Journal of Cellular Biochemistry 37:151–159 (1988)
Signal Transduction in Cytoplasmic Organization
and Cell Motility 131–139

Vanadate-Treated Baby Hamster Kidney Fibroblasts Show Cytoskeleton and Adhesion Patterns Similar to Their Rous Sarcoma Virus-Transformed Counterparts

Pier Carlo Marchisio, Nicoletta D'Urso, Paolo M. Comoglio, Filippo G. Giancotti, and Guido Tarone

Dipartimento di Scienze Biomediche e Oncologia, Facoltà di Medicina, Università di Torino, 10126 Torino, Italy

Rous sarcoma virus-transformed baby hamster kidney fibroblasts (RSV/B4-BHK) adhere to a fibronectin-coated substratum by means of dot-like adhesion sites called podosomes in view of their shape and function as cellular feet (Tarone et al.: *Exp Cell Res* 159:141, 1985). Podosomes concentrate tyrosine-phosphorylated proteins, including $pp60^{v\text{-}src}$, and appear in many cells transformed by oncogenes coding for tyrosine kinases. In this paper we used orthovanadate, an inhibitor of phosphotyrosine phosphatases, in order to increase the cellular concentration of phosphotyrosine and to study whether this treatment induced the cytoskeleton remodeling leading to the formation of podosomes. Indeed, orthovanadate (10–100 μM) induced in a time- and dose-dependent manner the redistribution of F-actin and the formation of podosomes in BHK cells. Cytoskeleton remodeling occurred along with a marked increase of tyrosine phosphorylated proteins. The vanadate effect on the cytoskeletal phenotype was enhanced by the simultaneous treatment of cells with a phorbol ester. Under the latter conditions almost all BHK cells showed podosomes. The vanadate effect was reversible insofar as podosomes and tyrosine-phosphorylated proteins disappeared. Then, vanadate treatment of normal cells induced the cascade of events leading to the cytoskeletal changes typical of transformation and suggested that the transformed cytoskeletal phenotype may be primarily induced by the tyrosine phosphorylation of unknown target(s) operated by endogenous kinases.

Key words: vanadate, phosphotyrosine, transformation

The most evident aspect of neoplastic transformation is a marked change in cell shape caused by an extensive remodeling of the cytoskeleton and notably of its microfilamentous component [1,2]. At the same time, transformation causes a change of adhesion witnessed by the reduction of focal contacts [3,4]. In fibroblasts transformed by oncogenes coding for tyrosine kinases [5], the disappearance of adhesion plaques is followed by the formation of peculiar dot-like adhesion structures [3,4,6–9] which have

Received February 21, 1987; revised and accepted September 11, 1987.

been extensively characterized and termed podosomes in view of their possible function as cellular feet [10,11]. Podosomes display a peculiar distribution of cytoskeletal proteins different from that found in adhesion plaques and are structurally identical with the adhesion structures found in monocytes and monocyte-derived cells [11–13]. In transformed cells podosomes accumulate tyrosine-phosphorylated proteins [6,7,10].

Since podosomes are absent from cells transformed by oncogenes devoid of tyrosine kinase activity [10], the possibility exists that the mechanism underlying their formation has to be sought in the enhancement of this enzymatic activity. In order to test this possibility nontransformed baby hamster kidney (BHK) fibroblasts have been exposed to conditions known to enhance the level of endogenous phosphotyrosine—namely, treatment with vanadate which induces the onset of several transformation parameters in normal cultured cells [14]. Vanadate acts on cells by inhibiting phosphatases that remove the phosphate group from phosphotyrosine [15–18]. Then, this ion, on one hand, inhibits the rapid physiological breakdown of tyrosine phosphoproteins and, on the other hand, increases the tyrosine kinase activity which depends on phosphorylation of tyrosine residues [19–21, for review see 22].

In this paper we report that vanadate treatment of nontransformed cells induced 1) changes in cytoskeletal architecture identical with those reported [10] for their RSV-transformed counterparts (RSV-B4 BHK), including the formation of podosomes; 2) a phosphotyrosine protein pattern similar to that of RSV-B4 BHK cells; 3) the enrichment of phosphotyrosine at adhesion structures. The vanadate effect was dose- and time-dependent, reversible, and enhanced by a phorbol ester which, per se, may enhance the tyrosine kinase activity of $pp60^{src}$ via a protein kinase C-induced phosphorylation at serine [23].

MATERIALS AND METHODS

Cells

Baby hamster kidney (BHK) fibroblasts were obtained from the stock of Dr. I. Macpherson (London, UK). BHK fibroblasts transformed by the Bryan high titer strain of Rous sarcoma virus (RSV/B4-BHK) were a gift of Dr. L. Warren (Philadelphia, PA). These cell lines were propagated at 37°C, 5% CO_2, and 100% humidity in Dulbecco's modified Eagle medium (DMEM) containing penicillin-streptomycin and supplemented with 10% fetal calf serum (FCS). Cells were routinely tested for mycoplasma and found to be negative.

In order to study adhesion and adhesion-related cytoskeletal structures in controlled conditions, RSV/B4-BHK and BHK cells were seeded on fibronectin-coated coverslips as previously reported [10]. Briefly, glass coverslips were extensively acid-washed and coated with purified human plasma fibronectin (10 μg/ml) in 150 mM sodium chloride and 10 mM sodium phosphate buffer, pH 7.4 (PBS), for 60 min at room temperature. Residual protein binding sites on the glass surface were blocked by further incubation with 0.2% bovine serum albumin (BSA) in PBS. Fibronectin was purified from human plasma as previously reported [24]. Cells harvested from culture dishes by EGTA treatment were plated either in serum-free DMEM or in DMEM containing 2% FCS.

Experimental Procedures

Sodium orthovanadate (Sigma, St. Louis, MO) was freshly prepared immediately before addition to cell cultures in order to avoid storage of concentrated solutions which

may result in polymerization [25]. Sodium orthovanadate was added to semiconfluent cell cultures at the concentrations of 12.5, 25, 50, and 100 μM and incubated for variable times. In order to test the reversibility, orthovanadate-containing DMEM was removed and replaced by fresh medium.

In some experiments BHK cells were simultaneously treated with orthovanadate and 10–200 nM 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a selective activator of protein kinase C [26,27].

The viability of orthovanadate-treated or orthovanadate-plus-TPA-treated BHK cells was monitored by trypan blue exclusion test and by measuring the incorporation of ^{35}S-labeled methionine into proteins.

Preparation of Antibodies Cross-Reacting With Phosphotyrosine

Azobenzyl phosphonate (ABP) antibodies were raised, affinity-purified, and characterized as previously reported [28]. Briefly, ABP antisera were raised in rabbits by three monthly injections of 1 mg of keyhole limpet hemocyanin to which ≈30 p-azobenzyl phosphonate groups were covalently linked per 100,000 molecular weight units. Antibodies were purified by affinity chromatography on ABP-BSA coupled to cyanogen bromide-activated Separose 4B (Pharmacia, Uppsala, Sweden; 4 mg of protein/g of Sepharose) in 0.1 M sodium bicarbonate. Immunoglobulins from ABP antisera were precipitated with 40% saturated ammonium sulfate and applied to the column at room temperature. After washing the column with PBS, bound antibodies were eluted with 3 M potassium isothiocyanate, pH 7, at room temperature and dialyzed against PBS. The antibodies were found to cross-react with phosphotyrosine by radioimmunoassay and to precipitate selectively tyrosine phosphorylated proteins [e.g., 29].

Fluorescence and Interference Reflection Microscopy

Coverslips were treated for fluorescence microscopy essentially as described in other papers [10,13]. Cells were fixed for 5 min at room temperature in 3% formaldehyde (from paraformaldehyde) in PBS (pH 7.6) containing 2% sucrose and permeabilized (5 min, 0°C) in HEPES-Triton X-100 buffer (20 mM HEPES, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$ and 0.5% Triton X-100). This fixation and permeabilization procedure has been successfully used for other studies of cytoskeleton and adhesion [10,13].

In order to visualize F-actin, fixed and permeabilized cells were stained with 2 μg/ml rhodamine-labeled phalloidin (R-PHD, a kind gift of Dr. Th. Wieland, Max Planck Institute for Experimental Medicine, Heidelberg, FRG) for 30 min at 37°C. R-PHD has been shown to bind firmly to F-actin [30].

Indirect immunofluorescence experiments were performed essentially as reported [10]. Briefly, primary antibodies were layered on fixed and permeabilized cells and incubated in a humid chamber for 30 min. After rinsing in PBS containing 0.2% BSA, coverslips were incubated in swine antirabbit IgGs (100 μg/ml) for 30 min at 37°C in the presence of 2 μg/ml of fluorescein-labeled phalloidin (F-PHD, a gift of Dr. Th. Wieland). Coverslips were then mounted in 50% glycerol-PBS.

Observations were carried out in a Leitz Orthoplan microscope equipped for epifluorescence and interference reflection microscopy (IRM). Fluorescence images were recorded on Kodak Tri X films exposed at 1,000 ISO and developed in Nucleol

BF 200 (Chimifoto Ornano, Milano, Italy). IRM pattern were simultaneously recorded on Agfa Ortho 25 films developed in Agfa Neutol NE.

Immunoblotting

Immunoblotting analysis was performed on total extracts of cells solubilized in hot 2.5% SDS in 125 mM TRIS-HCl, pH 6.8.

Protein content per cell, as determined by Lowry's method, was equalized in all samples. The latter, corresponding to 0.1 mg protein per lane, were subjected to electrophoresis on a 5–15% gradient polyacrylamide gel slab in presence of sodium-dodecylsulfate (SDS) and 2% β-mercaptoethanol as described by Laemmli [31]. After electrophoresis, proteins were transferred to nitrocellulose sheets (Bio-Rad Laboratories) by Western blotting [32]. Immunostaining of blotted membrane proteins was performed essentially as previously described [29] with 16 μg/ml of affinity-purified ABP antibodies. After rinsing, bound antibodies were revealed by ^{125}I-labeled protein A.

RESULTS

Phenotypic Changes

The changes of microfilament distribution occurring upon orthovanadate and orthovanadate + TPA treatment are shown in Figure 1. Control BHK fibroblasts showed

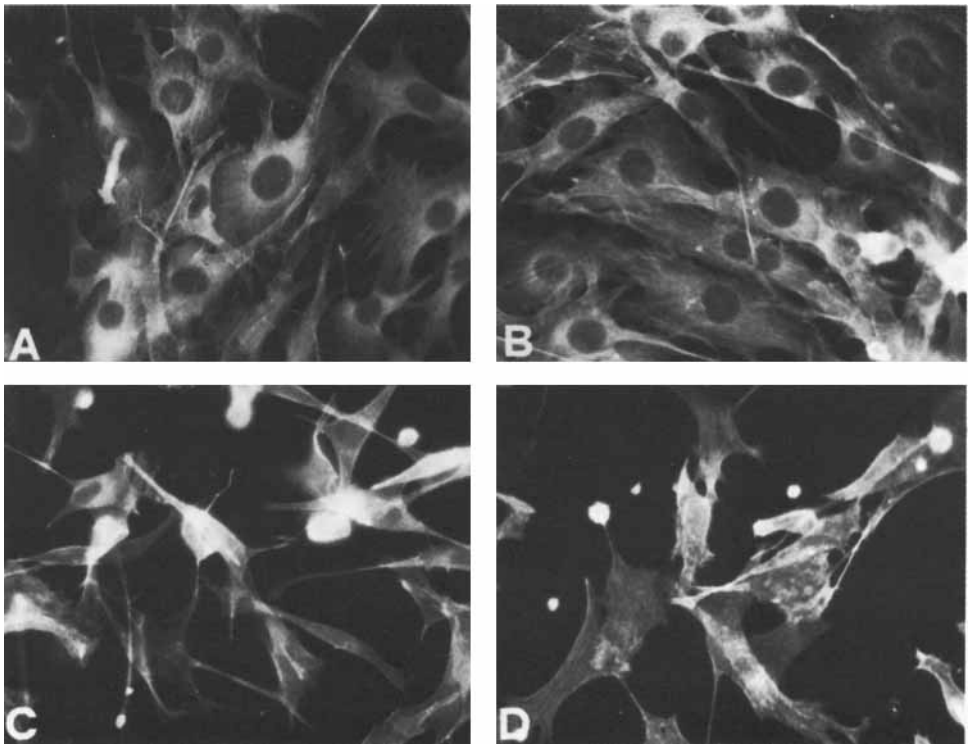

Fig. 1. Cultured BHK fibroblasts stained for F-actin by R-PHD. Control (**A**), after 80 nM TPA (**B**), after 50 μM orthovanadate (**C**), and after 50 μM orthovanadate plus 80 nM TPA (**D**). ×750.

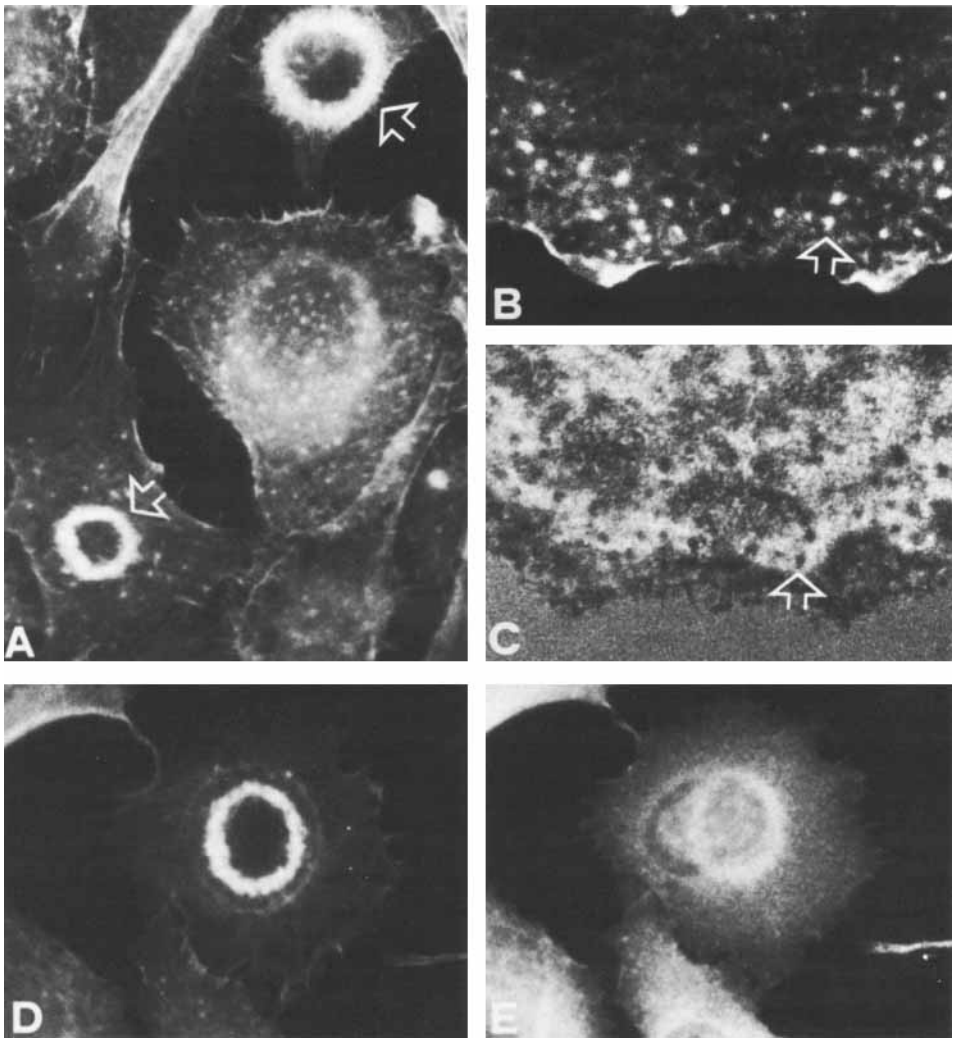

Fig. 2. Cultured BHK fibroblasts exposed to 50 μM orthovanadate overnight and stained for F-actin with PHD (**A**, **B**, **D**) and immunodecorated with antibodies cross-reacting with phosphotyrosine (**E**). **C** represents the IRM picture of B. The simultaneous treatment of BHK fibroblasts with orthovanadate and TPA enhances the formation of individual podosomes and rosettes (A, arrowheads). Individual podosomes correspond to close-contact adhesion sites (B and C, gray spots surrounded by clear halo in IRM). Individual podosomes or rosettes (D and E) are sites of marked concentration of phosphotyrosine-containing proteins. ×2,500 (A, D, E); ×3,750 (B, C).

their typical pattern of stress fibers stained for F-actin by R-PHD (Fig. 1A). Stress fibers progressively disappeared after exposure to orthovanadate (Fig. 1C). Many cells lost their spread shape and showed elongated or rounded shapes typical of transformed BHK cells. Eventually, in a dose-dependent manner, a considerable fraction of orthovanadate-treated BHK cells detached from the substratum and were lost in the staining procedure. TPA (80 nM) per se did not alter control cell shape and microfilament pattern in this type of cultured cells (Fig. 1B). However, in conjunction with 50 μM orthovanadate,

TPA induced an even more marked change of the cytoskeleton and the appearance of dot adhesions sites (Fig. 1D; see also Fig. 2).

Exposure to 25–50 μM orthovanadate for about 12 hr caused the loss of adhesion plaques and their replacement by dot-like adhesion structures of the close contact type either isolated or clustered in rosettes (Fig. 2A). These structures were qualitatively identical with podosomes found in RSV-B4 BHK cells [10] insofar as they corresponded to an IRM-gray dot surrounded by a clear halo (Fig. 2B,C), had a vinculin-containing ring (not shown), and were intensely positive in immunofluorescence with the antibody cross-reacting with phosphotyrosine (Fig. 2D,E).

Only about 10% of BHK cells incubated overnight with 25–50 μM orthovanadate showed clearly recognizable podosomes scattered at their ventral membrane. However, when the above treatment was combined with nM TPA, nearly all cells showed podosomes and rosette-like podosome clusters such that their pattern could not be distinguished from that of RSV-B4 BHK cells [10]. In contrast to other cell types [e.g., see 35], TPA did not cause cytoskeletal changes in BHK fibroblasts under these experimental conditions.

Upon removal of orthovanadate or orthovanadate plus TPA from the culture medium BHK fibroblasts recovered their normal cytoskeletal pattern in about 24 hr and were indistinguishable from those shown in Figure 1A.

Immunodecoration of Phosphotyrosine Proteins

Control, RSV-B4 BHK, and 12-hr-vanadate-treated BHK cells were solubilized and subjected to SDS polyacrylamide gel electrophoresis in the presence of β-mercapto ethanol as a reducing agent. After Western blotting, nitrocellulose stripes were immunodecorated with ABP antibodies cross-reacting with phosphotyrosine (Fig. 3). Control untreated BHK cells (lane A) did not show detectable tyrosine phosphorylated bands; phosphotyrosine was also barely detectable in 12.5-μM-treated (lane B) and in 25-μM-treated (lane C) cells. Fifty-micromolar- and 100-μM-treated cells (lanes D and E) showed a much higher content of blotted proteins recognized by ABP antibodies. No difference was found after cotreatment of cells with 200 nM TPA in conjunction with 50 or 100 μM orthovanadate. In general, the band pattern was similar but not identical with that of RSV-transformed BHK fibroblasts (lane F).

The immunodecoration pattern was reversible upon removal of orthovanadate from the culture medium. Figure 4 shows control untreated cells (lane A), cells treated with 25 μM (lane B) and 50 μM (lane C) orthovanadate for 24 hr; after rinsing with fresh medium and culturing for 24 hr in orthovanadate-free medium the amount of phosphotyrosine cross-reacting proteins was drastically reduced (lanes D and E).

DISCUSSION

The exposure of nontransformed cultured fibroblasts to vanadate induces reversible phenotypic changes similar to those occurring upon infection with retroviruses coding for tyrosine kinases [14]. In BHK fibroblasts straight orthovanadate treatment seems to induce transformation-like phenotypic changes only in a 10% fraction of cells, but all cells respond when a TPA stimulus is added; this suggests that there is some heterogeneity within the BHK cell population in the orthovanadate response. Since ions deriving from sodium orthovanadate in treated cells are known to inhibit phosphatases acting on phosphotyrosine with higher specificity [15–18], it was concluded that phenotypic transformation was caused by an artificially increased content of tyrosine-phosphorylated proteins

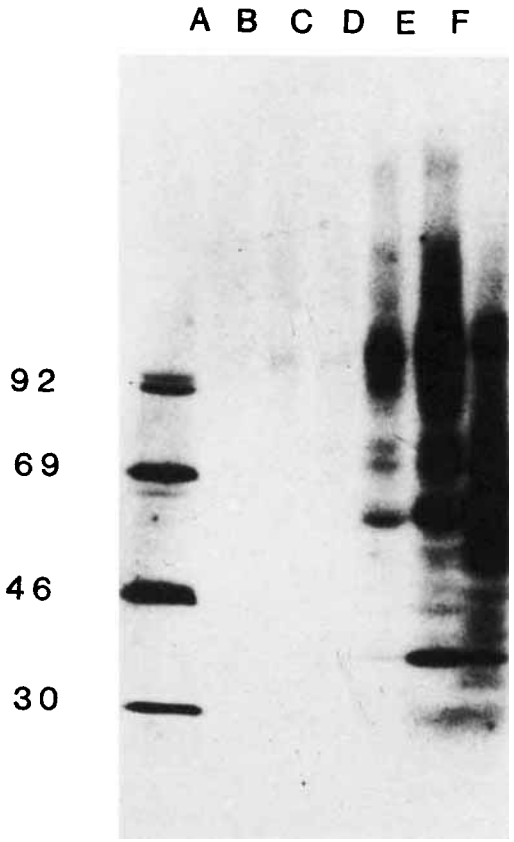

Fig. 3. Immunodecoration of phosphotyrosine-containing proteins by cross-reacting antibodies (ABP antibodies). **Lane A**: BHK, control. **Lane B**: BHK, 12.5 μM orthovanadate. **Lane C**: BHK, 25 μM orthovanadate. **Lane D**: BHK, 50 μM orthovanadate. **Lane E**: BHK, 100 μM orthovanadate. **Lane F**: RSV/B4-BHK, control. Orthovanadate was kept overnight in the culture medium.

[14] mimicking the effects of class 1 oncogene kinases. A major effect of the latter type of transformation involves the cytoskeleton and notably the membrane-microfilament complex controlling cell adhesion [3,4,6,7,9,10]. At early stages of transformation, ventrally located adhesion plaques concentrate tyrosine phosphorylated proteins [33], including $pp60^{v\text{-}src}$ [6,7]. Later, stress fibers disappear and cells change their F-actin distribution [34] and develop dot-like adhesion devices which contain several adhesion-related molecules [3,4,6,7,9–11], $pp60^{v\text{-}src}$ [6,7] and most probably other tyrosine phosphorylated molecules [10]. In our laboratory, such dot adhesion structures have been termed podosomes in view of their assigned function of cellular feet [10] and of their specific molecular architecture identical with structures described in monocytes and monocyte-derived cells [11–13].

The redistribution of adhesion-related cytoskeletal molecules leading to the appearance of podosomes in RSV-transformed cells (and not in cells transformed by agents that do not code for tyrosine kinases [10]) has been tentatively attributed to the phosphorylation at tyrosine of some target molecules. The tyrosine phosphorylated proteins, which appear after vanadate treatment, are in fact similar to those described in cells

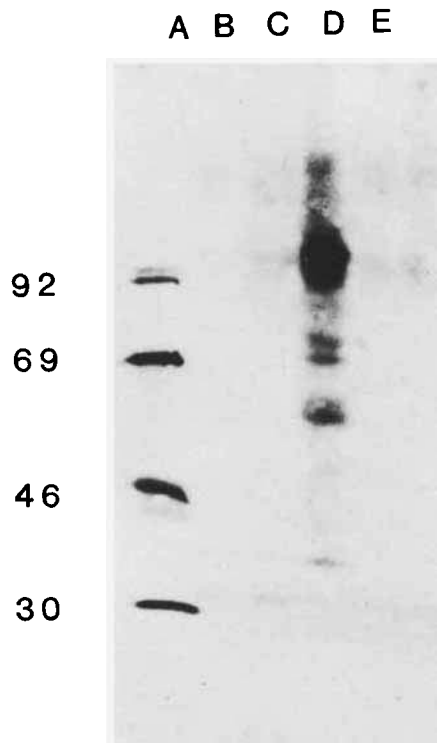

Fig. 4. Immunodecoration of phosphotyrosine-containing proteins by cross-reacting antibodies (ABP antibodies). **Lane A**: BHK, control. **Lane B**: BHK cells exposed for 24 hr to 25 μM orthovanadate. **Lane C**: Same with 50 μM. **Lane D**: 24-hr recovery in orthovanadate-free medium after 25 μM. **Lane E**: Same after 50 μM.

transformed by different retroviruses [28,29]: among these particularly relevant are proteins whose M_r is on the order of 130, 90, and 70 kilodaltons (Kd). Vinculin was originally suspected to be one of such molecules [36, see also 28]. At present, a candidate for the alterations of the membrane-microfilament interaction complex may be indicated in a transmembrane fibronectin receptor which is a major target of tyrosine kinases [37], also in view of its direct connection with talin [38], a cytoskeletal protein of the membrane-microfilament interaction complex [39,40].

In this paper we show that, by simply increasing the intracellular content of phosphotyrosine in normal cells, the molecular mechanism leading to the redistribution of the adhesion-related cytoskeletal molecules into podosomes can be triggered. So far we cannot indicate the primary target. We can just suggest that such a target is present in normal cells and may undergo phosphorylation at tyrosine residues even in cells which do not express virus encoded kinases. The effect of vanadate in BHK cells, in different cell lines and in human primary cells (in preparation), is promptly and completely reversible.

ACKNOWLEDGMENTS

This work was partially supported by the Italian Association for Cancer Research (AIRC), by Regione Piemonte, and by CNR Progetto Finalizzato "Oncologia" (CNR CT 85.02231.44 and CT 86.02612.44).

REFERENCES

1. Edelman GM, Yahara I: Proc Natl Acad Sci USA 73:2047, 1976.
2. Wang E, Goldberg AR: Proc Natl Acad Sci USA 73:4065, 1976.
3. Rohrschneider LR: Proc Natl Acad Sci USA 77:3514, 1980.
4. David-Pfeuty T, Singer SJ: Proc Natl Acad Sci USA 77:6687, 1980.
5. Bishop JM: Annu Rev Biochem 52:301, 1983.
6. Shriver K, Rohrschneider LR: J Cell Biol 89:525, 1981.
7. Nigg EA, Sefton BM, Hunter T, Walter G, Singer SJ: Proc Natl Acad Sci USA 79:5322, 1982.
8. Carley WW, Barak SL, Webb WW: J Cell Biol 90:797, 1981.
9. Marchisio PC, Capasso O, Nitsch L, Cancedda R, Gionti E: Exp Cell Res 151:332, 1984.
10. Tarone G, Cirillo D, Giancotti FG, Comoglio PM, Marchisio PC: Exp Cell Res 159:141, 1985.
11. Marchisio PC, Cirillo D, Teti A, Zambonin-Zallone A, Tarone G: Exp Cell Res 169:202, 1987.
12. Zambonin-Zallone A, Teti A, Primavera MV, Naldini L, Marchisio PC: J Anat 137:57, 1983.
13. Marchisio PC, Cirillo D, Naldini L, Primavera MV, Teti A, Zambonin-Zallone A: J Cell Biol 99:1696, 1984.
14. Klarlund JK: Cell 41:707, 1985.
15. Leis JF, Kaplan NO: Proc Natl Acad Sci USA 79:6507, 1982.
16. Swarup G, Speeg KV Jr, Cohen S, Garbers DL: J Biol Chem 257:7298, 1982.
17. Swarup G, Cohen S, Garbers DL: Biochem Biophys Res Commun 107:1104, 1982.
18. Nelson RL, Branton PE: Mol Cell Biol 4:1103, 1984.
19. Purchio AF, Wells SK, Collett MS: Mol Cell Biol 3:1589, 1983.
20. Rosen OM, Herrera R, Olowe Y, Petruzzelli LM, Cobb MH: Proc Natl Acad Sci USA 80:3237, 1983.
21. Brown DJ, Gordon JA: J Biol Chem 259:9580, 1984.
22. Hunter T, Cooper JA: Annu Rev Biochem 54:897, 1985.
23. Gould KL, Woodgett JR, Cooper JA, Buss JE, Shalloway D, Hunter T: Cell 42:849, 1985.
24. Tarone G, Galetto G, Prat M, Comoglio PM: J Cell Biol 94:179, 1982.
25. Rubinson KA: Proc R Soc Lond [Biol] 212:65, 1981.
26. Niedel JE, Kuhn LJ, Vanderbank GR: Proc Natl Acad Sci USA 80:36, 1983.
27. Leach KL, James ML, Blumberg PM: Proc Natl Acad Sci USA 80:4208, 1983.
28. Comoglio PM, Di Renzo MF, Tarone G, Giancotti FG, Naldini L, Marchisio PC: EMBO J 3:483, 1984.
29. Di Renzo MF, Ferracini R, Naldini L, Giordano S, Comoglio PM: Eur J Biochem 158:383, 1986.
30. Wulf E, Deboben A, Bautz FA, Faulstich H, Wieland T: Proc Natl Acad Sci USA 76:4498, 1979.
31. Laemmli UK: Nature 227:680, 1970.
32. Towbin H, Stahelin T, Gordon J: Proc Natl Acad Sci USA 76:4350, 1979.
33. Marchisio PC, Di Renzo MF, Comoglio PM: Exp Cell Res 154:112, 1984.
34. Boschek CB, Jockhusch BM, Friis RR, Back R, Grundmann E, Bauer H: Cell 24:175, 1981.
35. Schliwa M, Nakamura T, Porter KR, Euteneuer U: J Cell Biol 99:1045, 1984.
36. Sefton BM, Hunter T, Ball EH, Singer SJ: Cell 24:165, 1981.
37. Hirst R, Horwitz A, Buck C, Rohrschneider LR: Proc Natl Acad Sci USA 83:6470, 1986.
38. Horwitz A, Duggan K, Buck C, Beckerle M, Burridge K: Nature 320:531, 1986.
39. Burridge K, Connell L: J Cell Biol 97:359, 1983.
40. Burridge K, Connell L: Cell Motil 3:405, 1983.