COMMENTARY

Cytoskeletal targets for oncogenic tyrosine kinases

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Introduction

The past decade has seen intense investigation into the role of tyrosine phosphorylation in cell growth and tumorigenesis (Cooper and Hunter, 1983). Whilst it is now well established that receptor occupancy by a number of growth factors is associated with tyrosine phosphorylation, the signal transduction pathways and the mechanism by which oncogenic tyrosine kinases induce cell growth and neoplastic changes remain elusive (Jove and Hanafusa, 1987; Yarden and Ullrich, 1988). The acutely transforming avian sarcoma viruses have been invaluable tools for examining the molecular basis underlying the changes in cell morphology and growth regulation that occur during neoplasia. The *src* gene product of Rous sarcoma virus (RSV) is probably the best-characterised oncogenic tyrosine kinase, and we shall review the evidence showing that components of the cytoskeleton are functional targets for this enzyme.

Tumour formation *in vivo* and transformation of cells *in vitro* by RSV is due to the expression of the viral *src* gene, which codes for a $60\times10^3 M_r$ membrane-associated phosphoprotein whose only known function is that of a tyrosine kinase (pp60$^{v\text{-}src}$). Cells transformed by RSV are characterised by anchorage-independent growth, loss of growth-regulation and changes in metabolite transport and utilization. In addition, transformed cells have a rounded cell shape and exhibit cytoskeletal changes, including a reduction in microfilament bundles, a redistribution of actin-associated proteins such as α-actinin, vinculin and talin, and changes in adhesion plaques, the structures responsible for cell attachment to substrata. There is also a loss of the extracellular matrix protein fibronectin, which may be related to the reduced adherence of these cells (Jove and Hanafusa, 1987).

The investigation of targets for pp60^{src} within whole cells has proved complex. The comparison of phosphoproteins from normal and RSV-transformed cells by two-dimensional SDS–PAGE has yielded patterns difficult to interpret, even with the aid of computer analysis. Another approach has been to ask what the likely candidates for pp60$^{v\text{-}src}$ targets within cells might be and to investigate these defined proteins, and this has led to a series of interesting findings.

The localisation of pp60$^{v\text{-}src}$ in transformed cells

It is now well established that pp60$^{v\text{-}src}$ is localised preferentially in the plasma membrane, and the investigation of mutant proteins has demonstrated a prerequisite for plasma membrane localisation before efficient transformation can occur (Cross *et al.* 1984; Kamps *et al.* 1986; Wyke and Stoker, 1987). Recently, Kaplan *et al.* (1990) have described three domains that are capable of mediating the attachment of pp60$^{v\text{-}src}$ to different subcellular membranes, and a $32\times10^3 M_r$ plasma membrane protein that binds to myristylated pp60$^{v\text{-}src}$ has also been identified (Resh and Ling, 1990). pp60$^{v\text{-}src}$ is concentrated in the remnant adhesion plaques and related structures such as 'rosettes' and 'podosomes' where it co-localises with vinculin and talin (Rohrschneider, 1980). It is not extracted from transformed cells by nonionic detergents, indicating a physical association with the detergent-insoluble cytoskeleton (Burr *et al.* 1981; Felice *et al.* 1990). This feature, allied to the observations that cytoskeletal changes occur rapidly (within 15 min) after pp60$^{v\text{-}src}$ is activated (Boschek *et al.* 1981), has led to speculation that cytoskeletal proteins might be specific targets for pp60$^{v\text{-}src}$ and that tyrosine-specific phosphorylation of one or more of these proteins would lead to the cytoskeletal disruption that characterises transformation. In support of this hypothesis, Hamaguchi and Hanafusa (1987, 1989) have reported a correlation between the association of pp60$^{v\text{-}src}$ with the cytoskeleton and transformation, and several cytoskeletal proteins whose phosphorylation correlates with transformation have recently been described (Glenney and Zokas, 1989). Whilst several membrane and cytoskeletal targets for pp60$^{v\text{-}src}$ have now been identified, the functional significance of tyrosine-specific phosphorylation of these proteins is still debatable (Kellie, 1988), although there are now some indications that the tyrosine-specific phosphorylation of at least one of these proteins has a functional consequence.

Cytoskeletal substrates for pp60$^{v\text{-}src}$

In vitro, several cytoskeletal proteins have been shown to be substrates for pp60$^{v\text{-}src}$: these include tubulin, MAP-2, fodrin and spectrin (Akiyama *et al.* 1986). Although this may be physiologically relevant, the phosphorylation of these proteins within whole cells has yet to be demonstrated. On the other hand, several other cytoskeletal proteins have been found to be phosphorylated on tyrosine

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in response to transformation. A Ca^{2+}- and phospholipid-dependent actin-binding protein variously termed p36, calpactin I or lipocortin II (now annexin II, according to new nomenclature; Crumpton and Bedman, 1990) is a major substrate. Investigations using partial transforming mutants have revealed no correlation between the tyrosine-specific phosphorylation of p36 and any of the morphological events associated with transformation (Nakamura and Weber, 1982; Stoker *et al.* 1986). Since it is now clear that p36 is one of a family of these molecules within cells, it may be that other isoforms or subpopulations within transformed cells are functionally affected. One putative function of the lipocortin/annexin family is the ability to inhibit phospholipase A_2 activity, and it is possible that phosphorylation might alter this, leading to changes in arachidonic acid metabolism and second messenger generation; however, evidence for this is lacking. Glenney (1985) has shown that tyrosine phosphorylation of p36 reduces its ability to interact with actin *in vitro*; however, the consequences of this within whole cells are unknown. Clathrin, the major protein of coated vesicles, ezrin (an 81×10^3 M_r protein originally found in brush border membranes) and calmodulin are all phosphorylated on tyrosine after RSV transformation (Fukami *et al.* 1986; reviewed by Wyke and Stoker, 1987). However, there are no data showing whether these are fortuitous phosphorylations due to the promiscuous substrate specificity of $pp60^{v\text{-}src}$, or whether these lead to cellular changes associated with transformation.

Another set of proteins have been shown to be phosphorylated on tyrosine in RSV-transformed cells. These include the proteins vinculin and talin, and the transmembrane receptor for fibronectin, a member of the integrin superfamily (Sefton *et al.* 1981; Pasquale *et al.* 1986; Hirst *et al.* 1986; DeClue and Martin, 1987). For the purposes of this review, since integrin can be localised with cytoskeletal structures, we will assume that this is a cytoskeletal protein, even if it does not fulfil all the requirements, such as resistance to nonionic detergents. These proteins are found in the cellular adhesion plaque, where $pp60^{v\text{-}src}$ is concentrated (Rohrschneider, 1980), thus there is a physical co-distribution of substrates with enzyme in the cell (Burridge, 1986). Vinculin, talin and the fibronectin receptor are thought to form a transmembrane bridge linking the extracellular matrix with the intracellular actin-based cytoskeleton (Burridge *et al.* 1988; Jones *et al.* 1989; Nuckolls *et al.* 1990). It is possible that phosphorylation of any one of these proteins might disrupt the adhesion plaque/cytoskeleton integrity resulting in the rounding up of the cells. Mutant viruses that express variant $pp60^{v\text{-}src}$ proteins and confer different transformed phenotypes have been useful in examining this question. Several groups have investigated the tyrosine-specific phosphorylation of vinculin in cells transformed by such mutants and have found no correlation between vinculin phosphorylation and cellular morphology (Rohrschneider and Rosok, 1983; Antler *et al.* 1985; Nigg *et al.* 1986; Kellie *et al.* 1986*a*). The addition of fibronectin to RSV-transformed cells, which induces a flatter morphology, had no effect on vinculin phosphorylation (Kellie *et al.* 1986*b*) and the observation that there is no change in vinculin phosphorylation during normal cell division (Rosok and Rohrschneider, 1983) has lead to the conclusion that tyrosine-specific phosphorylation of vinculin does not play a crucial role in the loss of adhesion plaque integrity during transformation. Tyrosine-specific phosphorylation of talin has not been examined so extensively, but the few studies that have been performed indicate a similar lack of correlation between talin phosphorylation and cell rounding or cytoskeletal disruption (DeClue and Martin, 1987). A novel adhesion plaque protein, paxillin, has recently been described that binds to the tail region of vinculin (Turner *et al.* 1990). This protein is phosphorylated on tyrosine in RSV-transformed cells with a stoichiometry of 20–30% (about 20-fold that of vinculin or talin), suggesting that this may have a functional consequence, although it remains to be demonstrated whether this intermolecular association is affected (Glenney and Zokas, 1989). There have been only a limited number of studies of tyrosine phosphorylation of the fibronectin receptor. Hirst *et al.* (1986) found that after transformation by RSV the β chains of the avian fibronectin receptor were phosphorylated on tyrosine and that this also occurred upon transformation with other tyrosine kinases such as those encoded by Fujinami sarcoma virus, Y73 or avian erythroblastosis virus. In agreement, we have recently found that the fibronectin receptor is tyrosine-phosphorylated in rounded RSV-transformed cells, which lose surface fibronectin, but is not phosphorylated in transformed cells with a flatter phenotype, which retain a surface fibronectin network (Horvath *et al.* 1990). One of the problems of studying these cytoskeletal proteins has been the lack of a functional assay; however, in recent years *in vitro* binding studies have been used to demonstrate a physical interaction between vinculin and talin, and between talin and the fibronectin receptor (Burridge and Mangeat, 1984; Horwitz *et al.* 1986). Using such methods it has been possible to show that tyrosine-phosphorylated fibronectin receptor from RSV-transformed cells has a reduced capacity to interact with either talin or fibronectin (Tapley *et al.* 1989). *In vitro* α-actinin can interact with integrin β subunits, and so phosphorylation of integrin might also disrupt this association within cells, leading to adhesion plaque dissociation (Otey *et al.* 1990). Evidence is beginning to accumulate that tyrosine-specific phosphorylation of the fibronectin receptor may play a role in both the disruption of adhesion plaques and the reduced ability of RSV-transformed cells to interact with fibronectin. This role of matrix receptors is further complicated, however, by the reports of differential expression of high-affinity and low-affinity fibronectin receptors occuring after transformation (Plantefaber and Hynes, 1989).

The presence of $pp60^{v\text{-}src}$ in adhesion plaques is insufficient to disrupt adhesion structures

The use of morphological mutants of RSV has permitted the examination of the effect of the presence of $pp60^{v\text{-}src}$ on adhesion plaque integrity. Whilst many mutants show a correlation between the presence of $pp60^{v\text{-}src}$ and adhesion plaque disruption (Rohrschneider and Rosok, 1983; Rohrschnieder and Reynolds, 1985; Nigg *et al.* 1986), there are several important examples where this is not the case. The RSV mutant CU2 induces a flat morphology but these cells have an overproduction of $pp60^{v\text{-}src}$-containing adhesion plaques; the ASV variants 1702, 157 and 2234.3 all encode active proteins that are localised in well-developed adhesion plaques (Kreuger *et al.* 1983; Kellie *et al.* 1986*a*; Horvath *et al.* 1990) and cells infected with RD10 or the temperature-sensitive mutants CU11 (at the permissive temperature) and LA32 (at the restrictive temperature) all have near-normal adhesion plaques that contain

pp60$^{v\text{-}src}$ (Rohrschneider and Rosok, 1983; Stoker *et al.* 1986). Thus the presence of pp60$^{v\text{-}src}$ in adhesion plaques is insufficient to disrupt them. The examination of the effect of pp60$^{v\text{-}src}$ on adhesion plaques leads us to a somewhat tautological argument: if the major action of pp60$^{v\text{-}src}$ was to break down adhesion plaques, then logically the residual structures found in transformed cells must be anomalies resistant to disruption. Therefore, the most informative adhesion structures would be those that no longer exist because they are pp60$^{v\text{-}src}$-sensitive (making their study rather difficult). An alternative explanation for the presence of pp60$^{v\text{-}src}$ in residual adhesion plaques is that it actually protects plaques from degradation; however, this property conflicts with its other roles in positively promoting cell transformation, and so seems less likely.

The effect of transformation on the proteins of membrane–cytoskeletal structures

If tyrosine-specific phosphorylation of membrane–cytoskeletal proteins is involoved in maintainence or induction of the transformed phenotype, what would be the functional consequences of this modification? We can postulate at least two possible mechanisms for cell rounding (see Fig. 1): (1) tyrosine phosphorylation of all adhesion plaque proteins results in a decreased intermolecular association of these proteins, leading to a break-up of adhesion structures and thence to cell rounding (model 1); or (2) tyrosine phosphorylation of integrin results in decreased affinity for fibronectin, leading to cell rounding, with the break-up of adhesion structures as a secondary process (model 3), i.e. adhesion plaque disruption is an effect of cell rounding, not a cause. An intermediate case would be the disruption of both fibronectin and talin association after phosphorylation of integrin (model 2).

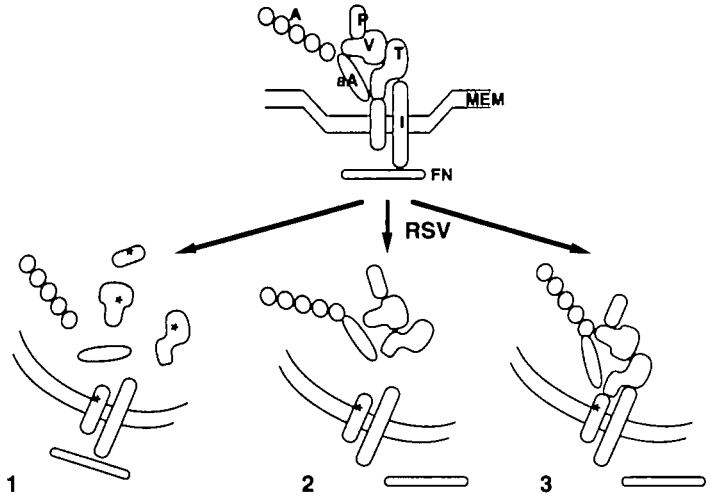

Fig. 1. Putative relationships between adhesion plaque proteins in untransformed or RSV-transformed cells. A, actin; aA, alpha-actinin; V, vinculin; T, talin; I, integrin (fibronectin receptor); P, paxillin; FN, fibronectin; MEM, membrane. Model 1: breakup of linkages between all adhesion plaque proteins. Model 2: breakup of linkages between adhesion plaque proteins and integrin, and integrin and fibronectin. Model 3: breakup of linkages between integrin and fibronectin. * Denotes tyrosine-phosphorylated protein.

The interaction of vinculin and talin with adhesion structures in RSV-transformed cells

The suggestion that vinculin and talin cannot interact with each other or with adhesion structures in RSV-transformed cells is not well supported in the literature. Vinculin has been found in the residual focal contacts, podosomes and rosettes of transformed cells (David-Pfeuty and Singer, 1980; Kellie *et al.* 1986*a*; Nigg *et al.* 1986; Marchisio *et al.* 1987; Gavazzi *et al.* 1989; Felice *et al.* 1990), and is co-localised with actin in these structures. Talin is also localised in these adherens structures; therefore, the molecular composition of adhesion structures in transformed cells is similar to that in untransformed cells, despite the ultrastructural changes. These observations indicate that although some of the proteins are lost the ability of vinculin to interact with talin and/or adhesion structures is at least partly retained after transformation. The situation is probably more complex than this, since vinculin appears to be lost more rapidly than talin from adhesion plaques after transformation (Brands *et al.* 1990). Thus there may be sequential disruption of adhesion plaque proteins.

The interaction of the fibronectin receptor with adhesion structures in RSV-transformed cells

In untransformed cells the fibronectin receptor is concentrated in adhesion plaques with an 'eye of the needle' configuration and is also co-localised with fibronectin fibrils. However, studies of RSV-transformed cells have revealed it to be more randomly distributed throughout the plasma membrane with little or no adhesion plaque localisation (Giancotti *et al.* 1986; Chen *et al.* 1986; Horvath *et al.* 1990). This is consistent with the suggestion that phosphorylation results in a decreased interaction of the fibronectin receptor with fibronectin (Tapley *et al* 1989). Since talin is not redistributed, this implies that: (1) the interaction of the fibronectin receptor with talin is also disrupted; and (2) the fibronectin receptor is not responsible for anchoring talin to adhesion structures. However, this is an oversimplification of the phenomenon. Whilst it is true that most adhesion plaques are disrupted upon transformation, some colocalisation of actin–vinculin–talin–integrin–pp60$^{v\text{-}src}$ in the remaining adhesion structures is preserved. This leads us back to the problem discussed before, i.e. are these remaining structures abnormal in being resistant to the influence of pp60$^{v\text{-}src}$? Alternatively, are the proteins in these remaining structures resistant to tyrosine phosphorylation? The answer to this second question is unknown, since the phosphorylation profile of specific proteins in the remaining adhesion plaques has not been investigated. However, immunofluorescence using anti-phosphotyrosine antibodies has demonstrated the presence of some tyrosine-phosphorylated proteins within residual adhesion structures, although these have not been identified (Comoglio *et al.* 1984; Felice *et al.* 1990; Horvath *et al.* 1990). Other investigations have also suggested that functional cytoskeletal interactions are not completely disrupted after transformation. The co-capping of talin with integrin was unaffected by transformation with RSV (Horvath *et al.* 1990) and no differences were found in the ability of normal and transformed cells to cluster talin and actin in response to fibronectin-coated beads (Mueller *et al.* 1989). These reports are consistent with talin being more tightly associated with adhesion plaques than vinculin in transformed cells (Brands *et al.* 1990).

These results suggest that a consequence of tyrosine phosphorylation is to disrupt the interaction of the fibronectin receptor with fibronectin, without necessarily affecting talin–vinculin or talin–vinculin–adhesion plaque interactions. However, whilst there are still unidentified components in this system that may be important in the induction of the transformed cytoskeletal changes, at present none of the proposed models wholly fits the observations, but features of all have been found.

Cytoskeletal substrates for pp60$^{c\text{-}src}$

Compared to the v-*src* protein, little is known about the physiological role of the normal cellular homologue, pp60$^{c\text{-}src}$. Only two cell types express the cellular *src* gene product to easily detectable levels: neurones and blood platelets (Cotton and Brugge, 1983; Brugge *et al.* 1985; Golden *et al.* 1986). In neurones pp60$^{c\text{-}src}$ is concentrated in the growth cone, leading to speculation that tyrosine phosphorylation might be involved in neurite extension (Maness *et al.* 1988). α and β tubulin and the synaptic vesicle protein synaptophysin have been shown to be substrates for pp60$^{c\text{-}src}$, and so it may play a role in neurosecretion in these cells (Cheng and Sayhoun, 1988; Pang *et al.* 1988; Barnekow *et al.* 1990; Matten *et al.* 1990). The effect of tyrosine phosphorylation on the functions of these proteins has yet to be examined. Tyrosine phosphorylation occurs rapidly during platelet activation when these cells are induced to secrete, and it has been reported that pp60$^{c\text{-}src}$ is located in platelet-dense granules, suggesting a role for secretion in these cells like that in neurones (Rendu *et al.* 1989). A later study, however, has concluded that most pp60$^{c\text{-}src}$ is located on the platelet plasma membrane or on the surface-associated canalicular membranes (Ferrell *et al.* 1990), and so this protein may have a more direct role in transmembrane signalling. Little is known about the substrates for pp60$^{c\text{-}src}$ within platelets; however, two recent studies have identified the platelet fibrinogen receptor glycoprotein IIb-IIIa as a substrate (Elmore *et al.* 1990; Findik *et al.* 1990). This molecule is a transmembrane glycoprotein comprising two subunits, gpIIb and gpIIIa, which can also bind fibronectin, laminin and thromospondin and belongs to the integrin superfamily of adhesion receptors. Findik *et al.* (1990) reported that purified platelet pp60$^{c\text{-}src}$ can phosphorylate both subunits of glycoprotein IIb-IIIa; however, Elmore *et al.* (1990) have recently shown that in platelet membranes only the IIIa chain of gpIIb-IIIa is phosphorylated on tyrosine. Immunopurified platelet pp60$^{c\text{-}src}$ could phosphorylate a peptide containing the tyrosine phosphorylation site of fibroblast integrin β_1 subunit, which is partially conserved in the IIIa chain. Since thrombin-induced tyrosine-specific phosphorylation can be inhibited by RGDS peptides (Ferrell and Martin, 1989), this implies a close functional association of pp60$^{c\text{-}src}$ with adhesion receptors during platelet activation. The findings that integrins can act as substrates for both the transforming and nontransforming *src* proteins, with consequences that include changes in cell–matrix interactions and cytoskeletal reorganisation, strongly suggest that tyrosine-specific phosphorylation of integrin receptors plays a crucial role in the regulation of cell shape.

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