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ARTICLE

Ocadaic acid treatment alters the intracellular localization of caveolin-1 and caveolin-2 in HepG2 cells

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ABSTRACT In this paper we provide evidences that protein phosphatases could regulate the intracellular localization of caveolin isoforms in a hepatoma cell line (HepG2). Ocadaic acid (OA) - a serine/threonine phosphatase inhibitor – was used in various concentrations (4nM and 100nM) to study the localization of caveolin-1 and caveolin-2 in HepG2 cells. Using fluorescent and confocal immunocytochemistry we have found that OA in both concentrations has significantly altered the intracellular localization and distribution of the caveolin-1 and caveolin-2 as well. In control (-OA treatment) the caveolin-1 was present in discrete punctate structures in the cytoplasm and also on the cell membrane. Caveolin-2 has partly overlapped with caveolin-1, but a significant amount caveolin-2 was detected around the nucleus. After OA (4 and 100 nM) treatment caveolin-1 has disappeared from the cell membrane, it was present mainly in the cytoplasm in larger vesicle or vacuole-like structures that were arranged along the cables of the cytoskeleton. In many cases caveolin-2 was found to colocalize with caveolin-1, but there was always a significant amount of caveolin-2 present around the nucleus. Immunoprecipitation and Western blot analysis revealed that in OA-treated cells a ~24 kDa protein identified as caveolin-2 was strongly phosphorylated on tyrosine residues. The effect of OA was not reversible, since the removal of OA has not resulted in the dephosphorylation of caveolin-2 and the perinuclear localization of caveolin-2 remained. Our data indicate that phophorylation of caveolin-2 can alter not only the intracellular localization of caveolin isoforms but also the distribution of caveolae. The cytoskeleton seems to play an important role in the normal and altered distribution of caveolae, and the tyrosine phosphorylation or the absence of dephosphorylation of caveolin-2 isoform can inhibit the recycling of caveolae.
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KEY WORDS

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Caveolae have been morphologically characterized as omega- or flask-shaped plasma membrane invaginations and biochemically as caveolin- and cholesterol-rich membrane domains (Kurchalia and Parton 1999). Caveolins are essential for caveolae formation, they constitute the structural framework of caveolae (Parton 1996). Three members of the caveolin gene family have been identified so far: caveolin-1, caveolin-2 and caveolin-3. Caveolin-1 induces caveolae formation, binds cholesterol, and interacts with signaling molecules. Caveolin-3, expressed in limited kinds of cells (muscle cells, Tang et al. 1994; Song et al. 1996; Way and Parton 1999; glials cells, Nishiyama et al. 1999; neurons, unpublished data, etc.), is thought to play similar role to that of caveolin-1. The function of caveolin-2 has not yet been defined in details. Data indicate its accessory role in caveolae formation, as well as its involvement in forming deep caveolae invaginations (Scheiffele et al. 1998; Fujimoto et al. 2000; Kiss et al. 2000).

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Caveolae seem to have multiple functions. Wide variety of signaling molecules (GPI-anchored proteins, intermediates of the MAP kinase phosphorylation cascade including h-Ras, Fyn, Src family tyrosine kinases, eNOS, heterotrimeric G proteins, G-protein-coupled receptors) have been found to be accumulated in caveolae (Anderson, 1998; Smart et al. 1999). These signaling molecules bind to caveolin itself (Couet et al. 1997). As a result of this binding the signaling molecules become inactive (Li et al. 1995), thus caveolae assumed to function as preassembled signaling complexes, message centers, signaling organelles (Smart et al. 1999). Caveolae also play an important role in the regulation of cellular cholesterol homeostasis (Fielding and Fielding 2000).

Caveolae take part in cellular transport as well. Increasing number of evidences confirm that caveolae are directly involved in the internalization of membrane components, extracellular ligands such as cholera toxin (Montesano et al. 1982; Parton et al. 1994), folic acid (Rothberg et al. 1990; Anderson et al. 1992), serum albumin (Scnitzer et al. 1994), autocrine motility factor-AMF (Berliname et al. 1998), GPI-

anchored proteins (Anderson 1992) green fluorescent protein (Nichols et al. 2001), urokinase receptors (Stahl amd Mueller 1995). Certain filamentous adhesin (FimH)-expressing bacteria are also internalized in caveolae-dependent pathway in immune cells (reviewed by Harris et al. 2002). Several nonenveloped viruses (Simian virus 40, Polyoma virus) enter cells through caveolae (Pelkmans et al. 2001; Pelkmans and Helenius 2002).

It has been postulated that phosphatases and kinases can regulate the internalization, and probably, the recycling of caveolae. In our experiments ocadaic acid (OA) was used to study the internalization and possible recycling of caveolae in HepG2 cell line. When hepatoma cells were treated with OA (4 and 100 nM), the cytoplasmic and plasma membrane distributions of caveolin-1 and caveolin-2 have changed. Caveolin-1 has disappeared from the cell surface and it was detected in vesicles arranged in rows in the cytoplasm. Since caveolae can be characterized by the presence of caveolin-1 and caveolin-2 (caveolin-2 seems to play an accessory role in caveolae-formation), the cytoplasmic route of caveolae can be followed by detection (localization) of these isoforms, so we think that caveolae have also disappeared from the cell surface. Although caveolin-2 was partly colocalized with caveolin-1, there was a significant amount of this isoform present in the perinuclear region. When OA was removed and the cells were incubated with an OA-free medium for 3 hours caveolin-1 was still arranged along cables, while caveolin-2 was detected around the nucleus. This perinuclear caveolin-2-containing ring was more prominent then in control cells. We were not able to detect caveolin-1 and –2 in punctate structures on the cell membrane. Our immunoprecipitation and Western blot analyses showed that OA treatment has resulted in tyrosine phosphorylation of a ~24 kDa caveolin-2. When the cells were incubated with OA-free medium for 3 hours, the tyrosine phosphorylation of caveolin-2 became stronger indicating that the effect of OA was not reversible. From these data we conclude that tyrosine phosphorylation of caveolin-2 plays an important role in the regulation of caveolin and caveolae distribution/localization in the cyto-plasm. Cytoskeleton seems to be involved in this procedure.

Materials and Methods

Materials

HepG2 hepatoma cell line was used for all experiments. The monoclonal antibodies (anti-caveolin-1 and anti-caveolin-2) were purchased from Transduction Laboratories, (Lexington, KI, USA). Anti-caveolin-1N, anti-caveolin-2 antibodies as well as anti-phosphotyrosine caveolin-1 have been purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibody against phosphotyrosine (4G40) was obtained from Upstate Biotechnology. The horseradish peroxidase-conjug-ated (HRP) anti-mouse and anti-rabbit IgG and the pre-stained standard protein markers were Bio-Rad (Hercules, CA, USA) products. The ECL nitrocellulose filter and the ECL reagent were manufactured by Amersham Bioscience Trading GmbH (Vienna, Austria). Protein phos-phatase 2A1 and protein tyrosine phosphatase (LAR) were purchased from Calbiochem (Lucerne, Switzerland).

Biotinylated anti-mouse IgG was obtained from Vector Laboratories (Burlingame, CA, USA), Alexa-conjugated anti-rabbit antibody (488 nm) and Alexa-conjugated avidin (594 nm) were purchased from Molecular Probes (Eugene, OR, USA).

Western blot analysis

HepG2 cells were solubilized with 1% SDS in 20 mM TRIS-HCL buffer, (pH 7.4) and boiled for 4-5 min. The protein contents of the lysates were measured with Lowry's method. 10-30 mg protein was separated on gradient (8-12% acrylamide) SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked with 3% BSA (dissolved in 0.1% Tween-PBS). Incubation was carried out with anti-caveolin-1 (VIP21) IgG (1:250), or anti-caveolin-2 (1:250) antibody. The second antibody (anti-rabbit IgG-HRP) was diluted to 1:10,000 (for anti-caveolin-1) and 1:3,000 (for anti-caveolin-2). The conditions of immunoblotting (incubation time, washing, ECL detection) were chosen as suggested by the manufacturer (Amersham Bioscience). After ECL detection the results were evaluated with an LKB Laser Densitometer using the GelScan program.

Immunoprecipitation

Cells (10^7) were lysed in 200 ml solubilization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 10% glycerol, 0.5% Nonidet P40, 0.1 mM PMSF, 10 mg/ml aprotinin). The lysates were incubated with specific antibodies, anti-caveolin-1 and anti-caveolin-2, for 5 h at 4°C. Immune complexes were formed by addition of protein-A-Sepharose 4B and incubated for 1 h at 4°C. The immune complexes were then sedimented by centrifugation at 12,000 g, followed by 4-5 washes in lysis buffer. Bound proteins were solubilized and analyzed on SDS-PAGE, follwed by immunoblotting. In some exper-imental groups the immunoprecipitates were treated with tyrosine phophatase (15 min at 25°C) dissolved in a buffer containing 20 mM Tris-HCL and 1 mM EDTA (pH 7.4).

Immunocytochemistry

HepG2 cells were fixed in methanol-aceton mixture at -20°C. After fixation endogenous biotin was blocked, then the cells were washed. To prevent aspecific binding the cells were treated with 1% BSA/PBS and incubated with anti-caveolin-1 (1:200) and anti-caveolin-2 (1:100) antibodies for over-night. After washig (3 times in 0.1% Triton-X 100 containing PBS)

anti-caveolin-1 was detected with Alexa-conjugated anti-rabbit IgG (488 nm, 1:100) To detect caveolin-2 a second antibody (biotinylated anti-mouse IgG in 1:100 dilution) was used and it was visualized with Alexa-conjugated avidin (594 nm, 1:100). Fluoromont was used to cover the cells. The samples were studied with Zeiss Axiophot microscope and with MRC 1024 Bio-Rad confocal scanning microscope.

Results

The effect of ocadaic acid on the intracellular distribution of caveolin-1 and caveolin-2 isoforms

When we used fluorescent and confocal immunocytochemistry we have found that OA in both 4 and 100nM concentrations has significant effect on the intracellular distribution of the caveolin-1 and caveolin-2 as well. In control (-OA treatment) the caveolin-1 was found in small punctate structures on the plasma membrane, but it was also detectable all over the cytoplasm. (Fig.1b) There was no preferential localization or cluster of these isoform. Caveolin-2 showed some colocalization with caveolin-1, but a significant amount caveolin-2 was detected around the nucleus (Fig 1c). After OA (4 and 100 nM) treatment caveolin-1 has disappeared from the cell membrane, it was present mainly in the cyto-plasm in larger vesicles or vacuole-like structures (Fig. 2b and Fig. 3b). Most of these vesicles were arranged in rows, along cable-like structures suggesting that the cytoskeleton must be involved in remodelling of the caveolin distribution pattern. Although in many cases caveolin-2 was found to colocalize with caveolin-1, there was always a significant amount of caveolin-2 present around the nucleus (Fig. 2c and Fig 3c). When OA was removed and the cells were incubated for 3 hours in an OA-free culture medium the perinuclear localization of caveolin-2 remained (Fig 4c and Fig 5c) while caveolin-1 was found to follow the arrangement of the cytoskeleton. (Fig 4b and Fig 5b).

The effect of ocadaic acid on the phosphorylation of caveolin isoforms

When we immunoprecipitate proteins from the lysate of HepG2 cells with anti-caveolin-2 antibody a 24 kDa protein was the only one immunoprecipited with this antibody. When we used anti-caveolin-2 antibody this 24 kDa band showed a strong labelling indicating that this protein is caveolin-2. Our Western blot analysis revealed that in OA-treated cells this 24 kDa protein (caveolin-2) was strongly phosphorylated on tyrosine residues (Fig 6 H/4 and H/100), giving evidence that OA treatment induces a strong tyrosine phosphorylation of caveolin-2 in HepG2 cells. The effect of OA was not reversible, since the removal of OA has not resulted in the dephosphorylation of caveolin-2 (Fog 6 RH/4 and RH/100).

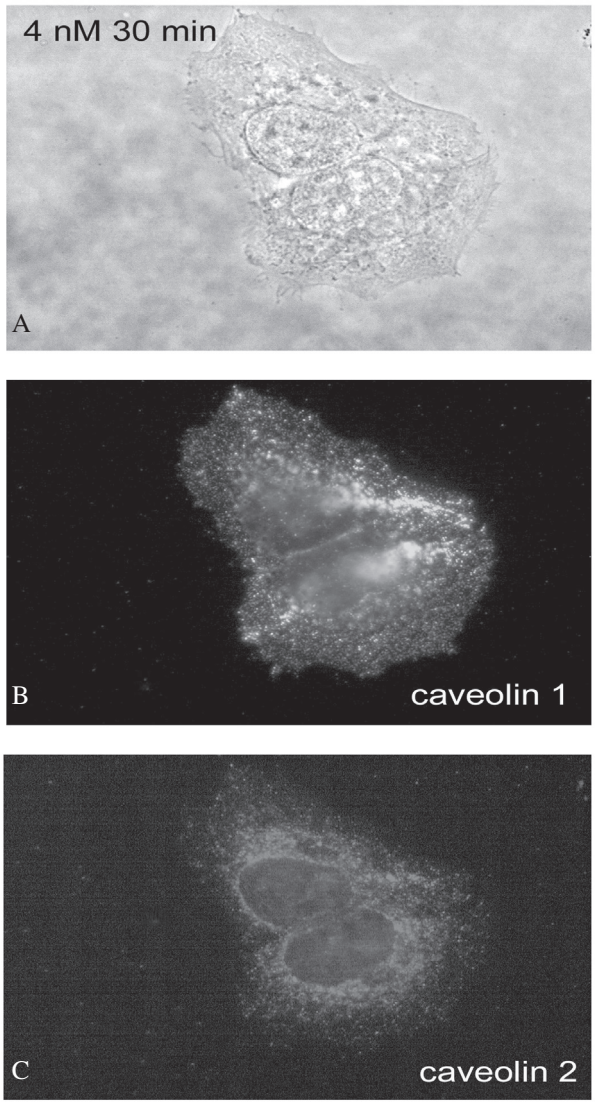

Figure1. The intracellular localization of caveolin-1 (**B**) and caveolin-2 (**C**) in control (no OA treatment cells. (**A**) phase contrast micrograph of a HepG2 cell. Caveolin-1 was found on discrete punctate structures on the cell surface and also in the cytoplasm (**B**). Caveolin-2 has partly overlapped with caveolin-1, but there is a preferential localization around the nucleus (**B**). Magnification: 1000x

Discussion

It has been generally accepted that besides clathrin-coated uptake mechanism alternative endocytotic pathways also exist. There are several candidates by which this alternative uptake can occur, one of them is the endocytosis via caveolae. Although it has been debated for a long time that caveolae can pinch off from the plasma membrane to form primary endocytotic vesicles (Anderson 1993; Van Deurs et al. 1993), there is an increasing body of evidence confirming that caveolae do pinch off from the plasma membrane and are directly involved

in the internalization of membrane com-ponent, extracellular ligands (Parton et al. 1994; Schnitzer et al. 1994; Berliname et al. 1998; Monteasno et al. 1998; Nichols et al. 2001; Stahl and Mueller 1995).

The caveolae-mediated endocytosis differs from clathrin-mediated pathway in many respects. In contrast to clathrin-coated endocytosis, the internalization of caveolae seems to be regulated by kinases and phosphatases. The increased phosphorylation of proteins associated with caveolae (Parton, 1994; Smart et al. 1995) or caveolin isoforms themselves (Aoki et al. 1999) stimulate caveolae to pinch off, and dephosphorylation of these protein might be required for recycling of these vesicles to the cell surface (Smart et al. 1995). The precise mechanism, substrates as well as kinases and phophatases taking part in this dephosphorylation procedure are not known. In order to study the effect of phosphorylation/dephosphorylation on the surface distribution and cytoplasmic localization of caveolin isoforms we have used ocadaic acid (OA). OA is a well-known serine/threonine (PP1 and PP2) protein phosphatase inhibitor (Wera and Hemmings 1995).

Our immunocytochemical studies showed that as a result of OA treatment in HepG2 cells the cytoplasmic and plasma membrane distributions of caveolin-1 and caveolin-2 have changed. Caveolin-1 has disappeared from the cell surface and was detected in vesicles arranged in rows in the cyto-

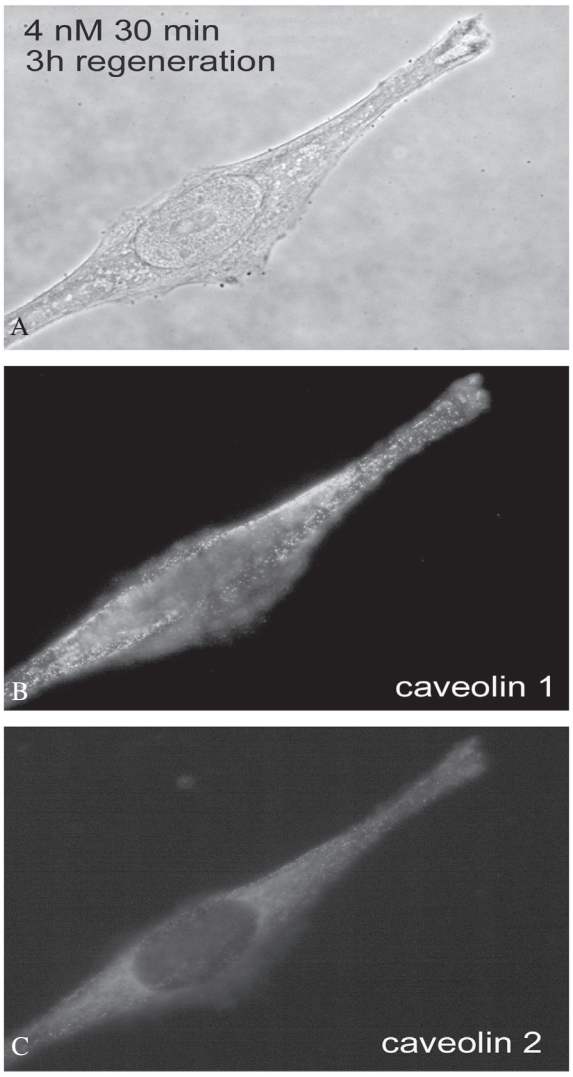

Figure 2. The effect of 4 nM OA treatment on the cellular distribution of caveolin-1 and caveolin-2. (**A**) phase contrast micrograph of HepG2 cells. After 30min OA treatment caveolin-1 has disappeared from the plasma membrane and was present in rows (**B**). Caveolin-2 was found in similar localization, but caveolin-2 containing perinuclear ring was still present (**C**). Magnification: 1000x

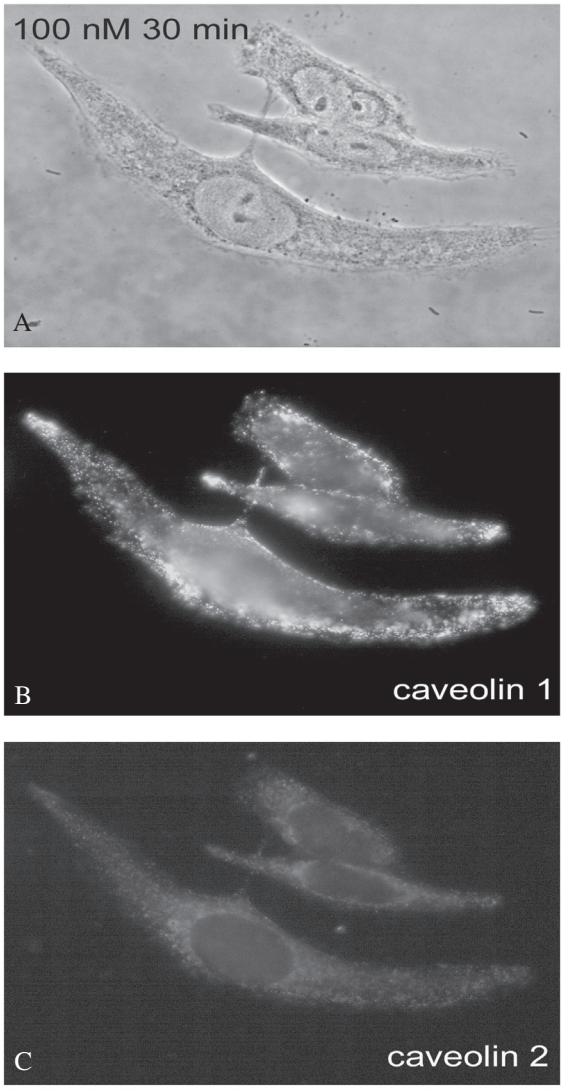

Figure 3. The effect of 100 nM OA on the cellular distribution of caveolin-1 and caveolin-2. (**A**) phase contrast micrograph of HepG2. Caveolin-1 was present in the periphery of the cytoplasm and it was arranged in rows (**B**). The caveolin-2 localization was similar to the 4 nM OA-treated cells (**C**). Magnification: 1000x

plasm. Since caveolae can be characterized by the presence of caveolin-1 and caveolin-2 (caveolin-2 seems to play an accessory role in caveolae-formation), the cytoplasmic route of caveolae can be followed by detection (localization) of these isoforms. From these data we think that caveolae have also disappeared from the cell surface. Although caveolin-2 was partly colocalized with caveolin-1, there was a significant amount of this isoform present in the perinuclear region. When OA was removed and the cells were incubated with an OA-free medium for 3 hours the localization pattern of the caveolin isoforms has changed again. Caveolin-1 was still arranged along cables, while caveolin-2 was detected around the nucleus. This perinuclear caveolin-2-containing ring was more prominent then in control cells. We were not able to detect caveolin-1 and –2 in punctate structures on the cell membrane. Our immunoprecipitation and Western blot analysis showed that OA treatment has resulted in tyrosine phosphorylation of a ~24 kDa protein which was identified as caveolin-2. When the cells were incubated with OA-free medium for 3 hours the tyrosine phosphorylation of the ca-

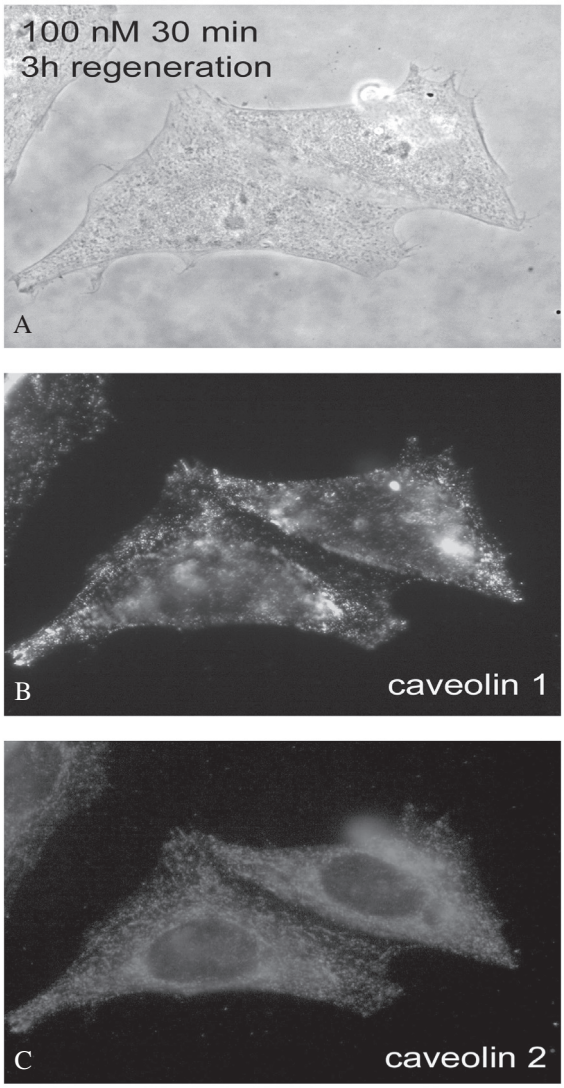

Figure 4. When 4 nM OA was removed and the cells were incubated in OA-free medium for 3 hours caveolin-1 was still present in the cytoplasm (in rows) and not on the cell membrane (**B**). The distribution of caveolin-2 has not changed (**C**) Magnification: 1000x

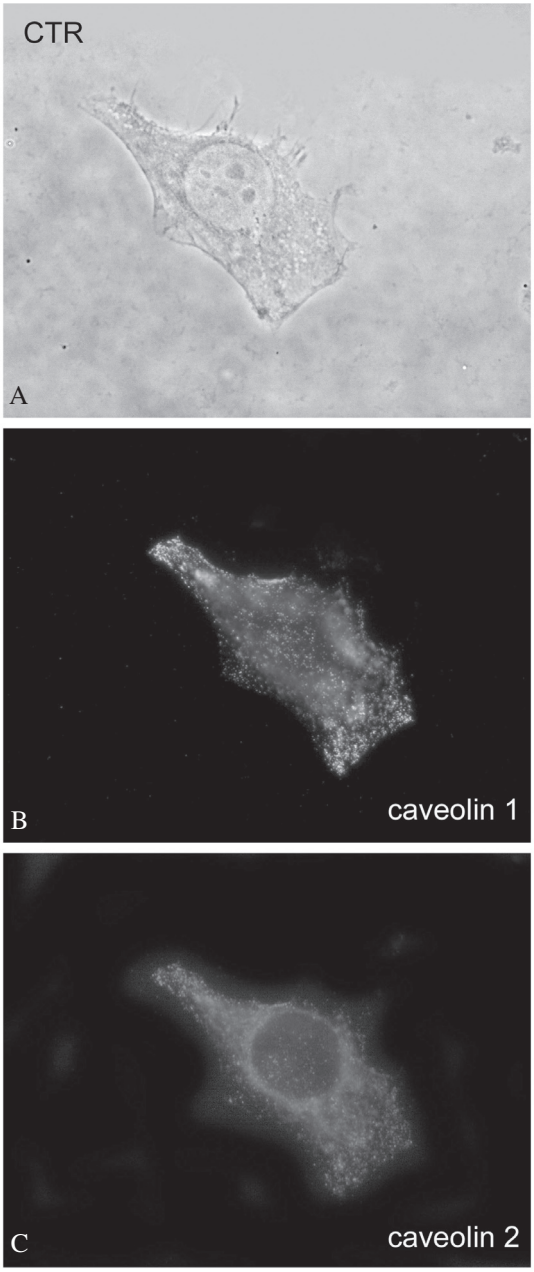

Figure 5. After 3 hours incubation in OA-free medium the effect of 100 nM OA was not found to be reversible. There was no caveolin-1 containing vesicles present on the plasma membrane (**B**), and caveolin-2 was found around the nucleus (**C**). Magnification: 1000x

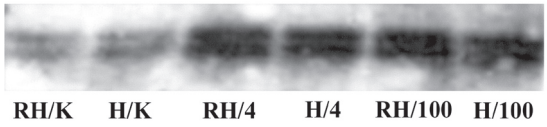

Figure 6. Immunoprecipitation with anti-caveolin-2 and Western blot analysis with anti-phosphotyrosine antibodies. Caveolin-2 (24 kDa) was strongly labelled on tyrosine residue when the cells were incubated with 4 and 100 nM ocadaic acid. 3 hours incubation without OA has not resulted in the dephosphorylation of caveolin-2. (RH/K: Control HepG2 cells 3 h in culture medium; H/K: control HepG2 cells 30min in culture medium; RH/4: HepG2 cells treated with 4 nM OA and 3 h in OA-free medium; H/4: HepG2 cells treated with 4 nM OA; RH/100: HepG2 cells treated with 100 nM OA and 3 h in OA-free medium; H/100: HepG2 cells treated with 100 nM OA.)

veolin-2 became stronger indicating that the effect of OA was not reversible. Although OA is a serine/threonine phophatase inhibitor, we propose that OA treatment causes tyrosine phosphorylation of caveolin-2 indirectly, through phosphorylation of other members of a phosphorylation cascade. It is known that the Src family tyrosine kinases can be efficiently blocked by PP1 and PP2 phosphatases (Marinissen and Gutkind 2001). Since caveolin was first identified as a substrate for phosphorylation by v-src (Glenney and Zokas 1989), and Src kinases were also found to be associated with caveolae (reviewed by Anderson 1998), we suggest that OA causes tyrosine phos-phorylation of caveolin isoforms through this phosphorylation cascade.

From these results we conclude that the tyrosine phosphorylation of caveolin-2 can be responsible for removal of caveolae from the cell surface. Since there are data suggesting that dephosphorylation of proteins associated with caveolae would be necessary for caveolae recycling (Smart et al. 1995), we think that in OA treated cells caveolae can not recycle back to the cell surface. Our data strongly suggest that tyrosine phosphorylation of caveolin-2 plays an im-portant role in the regulation of caveolin and caveolae distribution/localization in the cytoplasm. Cytoskeleton seems to be involved in this procedure.

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