Journal of Cellular Biochemistry 71:524–535 (1998)

Early Effects of PP60$^{v\text{-}src}$ Kinase Activation on Caveolae

Young-Gyu Ko, Pingsheng Liu, Ravindra K. Pathak, Leonard C. Craig, and Richard G. W. Anderson*

Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, Texas 75235–9039

Abstract Members of the nonreceptor tyrosine kinase family appear to be targeted to caveolae membrane. We have used a Rat-1 cell expressing a temperature sensitive pp60$^{v\text{-}src}$ kinase to assess the initial changes that take place in caveolae after kinase activation. Within 24–48 h after cells were shifted to the permissive temperature, a set of caveolae-specific proteins became phosphorylated on tyrosine. During this period there was a decline in the caveolae marker protein, caveolin-1, a loss of invaginated caveolae, and a 70% decline in the sphingomyelin content of the cell. One of the phosphorylated proteins was caveolin-1 but it was associated in coimmunoprecipitation assays with both a 30 kDa and a 27 kDa tyrosine-phosphorylated protein. Finally, the cells changed from having a typical fibroblast morphology to a rounded shape lacking polarity. In light of the recent evidence that diverse signaling events originate from caveolae, pp60 $^{v\text{-}src}$ kinase appears to cause global changes to this membrane domain that might directly contribute to the transformed phenotype. J. Cell. Biochem. 71:524–535, 1998.

Key words: caveolae; caveolin-1; tyrosine kinase; cell transformation

There is increasing evidence that caveolae play a major role in organizing signal transduction at the cell surface [Anderson, 1993, 1998]. Immunocytochemistry and cell fractionation first localized a number of different hormone receptors and signal transducers to caveolae [Lisanti et al., 1994]. Several studies identified specific signaling events that originate in caveolae, including the EGF dependent activation of Raf-1 [Mineo et al., 1996], IL-1 β stimulated production of ceramide [Liu and Anderson, 1995], and a PDGF receptor kinase cascade [Liu et al., 1996]. Finally, caveolin-1, a resident protein of caveolae, has been found to modulate the activity of heterotrimeric GTP binding proteins in vitro [Li et al., 1995].

The organization of these molecules in caveolae may be necessary for the integration of diverse sources of information during signal transduction. If so, cell behavior should be altered by perturbations that affect the structure and molecular composition of caveolae. Recently, Koleske et al. [1995] reported that oncogenically transformed cells tend to have reduced levels of caveolin-1 along with fewer invaginated caveolae. This change in caveolae structure may either cause the transformed phenotype or be the consequence of transformation. The tyrosine phosphorylation of caveolin-1, specifically by membrane associated pp60$^{v\text{-}src}$ kinase, has been linked to transformation of chick embryo fibroblasts [Glenney, 1989; Glenney and Zokas, 1989] and expression of caveolin-1 can reverse the transformed phenotype [Engelman et al., 1997]. These findings suggest a more causal relationship exists between caveolae structure and cell behavior.

The selection of a cell line transformed by a viral oncogene can take several weeks. This makes it difficult to distinguish between direct and indirect effects of transformation on caveolae structure and function. For this reason we have used a Rat 1 cell line expressing a temperature sensitive pp60$^{v\text{-}src}$ kinase to identify some of the initial changes that take place in caveolae upon kinase activation. We find that within 24 to 48 h after activation of the kinase, caveolae undergo profound changes in morphology, lipid composition, and protein phosphorylation. At the same time, the cells transform from a flat, highly organized morphology to a rounded shape with significantly fewer invaginated caveolae. This cell line may be useful for studying

Contract grant sponsor: National Institutes of Health; Contract grant numbers: HL 20948 and GM 52016.

*Correspondence to: Richard G. W. Anderson, Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75235–9039.

Received 14 April 1998; Accepted 19 June 1998

how disruption of caveolae-based signaling events leads to cell transformation.

MATERIALS AND METHODS

[^{3}H]-palmitate (36 Ci/mmol) and [^{35}S]-EXPRESS protein labeling mix (1,175 Ci/mmol) was purchased from Du Pont (Wilmington, DE). Sphingomyelin, ceramide, and diacylglycerol (DAG) standards were obtained from Sigma (St. Louis, MO). Thin layer chromatography plates were purchased from J.T. Baker Inc. (Phillipsburg, NJ). Anti-phosphotyrosine IgG was from Upstate Biotechnology Inc. (Lake Placid, NY).

Cell culture. *tsLA29* cells [Stoker et al., 1984, 1986], a Rat-1 fibroblast transfected with a temperature sensitive pp60$^{v\text{-}src}$ kinase, were grown in Dulbecco's Modified Eagle Medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Sigma), 50 U/ml penicillin, and 50 mg/ml streptomycin. The cells were grown either at 35°C (permissive temperature) or 39.5°C (nonpermissive temperature) in 5% CO_2 and recultured every 5 days. All experiments were carried out on confluent cell monolayers. The growth temperature did not cause any detectable change in the number of cells per dish.

Antibody production. Polyclonal antibodies K617 and K618 were raised against peptides whose sequences were derived from the N-terminal (REQGNIYKPNNK) and C-terminal (IFSNVRINLQKEI) regions of human caveolin-1. Peptides were coupled to Tuberculin PPD (Statens Seruminstitut, Copenhagen, Denmark) by incubating with 0.04% glutaraldehyde. New Zealand white rabbits were injected with 0.2 mg coupled peptide in complete Freund's adjuvant. The rabbits were boosted with 0.1 mg coupled peptide in incomplete Freund's adjuvant 3 weeks after the initial injection and thereafter when the antibody titer began to decrease as determined by Western blot. A total of three boosts were administered. Serum IgG was purified using Econo-Pac serum IgG purification columns (Bio-Rad, Hercules, CA). The IgG fractions were concentrated using Centricon-30 concentrators (Amicon, Beverly, MA).

Labeling experiments. Cells grown at 39.5°C were washed three times in prewarmed PBS and incubated for 30 min in methionine-deficient media (ICN, Costa Mesa, CA). After adding 100 µCi/ml [^{35}S]-EXPRESS protein labeling mix, the cells were incubated for 1 h, washed three times with prewarmed PBS, and chased in media containing 200-fold excess unlabeled methionine and 30 µg/ml cycloheximide (Sigma) at either 35°C or 39.5°C for various times. Cells were labeled with [^{3}H]-palmitate by culturing them in medium for 48 h that contained 10 µCi/well of [^{3}H]-palmitate.

Protein extraction. Cells grown in 100 mm dishes were washed three times with ice-cold PBS buffer and lysed with 1 ml of 150 TETN/OG (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% TX-100, and 60 mM octylglucoside) containing protease inhibitors (10 µg/ml soy bean trypsin inhibitor, 10 µM leupeptin, 1 µg/ml pepstatin A, 0.5 mM benzamidine, and 1 mM PMSF), and phosphatase inhibitors (1 mM NaF, 1 mM Na_3VO_4) by shaking the dish on a rocking platform for 40 min at 4°C. Lysates were centrifuged at 10,000*g* for 10 min at 4°C and the supernatant fractions were saved at −80°C for further analysis. Protein concentrations were determined by the Bradford Assay [Bradford, 1976].

Lipid extraction, separation, and detection. Lipids were extracted by the method of Bligh and Dyer [1959]. A 1 ml aliquot of the sample was mixed with 1.2 ml of methanol containing 2% acetic acid and 2 ml of chloroform. This mixture was vortexed three times (10 sec each time) and centrifuged for 10 min at 10,000*g*. The organic phase was collected and dried under nitrogen gas. The sample was redissolved in 40 µl of chloroform, mixed with 5 µg of unlabeled standard, and loaded onto a TLC plate. The TLC plate was developed in a solvent system consisting of either chloroform:acetone:methanol:acetic acid:H2O (10:4:3:2:1, v/v) for separation of sphingomyelin or benzene:ethyl ether:ethanol:acetic acid (50:40:4:0.4, v/v) for separation of ceramide and DAG. The separated lipids were either visualized by autoradiography or scraped and counted in 10 ml of scintillation fluid.

Preparation of caveolae. *tsLA29* cells grown in three 150 mM dishes were washed three times with ice-cold PBS, scraped from the plates in 15 ml PBS, and collected by centrifugation (600*g*, 5 min, 4°C). The collected cells were incubated with 1 ml HEPES buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, and 1% TX-100) containing protease inhibitors and phosphatase inhibitors for 20 min at 4°C and dounced 20 times. The extract was mixed with 1 ml of 2.5 M sucrose, transferred to a centrifuge tube,

and overlaid with 9 ml of a linear 10–30% sucrose gradient in HEPES buffer. The gradients were centrifuged for 21 h at 4°C in a TH 641 rotor at 100,000*g*. The gradient was fractionated into 15 fractions from top to bottom and the proteins from each fraction concentrated by TCA precipitation before analysis by gel electrophoresis.

Immunoprecipitation. Protein extracts (400 µg) prepared in 150 ml TETN/OG containing protease and phosphatase inhibitors were pre-cleared by mixing with 50 µl of protein A-Sepharose 4LB (Sigma) plus 25 µg of rabbit IgG in 150 TETN/OG. After 4 h incubation at 4°C, the beads were removed by centrifugation for 5 min at 15,000*g*. The supernatant was transferred to a fresh tube and 50 µl of fresh protein A-Sepharose was added together with either anti-caveolin-1 IgG (designated K617, 25 µg) or preimmune IgG (5 µl). Samples were then incubated overnight at 4°C. Beads were recovered by centrifugation and washed twice sequentially in buffer A (500 mM NaCl, 5 mM EDTA, 25 mM Tris-HCl, pH 7.5, 1% TX-100), buffer B (250 mM NaCl, 5 mM EDTA, 25 mM Tris-HCl, pH 7.5, 1% TX-100), and buffer C (25 mM Tris-HCl, pH 7.4 and 5 mM EDTA) before analysis by SDS-PAGE.

Electrophoresis and immunoblotting. Proteins were separated in SDS-polyacrylamide gels using the method of Laemmli [1970] and transferred to Immobilon (Milipore, Bedford, MA) in buffer D (25 mM Tris, and 192 mM glycine, pH 8.3). After transfer, the membrane was incubated overnight at 4°C in 5% nonfat dry milk prepared in PBS. Caveolin-1 and phosphotyrosine protein were visualized by sequential treatment with specific antibodies (anti-caveolin-1 IgG designated K618), HRP-conjugated secondary antibodies, and an enhanced chemiluminescence (ECL) substrate kit (Amersham, Buchinghamsher, England).

RNase protection assay. A cDNA fragment (156 bp) corresponding to the 5' end of human caveolin-1 was inserted in KS plasmid in a T3 anti-sense orientation. The plasmid was transformed into DH5 α, purified using Qiagen Midiprep Kit, and linearized by EcoR1 digestion. Anti-sense RNA fragment was then synthesized using T3 polymerase and [^{32}P]-UTP. A 10 µg sample of total RNA from each cell type was hybridized overnight at 50°C with the [^{32}P]-anti-sense RNA probe. The reaction was digested with RNase A and RNase T1 for 30 min at room temperature. SDS and proteinase K were added to the mixture to stop the digestion and the protected [^{32}P]-RNA fragments were extracted and separated on a 6% acrylamide, DNA sequencing gel.

Electron microscopy. Cells grown on 60 mM dishes were fixed in 3% glutaraldehyde, 100 mM Na-cacodylate, pH 7.4, and 0.5 mM $CaCl_2$ at room temperature for 2 h. The samples were washed overnight in 100 mM Na-cacodylate, pH 7.4 before further fixation with 2% OsO_4 in 100 mM Na-cacodylate, pH 7.4. Samples were washed in distilled water, dehydrated, and embedded in Spur's Resin.

RESULTS

Caveolae membrane is partially insoluble in Triton X-100 at 4°C [Brown and Rose, 1992; Sargiacomo et al., 1993]. This property can be used to separate caveolae from the rest of the cell. We grew *tsLA29* cells at the nonpermissive temperature (39.5°C), scraped them from the dish in Triton X-100 at 4°C and loaded the mixture on the bottom of a linear sucrose gradient. We centrifuged the sample for 21 h at 4°C and collected 15 fractions from the top of the gradient. A constant volume from each fraction was separated by gel electrophoresis and immunoblotted with an anti-caveolin-1 IgG directed against the C-terminal end of the molecule (Fig 1A, anti-caveolin-1 IgG). Two caveolin-1-specific bands were concentrated in fractions 3–6, indicating the position of the caveolae fractions on the gradient. Previously we showed this fraction contains less than 0.7 % of the cell protein with the bulk of the protein and phospholipid at the bottom of the gradient in fractions 11–15 [Liu and Anderson, 1995].

To see if any of the proteins in these fractions contained phosphotyrosine, a companion set of fractions from 39.5°C grown cells was immunoblotted with an anti-phosphotyrosine mAb (Fig. 1A, anti-phosphotyrosine IgG). The caveolae fraction was nearly devoid of any bands that reacted with the antibody but there were a few reactive bands in fractions 13–15. A dramatically different staining pattern was seen in immunoblots of cells grown at the permissive temperature (35°C) for 48 h (Fig 1B, anti-phosphotyrosine IgG). Both the caveolae fractions (3–6) and the bulk cell fractions (11–15) contained numerous reactive bands. The molecular weights of the two populations of tyro-

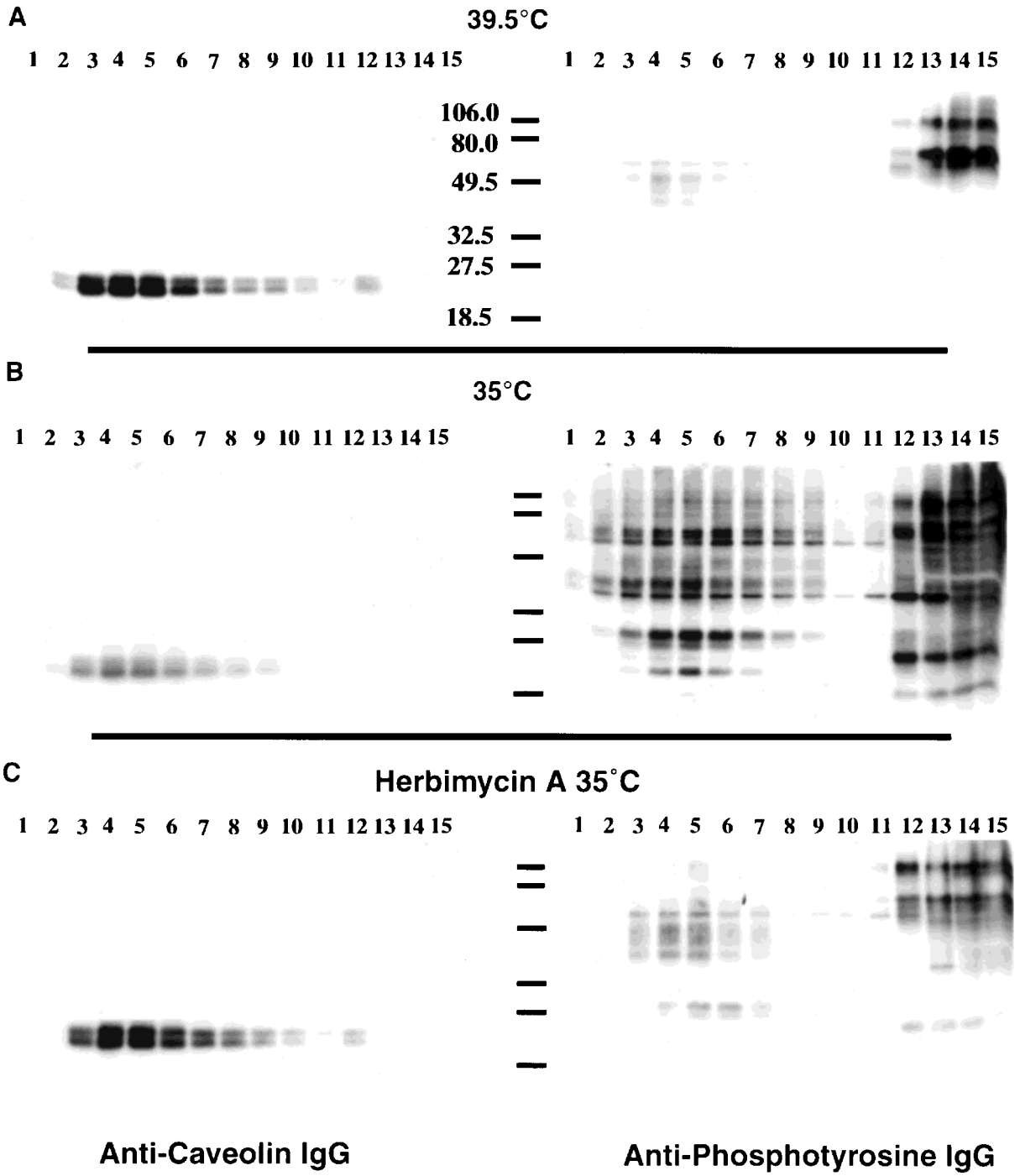

Fig. 1. Effect of pp60$^{v\text{-}src}$ kinase activation on tyrosine phosphorylation of caveolae proteins. *tsLA29* cells were grown at 39.5°C for 3 days. Individual sets of dishes were incubated either at 39.5°C (**A**), 35°C (**B**), or 35°C in the presence of herbimycin A (**C**) for 48 h. Triton X-100 insoluble caveolae were separated from detergent soluble components of the cell on sucrose gradients as described. A constant volume of each fraction was separated by gel electrophoresis and immunoblotted with either anti-caveolin-1 IgG (left) or anti-phosphotyrosine IgG (right).

sine phosphorylated proteins, however, were different. Many of the phosphoproteins in the caveolae fraction were not present in the bottom fractions including proteins with sizes of 22 kDa, 27 kDa, 30 kDa, 35 kDa, 60 kDa. Therefore, activation of pp60$^{v\text{-}src}$ kinase leads to the phosphorylation of multiple proteins, some of which are specific to caveolae.

Coincident with the appearance of phosphotyrosine-containing proteins, there was a marked decline in the amount of caveolin-1 in the caveolae fraction (Fig. 1B, anti-caveolin-1 IgG). Although both caveolin-1 isoforms were decreased, there usually was a greater loss of the high molecular weight form. The loss of caveolin-1 appeared to be directly related to pp60$^{v\text{-}src}$ kinase activation because cells grown at the permissive temperature in the presence of the kinase inhibitor herbimycin A had normal levels of caveolin-1 (Fig. 1C, anti-caveolin-1 IgG) along with a reduced number of phosphotyrosine proteins (Fig. 1C, anti-phosphotyrosine IgG).

The loss of caveolin-1 was reversible (Fig. 2). Cells grown at 39.5°C for 3 days had normal levels of caveolin-1 (lane 1). After 48 h of incubation at 35°C, the amount of caveolin-1 declined (lanes 2–4). Cells grown continuously at 35°C, by contrast, had reduced amounts of caveolin-1 (lane 5) but the amount detected returned to normal after the cells were shifted to the nonpermissive temperature for 24–48 h (lanes 7–8).

One way that pp60$^{v\text{-}src}$ kinase activation could reduce the amount of caveolin-1 in the cell is by stimulating protein degradation. Cells grown at the nonpermissive temperature (39.5°C) were incubated in the presence of [^{35}S]-EXPRESS protein labeling mix for 1 h. The cells were washed and fresh media containing excess unlabeled methionine was added to the dish before incubating the cells for various times at either the permissive (35°C) or nonpermissive temperature (39.5°C). At the end of each incubation period, caveolin-1 was immunoprecipitated, separated by gel electrophoresis and visualized by autoradiography (Fig. 3). The amount of radiolabeled caveolin-1 in the immunoprecipitate was similar in the two sets of cells. Moreover, during the 9 h incubation there was no significant decline in the amount of labeled caveolin-1 in either sample.

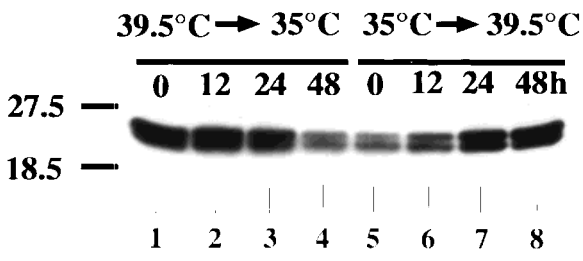

Fig. 2. Temperature dependent loss and return of caveolin-1. *tsLA29* cells were grown at either 39.5°C or 35°C for 3 days before the temperature was shifted to either 35°C or 39.5°C for 0, 12, 24, and 48 h, respectively. Proteins were separated by gel electrophoresis and blotted with anti-caveolin-1 IgG.

We next used an RNAase protection assay to determine if activation of pp60$^{v\text{-}src}$ kinase caused a reduction in the amount of caveolin-1 mRNA. A 156 base pair, anti-sense RNA fragment from the 5' end of human caveolin-1 mRNA was synthesized in the presence of [^{32}P] UTP using T3 polymerase. The radiolabeled probe was hybridized overnight at 50°C with total RNA from cells grown at either permissive (35°C) or nonpermissive temperatures (39.5°C; Fig. 4). At the end of the incubation, samples were digested with RNase A and T1 for 30 min and analyzed on a 6% DNA sequencing gel. Two protected fragments (156 bp and 126 bp) were present in both sets of cells, suggesting the presence of two mRNA isoforms for caveolin-1. The larger fragment, however, was significantly reduced in cells grown at permissive temperatures. The smaller protected fragment was not reduced. On the other hand, both bands were reduced in cells transfected with a constitutively active form of pp60$^{v\text{-}src}$ kinase (data not shown). Therefore, activation of pp60$^{v\text{-}src}$ kinase appears to inhibit the synthesis of the mRNA for the larger isoform of caveolin-1.

An anti-phosphotyrosine reactive band (22 kDa) comigrating with caveolin-1 was present in the caveolae fractions from cells grown at 35°C (Fig. 1B, compare anti-caveolin-1 with anti-phosphotyrosine). We used immunoprecipitation to see if this was caveolin-1. Cells grown at the non-permissive temperature (39.5°C) were shifted to 35°C for various times before we lysed the cells and prepared immunoprecipitates using either anti-caveolin-1 IgG or preimmune IgG (Fig. 5). The samples were separated by gel electrophoresis and immunoblotted with either anti-phosphotyrosine IgG (A) or anti-caveolin-1 IgG (B). Before the temperature was shifted (0 time, B), the two caveolin-1 isoforms were present in the immunoprecipitate but these bands reacted only weakly with anti-phosphotyrosine IgG (0 time, A). Within 12 h after the temperature was shifted, however, three anti-phosphotyrosine reactive bands appeared (A). One of these bands had the same electrophoretic mobility as the low molecular weight isoform of caveolin-1. The other two bands were coprecipitating proteins with apparent sizes of 30 and 27 kDa. This phosphotyrosine pattern persisted throughout the 12–48 h period at 35°C. At the same time, there was a

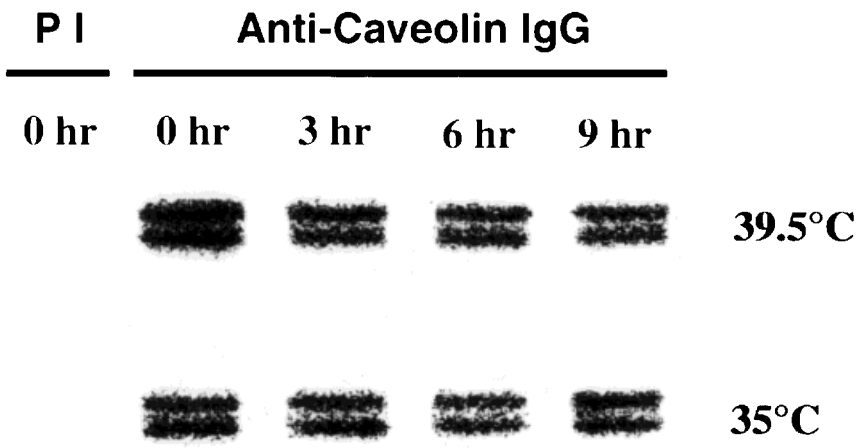

Fig. 3. Activation of pp60$^{v\text{-}src}$ kinase does not accelerate degradation of caveolin-1. Cells were grown at 39.5°C for 4 days before incubating in the presence of [^{35}S]-EXPRESS protein labeling mix for 1 h at 39.5°C. The cells were washed three times before adding 200-fold excess methionine plus 30 μg/ml of cycloheximide to the dish and incubating at either 39.5°C or 35°C for the indicated time. At the end of each incubation, samples were prepared for immunoprecipitation using either anti-caveolin-1 or preimmune IgG. Each immunoprecipitate was separated on 12.5% gels and autoradiograms prepared.

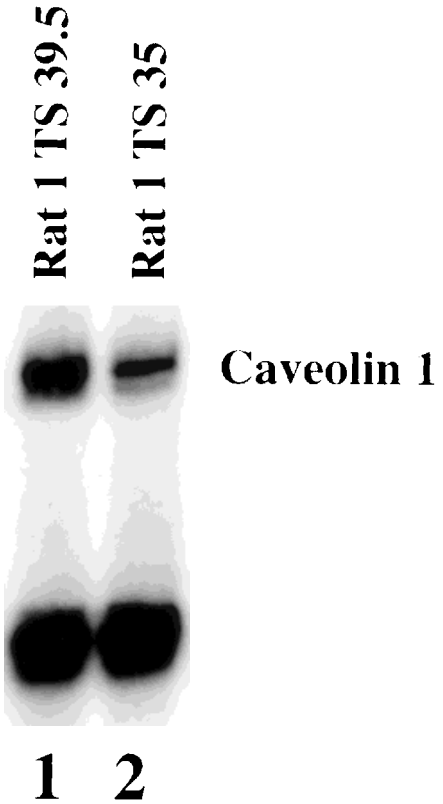

Fig. 4. Activation of pp60$^{v\text{-}src}$ kinase stimulates loss of caveolin-1 mRNA. Cells were grown at 39.5°C for 4 days and then incubated at either 39.5°C or 35°C for 48 h. Total RNA extracted from each cell type was used to protect freshly made [^{32}P]-labeled anti-sense caveolin-1 mRNA fragment during RNAase digestion. After the digestion, protected fragments were separated on 6% acrylamide, DNA sequencing gel.

progressive decline in the amount of caveolin-1 in the cells (B, 12–48). The higher molecular weight isoform, which presumably is synthesized by the larger of the two mRNAs for caveolin-1, was more rapidly lost from cells than the smaller form (B).

Previously we showed that the caveolae fraction from normal human fibroblasts contains about 50% of the sphingomyelin, DAG, and ceramide in the cell [Liu and Anderson, 1995]. A portion of the sphingomyelin in this fraction is converted to ceramide when cells are incubated in the presence of IL-1 β and this ceramide inhibits PDGF-stimulated DNA synthesis [Liu and Anderson, 1995]. We used radiolabeling to determine how pp60$^{v\text{-}src}$ kinase activation affected the lipid composition of *tsLA* 29 cells (Fig. 6). Cells were grown in the presence of [^{3}H]-palmitate for 48 h to label various lipids. Equal amounts of radioactivity were separated by thin layer chromatography and the quantity of radiolabeled sphingomyelin (A), ceramide (B), and DAG (C) measured. Normal Rat-1 cells grown at 37°C incorporated 50,000 DPM into the sphingomyelin band (bar 1, A), 15,000 DPM into the ceramide band (bar 1, B), and 12,500 DPM into the DAG band (bar 1, C). The amount of radioactivity in each of these lipids was slightly lowered by growing the Rat-1 cells at both 39.5°C (bar 2, A–C) and 35°C (bar 3, A–C). *tsLA29* cells grown at the nonpermissive temperature (39.5°C) had markedly lower amounts

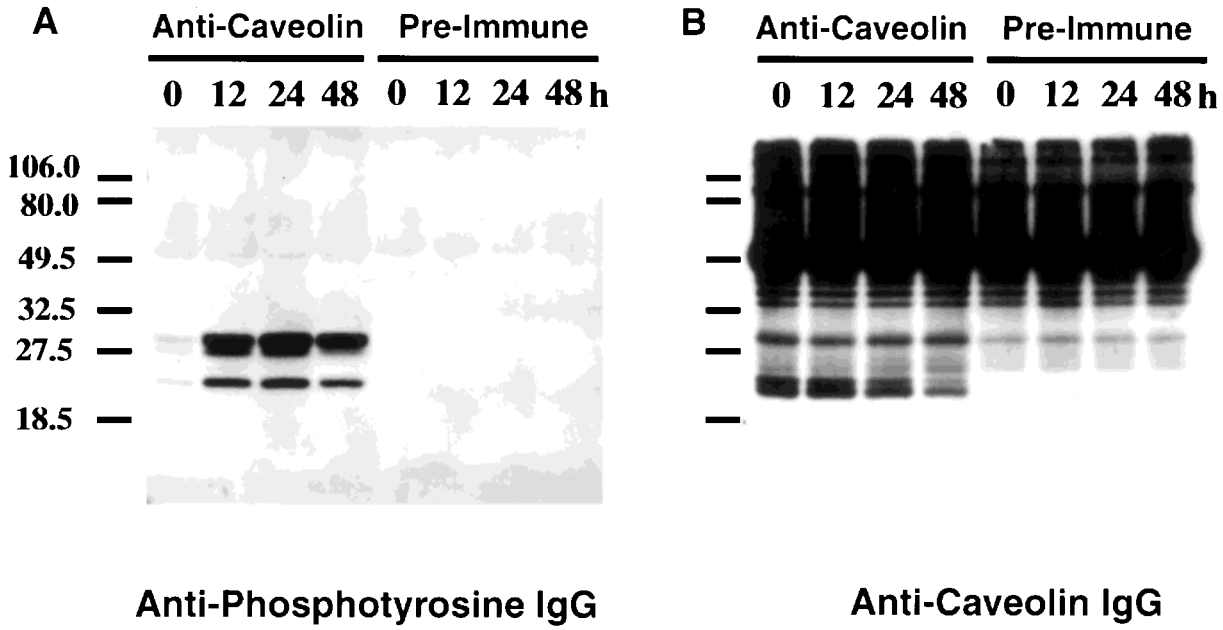

Fig. 5. Both caveolin-1 and caveolin-1-associated proteins are phosphorylated by pp60$^{v\text{-}src}$ kinase. Cells were grown at 39.5°C for 3 days before the temperature was shifted to 35°C for 0, 12, 24, and 48 h. At the end of each incubation, samples were prepared for immunoprecipitation using either anti-caveolin-1 or preimmune IgG. Each immunoprecipitate was separated on 12.5% gels and immunoblotted with either anti-phosphotyrosine (**A**) or anti-caveolin-1 (**B**) IgG. In addition to caveolin-1 the immunoprecipitates contained a 30 kDa and a 27 kDa protein that reacted with the anti-phosphotyrosine IgG. The lower molecular weight isoform of caveolin-1 matched the phosphorylated caveolin-1 band (compare lower band A with lower band B).

of both ceramide (bar 4, B) and DAG (bar 4, C) compared to Rat-1 cells grown at the same temperature (compare with bar 2, B and C). By contrast, the amount of radiolabeled sphingomyelin was about the same (compare bar 4 with bar 2, A). When *tsLA29* cells were grown at 35°C, where the pp60$^{v\text{-}src}$ kinase is active, there was no change in the amount of either ceramide (bar 5, B) or DAG (bar 5, C) but there was a dramatic decline in the amount of radiolabeled sphingomyelin (compare bar 4 with 5, A). Under kinase activation conditions, there was ~70% decline in the amount of sphingomyelin in the *tsLA29* cells.

We prepared autoradiograms of the thin layer chromatography plates to look for other changes in the lipid profile as a function of kinase activation (Fig. 6D, E). In addition to the specific change in sphingomyelin (SM lane 9,10, D), we saw an increase in phosphatidic acid (PA, lanes 9,10, D) as well as five sets of unidentified lipids (top three arrows, D and two arrows, E). Two other unidentified lipids were reduced in *tsLA29* cells grown at 35°C (lower two arrows, compare lane 5,6 with lane 9,10, D).

Commensurate with the changes in the protein and lipids in caveolae, the morphology of the cells was altered by pp60$^{v\text{-}src}$ kinase activation (Fig. 7). Normally *tsLA29* cells grow like Rat-1 cells, which are typical fibroblasts in appearance. The spread, polarized morphology of these cells (A) was dramatically altered by growing them for 48 h at 35°C (B). Under these conditions the cells became round and lost polarity. The change in overall morphology was accompanied by a substantial loss in the number of invaginated caveolae. Cells grown at the nonpermissive temperature had 1.5 invaginated caveolae/μm of membrane (arrows, Fig. 7A) while 35°C grown cells only had 0.2 invaginated caveolae/μm. The cells regained normal morphology when the temperature was shifted back to 39.5°C. Furthermore, cells grown at 35°C in the presence of herbimycin A did not undergo these morphological changes (data not shown).

DISCUSSION

Previous studies have localized a number of pp60$^{v\text{-}src}$ kinase substrates to Triton X-100 insoluble portions of the cell [Hamaguchi and Hanafusa, 1987, 1989]. We now find that some of these substrates are in a fraction of mem-

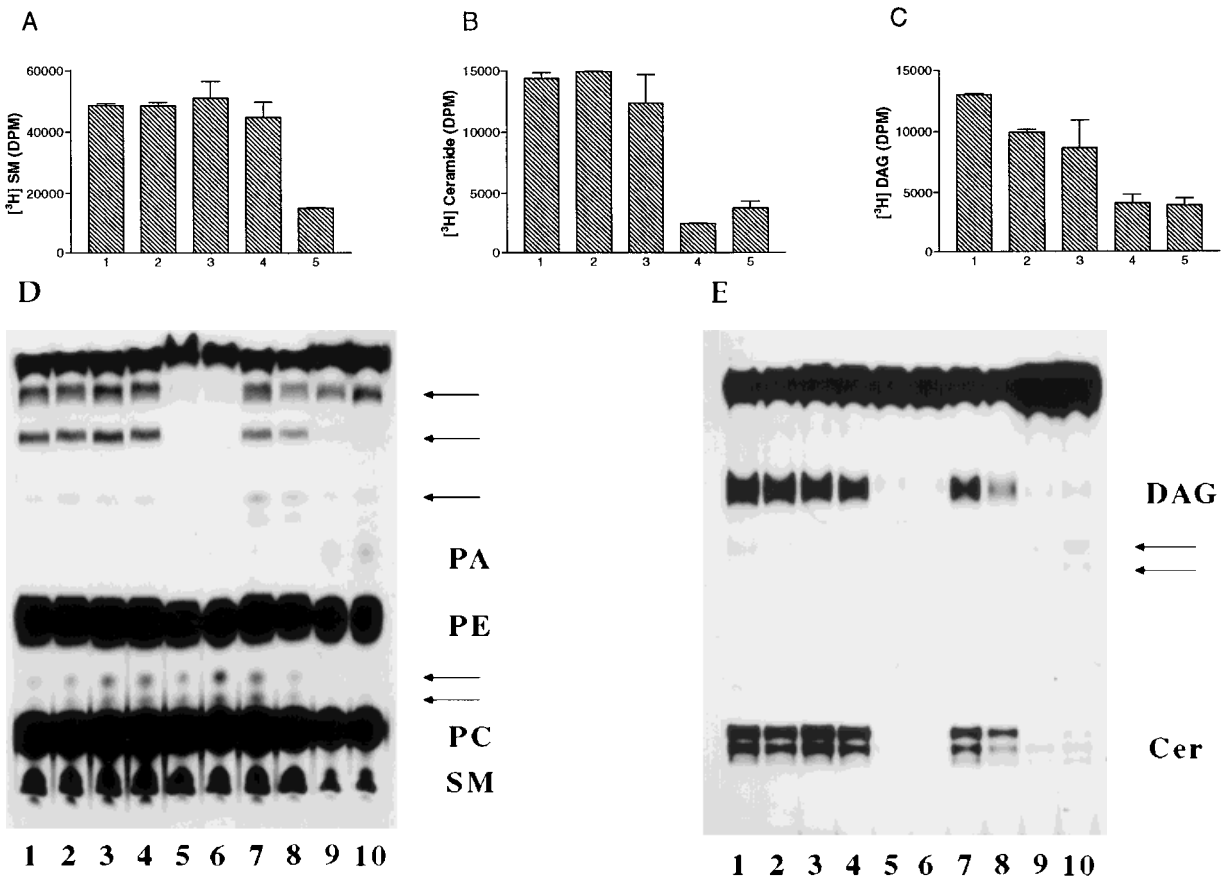

Fig. 6. Effect of pp60$^{v\text{-}src}$ kinase activation on lipid composition of Rat-1 cell. Either Rat-1 cells or *tsLA29* cells were grown at the indicated temperature for 4 days. For the final 48 h of culture, [^{3}H]-palmitate (10 μCi/well) was present in the medium. **A–C**: Rat 1B fibroblasts were cultured at 37°C (bar 1), 39.5°C (bar 2), or 35°C (bar 3). *tsLA29* cells were cultured at 39.5°C (bar 4) or 35°C (bar 5). Equal amounts of radioactive lipids (0.6×10^6 DPM) were separated by TLC and the sphingomyelin, ceramide, and DAG spots scraped and counted. Each measurement is the mean ± S.D(n = 3). **D,E**: Duplicate sets of Rat 1 cells were grown at 37°C (lane 1 and 2), 39.5°C (lane 3 and 4) or 35°C (lane 7 and 8). Duplicate sets of *tsLA29* cells were cultured at 39.5°C (lane 5 and 6) or 35°C (lane 9 and 10). The lipids were separated by thin layer chromatography and visualized by autoradiography. DAG, diacylglycerol; Cer, ceramide; SM, sphingomyelin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

brane with the properties of caveolae. The method of isolation we used does not separate caveolae from detergent insoluble membrane that might be associated with the Golgi apparatus [Brown and Rose, 1992]. Nevertheless, the insoluble phosphotyrosine-containing proteins most likely were in caveolae because pp60$^{c\text{-}src}$ kinase is enriched in caveolae isolated from plasma membrane [Liu et al., 1996] as are other members of the nonreceptor tyrosine kinase family [Sargiacomo et al., 1993]. This suggests that cellular *src* kinase ordinarily functions in caveolae membrane and when cells are infected with the Rous sarcoma virus a portion of the viral enzyme is targeted to this location.

Cell transformation by pp60$^{v\text{-}src}$ kinase may be the direct or indirect result of an unscheduled phosphorylation event. Among the many substrates for the kinase only a few have been linked to pp60$^{v\text{-}src}$ kinase-induced cell transformation [Glenney, 1989; Kozma et al., 1990; Kozma and Weber, 1990; Peterson et al., 1994; Wasilenko et al., 1991]. At least two of these substrates, caveolin-1 [Rothberg et al., 1992] and the EGF receptor [Mineo et al., 1996; Smart et al., 1995], are located in caveolae. In addition, the interaction of pp60$^{v\text{-}src}$ kinase with phosphatidylinositol 3-kinase, another protein enriched in caveolae [Liu et al., 1996], appears to be required for complete cell transformation [Wages et al., 1992]. This suggests that the inappropriate phosphorylation of multiple caveolae substrates contributes directly to *src* transformation.

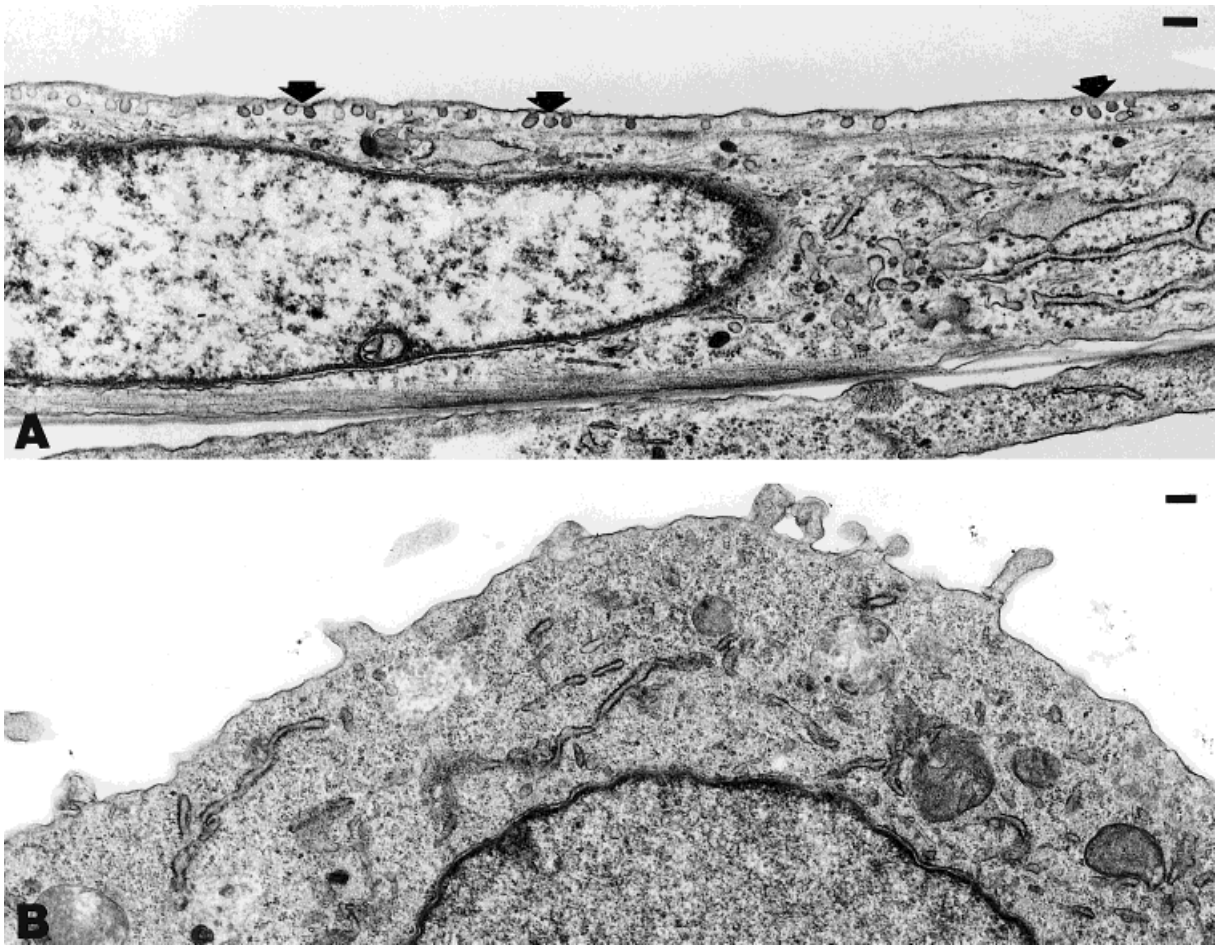

Fig. 7. Morphology of *tsLA29* cells at 39.5°C (**A**) and 35°C (**B**). Cells were grown at the indicated temperature for 2 days. The cells were fixed and processed for electron microscopy as described. Arrows indicate clusters of caveolae. Scale bar = 0.025 μm.

The tyrosine phosphorylation of both caveolin-1 and the coprecipitating 27 and 30 kDa proteins also occurs in response to insulin stimulation [Mastick et al., 1995; Mastick and Saltiel, 1997]. The insulin receptor has been localized by electron microscopy to caveolae-like structures in rat adipocytes [Smith and Jarett, 1988, 1990] and is concentrated in caveolae-like membrane from rat brain [Wu et al., 1997]. However, it is not present in the Triton X-100 insoluble portion of caveolae membrane [Mastick et al., 1995], possibly because it is removed by the Triton X-100 treatment [Smart et al., 1995]. Our results suggest pp60$^{v\text{-}src}$ kinase is directly responsible for the phosphorylation of these two proteins. Therefore, in its phosphorylation reaction the insulin receptor may normally act through pp60$^{c\text{-}src}$ kinase, or other non-receptor tyrosine kinases [Mastick and Saltiel, 1997]. Not all receptor tyrosine kinases cause the phosphorylation of these proteins [Liu et al., 1996] nor does the insulin receptor stimulate their phosphorylation in every cell [Liu et al., 1996]. Cell specific phosphorylation patterns generated by pp60$^{c\text{-}src}$ kinase may depend on how the participating molecules are organized in caveolae.

Caveolin-1 was originally detected as a substrate for pp60$^{v\text{-}src}$ kinase [Glenney and Zokas, 1989]. There are eight tyrosine residues that are potential sites for phosphorylation [Glenney, 1992]. Li et al. [1996] reported that the high molecular weight isoform of caveolin-1 is selectively phosphorylated by pp60$^{v\text{-}src}$ kinase both in vivo and in vitro. It has an additional 31 amino acids at the amino terminus containing three of the eight tyrosine residues in the molecule. We only detected phosphorylation of the

low molecular weight isoform (Fig. 5) and Mastick et al. [1995] found that insulin stimulated the tyrosine phosphorylation of both isoforms. Three different cell lines were used in these studies; NIH 3T3 cells [Li et al., 1996], 3T3-L1 cells [Mastick et al., 1995], and Rat-1 cells (current study). The *src* kinase is probably responsible for caveolin-1 phosphorylation in all of these cells but cell specific factors control which isoform becomes the substrate.

pp60$^{v\text{-}src}$ kinase activation had a dramatic effect on the amount of sphingomyelin in the cell. Forty-eight hours after kinase activation the sphingomyelin content was decreased by ~70% (Fig. 6). Since >50% of the cellular sphingomyelin appears to be in the caveolae fraction [Brown and Rose, 1992; Liu and Anderson, 1995], the concentration of sphingomyelin in this membrane domain must also have declined. The loss did not appear to be due to depressed levels of the sphingomyelin precursor, ceramide, because this lipid was equally low at both the permissive and nonpermissive temperatures. Within 30 min after pp60$^{v\text{-}src}$ kinase is activated in *tsL29 cells* there is an increase in the formation of DAG accompanied by a release of water soluble choline [Wyke et al., 1992]. Since DAG can stimulate the conversion of sphingomyelin to ceramide in caveolae [Liu and Anderson, 1995], the low sphingomyelin at 48 h may be due to the over production of DAG [Wiegmann et al., 1994]. Without an adequate pool of sphingomyelin *src* transformed cells may be unable to respond properly to modulatory events that use ceramide as a signaling intermediate. This could be an especially critical factor in transformation since ceramide produced in caveolae can suppress mitogenesis [Liu and Anderson, 1995].

The decline in sphingomyelin after pp60$^{v\text{-}src}$ kinase activation was accompanied by a decrease in caveolin-1 protein (Fig. 2). The higher molecular weight caveolin-1 isoform seemed to be lost more rapidly than the smaller isoform. This correlated with a decline in the larger of two caveolin-1-specific RNAase protected RNA fragments (Fig. 4). These cells apparently contain two different mRNAs for caveolin-1 and each is differently regulated in response to activation of pp60$^{v\text{-}src}$ kinase.

We observed a dramatic decline in the number of invaginated caveolae in pp60$^{v\text{-}src}$ kinase activated cells (Fig. 7). This altered morphology can not be accounted for by the simple loss of caveolin-1, as may be the case in other transformed cells [Koleske et al., 1995]. Since the more persistent isoform of caveolin-1 was phosphorylated, another explanation is that phosphorylation of caveolin-1 causes the cholesterol content of caveolae membrane to decline. Caveolin-1 is a cholesterol binding protein [Murata et al., 1995] and the traffic of this molecule between the ER and Golgi apparatus has been implicated in the transport of cholesterol to caveolae [Conrad et al., 1995; Fielding and Fielding, 1995; Smart et al., 1994, 1996]. If phosphorylated caveolin-1 is unable to transport cholesterol, the cholesterol content of caveolae membrane will become depleted. Cholesterol appears to be essential for the normal structure and function of caveolae since experimentally lowering the cholesterol of the cell reduces the number of invaginated caveolae [Chang et al., 1992; Rothberg et al., 1990].

Many studies have focused on identifying the key pp60$^{v\text{-}src}$ kinase substrates responsible for the transformed phenotype. Our results suggest a global effect pp60$^{v\text{-}src}$ kinase activation on caveolae membrane lipids and proteins. The likely cause of these changes is the targeted action of the kinase at this membrane site. The mere presence of this enzyme in caveolae causes the phosphorylation of multiple substrates involved in cell signaling. The unscheduled phosphorylation of these proteins obviously will affect signal transduction. In addition, the altered lipid composition of caveolae could affect the uptake of essential nutritional and informational molecules by caveolae [Anderson, 1998].

ACKNOWLEDGMENTS

We thank William Donzell and Grace Liao for their valuable technical expertise and Stephanie Baldock for administrative assistance. We are also grateful to Dr. Susie Mumby for assistance with the production of peptide antibodies and Dr. Michael Weber for providing us with the *tsLA29* cells. This work was supported by NIH and the Perot Family Foundation.

REFERENCES

Anderson RGW (1993): Caveolae: Where incoming and outgoing messengers meet. Proc Natl Acad Sci USA 90: 10909–10913.

Anderson RGW (1998): The caveolae membrane system. Annu Rev Biochem 67:199–225.

Bligh EG, Dyer WJ (1959): A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Brown DA, Rose JK (1992): Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 68:533–544.

Chang W-J, Rothberg KG, Kamen BA, Anderson RGW (1992): Lowering the cholesterol content of MA104 cells inhibits receptor mediated transport of folate. J Cell Biol 118:63–69.

Conrad PA, Smart EJ, Ying Y-S, Anderson RGW, Bloom GS (1995): Caveolin cycles between plasma membrane caveolae and the Golgi complex by microtubule-dependent and microtubule-independent steps. J Cell Biol 131:1424–1433.

Engelman JA, Wykoff CC, Yasuhara S, Song KS, Okamoto T, Lisanti MP (1997): Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. J Biol Chem 272:16374–16381.

Fielding PE, Fielding CJ (1995): Plasma membrane caveolae mediate the efflux of cellular free cholesterol. Biochemistry 34:14288–14292.

Glenney JR (1989): Tyrosine phosphorylation of a 22-kDa protein is correlated with transformation by Rous sarcoma virus. J Biol Chem 264:20163–20166.

Glenney JR (1992): The sequence of human caveolin reveals identity with VIP21, a component of transport vesicles. FEBS Lett 314:45–48.

Glenney JR, Zokas L (1989): Novel tyrosine kinase substrates from Rous sarcoma virus transformed cells are present in the membrane skeleton. J Cell Biol 108:2401–2408.

Hamaguchi M, Hanafusa H (1987): Association of $p60^{src}$ with Triton X-100-resistant cellular structure correlates with morphological transformation. Proc Natl Acad Sci USA 84:2312–2316.

Hamaguchi M, Hanafusa H (1989): Localization of major potential substrates of $p60^{v\text{-}src}$ kinase in the plasma membrane matrix fraction. Oncogene Res 4:29–37.

Koleske AJ, Baltimore D, Lisanti MP (1995): Reduction of caveolin and caveolae in oncogenically transformed cells. Proc Natl Acad Sci USA 92:1381–1385.

Kozma LM, Reynolds AB, Weber MJ (1990): Glycoprotein tyrosine phosphorylation in Rous sarcoma virus-transformed chicken embryo fibroblasts. Mol Cell Biol 10:837–841.

Kozma LM, Weber MJ (1990): Constitutive phosphorylation of the receptor for insulin-like growth factor I in cells transformed by the *src* oncogene. Mol Cell Biol 10:3626–3634.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Li S, Okamoto T, Chun M, Sargiacomo M, Casanova JE, Hansen SH, Nishimoto I, Lisanti MP (1995): Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. J Biol Chem 270:15693–15701.

Li S, Seitz R, Lisanti MP (1996): Phosphorylation of caveolin by *src* tyrosine kinases. J Biol Chem 271:1–6.

Lisanti MP, Scherer P, Tang ZL, Sargiacomo M (1994): Caveolae, caveolin and caveolin-rich membrane domains: A signalling hypothesis. Trends Cell Biol 4:231–235.

Liu P, Anderson RGW (1995): Compartmentalized production of ceramide at the cell surface. J Biol Chem 45:27179–27185.

Liu P, Ying Y-S, Ko Y-G, Anderson RGW (1996): Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. J Biol Chem 271:10299–10303.

Mastick CC, Brady MJ, Saltiel AR (1995): Insulin stimulates the tyrosine phosphorylation of caveolin. J Cell Biol 129:1523–1531.

Mastick CC, Saltiel AR (1997): Insulin-stimulated tyrosine phosphorylation of caveolin is specific for the differentiated adipocyte phenotype in 3T3-L1 cells. J Biol Chem 272:20706–20714.

Mineo C, James GL, Smart EJ, Anderson RGW (1996): Localization of EGF stimulated Ras/Raf-1 interaction to caveolae. J Biol Chem 271:11930–11935.

Murata M, Peränen J, Schreiner R, Wieland F, Kurzchalia TV, Simons K (1995): VIP21/caveolin is a cholesterol-binding protein. Proc Natl Acad Sci USA 92:10339–10343.

Peterson JE, Jelinek T, Kaleko M, Siddle K, Weber MJ (1994): c phosphorylation and activation of the IGF-I receptor in *src*-transformed cells. J Biol Chem 269:27315–27321.

Rothberg KG, Heuser JE, Donzell WC, Ying Y-S, Glenney JR, Anderson RGW (1992): Caveolin, a protein component of caveolae membrane coats. Cell 68:673–682.

Rothberg KG, Ying Y-S, Kamen BA, Anderson RGW (1990): Cholesterol controls the clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. J Cell Biol 111:2931–2938.

Sargiacomo M, Sudol M, Tang Z, Lisanti MP (1993): Signal transducing molecules and GPI-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J Cell Biol 122:789–808.

Smart EJ, Ying Y-S, Donzell WC, Anderson RGW (1996): A role for caveolin in transport of cholesterol from ER to plasma membrane. J Biol Chem 271:29427–29435.

Smart EJ, Ying Y-S, Mineo C, Anderson RGW (1995): A detergent free method for purifying caveolae membrane from tissue culture cells. Proc Natl Acad Sci USA 92: 10104–10108.

Smart EJ, Ying Y-U, Conrad PA, Anderson RGW (1994): Caveolin moves from caveolae to the Golgi apparatus in response to cholesterol oxidation. J Cell Biol 127:1185–1197.

Smith RM, Jarett L (1988): Receptor-mediated endocytosis and intracellular processing of insulin. Lab Invest 58:613–629.

Smith RM, Jarett L (1990): Differences in adenosine triphosphate dependency of receptor-mediated endocytosis of alpha2-macroglobulin and insulin correlate with separate routes of ligand-receptor complex internalization. Endocrinology 126:1551–1560.

Stoker AW, Enrietto PJ, Wyke JA (1984): Functional domains of the $pp60^{v\text{-}src}$ protein as revealed by analysis of temperature-sensitive Rous sarcoma virus mutants. Mol Cell Biol 4:1508–1514.

Stoker AW, Kellie S, Wyke JA (1986): Intracellular localization and processing of pp60$^{v\text{-}src}$ proteins expressed by two distinct temperature-sensitive mutants of Rous sarcoma virus. J Virol 58:876–883.

Wages DS, Keefer J, Rall TB, Weber MJ (1992): Mutations in the SH3 domain of the *src* oncogene which decrease association of phosphatidylinositol 3'-kinase activity with pp60$^{v\text{-}src}$ and alter cellular morphology. J Virol 66:1866–1874.

Wasilenko WJ, Payne DM, Fitzgerald DL, Weber MJ (1991): Phosphorylation and activation of epidermal growth factor receptors in cells transformed by the *src* oncogene. Mol Cell Biol 11:309–321.

Wiegmann K, Schütze S, Machleidt T, Witte D, Krönke M (1994): Functional dichotomy of neutral and acidic sphingomyelinase in tumor necrosis factor signaling. Cell 78: 1005–1015.

Wu C, Butz S, Ying Y-S, Anderson RGW (1997): Tyrosine kinase receptors concentrated in caveolae-like dmains from neuronal plasma membrane. J Biol Chem 272:3554–3559.

Wyke AW, Cook SJ, MacNulty EE, Wakelam MJ (1992): *v-src* induces elevated levels of diglyceride by stimulation of phosphatidylcholine hydrolysis. Cell Sign 4:267–274.