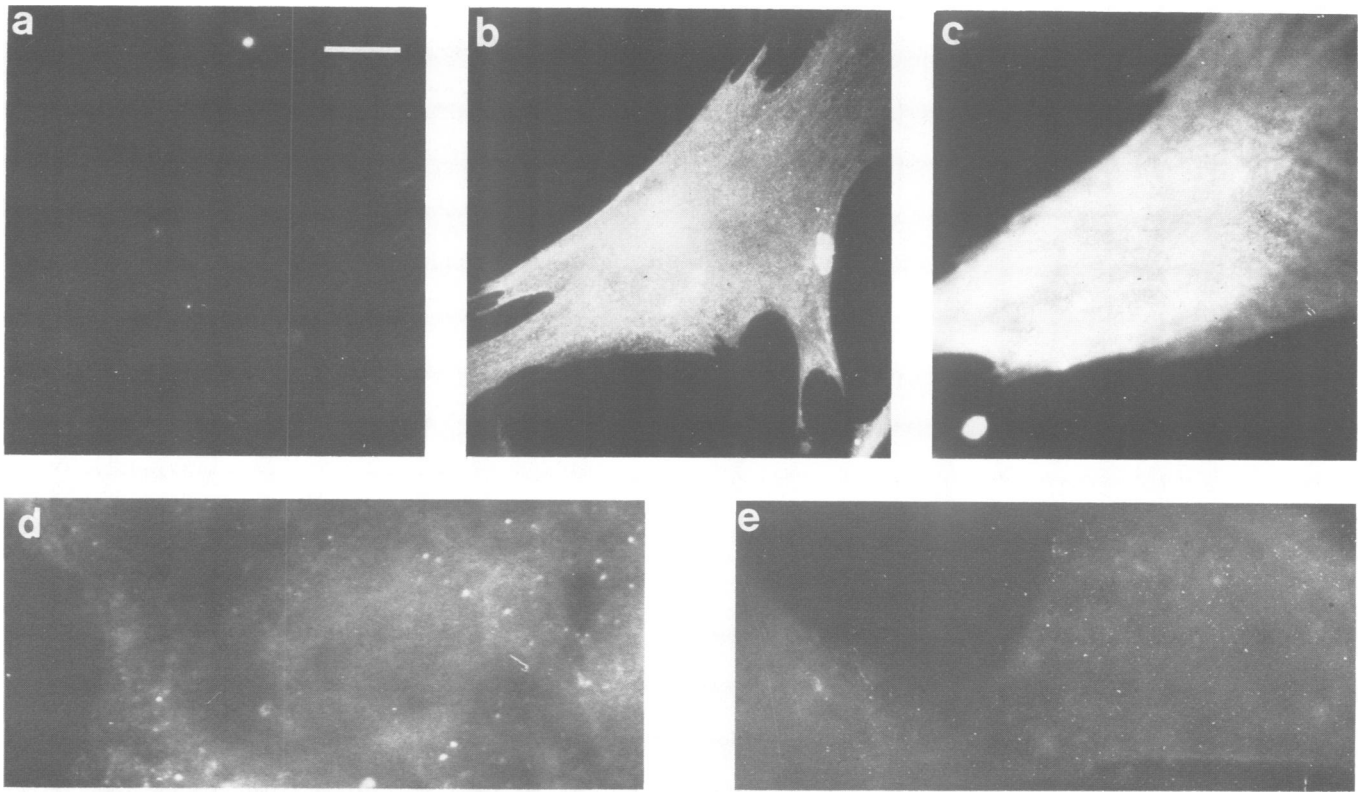

Fig. 3. Immunofluorescence microscopy showing reaction of anti-p36 antibodies on paraformaldehyde-fixed cells **(a)** on paraformaldehyde-NP-40-fixed cells **(b)**, and of rabbit anti-p230 antibodies on paraformaldehyde-NP-40-fixed cells **(c)**. **(d)** and **(e)** show the staining reaction of paraformaldehyde-NP-40-fixed cells with anti-p36 **(d)** and anti-P230 antibodies **(e)** preabsorbed with the corresponding antigens. The prints in **(d)** and **(e)** are slightly underdeveloped to allow the visualization of the cell contours. Scale bar = 10 μm.

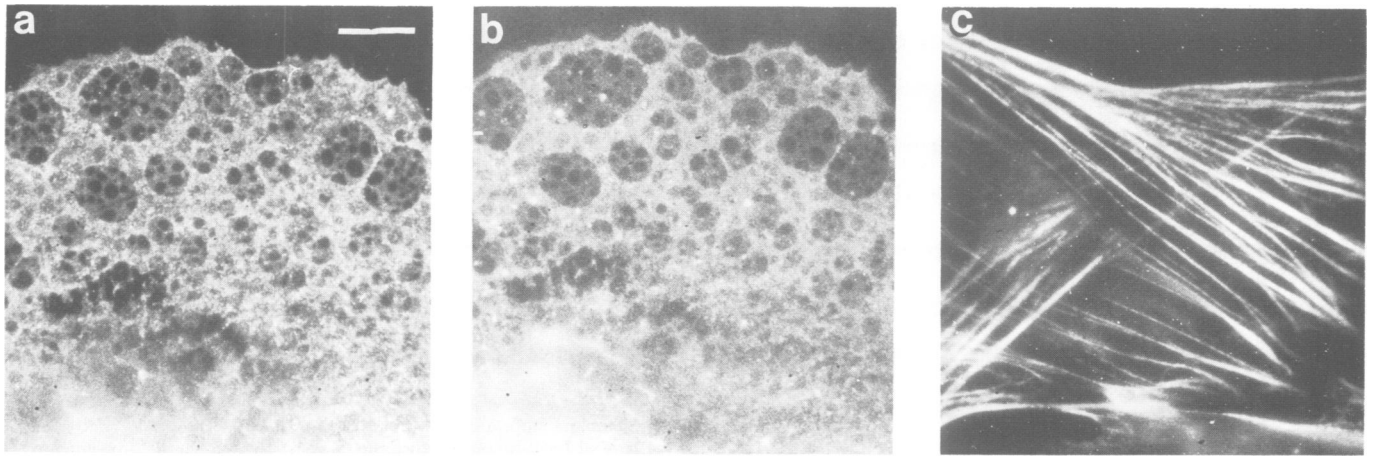

Fig. 4. Immunofluorescence microscopy of cytoskeletal preparations of cultured fibroblasts. Anti-p36 antibodies **(a)**, TRITC-WGA **(b)**, and anti-myosin antibodies **(c)**. **(a)** and **(b)** represent double-staining of the same section. Scale bar = 3 μm.

localization is most apparent in the Triton X100-extracted cytoskeletal preparations of the cells in which ~70% of the cellular protein has been removed (Lehto, 1983). The structural basis of the co-distribution of p36 and p230 is not clear. The fact that the plasma membrane-associated localization of both of these proteins can also be seen in prefixed cells, makes it improbable that the co-distribution is artefactual and due to fortuitous adherence of the proteins to each other. It is possible, however, that the reticular staining pattern seen in cytoskeletal preparations does not represent a native confinement of the proteins but is due to their lateral reorganization after extraction of most of the plasma membrane lipids with detergent (cf., Lehto *et al.*, 1983). The concomitant alterations in the distribution of both p36 and p230 upon detergent extraction strongly favor, however, the interpretation of either direct or indirect linkage between these proteins. This view is further strengthened by our more recent findings that both anti-p36 and anti-p230 fluorescence are excluded from the areas of microfilament bundles and that both antigens seem to undergo a similar partial redistribution upon

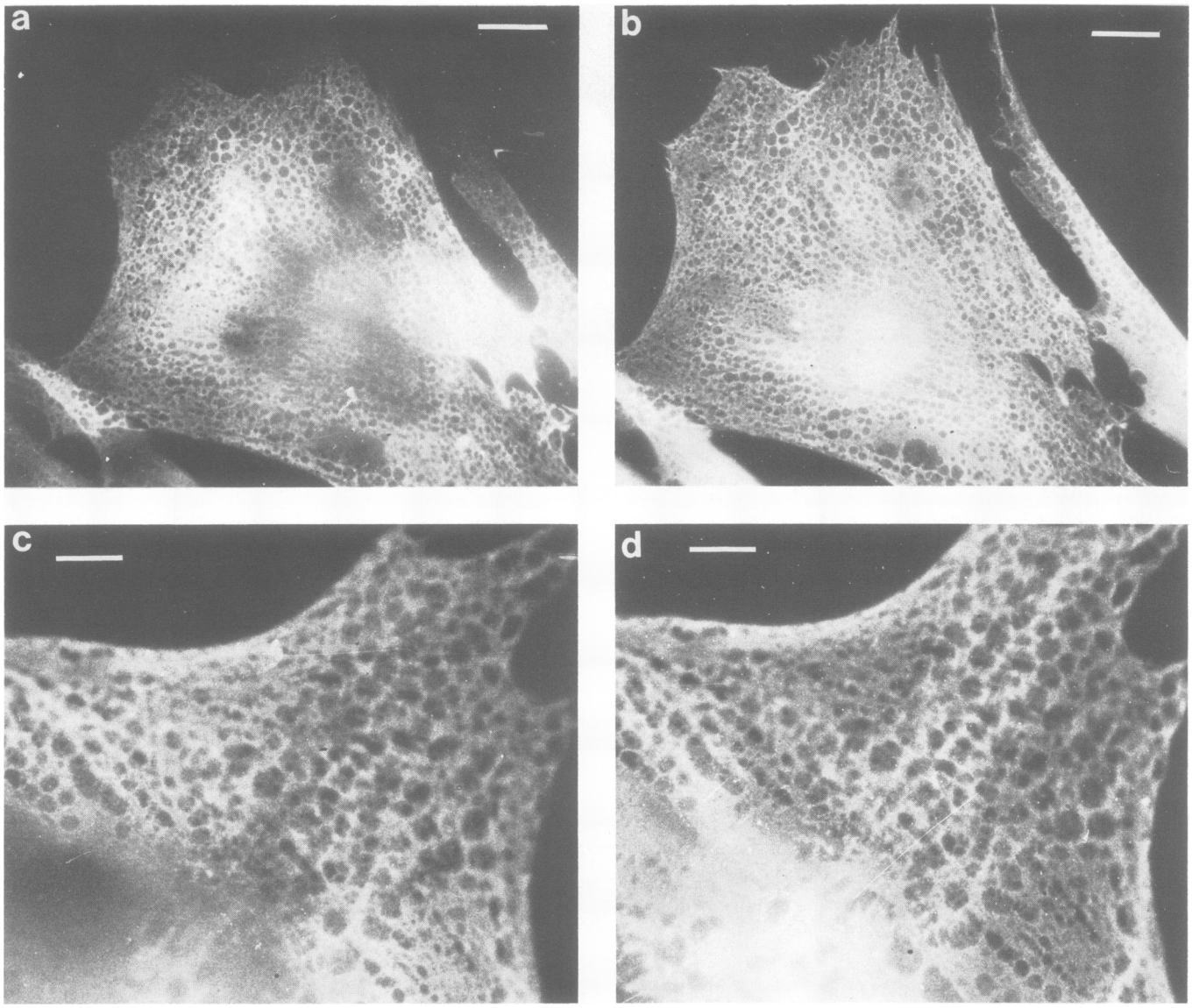

Fig. 5. Immunofluorescence microscopy of cytoskeletal preparations of cultured fibroblasts. Double-staining with anti-p36 antibodies (**a** and **c**) and monoclonal anti-p230 antibodies (**b** and **d**). Scale bar = 10 μm (**a** and **b**), 2 μm (**c** and **d**).

prolonged cytochalasin treatment (K. Alitalo, J. Keski-Oja, I. Virtanen and V.-P. Lehto, unpublished observations).

P230 appears to be an immunoanalogue of α-spectrin, a well-characterized component of erythrocyte membrane skeleton (Branton *et al.*, 1981; Marchesi, 1983). Recently, a family of spectrin-like molecules have been discovered in various types of non-erythroid cells in all of which they are peripherally distributed (Levine and Willard, 1981; Bennett *et al.*, 1982; Burridge *et al.*, 1982; Glenney *et al.*, 1982; Repasky *et al.*, 1982; Lehto and Virtanen, 1983). In view of the proposed role for p36 as a mediator of malignant transformation and of its co-distribution with p230, it is important that spectrin-like proteins are able to cross-link actin (Burridge *et al.*, 1982; Glenney *et al.*, 1982) and that they have been suggested to play a key role in the regulation of cell motility and surface phenomena (Lazarides and Nelson, 1982; Lehto and Virtanen, 1983) which all undergo major changes during transformation.

Materials and methods

Cells

Human embryonic fibroblasts were cultured in plastic Petri dishes in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal calf serum (FCS; Flow Laboratories, Irvine, Scotland) and antibiotics. Human red blood cells were obtained from the Finnish Red Cross Blood Transfusion Service (Helsinki).

Preparation of antisera

p230 was purified from fetal calf lens by hydroxyapatite column chromatography and preparative polyacrylamide gel electrophoresis (Lehto and Virtanen, 1983). The antibodies were raised in rabbits, and affinity-purified as described previously (Lehto and Virtanen, 1983). To produce monoclonal antibodies against p230, BALB/c mice were inoculated i.p. with 100 μg of crude p230 fraction of lens tissue in Freund's complete adjuvant, and boosted i.v. 21 days later with 15 μg of the same material in NaCl-P buffer (140 mM NaCl, 0.1 M phosphate buffer, pH 7.2). Three days later the mice were sacrificed, the spleens were excised and 1.25 x 10^8 suspended spleen cells were fused with 1.25 x 10^7 cells of P3/NS-7/1-Ag 41-1-myeloma line according to Köhler and Milstein (1975).

Supernatants from hybridoma cultures were screened primarily by an

enzyme-linked immunosorbent assay with the original antigen preparation as a ligand. The positive clones were further selected for specificity towards p230 in both immunofluorescence microscopy, using the previously characterized anti-p230 rabbit serum as a control, and in immunoblotting on electrophoretically separated polypeptides of human red blood cell ghosts and cultured fibroblasts. Rabbit antibodies to p36, purified from chicken fibroblasts by ion exchange and hydroxyapatite column chromatography (Erikson and Erikson, 1980; Courtneidge *et al.*, 1983), were raised as described previously (Courtneidge *et al.*, 1983). Anti-myosin antibodies were a kind gift from R.A. Badley (Unilever Research Laboratory, Sharnbrook, UK) (Badley *et al.*, 1978).

IF microscopy

Cells grown on glass coverslips were washed with NaCl-P buffer and then fixed with 2.5% paraformaldehyde in 0.01 M phosphate buffer, pH 7.2, at room temperature for 15 min. For some experiments, the paraformaldehyde-fixed cells were treated with 0.1% NP-40 (BDH-Chemicals Ltd., Poole, UK) in NaCL-P buffer for 5 min to permeabilize the cells (Laurila *et al.*, 1978). Cytoskeletal preparations of cultured fibroblasts were produced by treating the cells with 0.5% Triton X-100 (BDH-Chemicals) in 50 mM Tris-HCl, supplemented with 1 mM phenylmethyl sulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, MO) and 1 mM N-p-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma) on ice for 15 min, washed with ice cold NaCl-P buffer, and then fixed with 2.5% paraformaldehyde for 15 min.

For IF microscopy, the fixed cells or cytoskeletal preparations were exposed either to rabbit-anti-p230 antibodies or to rabbit anti-p36 antibodies followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antiserum (Cappel Laboratories, Cochraneville, CA). For double immunofluorescence stainings, the cells were first exposed to rabbit-anti p36 followed by FITC-conjugated goat anti-rabbit IgG and then to hybridoma anti-p230 antibodies followed by TRITC-conjugated goat-anti mouse IgG (Cappel). The specificity of the immunofluorescence staining was assessed by preabsorbing the antisera with the corresponding antigens in an approximate antigen/antibody ratio of 40:1. To visualize the surface lamina, the detergent-treated cultured fibroblasts were exposed to TRITC-WGA (Vector Laboratories, Burlingame, CA) as described previously (Lehto *et al.*, 1983). The specimens were mounted in 50% glycerol buffered with veronal acetate (pH 8.4) and examined in a Zeiss Universal microscope equipped with an epi-illuminator III RS, HBO 200 W lamp, phase contrast optics and filters for FITC- and TRITC-fluorescence.

Electrophoretic techniques

Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Laemmli (1970). Transfer of electrophoretically separated polypeptides onto a nitrocellulose paper was carried out with a commercial blotting apparatus (Trans Blot, Bio-Rad, Richmond, CA) according to the method of Towbin *et al.* (1979).

Immunoblotting

For immunoblotting, the nitrocellulose sheets were soaked in NaCl-P buffer, 3% bovine serum albumin (BSA), 10% normal swine or rabbit serum, 0.1% Triton X-100 for 2 h, washed and exposed to 50 μg/ml of the primary antiserum in NaCl-P buffer supplemented with 2% BSA and 0.2% Triton X-100 in the above medium for 60 min. After washing, the sheets were incubated with POX-conjugated swine-anti-rabbit IgG or POX-conjugated rabbit-anti-mouse IgG (50 μg/ml; Dakopatts, Copenhagen, Denmark) for 60 min, washed and then exposed to 3,3′-diaminobenzidine-tetrahydrochloride (DAB; Fluka AG, Buchs, Switzerland) in 10 mM Tris-HCl buffer, pH 7.5, 0.05% H_2O_2 to develop the peroxidase reaction.

Acknowledgements

The skilful technical assistance of Ms. Pipsa Kaipainen and R. Taavela is kindly acknowledged. This study was supported by grants from the Finnish Medical Research Council, the Finnish Cancer Foundation, The Sigrid Juselius Foundation and The Association of Finnish Life Insurance Companies.

References

Amini,S. and Kaji,A. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 960-964.
Arrigo,A.-P., Darlix,J.-Ö. and Spahr,P.-F. (1983) *EMBO J.*, **2**, 309-315.
Badley,R.A., Woods,A., Carruthers,L. and Rees,D.A. (1978) *Exp. Cell Res.*, **117**, 231-244.
Bennett,V., Davis,J. and Fowler,W.E. (1982) *Nature*, **299**, 126-131.
Ben Ze'ev,A., Duerr,A., Solomon,F. and Penman,S. (1979) *Cell*, **17**, 859-865.
Bishop,J.M. (1983) *Cell*, **32**, 1018-1020.
Branton,D., Cohen,C.M. and Tyler,J. (1981) *Cell*, **24**, 24-32.
Buhrow,S.A., Cohen,S. and Stavros,J.V. (1982) *J. Biol. Chem.*, **257**, 4019-4022.
Burridge,K., Kelly,T. and Mongeat,P. (1982) *J. Cell Biol.*, **95**, 478-486.
Cheng,Y.-S.E. and Chen,L.B. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2388-2392.
Chinkers,M. and Cohen,S. (1981) *Nature*, **290**, 516-519.
Cohen,S., Ushiro,H., Stoscheck,C. and Chinkers,M. (1982) *J. Biol. Chem.*, **257**, 1523-1531.
Collett,M.S. and Erikson,R.L. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2021-2024.
Collett,M.S., Purchio,A.F. and Erikson,R.L. (1980) *Nature*, **285**, 167-169.
Cooper,J.A. and Hunter,T. (1981) *Cell*, **24**, 741-752.
Cooper,J.A. and Hunter,T. (1982) *J. Cell Biol.*, **94**, 287-296.
Cooper,J.A. and Hunter,T. (1983) *J. Biol. Chem.*, **258**, 1108-1115.
Cooper,J.A., Reiss,N.A., Schwartz,R.J. and Hunter,T. (1983) *Nature*, **302**, 218-223.
Courtneidge,S., Ralston,R., Alitalo,K. and Bishop,J.M. (1983) *Mol. Cell. Biol.*, **3**, 340-350.
Erikson,E. and Erikson,R.L. (1980) *Cell*, **21**, 829-836.
Erikson,R.L., Collett,M.S., Erikson,E. and Purchio,A.F. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 6260-6264.
Ghosh-Dastidar,P. and Fox,C.F. (1983) *J. Biol. Chem.*, **258**, 2041-2044.
Gilmer,T.M. and Erikson,R.L. (1981) *Nature*, **294**, 771-773.
Glenney,J.R.,Jr., Glenney,P. and Weber,K. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4002-4005.
Hunter,T. and Cooper,J.A. (1981) *Cell*, **24**, 741-752.
Hunter,T. and Sefton,B.M. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 1311-1315.
Köhler,G. and Milstein,C. (1975) *Nature*, **256**, 495-497.
Kudlow,J.E., Buss,J.E. and Gill,G.N. (1981) *Nature*, **290**, 519-521.
Laemmli,U.K. (1970) *Nature*, **227**, 620-625.
Laurila,P., Virtanen,I., Stenman,S. and Wartiovaara,J. (1978) *J. Histochem. Cytochem.*, **26**, 251-256.
Lazarides,E. and Nelson,W.J. (1982) *Cell*, **31**, 505-508.
Lehto,V.-P. (1983) *Exp. Cell Res.*, **143**, 271-286.
Lehto,V.-P., Vartio,T., Badley,R.A. and Virtanen,I. (1983) *Exp. Cell Res.*, **143**, 287-294.
Lehto,V.-P. and Virtanen,I. (1983) *J. Cell Biol.*, **96**, 703-717.
Levine,J. and Willard,M. (1981) *J. Cell Biol.*, **90**, 631-643.
Levinson,A.D., Oppermann,H., Levintow,L., Varmus,H.E. and Bishop,M. (1978) *Cell*, **15**, 561-572.
Levinson,A.D., Oppermann,H., Varmus,E. and Bishop,J.M. (1980) *J. Biol. Chem.*, **255**, 11973-11980.
Maness,P.F., Hansjorg,E., Greenberg,M.E., O'Farrell,M., Gall,W.E. and Edelman,G.M. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 5028-5032.
Marchesi,V.T. (1983) *Blood*, **61**, 1-11.
McGrath,J.P. and Levinson,A.D. (1982) *Nature*, **295**, 423-426.
Mescher,M.F., Jose,M.J.L. and Balk,S.P. (1981) *Nature*, **289**, 139-144.
Nigg,E.A., Cooper,J.A. and Hunter,T. (1983) *J. Cell Biol.*, **96**, 1601-1609.
Radke,K., Gilmore,T. and Martin,G.S. (1980) *Cell*, **21**, 821-828.
Radke,K. and Martin,G.S. (1979) *Proc. Natl. Acad. Sci. USA*, **78**, 5212-5216.
Repasky,E.A., Granger,B.L. and Lazarides,E. (1982) *Cell*, **29**, 821-833.
Rubsamen,H., Saltenberger,K., Friis,R.R. and Eigenbradt,E. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 228-232.
Sefton,B.M., Hunter,T., Ball,E.H. and Singer,S.J. (1981) *Cell*, **24**, 165-174.
Sefton,B.M., Hunter,T., Nigg,E.A., Singer,S.J. and Walter,G. (1982) *Cold Spring Harbor Symp. Quant. Biol.*, **46**, 939-951.
Steck,T.L., Weinstein,R.S., Straus,J.H. and Wallach,D.H.F. (1970) *Science (Wash.)*, **168**, 255-257.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.
Zigmond,S.H., Otto,J.J. and Bryan,J. (1979) *Exp. Cell Res.*, **119**, 205-220.