Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1381–1385, February 1995
Cell Biology

Reduction of caveolin and caveolae in oncogenically transformed cells

(cancer/growth regulation/oncogene/potocytosis)

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Contributed by David Baltimore, November 28, 1994

ABSTRACT **Caveolae are flask-shaped non-clathrin-coated invaginations of the plasma membrane. In addition to the demonstrated roles for caveolae in potocytosis and transcytosis, caveolae may regulate the transduction of signals from the plasma membrane. Transformation of NIH 3T3 cells by various oncogenes leads to reductions in cellular levels of caveolin, a principal component of the protein coat of caveolae. The reduction in caveolin correlates very well with the size of colonies formed by these transformed cells when grown in soft agar. Electron microscopy reveals that caveolae are morphologically absent from these transformed cell lines. These observations suggest that functional alterations in caveolae may play a critical role in oncogenic transformation, perhaps by disrupting contact inhibition in transformed cells.**

Oncogenic transformation leads to many changes in cultured cells. These changes include the loss of a requirement for growth factors (1), alterations in cell-surface molecules and membrane fluidity (2–4), the loss of contact inhibition of cell division and motility (5, 6), and the loss of anchorage dependence for growth (7). Measurements of these parameters are used to evaluate the oncogenic potential of a gene or its mutant derivatives. Despite the utility of these assays in scoring oncogenic potential *in vitro*, the molecular mechanisms that regulate these changes are poorly understood. A dissection of the molecular mechanisms behind these changes should lead to enhanced understanding of tumor initiation and progression *in vivo*.

Caveolae are 50- to 100-nm flask-shaped invaginations of the plasma membrane (8, 9). Caveolae have been implicated in potocytosis and transcytosis of small molecules and ions in endothelial cells and sorting of surface proteins in polarized epithelial cells (8, 10–13). Several lines of evidence suggest that caveolae may also participate in signal transduction. For example, caveolin, a protein found on the inner striated coat of caveolae, is a major v-src substrate in Rous sarcoma virus-transformed chicken embryo fibroblasts (14, 15). Both the phosphorylation of caveolin and transformation are dependent on the myristoylation of v-src (16). Furthermore, several G-protein-coupled receptors and bacterial toxins that modify G proteins are localized to or internalized by caveolae (for review, see ref. 17). Glycosyl-phosphtidylinositol (GPI)-linked proteins and the muscarinic acetylcholine receptor cluster to caveolae in response to cross-linking antibodies or receptor agonists, respectively (18, 19). Finally, purified caveolar membrane domains are enriched in signaling proteins: Src-like nonreceptor tyrosine kinases, heterotrimeric G proteins, the ras-related GTPase Rap 1A, mitogen-activated protein kinases, other serine/threonine kinases, SH2–SH3 adaptor proteins, and other proteins with roles in signal transduction (20–22).

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Caveolae may regulate the proliferation of normal cells *in vitro*. We have recently observed that NIH 3T3 cell lines transformed by several different oncogenes express greatly reduced levels of caveolin. Furthermore, the reduction of caveolin in these cell lines correlates with the size of colonies they form upon growth in soft agar. Electron microscopy reveals that caveolae are missing from the transformed cells that express reduced levels of caveolin. The reduction in caveolin does not correlate with serum requirements for growth of these cell lines in culture. The loss of caveolin could contribute to a loss of contact inhibition of motility or mitosis in transformed cells that may be critical to allow cells to proliferate in soft agar.

MATERIALS AND METHODS

Cell Culture. Normal and transformed NIH 3T3 cell lines were cultured on 90- or 150-mm tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) donor calf serum (DCS) (GIBCO). The v-*abl*, polyoma virus middle-sized tumor antigen (mTAg)-, *bcr–abl* (p210)-, and *crk1*-transformed NIH 3T3 cell lines were the gifts of Warren Pear, Ruibao Ren, and Martin Scott (Massachusetts Institute of Technology). The H-*ras*^{G12V}-transformed cell line was the gift of David Sanders (Whitehead Institute). The v-*src*-transformed NIH 3T3 cell line was the gift of David Shalloway (Cornell University, Ithaca, NY). To assay serum requirements for growth and survival, 1000 cells of each cell line were plated onto multiple 60-mm plates in DMEM containing 10% DCS. The medium was replaced the following day with DMEM containing 10%, 5%, 2%, or 0.5% DCS. Cells were fed every 3 days. After 10 days, colonies arising from the plated cells were visualized by staining with 0.1% crystal violet in 50% (vol/vol) MeOH. Colony numbers were normalized to numbers observed on plates grown in 10% DCS. To assay growth in soft agar, 1000 cells of each line were suspended in 3 ml of DMEM containing 5% and 10% DCS and 0.33% SeaPlaque low-melting-temperature agarose (American Bioanalytical, Natick, MA). These cells were plated over a 2-ml layer of solidified DMEM containing 5% or 10% DCS and 0.5% agarose, and cells were allowed to settle to the interface between these layers at 37°C. After 20 min, plates were allowed to harden at room temperature for 30 min before returning to 37°C. The plates were fed every 3–4 days by overlaying with 2 ml of medium containing 0.33% agarose. After 2 weeks, the plates were stained with 0.1% crystal violet in 50% MeOH at 4°C. Plates were destained with cold water. Colonies were photographed under low (×4) magnification.

Western Blot Analysis. Cells were disrupted in RIPA buffer (20 mM Mes, pH 6.5/150 mM NaCl/1% Triton X-100/0.1% SDS/1 mM phenylmethylsulfonyl fluoride/2 mM benzamidine hydrochloride). Protein was quantitated with Bio-Rad protein

Abbreviations: DCS, donor calf serum; mTAg, middle-sized tumor antigen.

assay kit and bovine serum albumin as a standard. Blots were prepared by standard procedures (23). Blots were probed with anti-caveolin monoclonal antibodies (Transduction Laboratories, Lexington, KY) or anti-Hsp70 polyclonal antiserum (a gift of Peter Murray, Whitehead Institute). The proteins were visualized with horseradish peroxidase-conjugated secondary antibodies and the ECL system (Amersham). Amounts of caveolin in transformed cells were determined by comparison to standard dilutions of NIH 3T3 cell extract.

Northern Blot Analysis. Total RNA was extracted with TriReagent LS (Molecular Research Center, Cincinnati) by the manufacturer's instructions. Fifteen micrograms of total cellular RNA was denatured with glyoxal and subjected to Northern blot analysis with ^{32}P-labeled probes for the mouse caveolin mRNA (2.4 kb) and human 28S RNA (a gift of Pat Guilfoile, Whitehead Institute), as a control for loading.

Transmission Electron Microscopy. Cells were fixed with glutaraldehyde, postfixed with OsO_4, and stained with uranyl acetate and lead citrate as described (20). Samples were examined under the Philips 410 transmission electron microscope. Cells were photographed at a magnification of ×115,500. Caveolae were identified by their characteristic flask shape, size (50–100 nm), and location at or near the plasma membrane.

RESULTS

Caveolin Is Reduced in Transformed NIH 3T3 Cell Lines. Caveolin, a principal component of the protein coat of caveolae, is phosphorylated on tyrosine in v-*src*-transformed fibroblasts, suggesting a relationship between alterations in the structural components of caveolae and oncogenic transformation (14–16). To determine whether alterations of caveolin structure or function occur during transformation by oncogenes other than v-*src*, the caveolin in extracts of oncogenically transformed NIH 3T3 cell lines was compared to caveolin in extracts of normal NIH 3T3 cell lines. The amount of caveolin in normal or transformed NIH 3T3 cell lines was assayed by Western blot analysis (Fig. 1*A*). The level of caveolin was clearly reduced in NIH 3T3 cell lines transformed by expression of the v-*abl*, *bcr–abl*,H-*ras*^{G12V}, polyoma virus mTAg, or *crk1* oncogenes. The relative amount of caveolin in the transformed cells was estimated by Western blot analysis with dilutions of NIH 3T3 protein extract as a standard (data not shown). Similar analysis was performed for the cytosolic heat shock protein Hsp70 as a control for loading. The amount of caveolin in transformed cells varied from about 30% of levels found in normal NIH 3T3 cells (in *crk1*-transformed cells) to less than 1% of normal levels (in v-*abl*-transformed cells) (see Fig. 2).

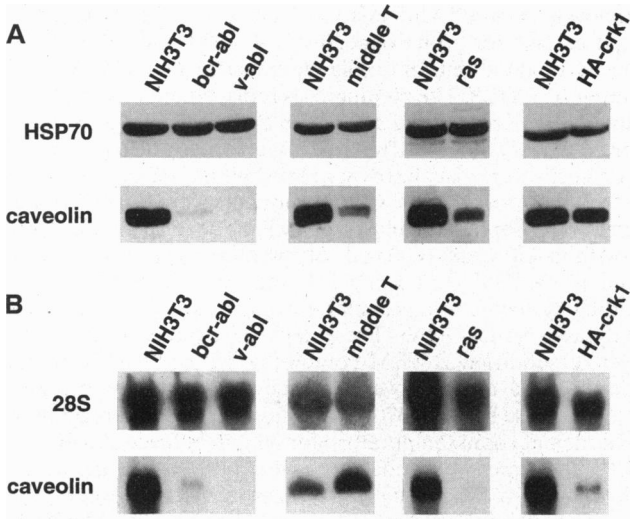

FIG. 1. Caveolin expression in normal and transformed NIH 3T3 cell lines. (*A*) Protein (50 μg) from wild-type NIH 3T3 cells or NIH 3T3 cell lines transformed with v-*abl* (v-abl), p210$^{bcr-abl}$ (bcr-abl), polyoma virus mTAg (middle T), H-*ras*^{G12V} (ras), or *crk1* (HA-crk1) was subjected to Western blot analysis with antibodies to caveolin or, as a control, cytosolic Hsp70. The proteins were visualized with horseradish peroxidase-conjugated secondary antibodies and the ECL system. (*B*) Total cellular RNA (15 μg) was denatured with glyoxal and subjected to Northern blot analysis with ^{32}P-labeled probes for caveolin mRNA (2.4 kb) and 28S RNA, as a control.

Northern blot analysis of total cellular RNA revealed that caveolin mRNA was reduced in the transformed NIH 3T3 cell lines in parallel with the amount of caveolin protein found in these cell lines (Fig. 1*B*). In the one noteworthy exception, there appeared to be a slight induction of caveolin mRNA in the NIH 3T3 cell line transformed by expression of polyoma mTAg. Western blot analysis revealed that these cells had only 4% the level of caveolin found in normal NIH 3T3 cells, suggesting that caveolin is either less stable or translated at a reduced rate in these cells. Identical results were obtained by S1 nuclease analysis with an oligonucleotide complementary to caveolin mRNA (data not shown).

Correlation with the Size of Colonies in Soft Agar. Oncogenically transformed cells are often able to grow under conditions that limit growth of normal cells. The normal or transformed NIH 3T3 cell lines analyzed above were assayed for growth under restrictive conditions to gain insight into possible roles of caveolin in regulating cell growth. When plated in soft agar, normal cells will cease division whereas transformed cells will continue to proliferate. The NIH 3T3 cell lines were plated at low density in soft agar and incubated for 2 weeks. Although the transformed cell lines formed colonies in soft agar with similar efficiencies (70–100%), the sizes of the colonies formed by these cell lines varied widely. Interestingly, the size of colonies formed by these cell lines in soft agar correlated very well with the reduction of caveolin (Fig. 2). For example, the v-*abl*-transformed cell line, which contained the least caveolin among those transformed lines examined, formed the largest colonies when grown in soft agar. Conversely, colonies formed by the *crk1*-transformed cell line are very small and these cells exhibited a less dramatic reduction in caveolin.

Transformed cells often exhibit a decreased requirement for growth factors for cell survival and proliferation. The NIH 3T3 cell lines were plated at low density on 60-mm plates. After incubation in medium containing 10% DCS serum for 1 day, the cells were incubated in medium containing 10%, 5%, 2%, or 0.5% DCS. After 10 days, the number of colonies arising on the plates was quantitated after staining with crystal violet (Fig. 3). Each cell line exhibited different survival and growth properties under conditions of reduced serum. Surprisingly, while two transformed cell lines (*bcr–abl*, HA-*crk1*) grew much better than normal NIH 3T3 cells, the other lines appeared to grow slightly worse than normal NIH 3T3 cells in conditions of reduced serum. Clearly, there is no correlation between the levels of caveolin and a decreased requirement for serum.

Transformed Cells Contain No Detectable Caveolae. Caveolae can be readily visualized in a wide range of endothelial and epithelial cells by transmission electron microscopy (14). Normal, v-*abl*-transformed, and H-*ras*^{G12V}-transformed NIH 3T3 cells were fixed, stained, and analyzed by transmission electron microscopy. Caveolae are abundant in normal NIH 3T3 cells and can be distinguished by their characteristic size (50–100 nm), flask-like shape, and location on, or juxtaposition to, the plasma membrane (Fig. 4*A*). Every field of sectioned NIH 3T3 cells examined in this way contained at least 5–10 caveolae and in many cases dozens of clustered caveolae could be found. In contrast, v-*abl*- and H-*ras*^{G12V}-transformed cells contained few if any detectable caveolae (Fig. 4 *B* and *C*). Despite viewing >20 fields of each of these cells, only one structure resembling a caveola was observed in the v-*abl*-transformed cells. Thus,

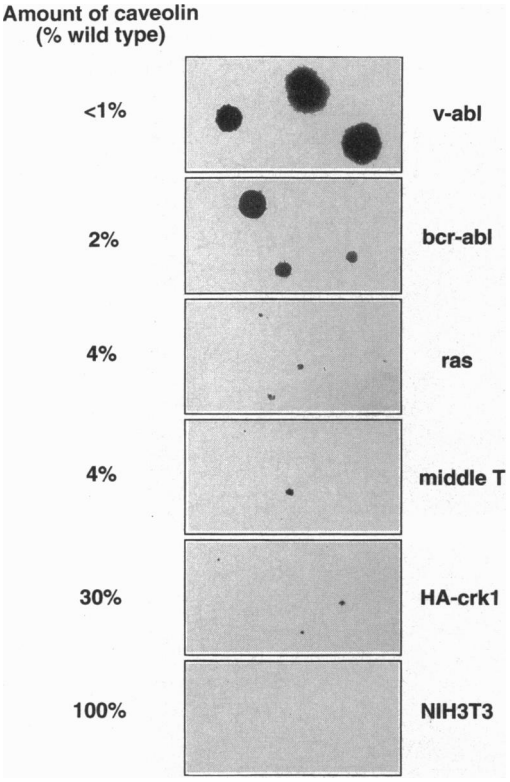

FIG. 2. Growth of normal and transformed NIH 3T3 cell lines in soft agar. Cell lines as described in Fig. 1 were plated at a density of 1000 cells per 60-mm dish in growth medium (DMEM) containing 10% DCS and 0.33% soft agar. Cells were fed twice weekly and were stained after 2 weeks with 0.1% crystal violet in 50% MeOH. Plates were destained and photographed under low magnification (×10). The amount of caveolin in each cell line was quantitated by Western blot analysis as in Fig. 1 with dilutions of NIH 3T3 protein extract as a standard (data not shown). Similar results were obtained with cells grown in DMEM containing 5% DCS. (×4.)

two transformed cell lines that contain reduced levels of caveolin also contain few caveolae.

DISCUSSION

The disruption of caveolar integrity appears to be a general feature of oncogenic transformation of fibroblasts. Although the v-*abl*, *bcr–abl*, H-*ras*^{G12V}, mTAg, and *crk1* oncogenes initiate transformation by different mechanisms (24), cellular transformation by each of these proteins leads to reductions in cellular levels of caveolin. It is unclear whether the reduction in caveolin levels is a direct result of an oncogenic stimulus or, rather, whether cells that express reduced levels of caveolin are preferentially selected for oncogenic transformation. These reductions in caveolin probably contribute to a loss of caveolae as observed in cells transformed by v-*abl* and H-*ras*^{G12V}. It is unclear, however, whether the loss of caveolae is solely due to reductions in caveolin levels. Future experimentation should indicate whether the loss of caveolin leads to a loss of caveolae. More importantly, these experiments should also determine whether the loss of caveolar integrity is necessary or sufficient for cellular transformation.

Three pathways exist to alter caveolin or reduce caveolin levels in transformed cells. In v-*src*-transformed chicken embryo fibroblasts, caveolin is phosphorylated on tyrosine residues, although the functional consequences of this modification are not understood (15, 16). In the course of this study, we also observed phosphorylation of caveolin in v-*src*-transformed NIH 3T3 cells (data not shown). In v-*abl*-, *bcr–abl*-,

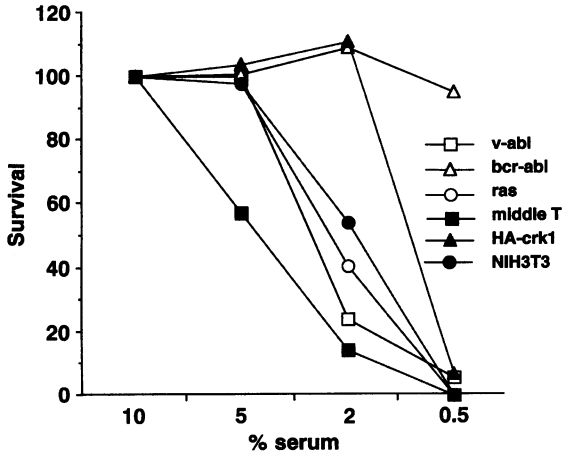

FIG. 3. Serum requirements of normal and transformed NIH 3T3 cell lines in soft agar. Cell lines as described in Fig. 1 were plated at a density of 1000 cells per 60-mm dish in growth medium (DMEM) containing 10% DCS. The medium was replaced the following day with DMEM containing 10%, 5%, 2%, or 0.5% DCS. Cells were fed every 3 days. After 10 days, colonies arising from the plated cells were visualized by staining with 0.1% crystal violet in 50% MeOH. Colonies were counted and normalized (as a percentage) to the number of colonies that grew on plates in medium containing 10% DCS (100%). The number of colonies surviving is an average from two plates. The number of surviving colonies are plotted as a function of serum concentration.

H-*ras*^{G12V}-, and *crk1*-transformed cells, reductions in steady-state levels of caveolin mRNA lead to the reduction in caveolin levels. Transformation by mTAg leads to reductions in caveolin protein to 4% of levels in normal cells without a parallel reduction in caveolin mRNA levels. These data imply another pathway that regulates the synthesis or stability of caveolin. The existence of multiple mechanisms that alter caveolin levels and/or structure suggests that caveolin may be a critical target in the pathway leading to cellular transformation.

A classic *in vitro* criterion of cellular transformation is the ability to grow in soft agar. It was therefore very striking that caveolin levels correlated with this parameter, suggesting there might be a causal relationship between caveolin levels and growth in soft agar. Despite this correlation, caveolin levels have no relationship to the serum requirements for growth of normal or transformed cells in culture. These data implicate caveolae specifically in the regulation of cell growth in soft agar.

How might caveolin regulate the ability of cells to grow in soft agar? In normal cells, both motility and mitosis are inhibited by contact with other cells (5, 6). A loss of inhibition of either of these processes may allow transformed cells to grow in soft agar. Although caveolin is distributed throughout the cell surface, it is concentrated on the leading edge of cells (14). Interestingly, contact between a cell's leading edge and adjacent cells mediates contact inhibition of movement and may also play a role in inhibiting proliferation (6). A reduction of caveolae in the leading edge of cancer cells may thus render them insensitive to contact inhibition of growth or motility.

Several observations are also consistent with a direct role for caveolae in the transduction of signals from the plasma membrane. Caveolar membrane domains are enriched in proteins with roles in signal transduction (20–22). Interestingly, the muscarinic acetylcholine receptor is clustered into caveolae upon treatment with agonists but not with antagonists (18). Similarly, antibody crosslinking of glycosyl-phosphatidylinositol (GPI)-linked proteins leads to their clustering in caveolae (19). Treatment of cells with activators of protein kinase C cause caveolae to flatten out, demonstrating a link between intracellular signaling and the structural integrity of caveolae

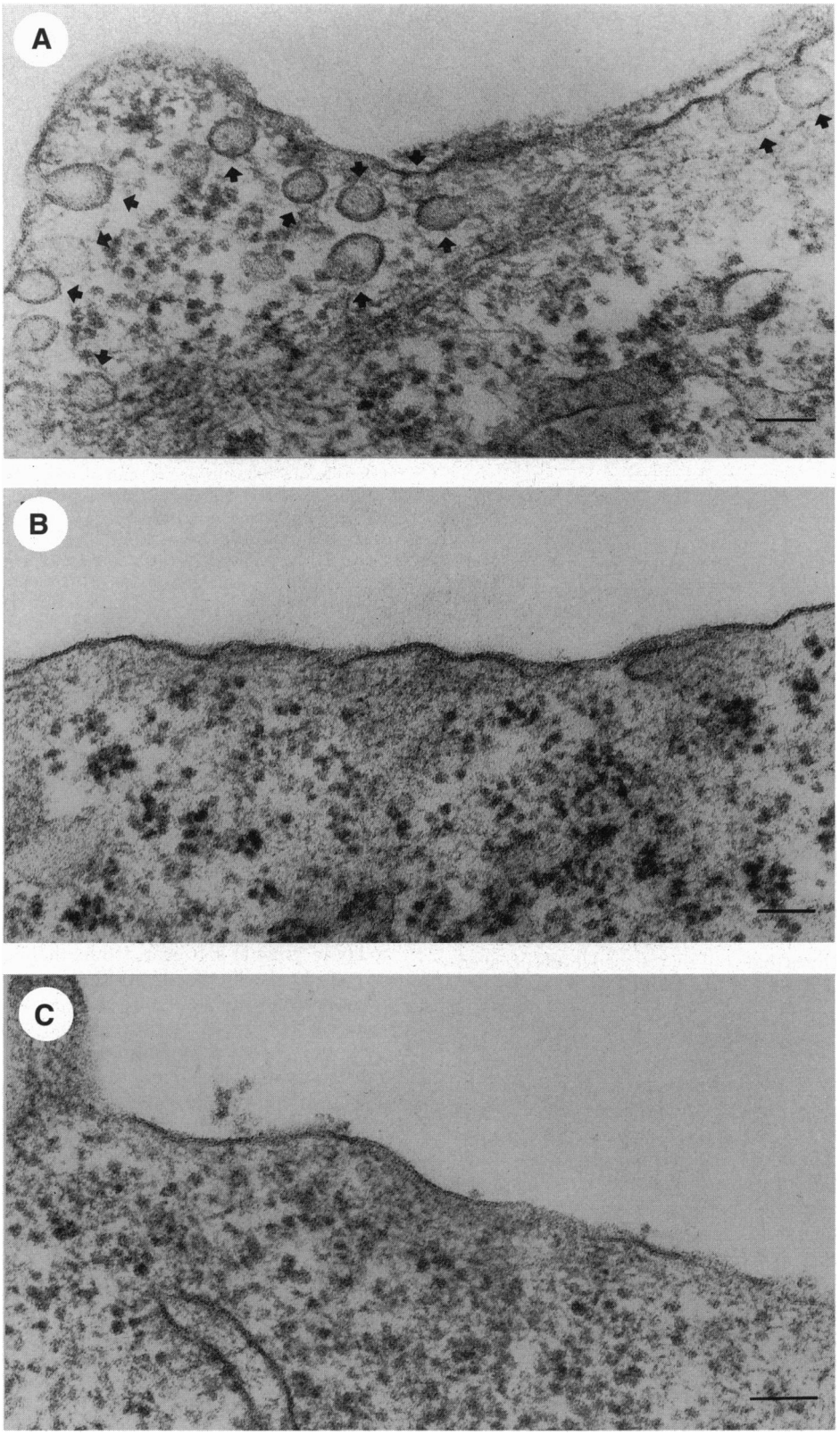

FIG. 4. Electron microscopy of normal and transformed NIH 3T3 cells. Cells were fixed with glutaraldehyde, postfixed with OsO_4, and stained with uranyl acetate and lead citrate. Cells were photographed at a magnification of ×104,000. Caveolae were located by their characteristic flask-shape, size (50–100 nm), and location at or near the plasma membrane. Caveolae are indicated by arrows. (Bar = ≈50 nm.) (*A*) NIH 3T3 cell. (*B*) H-*ras*^{G12V}-transformed NIH 3T3 cell. (*C*) v-*abl*-transformed NIH 3T3 cell.

(25). These observations seem to implicate caveolae in the potentiation of signals from the plasma membrane. However, these observations are also consistent with a role for caveolae in sequestering signaling molecules to attenuate signaling after receptor stimulation. The reduction of caveolae in transformed cells could prevent the downregulation of growth-promoting signals, thus contributing to uncontrolled proliferation.

Several proteins that mediate the internalization of extracellular or membrane-associated molecules are phosphorylated on tyrosine in Rous sarcoma virus-transformed cells: the gap junction protein connexin 43, the heavy chain of clathrin, and caveolin. Interestingly, the phosphorylation of connexin 43 by v-*src* has been correlated to a decrease in junctional permeability in *Xenopus* oocytes (26). Similarly, oncogenic stimuli may downregulate clathrin-coated pits and caveolae to limit the receptor-mediated and potocytotic uptake of small solutes and molecules. These modifications may insulate the cancer cell from molecules and signals that may be toxic or inhibitory to rapidly dividing cells.

These studies demonstrate a general reduction of caveolin and caveolae in fibroblasts transformed by various oncogenes. However, these studies do not show that caveolae directly modulate the oncogenic program in transformed cells. The inverse correlation between cellular caveolin levels and the ability to grow in soft agar does suggest that caveolae may directly regulate the ability of cells to grow in soft agar.

Note Added in Proof. Recent experiments show that in NIH 3T3 cells transformed by a temperature-sensitive Abelson virus, caveolin levels are increased by shift to the nonpermissive temperature. Thus, caveolin levels appear to be directly regulated by an oncogenic stimulus.

We thank Ya-Huei Tu for her expert assistance in electron microscopy. We thank Drs. R. O. Hynes and K. Alexandropoulos for critical review of this manuscript. A.J.K. thanks the members of the Baltimore and Lisanti labs for many helpful discussions. This work was aided by a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research to A.J.K. and by a grant from the W. M. Keck Foundation supporting the Whitehead Fellows Program to M.P.L. The research was supported by National Institutes of Health Grants GM-50443 to M.P.L. and CA-51462 to D.B.

1. Cherington, P. V., Smith, B. L. & Pardee, A. B. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3937–3941.
2. Hynes, R. O. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 3170–3174.
3. Gaffney, B. J. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 664–668.
4. Hakomori, S. (1975) *Biochim. Biophys. Acta* **417,** 55–89.
5. Holley, R. W. & Kiernan, J. A. (1968) *Proc. Natl. Acad. Sci. USA* **60,** 300–304.
6. Abercrombie, M. (1970) *In Vitro* **6,** 128–142.
7. Shin, S.-I., Freedman, V. H., Risser, R. & Pollack, R. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4435–4439.
8. Anderson, R. G. W. (1993) *Curr. Opin. Cell Biol.* **5,** 647–652.
9. Travis, J. (1993) *Science* **262,** 1208–1209.
10. Anderson, R. G. W., Kamen, B. A., Rothberg, K. G. & Lacey, S. W. (1992) *Science* **255,** 410–411.
11. Simionescu, N., Simionescu, M. & Palade, G. E. (1972) *J. Cell Biol.* **53,** 365–392.
12. Simionescu, N., Simionescu, M. & Palade, G. E. (1975) *J. Cell Biol.* **64,** 586–607.
13. Kurzchalia, T. V., Dupree, P., Parton, R. G., Kellner, R., Virta, H., Lehnert, M. & Simons, K. (1992) *J. Cell Biol.* **118,** 1003–1014.
14. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y.-S., Glenney, J. R. & Anderson, R. G. W. (1992) *Cell* **68,** 673–682.
15. Glenney, J. R. & Soppet, D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10517–10521.
16. Glenney, J. R. (1989) *J. Biol. Chem.* **264,** 20163–20166.
17. Lisanti, M. P., Scherer, P. E., Tang, Z. & Sargiacomo, M. (1994) *Trends Cell Biol.* **4,** 231–235.
18. Strosberg, A. D. (1991) *Eur. J. Biochem.* **96,** 1–10.
19. Mayor, S., Rothberg, K. G. & Maxfield, F. R. (1994) *Science* **264,** 1948–1951.
20. Sargiacomo, M., Sudol, M., Tang, Z. & Lisanti, M. P. (1993) *J. Cell Biol.* **122,** 789–807.
21. Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y.-H., Cook, R. F. & Sargiacomo, M. (1994) *J. Cell Biol.* **125,** 111–126.
22. Chang, W.-J., Ying, Y.-S., Rothberg, K. R., Hooper, N. M., Turner, A. J., Gambliel, H. A., De Gunzburg, J., Mumby, S. M., Gilman, A. G. & Anderson, R. G. W. (1994) *J. Cell Biol.* **126,** 127–138.
23. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 471–504.
24. Bishop, J. M. (1991) *Cell* **64,** 235–248.
25. Smart, E. J., Foster, D. C., Ying, Y.-S., Kamen, B. A. & Anderson, R. G. W. (1994) *J. Cell Biol.* **124,** 307–313.
26. Swenson, K. I., Piwnica-Worms, H., McNames, H. & Paul, D. L. (1990) *Cell Regul.* **1,** 989–1002.