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Protein and lipid lateral diffusion in normal and Rous sarcoma virus transformed chick embryo fibroblasts

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We measured the lateral diffusion of the fluorescent lipid analogue dioctadecylindocarbocyanine iodide (DiI) and of membrane glycoproteins labeled with tetramethylrhodamine (TRITC) succinyl concanavalin A (SConA) via fluorescence photobleaching recovery (FPR) at selected times during a temperature downshift experiment on transformation-defective temperature sensitive (td-ts) Rous sarcoma virus (RSV) NY68-transformed chicken embryo fibroblasts (CEF) and on identically treated CEF and RSV-transformed CEF. There were no significant differences in the lateral diffusion of DiI at any of the times measured. The lateral diffusion of TRITC-SConA on the RSV-transformed CEF, $(1.32 \pm 0.12) \cdot 10^{-10}$ cm^2 s^{-1}, was approximately two times faster than that observed in normal CEF, $(0.61 \pm 0.06) \cdot 10^{-10}$ cm^2 s^{-1}. In the cells undergoing RSV NY68-mediated transformation, TRITC-SConA diffusion increased over a 24-h period from a value comparable to that observed in normal CEF, $(0.72 \pm 0.13) \cdot 10^{-10}$ cm^2 s^{-1} to a value comparable to the RSV-CEF transformed cells, $(1.74 \pm 0.20) \cdot 10^{-10}$ cm^2 s^{-1}. All diffusion measurements reported were made at the permissive temperature for RSV-NY68 (35°C) unless stated otherwise. The changes in the lateral diffusion of TRITC-SConA occurred between the fifth and twelfth hour of the downshift course and could be associated with cytoskeletal disruption and/or fibronectin degradation, both known to occur at this time in RSV-transformed cells. To assess the contribution of extracellular matrix (ECM) degradation, SConA mobility was measured in normal and RSV-transformed cells treated with trypsin. This treatment increased SConA mobility approximately 4-fold in the normal cells relative to untreated controls and only 2-fold in the RSV-CEF transformed cells. No significant difference in SConA mobility between trypsinized spherical normal and transformed cells was apparent.

Introduction

One of the most intensely studied transformed cell systems is that of Rous sarcoma virus (RSV)-transformed chicken embryo fibroblasts (CEF). RSV is a replication-competent avian RNA tumor virus that causes fibrosarcoma in birds [1]. Transformation of cultured chicken embryo fibroblasts results in numerous alterations of cell structure and function. Early studies reported an increased agglutination of cells with both concanavalin A (ConA) and wheat germ agglutinin (WGA) [2]. Transformed CEF exhibit loss of density-dependent inhibition of growth and are capable of anchorage-independent growth [3]. The transformed cells exhibit a rounded or elongated morphology with accompanying decreased adhesion to various substrates [2]. Structural changes include disruption of the cytoskeleton microfilaments (stress fibers) [4–6] and decreased synthesis of tropomyosin [7]. The extracellular matrix (ECM) is modified by proteolytic degradation [8,9] and by decreased production of fibronectin [10–12] and collagen [13].

Much of the information gained to date in this cell system has been obtained with the use of transformation-defective temperature-sensitive (td-ts) mutants of RSV. In cells infected with such mutants, the transformed phenotype is expressed at the permissive temperature but not at the restrictive temperature. Viral replication continues at both temperatures [14]. Shifting cultures infected with these viruses from the restrictive to the permissive temperature makes it possi-

Abbreviations: DiI, 3,3'-dioctadecylindocarbocyanine iodide; TRITC, tetramethylrhodamine isothiocyanate; SConA, succinyl concanavalin A; FPR, fluorescence photobleaching recovery; RSV, Rous sarcoma virus; CEF, chicken embryo fibroblasts; td-ts, transformation-defective temperature-sensitive; HBSS, Hank's balanced salt solution; ECM, extracellular matrix; S.E., standard error; FP, fluorescence polarization; ConA, concanavalin A; WGA, wheat germ agglutinin.

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ble to initiate transformation synchronously allowing time-course studies of various transformation parameters. Such td-ts mutant systems have been used by several researchers to study changes in the transformed CEF membrane, cytoskeleton and extracellular matrix. Scanning electron microscopy demonstrates the transient formation of edge ruffles during the first hour of transformation [6]. This phenomena is followed by the formation of ruffle-like evaginations, flowers, on the dorsal surface of the transformed cell between 1 and 6 h after shift to the permissive temperature [15]. Others have reported changes in cytoskeletal organization 6–12 h after initiation of the transformation [4,5]. Boschcr et al. [6] confirmed these earlier reports utilizing a variety of microscopic techniques and specific immunochemical reagents in a time-course experiment with the td-ts mutant RSV NY68 [16]. Loss of the CEF main extracellular matrix protein fibronectin was shown to occur 3–12 after the initiation of transformation. The cell shape changes characteristic of CEF transformation occur following cytoskeletal disruption and loss of fibronectin.

We have used fluorescence photobleaching recovery (FPR) to examine the lateral mobility of 3,3′-dioctadecylindocarbocyanine iodide (DiI) and of membrane glycoproteins labeled with tetramethylrhodamine isothiocyanate-conjugated succinyl concanavalin A (TRITC-SConA) on adherent normal CEF, RSV-transformed cells and RSV-NY68 infected CEF undergoing temperature shift from non-permissive to permissive temperature. Our principle goal was to determine if measurable changes in the diffusion coefficients of the two probes occurred during the transformation process. All three cell samples were examined at relevant selected times and conditions in an effort to correlate such changes with previously reported transformation-induced changes in morphology and cellular organization [4–6,15]. In an effort to evaluate the contribution of the ECM to any change, FPR experiments were also performed on trypsin-treated cells.

Materials and Methods

Cell cultures and viruses. Chick embryo fibroblast cultures were established with cells obtained from SPAFAS, Incorporated (Norwich, CT). Cultures of CEFs infected with cloned stocks of the Schmidt-Ruppin strain of Rous sarcoma virus, subgroup A (SR-A), and its transformation-defective temperature-sensitive mutant NY68 were the kind gifts of Dr. H. Hanafusa, Rockefeller University, New York, NY, and Dr. R. Erickson, Harvard University, Cambridge, MA. Cultures were maintained in Medium 199 supplemented with 10% (v/v) tryptose phosphate broth, 5% (v/v) fetal calf serum, 200 units of penicillin per milliliter and 2 mg of streptomycin per milliliter. Primary and subsequent cultures of CEF and CEF infected with wild-type RSV were propagated at 37°C, 100% relative humidity and 5% CO_2. Cultures of CEF infected with RSV NY68 were maintained under identical conditions at 42°C, the non-permissive temperature for RSV NY68. All cultures were originally seeded or maintained in 75 cm^2 plastic tissue culture flasks. Culture supernatants from cells used in given mobility experiments were collected and assayed for the presence of RNA-directed DNA polymerase activity using $(A)_n(dT)_{12-18}$ to demonstrate the presence of infection [17]. Following verification of infection, the cultures were harvested and seeded in 25 cm^2 plastic petri dishes. All cultures were incubated for 24 h at 42°C. Following incubation at 42°C for 24 h one-half of the cultures were switched to 35°C, the permissive temperature of RSV NY68, initiating the temperature downshift experiments. Cultures were removed at selected times, labeled with DiI or TRITC-SConA as described below, and used immediately for lateral diffusion measurements. Care was taken to maintain the cultures at the appropriate temperature during labeling and data collection.

Lateral diffusion measurements were also performed on unattached normal CEF cells and RSV-transformed cells. In such cases cells were prepared by 2 min trypsinization in 1 : 4 diluted 0.25% trypsin, 0.02% EDTA at 35°C followed by suspension in growth medium supplemented with 5% calf serum for 5–6 h prior to labeling. Once again, cells were maintained at the appropriate temperature (35°C) during preparation, labeling and data gathering.

Cell labeling for FPR measurements. The phospholipid analog DiI was synthesized as previously described by Sims et al. [18]. The DiI was dissolved in absolute ethanol to a final concentration of 0.5 mg/ml. Maximal absorption of DiI in ethanol is at 553 nm with an extinction coefficient of $1.2 \cdot 10^5\ M^{-1}\ cm^{-1}$ and the fluorescence emission spectrum peaks at 565 nm. Cell cultures in 25 cm^2 petri dishes were washed twice with 2 ml of Hank's balanced salt solution (HBSS). Each culture was then incubated either with 1.98 ml of HBSS containing 20 μl DiI solution or with 2 ml HBSS containing 20 μg/ml SConA for 20 min at 35°C or 42°C. Following labeling each culture was washed twice in 2 ml of HBSS to remove excess label, covered with 0.5 ml HBSS and used immediately for mobility measurements. Trypsinized cultures were centrifuged at 1500 rpm for two min and resuspended in 2 ml of HBSS containing one of the above labels. The suspension was then vortexed gently and incubated at 35°C or 42°C for 5 min. Following incubation the cells were centrifuged in 2 ml HBSS at 1500 rpm twice to remove excess label and resuspended in 0.5 ml HBSS. Cells were then placed in siliconized well slides with a plastic cover slip and used immediately for FPR measurements.

FPR measurements. Descriptions of the fluorescence photobleaching recovery equipment and methods used in these experiments have been published elsewhere [19–27]. Measurements were made at 35°C or 42°C with a Zeiss Universal microscope equipped with a thermally controlled stage [23]. A 514.5 nm laser beam attenuated to 0.2 μW was focused on the dorsal surface of the cell through a III/RS fluorescence illuminator (Carl Zeiss, Inc., New York, NY), and emitted fluorescence was isolated by a standard filter/dichroic mirror set. Focusing on the dorsal surface of attached cells was facilitated by choosing an area of membrane near the nuclear bulge although measurements were successfully performed on the peripheral membrane as well. The focused laser beam was previously determined to have a $1/e^2$ radius of $8.57 \cdot 10^{-5}$ cm [27]. Morphology of each cell examined was determined visually and classified as normal, elongated or rounded as previous described by Boscher and co-workers [6]. Laser beam focusing was facilitated by an image-intensified video fluorescence microscope system consisting of a NITEC NVS-100 image intensifier (Optic-Electronic Corp., Dallas, TX) optically interfaced between the Zeiss microscope and a RCA TC 2511/U Ultricon closed circuit television camera. DiI molecules within the less than 1 μm^2 beam region were bleached by a 5 ms pulse of approx. 3 mW of 514.5 nm light. TRITC-SConA molecules were bleached with 5 ms pulses of 5–6 mW of light as outlined below. Difficulties in measuring the diffusion coefficient of the small fraction of mobile membrane proteins on fibronectin-laden cells were circumvented by two independent methods, removal of the fibronectin by trypsin and repeated bleaching of the same spot to eliminate interfering fluorescent lectin bound to the immobile fibronectin. In the latter experiments a total of four bleaches per spot was performed. We have previously utilized such repeated bleaching methods [27]. Both in that instance and in the current study, diffusion coefficients did not significantly vary following the first bleach.

Results

The time course of the various morphological changes associated with transformation of CEF with the td-ts mutant NY68 has been published previously [4–6,15]. Of particular interest to us was the transient appearance of edge ruffles in the first hour following temperature downshift, the transient peak of surface flower formation 3–6 h after initiation of transformation, the cytoskeletal disruption at 3–24 h and the loss of fibronectin observed 3–12 h after the downshift. In order to monitor the possible effects of these events on the lateral mobility of DiI and SConA receptors, we measured diffusion coefficients of these molecules in RSV NY68-infected CEF cells at 0, 5, 12 and 24 h following temperature downshift. In addition, mobility measurements of DiI and SConA receptors were performed in the cell membranes of identically treated normal CEF and RSV-transformed cells.

TABLE I

Effect of temperature downshift on DiI diffusion in the membranes of CEF, RSV-transformed CEF and RSV NY68-infected CEF

The growth and treatment of cells are described in Materials and Methods. The diffusion coefficients D for each cell culture subset at each time following initiation of transformation is presented as the mean ± S.E. of n individual cell measurements, where n is the number given in parenthesis following D. All measurements were performed at 35°C.

Time (h)	Diffusion coefficient (D) (10^{-8} cm^2 s^{-1})		
	CEF	RSV CEF	RSV NY68 CEF
0	1.51 ± 0.12 (22)	1.19 ± 0.24 (12)	0.98 ± 0.05 (16)
5	1.38 ± 0.03 (32)	1.43 ± 0.08 (20)	0.68 ± 0.21 (15)
12	1.27 ± 0.12 (17)	1.56 ± 0.05 (32)	1.13 ± 0.10 (12)
24	1.55 ± 0.13 (12)	1.33 ± 0.08 (17)	1.00 ± 0.05 (30)

The lateral mobilities of the DiI probe in the membranes of the RSV-NY68-infected CEF, RSV-transformed CEF and normal CEF during the temperature downshift experiment are reported in Table I. Percent recovery of fluorescence after photobleaching averaged 85 ± 15% for all sample sets examined. A small transient decrease in DiI mobility was observed in the RSV-NY68 CEF cells 5 h after the initiation of transformation. DiI mobility at all other times measured during the course of transformation was slightly but consistently slower than that measured in the membranes of the other two cell cultures. Corresponding DiI diffusion constants for the identically treated normal CEF and RSV transformed CEF may also be found in the same table. No change in DiI mobility following downshift to 35°C was noted in these cells. No significant difference in DiI mobility was observed in any of the three control cultures treated identically but maintained at 42°C throughout the 24-h experimental period. DiI mobility in the RSV NY68-infected cells was, once again, slightly slower throughout. The transient decrease in DiI mobility at the permissive temperature correlates temporally with the previously reported [15] appearance of flowers on the surface of the cells. Possible explanations for this apparent correlation of events are addressed in the Discussion of this paper.

As discussed above, we anticipated, and in fact encountered, difficulty measuring TRITC-SConA diffusion on the surface of normal CEF and cells infected with RSV NY68 early in the temperature downshift experiment. These differences arose due to ConA binding to the abundant fibronectin present in these cultures. In order to circumvent this problem, multiple

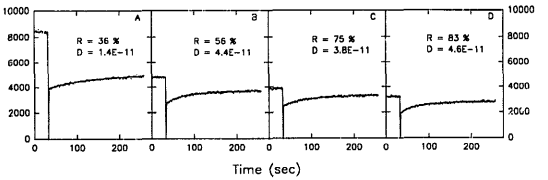

Fig. 1. Effect of successive bleaches at a single spot on SConA diffusion on normal CEFs at 35°C. Panels A–D illustrate the recovery of fluorescence after photobleaching for four bleaches following immediately one after the other. By the fourth bleach, the percent recovery has reached 83% while the SConA diffusion coefficient remains approximately constant at $4 \cdot 10^{-11}$ cm^2 s^{-1} for the last three bleaches.

bleaches were performed on the same interrogation spot. The rationale for this approach is that repeated bleaches should eliminate interfering fluorescence from TRITC-SConA bound to the essentially immobile fibronectin. Subsequent fluorescence would originate from the mobile cell surface proteins diffusing into the interrogation beam during the period of recovery. Repeated bleaching of the spot does in fact result in a stable diffusion constant, a decrease in total fluorescence and an increase in fluorescence recovery with each subsequent bleach (Fig. 1). Fig. 2 demonstrates effects of repeated bleaches of normal CEF on the diffusion constant and percent fluorescence recovery of TRITC-SConA. Following the initial bleach on the fibronectin-rich normal CEF, the diffusion constant increased to a reproducible value while the percent fluorescence recovery increased with each subsequent bleach from an initial value of $39 \pm 15\%$. In contrast, diffusion constants in the fibronectin-poor transformed

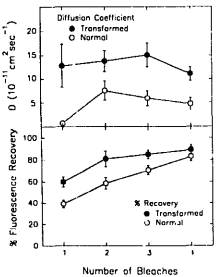

Fig. 2. Diffusion coefficients (top) and percent recovery (bottom) of TRITC-SConA as functions of serial bleaches at single interrogation spots in normal CEFs (○) and RSV transformed CEFs (△). Each data point represents the mean of 10 to 20 measurements. Error bar represents ± S.E. Measurements were performed at 35°C 24 h after temperature downshift from 42°C.

cells remained relatively constant and the percent fluorescence recovery, initially $60 \pm 15\%$, reached a stable value sooner. After four bleaches both fibronectin-rich and -poor cells exhibited > 80% fluorescence recovery. Total fluorescence in each instance decreased after the first bleach to relatively stable values.

The lateral mobilities of the SConA probe obtained over the 24-h period of the temperature downshift experiment for the three sets of cells are reported in Table II. The reported diffusion constants are the means of 10 to 20 measurements on individual cells. Each cell measurement is the average of the second, third and fourth bleaches of a single interrogation spot on the cell. In all instances, SConA receptors are 2.5–3-times more mobile in the RSV transformed cells than the normal CEF. Mobility of TRITC-SConA on cells infected with RSV-NY68 was statistically identical to the mobility of TRITC-SConA on the uninfected cells at early times in the temperature downshift experiment (0–6 h). However, at 12 h and 24 h diffusion constants were 2–3-times faster than the corresponding values for the normal cells and in agreement with those obtained for the wild-type RSV-transformed cells. The change in mobility between 6–12 h corresponds tempo-

TABLE II

Effect of temperature downshift on TRITC-SConA diffusion in the membranes of CEF, RSV-transformed CEF and RSV NY68-infected CEF

The growth and treatment of cells are described in Materials and Methods. The diffusion coefficients D for each cell culture subset at each time following initiation of transformation are presented as the mean ± S.E. of n serial bleach measurements, where n is the number given in parenthesis following D. All measurements were performed at 35°C.

Time (h)	Diffusion coefficient (D) (10^{-10} cm^2 s^{-1})		
	CEF	RSV CEF	RSV NY68 CEF
0	0.64 ± 0.09 (21)	1.74 ± 0.15 (10)	0.72 ± 0.13 (10)
5	0.58 ± 0.10 (17)	1.64 ± 0.18 (12)	0.57 ± 0.11 (12)
12	0.70 ± 0.09 (12)	1.48 ± 0.15 (12)	1.22 ± 0.08 (23)
24	0.61 ± 0.06 (42)	1.32 ± 0.12 (24)	1.74 ± 0.20 (22)

TABLE III

Effect of trypsin treatment on TRITC-SConA diffusion in the membrane of subconfluent CEF, RSV-transformed CEF and RSV MY68-infected CEF

The growth and treatment of cells are described in Materials and Methods. The diffusion coefficients D for each cell culture subset in the adherent or non-adherent state is presented as the mean ± S.E. of n individual cell measurements, where n is the number given in parenthesis following D. All measurements were performed at 35°C.

Trypsin	Diffusion coefficient (D) (10^{-10} cm^2 s^{-1})		
	CEF	RSV CEF	RSV NY68 CEF
+	2.86 ± 0.27 (13)	3.25 ± 0.21 (13)	3.16 ± 0.28 (9)
−	0.61 ± 0.06 (42)	1.32 ± 0.12 (24)	1.74 ± 0.20 (22)

rally with, and appears to be the result of, the disruption of the cytoskeleton and/or the loss of fibronectin observed during transformation of CEF by RSV-NY68 [6]. No significant difference in SConA receptor mobility was observed between RSV-NY68 infected cells and normal CEF treated identically but maintained at 42°C throughout the 24-h period of examination. The wild-type RSV-transformed cells, however, continued to exhibit slightly faster SConA mobilities at the non-permissive temperature of 42°C.

FPR mobility measurements of both SConA and DiI probe mobilities were then performed on trypsinized cells from all three subsets 24 h after temperature downshift to 35°C. Treatment with trypsin removes the exoskeleton, which in CEF cells is primarily composed of fibronectin, resulting in rounded, non-adherent cells. No difference in DiI mobility was observed in the trypsinized samples, relative to control cultures, at 24 h after temperature downshift. Hence, removal of the exoskeleton also eliminated the transformation induced differences in the SConA receptor mobility. All trypsinized samples exhibited a significant and consistent increase in SConA receptor mobility (Table III). The average diffusion constant for the receptors in these cells was approximately $3 \cdot 10^{-10}$ cm^2 s^{-1}, regardless of phenotype. This is approximately 2–4-times faster than that of the untreated cells. Such a phenomenon has previously been reported in trypsin-treated non-adherent 3T3 BALB/cA31 and Kirstin murine sarcoma virus transformed 3T3 BALB/cA31 cells [28,29].

Discussion

We have measured the lateral diffusion of the fluorescent lipid analogue DiI and TRITC-SConA glycoprotein complexes in the plasma membranes of normal CEF, RSV-transformed CEF and RSV NY68-infected CEF. All three cell samples were examined at selected times and conditions relevant to known events in the synchronized transformation of cells by the td-ts mutant RSV-NY68.

FPR measurements of the lateral diffusion of various lipid analogues and SConA in the membranes of normal chick embryo fibroblasts have been previously reported. Schlessinger et al. [30] measured the diffusion of DiI and a ganglioside analogue in the membranes of normal CEF and reported DiI diffusion constants in fibronectin-rich and fibronectin-poor cell surface areas which agree well with our results. Elson and Yguerbide [31] reported slightly slower DiI diffusion coefficients for normal CEF, perhaps the result of different laser spot sizes utilized in their FPR experiments [31]. Diffusion coefficients of SConA-labeled glycoproteins [30] in fibronectin-rich areas do not differ significantly from those of fibronectin and are similar to the diffusion coefficients we obtained on the initial photobleaching measurement at a given spot. Subsequent FPR measurements at that site produced results similar to those previously reported for fibronectin-poor areas ($\sim 6 \cdot 10^{-11}$) allowing investigation of SConA receptor mobility.

The lateral mobilities of DiI in the membranes of the three cell cultures did not differ markedly. However, DiI mobility in the membranes of the RSV NY68-transformed cells was slightly, but consistently, slower. The membranes of normal CEF have been compared to those of RSV-transformed CEF and RSV NY68-infected cells at permissive and non-permissive temperatures by other biophysical measurements. No significant differences in DPH fluorescence polarization among the membranes of the three cell cultures were observed [32]. Similarly, no significant differences in membrane fluidity were observed via fatty acid spin labelling [32] although cells infected with NY68 appeared slightly more rigid. This correlates with the slightly slower DiI mobility we observed. Since this phenomenon is observed at both the permissive and non-permissive temperatures with both techniques, it is clearly not attributable to transformation. In contrast, the transient decrease in DiI mobility 5 h after temperature downshift was observed in the RSV NY68-infected cells only at the permissive temperature. This transient decrease correlates temporally with the formation of flowers on the surface of these cells and may be secondary to the formation of these transient cell membrane evaginations. Edidin and co-workers [33] have reported a similar small difference in DiI diffusion between the microvilli-rich membranes of the main body of unfertilized mouse eggs and the smooth membranes of the budding polar bodies of the same cells. It has been suggested that diffusing molecules on the surface of cells might take longer to reach the tip of such evaginations resulting in decreased diffusion constants via FPR measurements.

We are confident that the differences we observed in TRITC-SConA mobility between normal and transformed CEFs are attributable to the transformation process. It is attractive to attribute the increase in TRITC-SConA receptor mobility in transformed CEFs to some specific morphological event known to occur concurrently. The increase in mobility observed correlated temporally with both the disruption of the cytoskeleton and the loss of organized fibronectin. It is likely that the change is attributable to one or both of these transformation-induced phenomena. To assess the effect of destruction of the extracellular matrix, we measured the mobility of TRITC-SConA on trypsinized normal CEF and their RSV-transformed counterparts. The ECM of CEF is composed mainly of fibronectin. The measured SConA diffusion constants on the trypsinized cells were significantly higher than the values obtained for their attached counterparts. Although we feel that this is primarily the result of fibronectin loss, we realize that trypsinization may produce this effect by the degradation of other extracellular or membranous proteins.

Similar results were reported by Swaisgood and Schindler in 3T3 BALB/cA31 cells and their Kirsten murine sarcoma virus-transformed counterparts [28]. These authors interpreted their data as evidence of perturbed membrane dynamics caused by a cellular shape change-induced alteration of a linked matrix of submembranous, intramembranous and cytoskeletal elements. The SConA diffusion coefficient they reported for the trypsinized cells is in good agreement with the value we obtained for trypsin-treated CEFs and is approx. 10-times slower than would be expected for unhindered diffusion. Swaisgood and Schindler identified a subpopulation of trypsinized transformed cells that exhibited such unhindered protein mobility [29] which they attributed to a transformation-induced modification in the organization of the linked submembranous and intramembranous matrices. Significant changes in the dynamics of membrane proteins have been previously observed in RSV-transformed CEF and during the transformation induced by RSV NY68 at its permissive temperature. Porat et al. [34] examined the dynamics of the membrane-bound enzyme adenylate deaminase (ADA) by multifrequency phase fluorometry. Their results clearly demonstrate that ADA in RSV-transformed cells rotates approximately twice as fast as in normal CEF and that this behavior is mirrored in NY68-cells grown at the permissive and non-permissive temperatures, respectively. Such observations tend to support the existence of a linked matrix as suggested by Swaisgood and Schindler. In addition, the observations reported in this paper suggest that extracellular matrix components such as fibronectin may play a role in such a structure. Transformation by the *SRC* gene product may induce modification of the dynamic interactions of the cell cytoskeleton, the adhesion plaque/podosome proteins and the extracellular matrix, resulting in the increased protein diffusion and rotation. This modification could be induced indirectly by structural changes secondary to the transformation or as a direct result of the *SRC* gene product. It is possible that tyrosine-specific phosphorylation of vinculin may play a role in such a direct modification.

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