et al., 1986) and chicken integrin band 2 (α2) (M. A. Stepp et al., unpublished work), show extensive homologies throughout the putative transmembrane and cytoplasmic domains of these molecules. In addition, monoclonal antibody studies have led to the identification of five distinct α-subunits for the VLA antigens, two of which are related to the fibronectin receptor α5 and chicken integrin band 2/α5 (Hemler et al., 1987). Table 1 summarizes these relationships.

The functional consequences of this large number of α-β combinations are not well understood. It is interesting to speculate that this structural diversity evolved as a way to modulate the interactions of integrins from several different cell types with a variety of structurally diverse ligands, many of which (if not all) possess the RGD sequence. This would be of particular importance during morphogenesis where cells are required to alter their adhesive properties to the extracellular environment and to one another in a spatio-temporal fashion, in addition to effecting changes in cell shape. The integrins may contribute to these processes by providing a transmembrane link to the cytoskeleton capable of interacting with a variety of extracellular ligands having analogous functions but limited homologies.

The significance of the integrins during development is suggested by experiments in both urodeles and Drosophila where microinjection of RGD-containing peptides into living embryos has been shown to prevent the massive cellular rearrangements associated with gastrulation (Boucaut et al., 1984; Naidet et al., 1987). Indeed, in Drosophila there exist complexes of integrin-like glycoproteins, having variable α-subunits and a smaller common β-subunit, that are differentially expressed during development (Wilcox & Leptin, 1985). It is intriguing to consider the possibility that the integrin family of related receptors may provide some of the variability necessary to accommodate the large spectrum of the adhesive interactions essential to normal development.


Membrane and cytoskeletal changes in cells after transformation by Rous Sarcoma virus

STUART KELLIE*, NOEL M. WIGGLESWORTH†, BIPIN PATEL‡, THOMAS C. HOLME*.
DAVID R. CRITCHLEY‡ AND JOHN A. WYKE†
*Department of Biochemistry, The Hunterian Institute, Royal College of Surgeons of England, Lincoln's Inn Fields, London, WC2A 3PN; †Imperial Cancer Research Fund Laboratories, St Bartholomew's Hospital, London, ECIA and ‡Department of Biochemistry, University of Leicester, University Road, Leicester, LE1 7RH, U.K.

Rous sarcoma virus (RSV) transforms cells by the production of a single viral gene product, pp60\(^{+ve}\). This protein is a 60 kDa phosphoprotein with tyrosine kinase activity and in several cell types is localized at the plasma membrane — in particular in areas of membrane–cytoskeletal interaction (Collett et al., 1980; Rohrschneider, 1980). Although several intracellular targets for pp60\(^{+ve}\) have been identified, in no case has tyrosine-specific phosphorylation of any of these substrates been shown to be necessary for transfor-

Abbreviations used: RSV, Rous sarcoma virus; TPA, 12-tetradecanoyl phorbol-13-acetate; CEF, chick embry fiberglass; ts, temperature sensitive; HPFN, human plasma fibronectin.

Received 11 May 1987
formed phenotype using several morphological mutants of RSV which confer a variety of phenotypes on transformed cells. We have also examined the relative importance of vinculin phosphorylation by pp60\textsuperscript{src} with the loss of surface-associated fibronectin in RSV-transformed cells by performing 'fibronectin add-back' experiments first described by Yamada et al. (1976) and Ali et al. (1977). In addition, we have examined in more detail the changes in the major microfilament protein, actin, after transformation and disruption of actin-promoter, TPA.

We have employed a biochemical method for quantifying microfilament protein, actin, after transformation and associated fibronectin in RSV-transformed cells by performing an analysis of the well-formed adhesion plaques of tsLA32-infected cells at 41°C which were morphologically normal. In contrast, tsLA29-infected cells at 41°C showed only diffuse cytoplasmic staining with no adhesion-plaque localization of pp60\textsuperscript{src} (Table 1).

**Tyrosine phosphorylation of vinculin.** The tyrosine-specific phosphorylation of vinculin by pp60\textsuperscript{src} in temperature-sensitive formed cells was investigated by analysis of the phospho-amino acid content of vinculin. After labelling cells for 3 h with \(^{32}P\), vinculin was immune precipitated; the band localized by autoradiography and excised. After hydrolysis the liberated \(^{32}P\)-labelled phospho-amino acids were separated by high-voltage thin-layer electrophoresis (Cooper et al., 1983). The phosphotyrosine content of vinculin increased from about 2% to over 20% in both wild-type transformed CEF and rASV 2234.3-transformed CEF. Vinculin from cells infected with tsLA29 contained 19% phosphotyrosine at 35°C, but only about 5% at 41°C. However, in contrast, vinculin from cells transformed by tsLA32 contained about 18% phosphotyrosine at 35°C, yet about 20% at 41°C (Table 1). Thus cells which were either fusiform or indistinguishable from normal CEF, contained vinculin whose phosphotyrosine levels were as high as in wild-type, rounded transformants.

**Comparison of the relative importance of vinculin phosphorylation and the loss of cell surface fibronectin in the maintenance of the transformed phenotype**

Although changes in the functional integrity of the cytoskeleton seem to play a major role in those morphological changes observed when cells are transformed by RSV, the rounded morphology might also be explained by the reduction in levels of surface-associated fibronectin. Consistent with this are reports that the addition of fibronectin to

**Table 1. Tyrosine-specific phosphorylation of vinculin in RSV-transformed CEF**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Morphology*</th>
<th>pp60\textsuperscript{src} in adhesion plaques</th>
<th>Phosphotyrosine content† of vinculin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>N</td>
<td>–</td>
<td>2.0</td>
</tr>
<tr>
<td>Wild-type RSV</td>
<td>T</td>
<td>–</td>
<td>22.1</td>
</tr>
<tr>
<td>rASV 2234.3</td>
<td>E</td>
<td>+</td>
<td>23.2</td>
</tr>
<tr>
<td>tsLA29, 35°C</td>
<td>T</td>
<td>+</td>
<td>18.9</td>
</tr>
<tr>
<td>tsLA29, 41°C</td>
<td>N</td>
<td>–</td>
<td>5.1</td>
</tr>
<tr>
<td>tsLA32, 35°C</td>
<td>T</td>
<td>+</td>
<td>17.6</td>
</tr>
<tr>
<td>tsLA32, 41°C</td>
<td>N</td>
<td>+</td>
<td>19.5</td>
</tr>
</tbody>
</table>

*N: normal; T: transformed; F: fusiform morphologies.

†Phosphotyrosine content expressed as a percentage of the total phospho-amino acids present.

**Table 2. Phospho-amino acid content of vinculin in fibronectin-supplement RSV–CEF cultures**

<table>
<thead>
<tr>
<th>Cells/treatment</th>
<th>Morphology*</th>
<th>pp60 in cell contacts</th>
<th>Phosphotyrosine content of vinculin† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEF</td>
<td>N</td>
<td>–</td>
<td>2.1</td>
</tr>
<tr>
<td>RSV CEF</td>
<td>T</td>
<td>+</td>
<td>23.5</td>
</tr>
<tr>
<td>RSV CEF + HPFN (50(\mu)g/ml)</td>
<td>N/F</td>
<td>+ +</td>
<td>24.3</td>
</tr>
</tbody>
</table>

*N: normal; T: transformed; F: fusiform morphologies.

†Phosphotyrosine expressed as a percentage of the total phospho-amino acids present.
RSV-transformed cells restores a morphology similar, though not identical, to untransformed cells (Yamada et al., 1976; Ali et al., 1977). These results indicate that the loss of fibronectin, rather than tyrosine-specific phosphorylation of vinculin, might be more directly related to the transformed phenotype. It is also possible, however, that this effect of exogenous fibronectin might in some way modify the phosphorylation state of vinculin or alter the activity of pp60
\(^{\text{src}}\). We have therefore investigated the phosphotyrosine levels in vinculin isolated from RSV-transformed cells which have reverted to a more normal morphology, by the addition of fibronectin to the growth medium.

The addition of human plasma fibronectin (HPFN) 50 µg/ml to RSV-transformed CEF induced a reversion to a flatter, more normal morphology over a period of 1-3 days. These cells, although slightly more fusiform than untransformed cells, recovered the ability to respect cell boundaries and did not overgrow in multilayers. Staining using an antibody specific for HPFN indicated that these cells had incorporated the exogenous HPFN into an extensive matrix (Kellie et al., 1976). In sparse cultures, the cells adopted a slightly more spread morphology, with pp60
\(^{\text{src}}\) evident in adhesion plaques. In more dense cultures, the morphological reversion was more evident and staining of these cultures indicated that pp60
\(^{\text{src}}\) was present in both cell-substratum contents and also cell-cell contacts. The phosphotyrosine content of vinculin from such treated cells was quantified. Untransformed cells contained only 2% phosphotyrosine in vinculin, however, both RSV-transformed cells and fibronectin-supplemented RSV-transformed cells contained over 22% phosphotyrosine in vinculin (Table 2). Thus although fibronectin partially restored a normal morphology, there was no change in the levels of phosphotyrosine in vinculin. We conclude that loss of cell surface fibronectin is more important than tyrosine-specific phosphorylation of vinculin in the maintenance of the transformed phenotype.

Changes in the functional state of actin after transformation by RSV

Experiments using temperature-sensitive mutants have indicated that cytoskeletal changes are early events which occur after transformation and which are maintained by an active pp60
\(^{\text{src}}\) (Boschek et al., 1981). We have examined in more detail the functional state of actin in RSV-transformed cells by quantifying the actin monomer-polymer equilibrium using a modification of the DNase I inhibition assay for G- and F-actin originally described by Blikstad et al. (1978). Rat-1 cells and RSV-transformed Rat-1 cells were fractionated into a detergent-containing buffer to produce a soluble 'cytosolic' fraction and an insoluble 'cytoskeletal core' fraction (Holme et al., 1986). The amount of G- and F-actin was quantified in the cytosolic fraction, and the amount of actin in the cytoskeletal core was also quantified, assuming this to be F-actin only. The cytosolic G: F-actin ratio of Rat-1 cells was 1.27 whereas in RSV-Rat-1 cells the cytosolic G: F-actin ratio was 2.75. This shift in G: F-actin ratios after transformation was found to be due mainly to a decrease in the cytosolic F-actin form, with little change in the cytosolic G-actin form. However, there was a marked increase in the amount of insoluble 'core' actin after transformation (Table 3).

The treatment of cells in vivo with a tumour promoter such as TPA often elicits responses similar to those of transformed cells, in particular changes in cellular morphology and the reduction of microfilament bundles, and so for comparison we have analysed the functional state of actin in MDCK cells after TPA treatment. These cells respond rapidly to TPA (within 1 h) by rounding up with concomitant dramatic disappearance of stress fibres (Kellie et al., 1986). In these cells there was no change in either the cytosolic G: F-actin ratio or the insoluble 'core' content (Table 3), thus we conclude that the reduction in microfilament bundles found in RSV-transformed cells is mediated by a mechanism distinct from the reduction in microfilament bundles induced by TPA.

Conclusions

The mechanism by which cytoskeletal integrity is altered after transformation by RSV is unknown, although it is likely to involve tyrosine-specific phosphorylation of some cytoskeletal component. Phosphorylation of vinculin by pp60
\(^{\text{src}}\) does not correlate with the transformed phenotype, therefore it is likely that phosphorylation of some other component is involved. The loss of cell surface fibronectin seems to play a major role in morphological changes induced by transformation. There is a major shift in the actin monomer-polymer equilibrium of fibroblasts after transformation by RSV, however, in contrast there is no such change in epithelial cells whose microfilament bundles have been induced to dissociate by treatment with TPA, thus there seem to be distinct mechanisms which lead to stress-fibre breakdown depending on the cell type and the treatment to which they are exposed.

We thank Drs J. Brugge and R. L. Erikson for generous gifts of anti-pp60
\(^{\text{src}}\), Professor N. Crawford and Drs M. J. Bisell and A. W. Stoker for constructive advice and Miss Heather Watson for preparation of this manuscript. We are grateful to the M.R.C. and Smith Kline and French Ltd for partial support of this work.

Table 3. Polymerization state of actin in RSV-transformed CEF or TPA-treated MDCK cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Total actin* (µg/10^6 cells)</th>
<th>Cytoskeletal† core actin</th>
<th>Cytosolic G‡ (%)</th>
<th>Cytosolic F‡ (%)</th>
<th>Cytosolic G: F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat-1</td>
<td>60 ± 9</td>
<td>12 ± 2</td>
<td>49 ± 5</td>
<td>38 ± 5</td>
<td>1.27</td>
</tr>
<tr>
<td>RSV-Rat-1</td>
<td>56 ± 16</td>
<td>24 ± 6</td>
<td>56 ± 9</td>
<td>21 ± 6</td>
<td>2.75</td>
</tr>
<tr>
<td>MDCK</td>
<td>87 ± 18</td>
<td>17 ± 4</td>
<td>58 ± 3</td>
<td>25 ± 5</td>
<td>2.32</td>
</tr>
<tr>
<td>MDCK + TPA</td>
<td>94 ± 15</td>
<td>18 ± 1</td>
<td>57 ± 3</td>
<td>24 ± 3</td>
<td>2.37</td>
</tr>
</tbody>
</table>

* n = 6, ± s.d.
† Actin expressed as percentage of total cellular actin.
‡ G or F actin was determined by the DNase I inhibition assay.
Isolation and characterization of a vinculin cDNA from chick embryo fibroblasts

PETER JONES, GLYN J. PRICE, MATTHEW D. DAVISON and DAVID R. CRITCHLEY
Department of Biochemistry, University of Leicester, Leicester, LE1 7RH, U.K.

Vinculin is a 130 kDa protein that is found in muscle and non-muscle cells. Vinculin has been detected in vivo near membrane/actin filament attachment sites in the zonula adherens of intestinal epithelium, dense plaques of smooth muscle, the fascia adherens of cardiac and intercalated discs and the costameres of cardiac and skeletal muscle (Burridge & Feramisco, 1980). In cultured cells, the protein is primarily located at the termini of microfilament bundles at focal contacts and intercellular junctions.

Two-dimensional gel electrophoresis has revealed that there are several isolectric variants with pI values of between 6.8 and 7.3. The isolectric variants have been divided into three classes on the basis of their pI values, the α-variant being the most acidic. It is the α-variant that is phosphorylated in vivo and remains associated with the cytoskeleton after extraction with Triton X-100. In comparison, greater than 90% of the β-isoform is found in the detergent-soluble extract (Geiger, 1982). It is therefore possible that isoform diversity is related to different functional interactions of vinculin. In both smooth and skeletal muscle, immunological studies and peptide mapping have shown that a protein with a molecular mass of 152 kDa is extremely homologous to vinculin, and it has been called metavinculin (Feramisco et al., 1982). Although metavinculin has been demonstrated to be a product of a separate mRNA to vinculin (Feramisco et al., 1982), it is unknown whether vinculin and metavinculin represent separate gene products.

Earlier studies (Siliciano & Craig, 1982) concluded that in contrast to vinculin, metavinculin had the solubility properties of an integral membrane protein. However, this observation has now been attributed to an experimental artefact (Siliciano & Craig, 1987) and although metavinculin has distinct solubility characteristics, all that can be said at present is that it may associate peripherally with the plasma membrane of muscle cells.

Some knowledge of the structure of vinculin has been gained from electron microscopy and rotary shadowing (Milam, 1985) and also from limited digests with V8 protease. These studies have shown that vinculin can be divided into two domains consisting of a globular head of approximately 100 kDa — 8 nm in diameter and a rod-like tail of approximately 30 kDa — 20 nm long. In high salt, vinculin self-associates into multimers containing two to six individual molecules. These molecules associate head to head and tail to tail, but the tail-to-tail association appears to be favoured. In rotary-shadowed complexes, the site of contact in the tail region often appears very dense, implying a region of association extending some distance.

The location of vinculin at the termini of microfilament bundles in the focal contacts, suggests that it may have a role to play in the attachment of the actin microfilaments to the cell membrane. Earlier evidence (Burridge & Feramisco, 1982; Wilkins & Lin, 1982) suggested that vinculin could interact directly with actin. However, it is now known that the actin-binding properties that were attributed to vinculin are due to co-purifying contaminants in the vinculin preparations (Wilkins & Lin, 1986). Vinculin has now been demonstrated to be only one in a small chain of proteins involved in the linkage of the components of the extracellular matrix to the microfilament system. The first protein in this chain is a transmembrane glycoprotein complex called integrin which consists of at least three subunits in chick embryo fibroblasts and is part of a larger family of cell surface receptors (for a review see Hynes, 1987). Integrin has been shown by gel equilibrium filtration techniques to form a complex with the next protein in the chain, talin, a rod-like protein of molecular weight 215 kDa found exclusively in cell–matrix adhesion junctions (Horton et al., 1986). Previously, Burridge & Mangeat (1984) had demonstrated by ultracentrifugation, immune precipitation and by gel overlay techniques that vinculin and talin form a complex of moderately high affinity. The talin-binding domain of vinculin is contained within the globular head region. Both vinculin and integrin bind to the 190 kDa domain of talin. After vinculin, the next link in the chain to the microfilaments remains elusive. Vinculin must associate with at least one other protein, but its identity remains to be determined. There is some evidence to suggest that it may be α-actinin (Craig, 1985). However, better candidates may be the high molecular mass proteins, progenitors of the HA1 group of polypeptides that interact with the ‘barbed end’ of actin filaments (Wilkins et al., 1986) or the 82 kDa protein that localizes to the focal contact (Beckerle, 1986).

Considerably less is known about the arrangement of proteins in the cell–cell adherens junction than in the focal contact, and although vinculin is found to be located near the ends of microfilament bundles, talin is not contained within these junctions. In addition, the transmembrane protein involved in intercellular adhesion at these sites is a Ca2+-dependent membrane glycoprotein called A–CAM (Volk & Geiger, 1986a, b). It is as yet unknown whether vinculin can associate directly with A–CAM or whether some other protein has the functional role of talin.

Much of the interest in vinculin arose when it was found that vinculin was a substrate for pp60c-src and that levels of phosphorysine in vinculin, but not in other cytoskeletal proteins, was markedly increased (8–10 fold) in cells transformed by RSV (Selton et al., 1981). It was therefore speculated that the phosphorylation of vinculin specifically

Abbreviations used: RSV, Rous sarcoma virus; CNBr, cyanogen bromide.

Received 11 May 1987


794 BIOCHEMICAL SOCIETY TRANSACTIONS