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Enhanced number of actin binding sites on plasma membranes of polyoma virus-transformed fibroblasts

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Plasma membranes, isolated from normal (C13) and polyoma virus-transformed (J1) cultured BHK cells were incubated with G-actin under polymerizing conditions, followed by a low-speed centrifugation. The amount of actin attached to the pelleted BHK-J1 plasma membranes was at least twice that on BHK-C13 membranes, indicating a greater number of actin attachment sites on the former. This result was confirmed by the observation that the plasma membranes from the transformed cells were also more active in nucleating polymerization of pyrene-labelled actin. Most of the actin attachment sites could be solubilized by Triton or low-salt extraction treatment.

Transformation of cultured cells is accompanied by dramatic changes in the distribution of the cytoskeletal elements, such as disappearance of stress fibers, reduction in the number of focal adhesions and the enlargement of cortical actin networks [1–3]. Changes in the content of actin and some of the actin-associated proteins have also been reported [4–6].

The following work extends our previous studies [6] of the effects of transformation on the content and state of actin in baby hamster kidney cells, and is concerned with the attachement of extraneous rabbit skeletal muscle G-actin to plasma membranes of normal (BHK-21/C13) and polyoma virus transformed (BHK-J1) cultured fibroblasts. The comparison of the amounts of actin, attached per mg of plasma membrane protein, reflects the relative numbers of actin attachment sites in normal and transformed cells. The capacities of the two types of the plasma membranes to nucleate polymerization of pyrene-labelled actin were also compared.

Materials and Methods. The cells. Normal (BHK-21/C13) and polyoma virus transformed (BHK-J1) cells [7] were grown in large roller bottles in Dulbecco's modified Eagles's medium with 10% foetal calf serum and treated with trypsin (0.5 mg/ml) in 0.5 mM EDTA before harvesting. The cells were washed three times with ice-cold 0.145 M NaCl in 10 mM Tris, 0.1 mM EGTA, 1 mM $MgCl_2$ (pH 7.8).

The membranes. Approximately 4 volumes of a hypotonic solution containing 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT, 0.2 mM ATP, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM Pipes (pH 7.4) (buffer A) were added to the washed cells, followed after 15 minutes by the same volume of 1 M sucrose in buffer A. A mixture of proteinase inhibitors, leupeptin, chymostatin, antipain and pepstatin (all from Sigma), at a final concentration of 1 μg/ml each, was also included in buffer A during the cell lysis.

The suspension was sonicated 2×10 seconds, homogenized in a motor-driven Potter homogenizer and the lysate centrifuged for 5 min at $3000 \times g$ to pellet the nuclei. The post-nuclear supernatant was then centrifuged for 90 min at 3°C $100000 \times g$. The pellet obtained by high-speed centrifugation was homogenized in 20 ml of 0.5 M sucrose in buffer A with the above proteinase inhibitors and the suspension overlaid (4×5 ml) on 5 ml of 50% sucrose in buffer A. After an overnight centrifugation at $100000 \times g$ in an SW41 rotor (Beckman L2-65 centrifuge), the plasma membranes were collected from the interface, diluted and homogenized with about 20 ml of 0.5 M sucrose in buffer A to remove any residual cytoplasmic contamination and pelleted by a 20 min centrifugation at $20000 \times g$. The pellets were homogenized in buffer A and the membrane protein concentration adjusted to about 1 mg/ml. The specific activity of 5'-nucleotidase (an enzyme marker for plasma membranes) was about 20-times higher than that in the cell lysate, well within existing literature values [8]. The yields, the degree of 5'-nucleotidase enrichment and the ratios of cholesterol

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to protein (approx. 110 μg/mg) were similar for both types of plasma membranes. Low salt-treated plasma membranes were prepared by extracting the membrane pellets with 0.1 mM EDTA, 0.6 mM Tris-HCl (pH 7.5) for 1 h at 30 °C.

Attachment of actin. Rabbit skeletal muscle actin was prepared according to Spudich and Watt [9]. The muscle G-actin (final concentration 1–3 μM) was incubated 2 h at 30 °C with the membranes (final protein concentration 0.3 mg/ml). The ionic conditions were those of buffer A. After the incubation, the mixtures were centrifuged for 20 min at 20000 × *g*, at 20 °C, the pellets rinsed with the buffer A, dissolved in an SDS-sample buffer and analyzed by SDS-PAGE on 5–17% gradient gels. To estimate the relative amounts of actin bound to the plasma membranes, Coomassic blue stained gel slices corresponding to actin bands were eluted with 1 ml of 60% formic acid and absorbance measured at 620 nm.

Nucleation of actin polymerization. The ability of plasma membranes to nucleate actin polymerization was assayed by fluorimetry using pyrene-G-actin, as described previously [10].

Results and Discussion. Attachment of actin to plasma membranes was studied by polymerization of exogeneous skeletal muscle G-actin in the presence of the membrane fraction, followed by a sedimentation and an SDS-PAGE analysis of the membrane pellets. Fig. 1 shows the increased amount of actin associated with the membranes after an incubation under actin polymerizing conditions (compare lanes 2 and 3). As expected, the attachment is sensitive to vigorous mechanical agitation (lane 4) due to the breakage of the filaments, which then fail to sediment with the membranes. We have therefore avoided any agitation of the sample during the experiment. The attachment was abolished when the membranes were denatured by boiling (lane 5). No actin attachment was observed when the incubation was performed in the actin depolymerizing buffer (0.2 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM DTT, 2 mM Tris-HCl, pH 7.5, not shown).

To compare the number of the actin attachment sites on plasma membranes from normal and polyoma virus-transformed BHK cells, the following experiment was performed: the two types of membranes were matched for protein concentration, incubated with G-

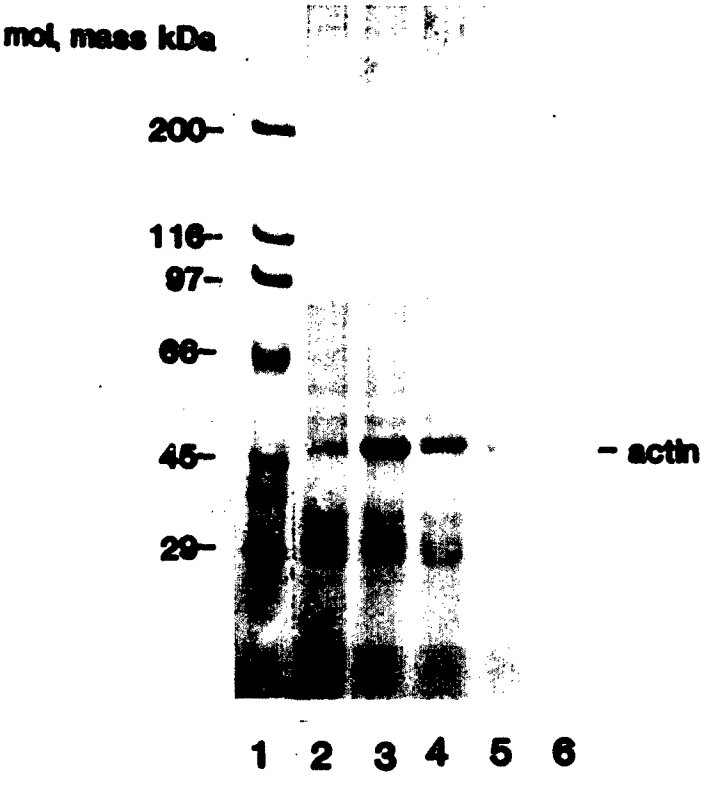

Fig. 1. Attachment of actin to plasma membranes isolated from BHK-C13 cells. G-actin (final concentration 3 μM) was incubated 100 min at 30 °C with the membranes (0.3 mg/ml) in buffer A, centrifuged 20 min at 20000 × *g* and the pellets analyzed by SDS-PAGE. (1) Molecular weight markers; (2) the membranes alone; (3) the membranes with actin; (4) as (3) but the sample was mixed vigorously prior to the centrifugation; (5) as (3) but the membranes were heated for 5 min in a boiling water bath prior to the incubation; (6) actin alone.

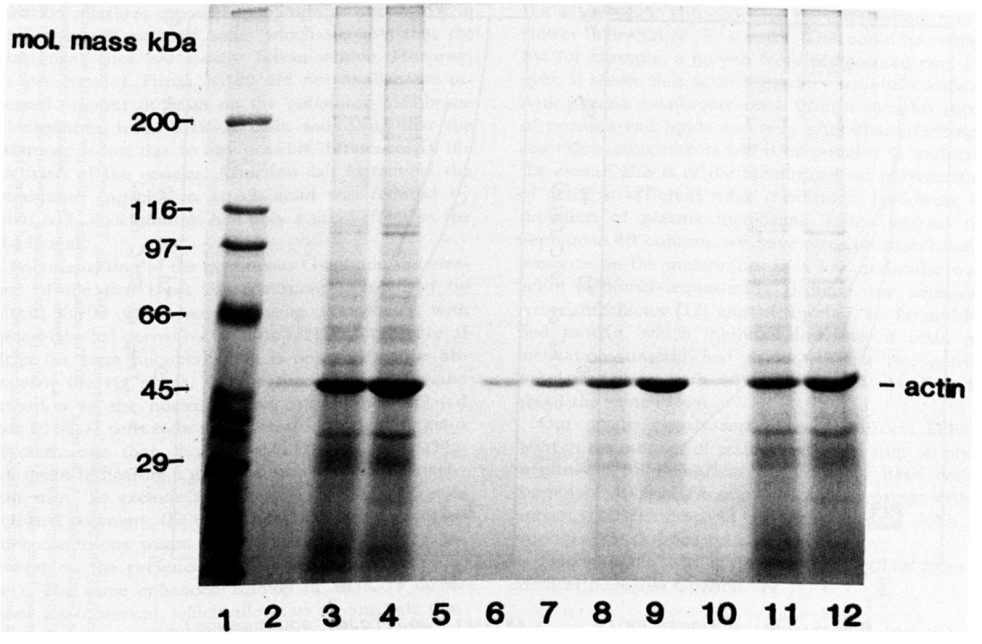

Fig. 2. Association of actin with plasma membranes from normal (C13) and polyoma virus transformed (J1) BHK cells. Experimental conditions were the same as in Fig. 1 except for the concentration of actin which was 2.2 μM. (1) Molecular weight markers; (2) actin alone; (3) the C13-membranes with actin; (4) the J1-,membranes with actin; (5), (6) and (7) as (2), (3) and (4). respectively, but in the presence of 1% Triton X-100; (8) and (9) actin with low-salt extracted C13- and J1-membranes, respectively, (10), (11) and (12) as (2), (3) and (4) but in the presence of 7 μM free calcium ions.

actin under polymerizing conditions and finally centrifuged at low speed. The pellets were analyzed by SDS-PAGE (Fig. 2, lanes 2, 3, 4). Similar experiment was performed in the presence of Triton X-100 (Fig. 2, lanes 5, 6, 7), calcium (Fig. 2, lanes 10, 11, 12) and with membranes extracted by low ionic strength buffer (Fig. 2, lanes 8,9).

After Coomassie blue staining, the gel slices corresponding to actin bands were cut out and the dye eluted. This allows a quantitation of the attached actin. The results are shown in Table I. It can be seen that in all cases, the amount of actin attached to the pelleted BHK-J1 membranes is at least twice that on BHK-C13 membranes. The presence of Triton X-100 in the in-

TABLE I

Estimation of the relative amounts of actin bound to the two types of plasma membranes

Coomassie blue stained gel slices corresponding to actin bands were cut out from the gel as shown in Fig. 2 and the stain eluted into 1 ml of 60% formic acid. The absorbance was measured at 620 nm.

Conditions:	Buffer A		Buffer A + 1% Triton X-100		Buffer A + low-salt extracted membranes		Buffer A + calcium	
Membranes:	C13	J1	C13	J1	C13	J1	C13	J1
Absorbance (620 nm) (control subtracted)	0.091	0.184	0.020	0.046	0.030	0.091	0.074	0.171
Attached actin: ratio J1/C13	2.0		2.3		3.0		2.3	
% of attached actin relative to conditions in Buffer A	100	100	22	25	33	49	81	93

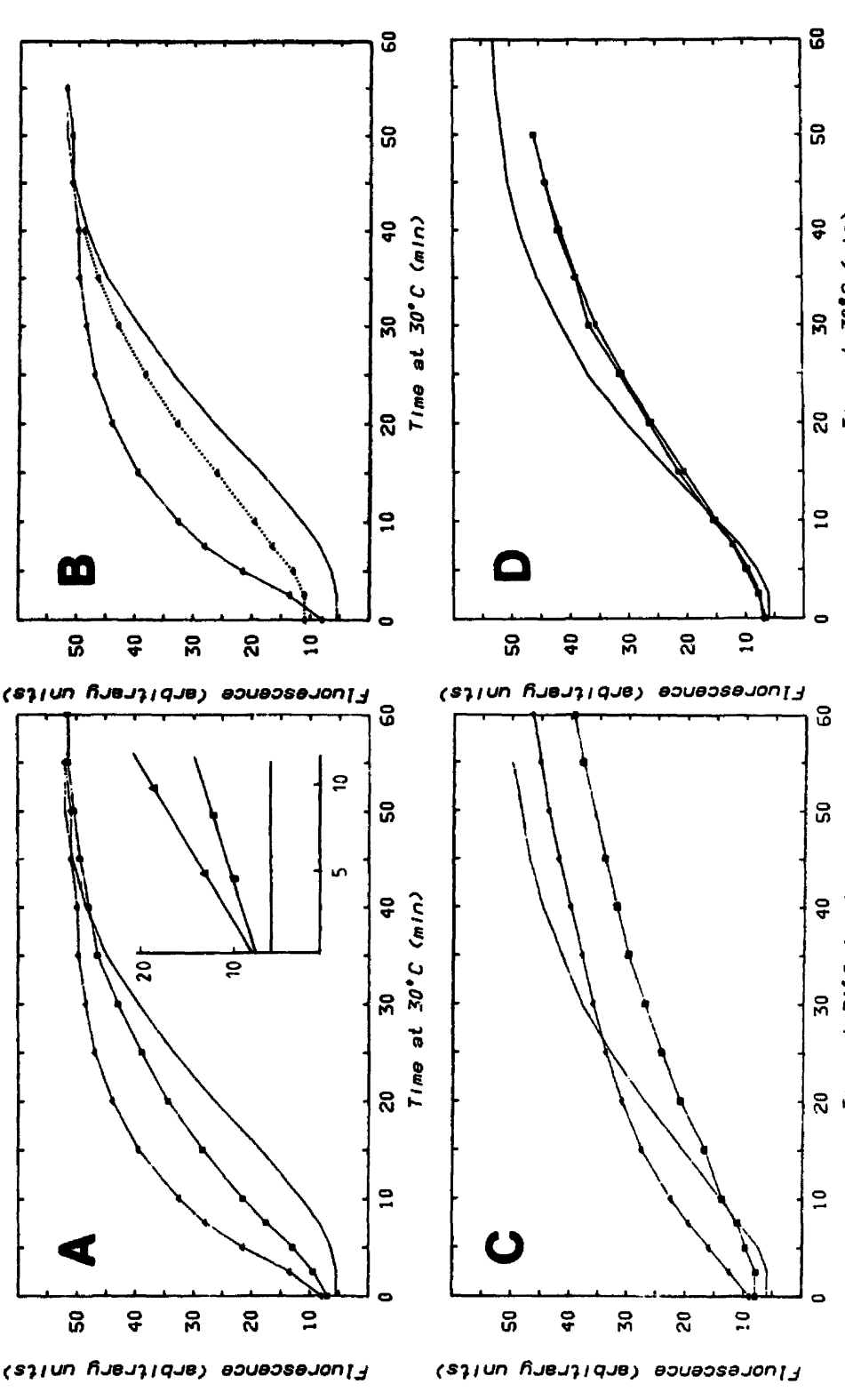

Fig. 3. Polymerization of pyrene-labelled G-actin in the presence of plasma membranes from normal (C13) and polyoma virus-transformed (J1) BHK cells. The fluorescence was measured in a Perkin-Elmer spectrofluorimeter. Samples contained the membranes (0.3 mg/ml) and 2.2 μM pyrene-G-actin in buffer A-1% Triton X-100. The actin was added at time zero. Control without the membranes (——); actin with C13-membranes (■——■); actin with J1-membranes (▲——▲); denatured membranes (▲·····▲). (A) Plasma membranes from polyoma virus-transformed cells are more efficient in nucleating actin polymerization than those from normal cells. Inset: Same membranes with 1 μM pyrene-G-actin. (B) The nucleating activity of the membranes is lost following denaturation by boiling. The higher initial fluorescence level with denatured membranes is due to Rayleigh scattering; the rate of polymerization and the lag phase were uneffected. (C) Slower polymerization rate in the presence of the membranes and calcium (7 μM free Ca^{2+} ions). (D) The membranes were extracted by a low-salt buffer prior to the incubation.

cubation mixtures caused a reduction of about 75% in the amount of pelleted actin, which suggests that the attachment sites are mostly Triton-soluble. However, the presence of Triton X-100 did not abolish the increased amount of actin on the remaining membrane cytoskeletons from BHK-J1 cells, indicating that the difference is not due to any possible differences in the sidedness of the vesicles. After low-salt extraction, the membranes capacity to attach actin was reduced by about 60%. Calcium ions had only a small effect on the attachment.

Polymerization of the extraneous G-actin in the presence of the two types of membranes solubilized by Triton X-100 was observed using fluorimetry with pyrene-labelled derivative of actin [11]. The presence of nuclei for actin polymerization is revealed by the absence of the lag phase, i.e. the time required for the formation of the nuclei. Plasma membranes isolated from BHK-J1 cells were more active in nucleating actin polymerization than those from BHK-C13 cells (Fig. 3A), again indicating a greater number of actin attachment sites. To exclude any lateral attachment of self-nucleated polymers, the experiment was also performed under conditions where polymerization is entirely dependent on the presence of the membranes (Fig. 3A, inset). The same enhanced activity of BHK-J1 membranes was observed, which allows us to conclude that the membranes of BHK-J1 cells contain a greater number of nucleation sites for the end-on attachment. Again, the presence of Triton in the incubation mixtures eliminates any problems connected to sidedness (such as a possibility of a greater number of 'inside-out' vesicles in the BHK-J1 membrane preparations).

Like the ability to attach actin filaments (Fig. 1, lane 5), the plasma membrane nucleating activity was lost on boiling (Fig. 3B). Thus, the attachment is specific and dependent on native nucleation sites on the membranes. After low salt extraction, most but not all of the nucleating activity has been lost and both types of plasma membranes exhibited a small inhibitory effect on the polymerization rate (Fig. 3D).

Such effects were sometimes observed even with the whole membranes, particularly in the presence of calcium (Fig. 3C). Under these conditions, the nucleation of actin polymerization by both BHK-J1 and -C13 membranes was clearly apparent (note the absence of the lag phase), although the polymerization rate was slower than that of the control. This could be explained by, for example, a growth from the pointed end. However, it seems that actin-regulatory activities associated with plasma membranes arise from a complex mixture of proteins and lipids and only after characterizing the individual components will it be possible to understand the overall effects of the membranes on polymerization of actin in different ionic conditions. Following fractionation of plasma membrane Triton extract on a Sepharose 4B column, we have already established the presence on the membranes of a low molecular weight actin monomer-sequestering protein, the actin-depolymerizing factor [12] and of another, so far unidentified protein, which inhibited the rate of actin polymerization (unpublished results). These two activities were present in both types of plasma membranes to about the same extent.

Our results clearly indicate a significant enhancement in the number of actin attachment sites on plasma membranes following transformation of BHK cells by the polyoma virus. These results are consistent with the previous observations of enriched cortical actin networks in transformed cells.

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