Focal-adhesion components are enriched in ventral membranes isolated from transformed keratinocytes in culture

Ronald E. GATES,*§ Steven K. HANKS‡ and Lloyd E. KING, Jr.†

* Research Service, Department of Veterans Affairs Medical Center and Departments of † Medicine (Dermatology) and ‡ Cell Biology, Vanderbilt University Medical Center, Nashville, TN, U.S.A

The lack of procedures for isolating or reconstituting intact focal adhesions has hindered studies of how focal adhesions are organized and of how their assembly/disassembly is controlled. A method for isolating large quantities of the ventral portion of plasma membranes from transformed keratinocytes (A-431 cells) in culture is described. Plasma membranes are stabilized using Zn^{2+} and the ventral portion isolated attached to the culture substratum after the body of the cell has been sheared away. Compared with complete plasma membranes isolated from cells scraped from the dish, these ventral-membrane

preparations are enriched 18.5-fold and 5.1-fold in the focal-adhesion components talin and vinculin respectively. While the epidermal-growth-factor receptor-kinase is less abundant in preparations of ventral membranes, a recently described tyrosine kinase which localizes to focal adhesions in mouse fibroblasts is enriched 19.9-fold. Extracellular matrix components, as well as their integrin receptors, are also enriched in these preparations of the ventral portion of plasma membranes compared with preparations of complete plasma membranes.

INTRODUCTION

Focal adhesions (FA) are discrete plasma membrane regions where cultured cells make close contact with the substratum and where stress fibres terminate (reviewed in [1]). They are enriched in members of the β_1 - and β_3 -subunit family of integrins [2,3] which act as specific receptors for extracellular matrix (ECM) components which coat the substratum. The cytoplasmic portions of these receptors interact with other components of FA including vinculin, talin and α -actinin which bind to the stress fibres [1].

The nature of the interaction between various FA components and the mechanism by which these interactions are modulated to control the assembly and disassembly of FA is poorly understood. While phosphorylation and dephosphorylation appear to regulate assembly and disassembly of FA, the kinases and substrates involved are not known [4]. Understanding of the structure and assembly/disassembly of FA has been hindered by the inability either to reconstitute them from individual components or to purify them from other membrane structures. We describe here a method for preparing large quantities of ventral membranes which retain native structure and biochemical activities and are enriched in known FA components including a recently identified FA protein tyrosine kinase, designated as Fad kinase [5].

MATERIALS AND METHODS

Materials

The A-431 epidermoid carcinoma cells were grown in DME/F-12 medium from Sigma Chemical Co. ^{35}S -Methionine as Tran ^{35}S -label came from ICN Biomedicals Inc. along with methionine-deficient Dulbecco's modification of Eagle's medium (DMEM). Both types of medium were supplemented with 5% (v/v) fetal

calf serum and 500 μ g/ml gentamicin sulphate, each of which was obtained from Grand Island Biological Co. Nitrocellulose was purchased from Millipore Corp. and fixed *Staphylococcus aureus* cells (Pansorbin) were purchased from Calbiochem. Affinity-purified rabbit antibody raised against laminin, and monoclonal antibodies raised against vinculin (clone VIN-11-5), α -actinin (clone BM-75.2), epidermal-growth-factor receptor (EGF-R) (clone 29.1.1), talin (clone 8d4) and fibronectin (clone FN-15) were obtained from Sigma Chemical Co. along with rabbit antibodies raised against mouse IgG and biotinylated goat antibodies raised against rabbit IgG, mouse IgG or mouse IgM. Telios provided the rabbit antibody against tenascin and mouse monoclonal antibodies against the α_2 (clone P1E6) and α_3 (clone P1B5) chains of human integrins. ^{125}I -Labelled donkey antibody against rabbit IgG came from the Amersham Corporation while fluorescein-conjugated goat anti-(rabbit IgG) antibody came from Vector Laboratories and rhodamine-conjugated goat anti-(mouse IgG) antibody came from Chemicon. Keith Burrig (University of North Carolina) supplied the rabbit antibody raised against chicken talin, and Martin Hemler (Dana Farber Cancer Institute) provided the monoclonal antibody (clone A-1A5) raised against the β_1 chain of human integrin. Avidin and biotinylated peroxidase were purchased from Vector Laboratories.

Antibodies against Fad kinase

Affinity-purified rabbit polyclonal antibodies raised against Fad kinase were prepared as described previously [5].

Membrane preparations

Plasma membranes were prepared according to Thom et al. [6] except that $CaCl_2$ in the buffer which was used to harvest the cells

Abbreviations used: FA, focal adhesion(s); ECM, extracellular matrix; DMEM, Dulbecco's modification of Eagle's medium; EGF-R, epidermal-growth-factor receptor; DPBS, Dulbecco's phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; Fad kinase, focal adhesion protein tyrosine kinase.

§ To whom correspondence should be addressed at F-325 ACRE Bldg., DVA Hospital, 1310 24th Avenue South, Nashville, TN 37212, U.S.A.

was replaced with 100 μ M each of EGTA and leupeptin to prevent the activation of calcium-activated neutral protease [7]. Ventral membranes were prepared from confluent culture dishes of A-431 cells using a modification of the procedure described by Avnur and Geiger [8]. In brief, attached cells were washed twice (15 s each wash with 15 ml per 100 mm-diam. dish) at room temperature with buffer A: 50 mM Mes, 5 mM MgCl_2 , and 3 mM EGTA. The cells were then incubated for 2 min at room temperature with 15 ml of buffer A which also contained 4 mM ZnCl_2 and then washed three times as above at room temperature with Dulbecco's phosphate-buffered saline (DPBS) with CaCl_2 omitted. The protease inhibitors, leupeptin and phenylmethanesulphonyl fluoride (PMSF), were added to these three solutions immediately before use to give final concentrations of 10 μ M and 100 μ M respectively. The cells were sheared from the culture dish using a 5 mm-wide spray produced by forcing DPBS (with CaCl_2 omitted) under 207 kPa of N_2 through a 20-gauge needle with the bevelled tip slightly bent. When all cells were sheared from the dish, the attached ventral membranes were processed as described below.

Photomicrography

Intact cells or ventral membranes were fixed at 4 °C for 4 h in 35 mm-diam. dishes using 4% (v/v) glutaraldehyde in DPBS (both CaCl_2 and MgCl_2 omitted). Photographs were taken of cells and ventral membranes as seen under phase contrast using a 40 $\times$ objective. Immunofluorescent staining of intact cells for Fad kinase and talin was performed as described previously [5], and photographs taken using a 100 $\times$ oil immersion Neofluor objective. All photomicrographs were made using a Zeiss Axioskop microscope equipped for phase-contrast and fluorescence microscopy.

Immunodetection on Western blots

Ventral membranes were removed from 100 mm-diam. dishes by floating the dish on water at 90 °C for 3 min after adding 2 ml of SDS harvesting buffer: 0.2% SDS, 0.2 mM EDTA, 0.1 mM leupeptin, 1 mM PMSF. The solubilized ventral membranes were combined with two water washes (1 ml) of each dish and dialysed against several changes of 0.05% SDS, freeze-dried to dryness, and reconstituted with water in 1/120 of the final volume after dialysis. After protein concentration was assayed as described previously [9], a 10% excess of sample for electrophoresis was prepared to contain 60 μ g of protein per 150 μ l of Laemmli [10] sample buffer, so that 150 μ l of sample could be loaded per lane on 5–10% gradient acrylamide gels and transferred to nitrocellulose according to Towbin et al. [11]. Individual proteins on the blot were identified with primary antibodies against the protein, followed by localization of bound antibody. Determination of bound antibody involved staining for bound peroxidase using diaminobenzidine and H_2O_2 according to Hsu et al [12], or the binding of ^{125}I -labelled donkey anti-(rabbit IgG) antibody followed by autoradiography.

Immunoprecipitation of labelled membranes

Labelled membranes were prepared in 100 mm-diam. culture dishes from cells grown for the final 12 h with 0.5 mCi of Tran^{35}S -label in 18 vol. methionine-deficient DMEM, 1 vol. calf serum and 1 vol. DMEM. Ventral membranes were prepared as above and harvested by sequentially scraping two dishes at 0 °C into 2 ml of Triton X-100 harvesting buffer: 0.2% Triton X-100,

20 mM Hepes, 0.5 mM EDTA, 100 μ M leupeptin, 1 mM PMSF, pH 7.5. After combining with two sequential water washes (1 ml each) of the two dishes, the ventral membranes were concentrated 10-fold using Millipore centrifugal ultra-free-20 filter units with a nominal 30 000 Da cut-off limit. Identically concentrated non-denaturing harvesting buffer was used to solubilize plasma membranes prepared from the same batch of labelled cells. Immunoprecipitations of aliquots of pairs of resuspended plasma and ventral membranes containing 10^6 c.p.m. of ^{35}S methionine were analysed simultaneously, essentially as described by others [13] using identical amounts of primary antibodies and of rabbit antibody raised against mouse IgG bound to fixed *Staphylococcus aureus* cells. Pairs of washed immunoprecipitates from ventral and plasma membranes were solubilized in 130 μ l of Laemmli [10] sample buffer and after centrifugation each supernatant was loaded into adjacent gel lanes and electrophoresed. Stained gels were soaked in 1 M sodium salicylate [14], dried and subjected to autoradiography.

Quantification

Areas under appropriate peaks in reflectance-densitometric scans of photographs of the peroxidase-stained blots, or in transmission densitometric scans of autoradiographs, were used to quantitate relative amounts of antigen. To minimize error when comparing ventral and plasma membranes, protein concentrations of each were determined together in the same protein assay. Comparisons for the amount of specific antigen were restricted to two different ventral membrane preparations and two different plasma membrane preparations electrophoresed, blotted and analysed for antigen side by side and simultaneously on the same gel and blot. This set of four comparisons was performed twice for each specific antigen, using different ventral membrane preparations in each set of comparisons. With immunoprecipitations, solubilized pairs of plasma and ventral membrane immunoprecipitates were electrophoresed in adjacent gel lanes on non-reducing gels and then compared. Each comparison was performed twice using different sets of plasma and ventral membranes.

To confirm that comparisons were made in the linear response range of photographs or autoradiographs we used the following approaches. For moderate differences in intensity between bands, different exposures were evaluated and only those in the mid-range, resulting in a constant ratio for a particular comparison, were used. With large differences in intensity between bands, the linear range was confirmed by the good agreement with results obtained by scanning light bands on longer exposures and dark bands on shorter exposures and referencing the scans to a common band of intermediate intensity. To reduce the bias toward the largest differences, the reciprocal of the average ratio of antigen in plasma membranes to ventral membranes was used for enrichment.

RESULTS

Isolation of ventral membranes

To isolate ventral membranes which contain FA for microscopy, Avnur and Geiger [8] used Zn^{2+} at pH 6 as a weak fixative to stabilize membranes and coalesce the cytoplasm around the nucleus [15]. A jet of DPBS was used to shear the rest of the cell from the slide on which the cells were grown while leaving the ventral membranes attached. We chose their approach for a large-scale isolation of ventral membranes because of its inherent advantages. The stabilizing process with Zn^{2+} does not affect most biochemical activities and is reversible to allow subsequent

Figure 1 Phase-contrast micrographs of intact A-431 cells (a) and of ventral membranes (b) prepared from these cells

processing and identification of individual FA components. To make large-scale preparations feasible, the steps in the isolation procedure were modified so that the procedure was faster and more reproducible. Monolayers of A-431 cells growing on plastic culture dishes were stabilized with Zn^{2+} and then sheared from the dish using a 5 mm-wide spray of DPBS from a buffer reservoir at 207 kPa of N_2 . This approach to isolating ventral membranes works well with cells that adhere tightly to the culture dishes (A-431 cells, keratinocytes) but was not successful with less adherent cells (fibroblasts, melanoma cells). Phase-contrast micrographs of ventral membranes of A-431 cells still attached to the dish are compared with the attached cells in Figure 1.

FA and ECM components

Sufficient protein was recovered from each 100 mm-diam. culture dish processed for ventral membranes (30–50 μg) to allow a direct comparison with isolated plasma membrane proteins. Figure 2(a) shows that while protein loading is comparable the overall pattern of proteins after SDS/PAGE was quite different for ventral and plasma membranes. These differences probably

Figure 2 Comparison of A-431 ventral membranes (V) and plasma membranes (M) for total proteins (a), cytoplasmic components of FA (b) and ECM components (c)

Identical amounts of protein (60 μg) from the two kinds of membrane preparations were electrophoresed in adjacent lanes on SDS/polyacrylamide gels and either stained for protein or transferred to nitrocellulose by Western blotting. The blots were incubated with (b) antibodies against vinculin (1:250 dilution) or talin (1:500), and (c) with antibodies against tenascin (1:400 dilution) or laminin (1:200). The arrowhead indicates the stained band corresponding to the indicated antigen and a–e indicate the position of molecular-mass markers: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase B (92.5 kDa), BSA (66 kDa) and ovalbumin (45 kDa) respectively.

reflect different membrane structures and functions in the two preparations such as FA in the ventral membranes. Indeed, Figure 2(b) shows that two known components of FA, talin and vinculin, are much more abundant in ventral membranes compared with plasma membranes. Similar enrichments in tenascin and laminin, ECM components which should be isolated with ventral membranes, are seen in Figure 2(c). Table 1 shows quantitative enrichments in ventral membranes for these FA cytoplasmic components and ECM components including those for α -actinin and fibronectin. When expressed as the ratio of amounts in plasma membranes to ventral membranes, the S.D.s are all within a third of their respective means, except for fibronectin where it is 1.2-fold greater than the mean. As a comparison between four sets of plasma- and ventral-membrane preparations, the quantitative results show that ventral membranes are consistently highly enriched in cytoplasmic FA and in ECM components.

The reduced amount of FA and ECM components in plasma membranes is not simply due to these components being left attached to the culture dish when cells are scraped from the dish. After cells were removed by scraping to prepare plasma membranes, culture dishes were treated with SDS harvesting buffer at 90 °C as is done to solubilize the ventral membranes. When compared with ventral membranes prepared at the same time from an identical number of dishes, only negligible amounts of talin or tenascin were detected.

Integrin subunits

Those α - and β -integrin subunits which form ECM receptors and which interact with the cytoplasmic FA components are integral components of FA and should also be enriched in ventral membranes. Figure 3 shows the results of immunoprecipitating α_2 -, α_3 - and β_1 -integrin subunits from solubilized ventral or plasma membranes prepared from the same batch of ^{35}S -methionine-labelled cells. The immunoprecipitates were analysed on reducing SDS gels (Figure 3a), where the β_1 and α_3 subunits have identical mobilities (lower arrow) while the α_2 subunit runs

Table 1 Enrichment of FA components in ventral membranes compared with plasma membranes

Average fold increase determined as described in the Materials and methods section from stained Western blots or from autoradiographs of Western blots similar to those shown in Figures 2, 3 and 4.

Component	Increase (fold)
Intracellular protein	
Talin	18.5
Vinculin	5.1
α -Actinin	2.0
Integrin subunits	
α_2	2.7
α_3	3.8
β_1	3.5
ECM components	
Laminin	5.4
Tenascin	11.8
Fibronectin	12.8
Protein tyrosine kinases	
Fad kinase	19.9
EGF-R kinase	0.5

Figure 3 Comparison of immunoprecipitates of different integrin subunits from A-431 ventral membranes (V) and plasma membranes (M)

The two membrane samples with identical amounts of radioactivity prepared from cells metabolically labelled simultaneously with ^{35}S -methionine were immunoprecipitated using antibodies against the α_2 (lanes 1 and 2), α_3 (lanes 3 and 4) and β_1 (lanes 5 and 6) subunits and the immunoprecipitates analysed on reducing gels (a) or non-reducing gels (b). Upper arrows indicate the position of α_2 subunit in (a) and of α_2 and α_3 subunits in (b). Lower arrows indicate the position of α_3 and β_1 subunits in (a) and of β_1 subunit in (b). For non-reducing gels, the β -mercaptoethanol in Laemmli [10] sample buffer was replaced with a solution of 100 mM *N*-ethylmaleimide resulting in a final 5 mM concentration. a-e indicate the position of the same molecular-mass-marker proteins described in the legend to Figure 1.

at a slower rate (upper arrow). Figure 3(b) shows the results using non-reducing gels where, compared with reducing gels, the β_1 subunit runs at a faster rate (lower arrow) and the α_1 subunit runs at a slower rate and co-migrates with the α_2 subunit (upper arrow). Clearly, the α_2 subunit in the first two lanes, the α_3 subunit in lanes 3 and 4 (and their associated β_1 subunits) along with the β_1 subunit in lanes 5 and 6 (and its associated α subunits) are enriched in ventral membranes compared with plasma membranes. This enrichment is quantitated in Table 1 as an

Figure 4 Comparison of A-431 ventral membranes (V) and plasma membranes (M) for Fad kinase (a) and for EGF-R kinase (b)

Western blots prepared as described in the legend to Figure 2 were incubated with antibodies against EGF-R (1:10⁴ dilution) or with affinity-purified immune serum against Fad kinase (1:125) or with pre-immune serum subjected to the same affinity-purification protocol. Bound antibodies were detected by the binding of ^{125}I -labelled second antibody and autoradiography (a) or by specific staining (b). Arrowheads and molecular-mass markers a-e are as described in the legend to Figure 2.

average for two sets of ventral and plasma membranes. The deviation of the range from the mean was less than 24 % of the mean for each of the three subunits. Hence, ventral membranes show a consistent enrichment of 3-4-fold for integrin receptors compared with plasma membranes.

Protein tyrosine kinases

Because high levels of phosphotyrosine have been found in FA [16], specific phosphotyrosyl kinases may be associated with FA and should be enriched in ventral-membrane preparations. Figure 4(b) shows that the EGF receptor, which is present at very high levels in A-431 plasma membranes, is actually less abundant in ventral membranes. When averaged over four sets of ventral- and plasma-membrane preparations, there is only 50 % as much EGF-R in ventral membranes as in plasma membranes (see Table 1). The results are quite different using an antibody raised against the recently discovered Fad kinase that selectively stains FA in mouse fibroblasts [5]. As seen in Figure 4(a), this antibody specifically recognizes a 115 kDa protein which is much more abundant in ventral membranes than in plasma membranes. Averaging over four sets of ventral- and plasma-membrane preparations gives a 19.9-fold enrichment of this tyrosine kinase in ventral membranes compared with plasma membranes (see Table 1). Hence, ventral membranes are consistently and substantially enriched in Fad kinase.

To confirm that Fad kinase is localized in FA in A-431 cells as it is in cultured mouse fibroblasts [5], the distributions of talin and of Fad kinase were determined in A-431 cells using immunofluorescence. Figure 5(a) shows the localization of monoclonal antibodies against talin to the FA in A-431 cells. Comparison of Figures 5(a) and 5(b), which show the distribution of affinity-purified polyclonal antibodies against Fad kinase, confirms that

Figure 5 Immunofluorescence micrographs of an A-431 cell showing the distribution of talin (a) and of Fad kinase (b)

Arrows indicate the positions of representative, identical FA staining for both talin and Fad kinase. A-431 cells that had been plated on glass coverslips coated with fibronectin (10 $\mu\text{g}/\text{ml}$) for 18 h before permeabilization and fixation, were exposed to mouse anti-(talin) antibody (diluted 1:500) and to affinity-purified rabbit antibodies raised against FA kinase (diluted 1:5). After washing, incubation with fluorescein-conjugated goat anti-(rabbit IgG) and rhodamine-conjugated goat anti-(mouse IgG) antibodies and further washing, the same cell was photographed using narrow-band filters specific for rhodamine (a) or fluorescein (b) fluorescence.

Fad kinase is also localized in FA in A-431 cells. While Figure 5 shows a cell with abundant FA, about half of the A-431 cells did not have detectable FA as determined by the absence of staining with antibodies raised against talin. While the reason for the absence of FA in many A-431 cells is not known, in no cases were A-431 cells seen in which the localization of talin and Fad kinase did not match. Appropriate control experiments confirmed the specificity of the staining reactions. Cross-reactivity with talin antibodies was eliminated by the staining of FA when A-431 cells were incubated only with antibodies against Fad kinase and the appropriate fluorescein-conjugated second antibody (results not shown). Also, the pre-immune serum subjected to the same affinity purification protocol as the immune serum against Fad kinase never localized to FA (results not shown).

DISCUSSION

Because of their lability, FA must be left attached to the substratum and stabilized in order to isolate them from the rest of the cell. The procedure of Avnur and Geiger [8] achieves both of these requirements and, as they have shown with

immunofluorescence micrography, isolates ventral membranes with intact FA. Our modification of this procedure to obtain large quantities of ventral membranes results in membrane preparations that are strikingly enriched in FA components. There are two major advantages to the methods described above for preparing ventral membranes enriched in FA. First, the stabilization with Zn^{2+} is reversible by chelation allowing solubilization and immunoprecipitation of individual FA components from the ventral membranes. Secondly, even without chelation, Zn^{2+} in intact ventral membranes still attached to the dish often does not compromise enzyme activities. Indeed, phosphorylating such ventral membranes with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of Mn^{2+} resulted in enhanced phosphorylation of EGF-R and several other proteins when EGF was added (results not shown). The results were virtually identical to similar phosphorylation experiments performed simultaneously using plasma membranes (results not shown).

Fad kinase is a 120 kDa protein tyrosine kinase identified using a homology-based cDNA cloning approach [5] that does not have a membrane-spanning region. Immunostaining of mouse fibroblasts showed that Fad kinase is primarily localized in FA [5]. As seen in Figure 5, anti-(Fad kinase) antibodies detect a 115 kDa human homologue of Fad kinase in A-431 cells that is greatly enriched in ventral membranes. Fad kinase may be a critical element in mediating cellular responses to the ECM. Aggregating integrin receptors by either plating cells on fibronectin [17] or treating them with antibodies against the β_1 -integrin chain [18] increases tyrosine phosphorylation on proteins in the 115–130 kDa range. When mouse fibroblasts are plated on fibronectin, similar increases are seen in the amount of phosphotyrosine in Fad kinase [5]. Hence, aggregation of the fibronectin receptor appears to activate Fad kinase, which then phosphorylates both itself and probably other unidentified substrates on tyrosine. After immunoprecipitation from A-431 cells, Fad kinase appears to phosphorylate itself (results not shown). However, we have not yet found conditions, either in intact A-431 cells or in A-431 ventral membranes, where Fad kinase phosphorylates itself or other proteins as it does in mouse fibroblasts.

There are considerable differences in the degrees of enrichment of the various marker components known to be associated with ventral membranes as compared with plasma membranes. The differences between the cytoplasmic FA components are probably explained by their known distributions outside FA. Vinculin, but not talin, is found in epithelial cell-cell junctions [19] and α -actinin is periodically distributed along stress fibres and coincides with talin and vinculin only at the FA [1]. Hence, as is observed, talin should be the most enriched cytoplasmic FA component in ventral membranes and α -actinin the least enriched. A similar explanation is likely to apply for the different distributions of the α - and β -integrin subunits. The $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrin receptors have been found in regions of cell-cell contact as well as in FA with non-transformed cultured keratinocytes [20–22]. Therefore, as seen in Table 1, these integrin subunits are not necessarily as enriched in ventral membranes as are components known to be specifically associated with FA, such as talin.

There are other explanations for the different degrees of enrichment of marker components in ventral membranes compared with plasma membranes. One such explanation is that some components which are not tightly associated with ventral membranes may dissociate as the plasma membranes are being isolated. These same components probably would not dissociate during the preparation of ventral membranes, because the membranes are left attached to the dish and are stabilized with Zn^{2+} . The selective loss of marker components from the plasma

membrane should therefore increase their apparent enrichment in the ventral membranes. This explanation may apply to highly enriched ECM components, such as fibronectin and tenascin, or to highly enriched cytoplasmic FA components such as talin and Fod kinase. Another explanation for the different degrees of enrichment is that FA components and ECM molecules could be left attached to the culture dish when cells are removed by scraping. This explanation is unlikely, because significant amounts of talin or tenascin were not recovered from culture dishes after the cells had been removed by scraping.

It is important to note that the two problems in determining the purification of ventral membranes are also fundamental to the advantages of isolating attached and stabilized ventral membranes. First, marker proteins expected to be enriched in ventral membranes may not be exclusive to ventral membranes, so the degree of purification is uncertain. Conversely, isolating ventral membranes eliminates protein molecules not actually involved in the adhesion of cells to the substratum, and ensures that only involved proteins are isolated. Secondly, some of the marker proteins in ventral membranes may dissociate when plasma membranes are isolated, so again the degree of purification of the ventral membranes is uncertain. Conversely, isolating attached and stabilized ventral membranes prevents the dissociation of weakly associated components, the association of which may actually depend on intact FA. Hence, ventral membranes should be well-suited for studying how cells attach to the substratum and how they regulate that attachment. Only those proteins actually involved in the attachment are isolated in the ventral membranes, while the labile structures, weak associations and enzymic activities are preserved.

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