Dynamics and interaction of caveolin-1 isoforms with BMP-receptors

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Accepted 7 July 2004
Journal of Cell Science 117, 643-650 Published by The Company of Biologists 2004
doi:10.1242/jcs.01402

Summary

Caveolae are small invaginations of the cell membrane that are thought to play a role in important physiological functions such as cell surface signaling, endocytosis and intracellular cholesterol transport. Caveolin-1 is a key protein in these domains and contributes to the organization of cholesterol and saturated lipids within these vesicular invaginations of the plasma membrane. Caveolae are thought to be involved in the signaling of tyrosine kinase receptors and serine threonine receptors. In this article we focus on the involvement of caveolae in the signal transduction of bone morphogenetic proteins (BMPs). BMPs play important roles during embryonic development and especially in chondrogenesis, osteogenesis, neurogenesis and hematopoiesis. The initiation of the signal tranduction starts by the binding of a BMP to a corresponding set of BMP receptors.

Using image cross-correlation spectroscopy, we show that the BMP receptors BRIa and BRII colocalize with caveolin-1 isoforms α and β on the cell surface. BRIa colocalizes predominantly with the caveolin-1 α isoform. Coexpression of BRII leads to a redistribution of BRIa into domains enriched in caveolin-1 β. After stimulation with BMP-2, BRIa moves back into the region with caveolin-1 α. BRII is expressed in regions enriched in caveolin-1 α and β. Stimulation of cells with BMP-2 leads to a redistribution of BRII into domains enriched in caveolin-1 α. Immunoprecipitation studies using transfected COS-7 cells indicate that BRII binds to caveolin-1 α and β. The binding of BRII to caveolin-1 was verified using A431 cells. Stimulation of starved A431 cells with BMP-2 lead to a release of caveolin-1 from the BMP receptors. We show further that the caveolin-1 β isoform inhibits BMP signaling whereas the α isoform does not.

Key words: Image correlation spectroscopy, BMP receptors, Caveolin-1, Fluorescence, Membrane distribution

Introduction

Caveolae are small invaginations of the cell membrane that are thought to play an important role in cell surface signaling, endocytosis and intracellular cholesterol transport (Parton, 1996; Anderson, 1998; Fujimoto et al., 1998). Caveolin-1, 2 and 3 were shown to be the major protein components of these membrane domains (Rothberg et al., 1992; Scherer et al., 1996; Tang et al., 1996). Caveolin-1 is expressed in two isoforms, caveolin-1 α and caveolin-1 β, which differ in their protein coding sequence starting at the methionine at position 1 and 32, respectively. Both isoforms have a common hydrophobic stretch of amino acids, a scaffolding domain and an acylated C-terminus (Scherer et al., 1995). The two caveolin isoforms show an overlapping, yet slightly different distribution on the cell surface (Fujimoto et al., 2000). Previous work has shown that the α and β isoforms have a slightly different role in caveolae formation (Fujimoto et al., 2000). The data suggest that there are caveolae composed only (or mainly) of the β isoform as well as caveolae with both isoforms present (Fujimoto et al., 2000; Nohe et al., 2004). With some notable exceptions (e.g. the interaction of caveolin-1 with the Insulin receptor, where the interaction stimulates phosphorylation of downstream targets) caveolin-1 binding to a protein inhibits the activity of that protein by maintaining it in an inactive state unless a stimulus is presented. For other signaling proteins, caveolin-1-binding serves to terminate signal transmission after activation (Schlegel and Lisanti, 2001). Since caveolin-1 is involved in regulation of receptor kinases our focus was to understand the influence of caveolin-1 on bone morphogenetic protein (BMP) signaling.

BMPs and growth differentiation factors (GDFs) belong to the transforming growth factor β (TGF β) superfamily. Both play important roles during embryonic development in chondrogenesis, osteogenesis, neurogenesis and hematopoiesis (Hogan, 1996; Bhatia et al., 1999; Celeste et al., 1990; Cunningham et al., 1995; Storm et al., 1994; Dunn et al., 1997; Padgett et al., 1987). They bind to BMP-receptors, which are serine threonine kinases. After ligand binding to at least one type-I and one type-II receptor, the type-II receptor phosphorylates the type-I receptor at the GS-box. This leads to the initiation of the Smad signaling cascade in which Smad1, 5 or 8 are phosphorylated. They in turn bind to Smad4 and are translocated to the nucleus (Kretschmar et al., 1997; Massague, 1998; Hoodless et al., 1996; Kawabata et al., 1995; Liu et al., 1995; Nohno et al., 1995). Previous studies have also shown

an activation of the p38 pathway upon ligand binding, but the mechanism for its activation is poorly understood (Kimura et al., 2000; Shirakabe et al., 1997; Nakamura et al., 1999; Nohe et al., 2002). BMP receptors exist on the cell surface as preformed hetero-oligomer complexes (PFCs), and as homo-oligomeric complexes. The ligand may bind to PFCs initiating the Smad signaling pathway, or to the high affinity binding receptor type-I (Evan et al., 1985) (BRI), which is then recruited by the type-II (BRII) into a new complex, the BMP-induced signaling complex (BISC), leading to the activation of the p38 pathway (Nohe et al., 2002). Recent data also indicate that BMP receptors are very flexible and dynamic on the cell surface and that the change of their aggregation and clustering is important for their signaling (Nohe et al., 2003).

We seek to understand whether these protein complexes are associated with other protein or lipid complexes that are represented in part by the caveolae. Here, we investigate the colocalization of the BMP receptors type-II (BRII) and type-I (BRIa) with caveolin-1 using image cross-correlation spectroscopy (ICCS). We show that BRII binds to caveolin-1 α and caveolin-1 β. Caveolin-1–BMP receptor interactions are released after stimulation with BMP-2, one of the most important BMPs in bone formation (Wozney et al., 1988). These results suggest that caveolin-1 inhibits the activation of BRIa in PFCs by binding to BRII. Reporter gene assays also indicate that, although caveolin-1 β inhibits BMP signaling, caveolin-1 α has no impact on the activation of the signaling cascade.

Materials and Methods

Recombinant BMP-2 was obtained from Wyeth Institute (Boston, NY). Biotinylated monoclonal antibody 9E10 directed against the myc tag (Evan et al., 1985) (anti-myc) was purchased from Babco (Berkely, CA). Antibody 12CA5 against the influenza hemaglutinin (HA) tag (Wilson et al., 1984) was provided by David Litchfield (Department of Biochemistry, The University of Western Ontario). The polyclonal antisera against the BMP receptors, BRIa and BRII, were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies recognizing either caveolin-1 α (clone 2234) alone or both of the caveolin-1 α and β isoforms (clone 2297) were purchased from Transduction Laboratories (New York, NY). The transferrin monoclonal antibody was obtained from Sigma (Oakville, ON, Canada). Secondary antibodies (donkey anti-goat RRX and goat anti-mouse Alexa Fluor 488) were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase- (HRP-) conjugated goat anti-mouse secondary antibody was from Santa Cruz. HRP-conjugated streptavidin was from Sigma. The cell lines, COS-7 (CRL 1651) and A431 (CRL 1555) were purchased from American Type Culture Collection (Manassa, VA). Plasmids encoding myc-BRIa, HA-BRIa, myc-BRII and HA-BRII were from P.K.'s laboratory as previously reported (Nohe et al., 2002; Nohe et al., 2003). Plasmids encoding caveolin-1 α-GFP and caveolin-1-β-GFP were provided by Hiroshi Kogo (Department of Anatomy, Nagoya University School of Medicine, Nagoya, Japan).

Transfection of COS-7 and A431 cells

COS-7 and A431 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GibcoBRL, Burlington, ON, Canada) and transfected by using the DEAE-dextran method (Seed and Aruffo, 1987). For immunoprecipitation, cells were grown in 35-mm dishes and transfected with 1 μg of DNA per construct.

Immunofluorescence labeling of cell surface receptors

The specificity of the polyclonal antisera against BRIa and BRII was tested by transfecting COS-7 cells with plasmids encoding HA-BRIa and myc-BRII. Cells were labeled with HA-FITC antibody (Santa Cruz Biotechnology) and the polyclonal antisera, followed by a donkey anti-goat RRX secondary antibody. The collected images show 100% colocalization with the receptors in addition to some background fluorescence. To measure the distribution of the BMP receptors on the cell surface, we used image correlation spectroscopy (ICS) and ICCS. A431 cells were cultured on 22-mm coverslips and either grown under normal conditions or serum-starved. Following 72 hours of serum-starvation, cells were either stimulated with BMP-2 for 2.5 hours or not, and fixed using acetone-methanol (Brown and Petersen, 1998) or paraformaldehyde-saponin treatment (Wang et al., 2003). Cells were blocked for 30 minutes with 5% BSA and then incubated with polyclonal antiserum, recognizing either BRII or BRIa, and with antibodies recognizing caveolin-1 α (clone 2234) or caveolin-1 α and β (clone 2297) according to manufacturers' protocols. Cells were washed 3× with PBS for 5 minutes and then incubated with the corresponding secondary antibodies at a concentration of 20 μg/ml. Following this, cells were washed again 3× for 5 minutes with PBS and the coverslips were mounted on airvol and dried overnight.

Immunoprecipitation

Transfected COS-7 or untransfected A431 cells were washed twice with ice-cold PBS and solubilized in lysis buffer (containing 10 mM Tris pH 7.5, 50 mM NaCl, 1% Triton X-100, 60 mM octyl gluCOSide, 1 mM PMSF, 10 mg/ml each of leupeptin, aprotinin, soybean trypsin inhibitor, benzamidine-HCl, pepstatin and antipain) at 4°C for 60 minutes. Epitope-tagged receptors were immunoprecipitated from extracts of transfected COS-7 cells by 12CA5 monoclonal antibodies (α-HA, 20 μg/ml; α-myc; 20 μg/ml) for 12 hours at 4°C, followed by incubation with protein G-Sepharose (30 μl of a 1:1 suspension in PBS) for 1 hour at 4°C. Endogenous receptors from A431 cells were precipitated with specific polyclonal antiserum (25 μg) for 2 hours at 4°C, followed by incubation with protein G-Sepharose (Pharmacia, Mississauga, ON, Canada) at 4°C for 1 hour. The G-Sepharose beads were washed three times with PBS. The bound protein was eluted by incubating the beads in SDS-PAGE sample buffer containing mercaptoethanol (5 minutes, 95°C). The protein solution was then subjected to electrophoresis on a 10-12% SDS-PAGE.

Western blots

Western blots were performed according to standard protocols. After electrotransfer and blocking (1 hour at room temperature in 10 mM Tris pH 7.9, 150 mM NaCl, 0.1% Tween 20, 3% BSA), the blot was incubated for 12 hours at 4°C in blocking buffer with monoclonal antibodies against the caveolin-1 α and β isoforms or the 9E10 antibody (anti-myc) (both antibodies were used at 10 μg/ml). Blots were washed 3× with blocking buffer and then incubated for 1 hour with the HRP-conjugated secondary antibody. Detection of adsorbed antibodies was performed by enhanced chemiluminescence (ECL) (Pierce, Brockville, ON, Canada), employing peroxidase-conjugated goat anti-mouse antibodies diluted 1:10,000 and HRP-conjugated streptavidin diluted at 1:25,000.

Luciferase reporter assay

A431 cells grown in 60-mm dishes were transfected with the pSBE, which is sensitive to the Smad signalling pathway (Jonk et al., 1998) and the BMP-receptor constructs using the DEAE dextran method. After transfection, cells were stimulated or not stimulated for 12 hours

with 20 nM BMP-2. Cells were lysed and luciferase activity was measured using a dual-luciferase assay system (Promega, Madison, WI).

Confocal microscopy

Labeled cells were visualized by using a Biorad MRC 600 Confocal microscope equipped with an argon-krypton mixed-gas laser and by using the appropriate filter sets for dual-fluorophore imaging. Cells expressing the receptors were selected under mercury lamp illumination with a 60× (1.4 NA) objective on an inverted Nikon microscope. An area of the cell, away from the nucleus, was enlarged and visualized. For measuring fluorescence of FITC, the filter was set for 488 nm laser excitation, and neutral density filters were used to attenuate the laser to 1% laser power. Fifteen scans were accumulated on photomultiplier tube 2 (PMT2) in the photon counting mode (to ensure linear scaling of the intensity). For measuring RRX-fluorescence, the filter was then shifted to allow excitation with the 568 nm laser line and 20 scans were accumulated on PMT1. The two photomultiplier tubes were set with the black level at 6.0 on the vernier scale, and the gain set at ten. After the collection of each set of 19 images, images were collected using identical settings but with the shutter to the sample closed, to obtain a measure of the dark current for each PMT.

Image correlation spectroscopy

Image Correlation Spectroscopy (ICS) and Image Cross-Correlation Spectroscopy are two techniques that can be used to study the distribution and localization of receptors (Brown and Petersen, 1998; Srivastava and Petersen, 1998; Wiseman and Petersen, 1999; Wiseman et al., 1997). ICS involves autocorrelation-analysis of the intensity-fluctuations within collected confocal images, in this case, of transfected cells that contain immunofluorescently labeled proteins.

The cluster density (CD) value gives the average number of receptor clusters per unit area, where g(0,0) is the amplitude of the autocorrelation function (Fig. 1H), w is the width of the function and N_p is the average number of particles available.

$$CD = \frac{1}{g(0,0)\pi w^2} = \frac{\overline{N}_p}{\pi w^2} . \quad (1)$$

In this work, the average CD values are normalized to the average CD value of cells transfected with either HA-BRIa or myc-BRII. For A431 cells, average CD values were normalized to the values for BRIa in normally cultured A431 cells and for BRII in serum starved A431 cells.

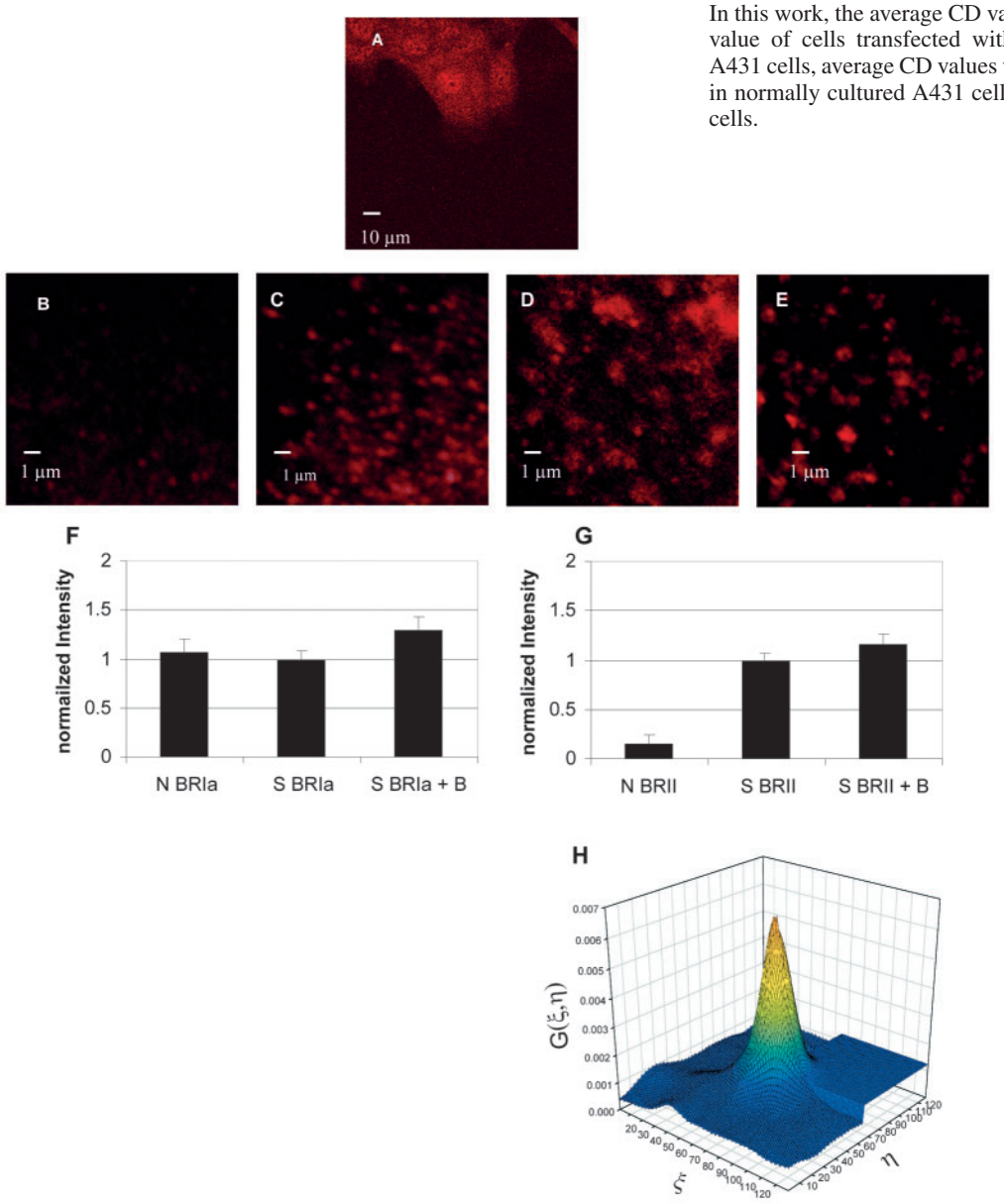

Fig. 1. BRII is upregulated when A431 cells are serum-starving. Serum-starved or non-serum-starved A431 cells were fixed and fluorescently labeled to test for the expression of (B,C,G) BRII or (D,E,F) BRIa , using a polyclonal antiserum against BRII and a secondary donkey anti goat RRX antibody, respectively. Cells were fixed and high magnification images of flat membrane regions were collected with a confocal microscope. (F,G) Fluorescence-intensities for quantification, calculated from a large number (~40) of images from different cells. Zoom-1 image of non-serum-starved A431 cells (A) labeled for BRIa, (B) stained for BRII. (C) Serum starvation of A431 cells leads to upregulation of BRII at the cell surface. (D) Non-serum-starved A431 cells that express BRIa. (E) Starved cells express comparable amounts of BRIa at the cell surface. (F) Expression levels of BRIa are the same for non-starved (N BRIa), starved (S BRIa) and stimulated (S BRIa + B) A431 cells. (G) Expression levels of BRII are very low for non-starved (N BRII) A431 cells, but is increased many-fold after starvation for 72 hours (S BRII) and remains constant upon stimulation with BMP-2 (S BRII + B). (H) A typical autocorrelation function for BRIa.

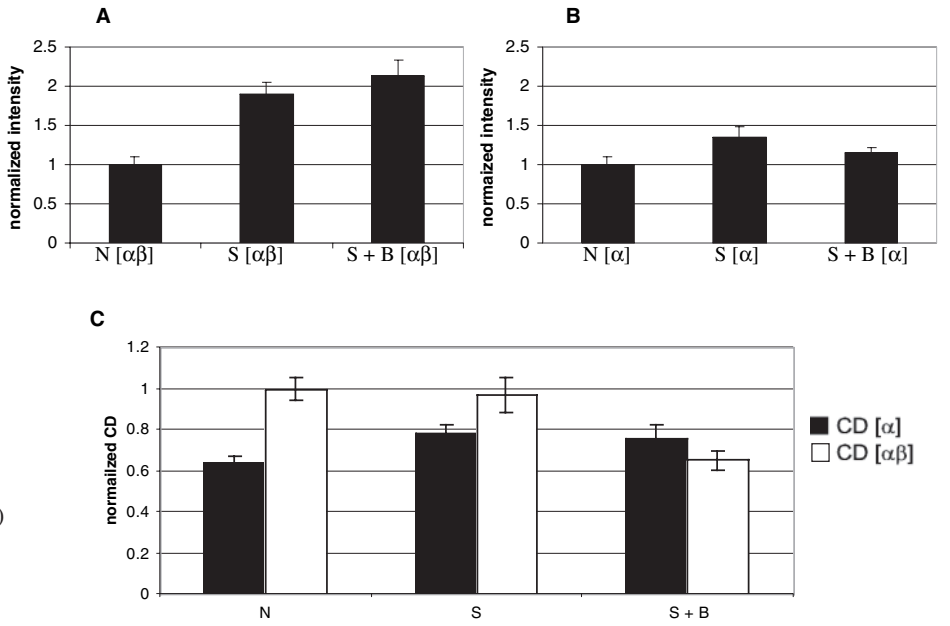

Fig. 2. Upon BMP-2 stimulation, caveolin-1 β moves into caveolae enriched in caveolin-1 α. A431 cells were either cultured normally in DMEM with 10% FBS (N) or were serum-starved (S). After 3 days, starved cells were stimulated (S+B), or not stimulated (S) with BMP-2, fixed and labeled for (B) caveolin-1 α [α] or (A) caveolin-1 α β [αβ] with monoclonal antibodies against the caveolin-1 isoforms and a secondary fluorescently labeled antibody against the caveolin-1 antibodies. Cell membrane expression was visualized by confocal microscopy. From 40 different cells 40 high-magnification images of the membrane were collected and the average intensity of the labeled caveolin-1 was calculated. For each image, the cluster density (CD) of each of the caveolin-1 isoforms was calculated by ICS (see Materials and Methods) and expressed as a ratio relative to the CD observed for the antibodies recognizing caveolin-1 α and β (C).

Image cross-correlation spectroscopy

ICCS is an extension of ICS, where a cross-correlation function is generated by calculating the coincident fluorescence-intensity-fluctuations in images collected from two different chromophores that are attached to two different protein or receptor types. One receptor type is labeled with a green probe and is imaged to give the intensity map, $i_g(x,y)$, whereas the second receptor type is labeled with a red probe and imaged to give the intensity map, $i_r(x,y)$. The fraction of each protein type associated with the other type can be calculated by looking at the ratios of CD values:

$$F(g/r) = \frac{CD_{gr}}{CD_g} \quad \text{or} \quad F(r/g) = \frac{CD_{gr}}{CD_r}, \qquad (2)$$

where F(g/r) represents the fraction of green labeled protein clusters that contain red labeled proteins. Correspondingly, F(r/g) is the fraction of red labeled protein clusters containing green labeled proteins.

Thus, ICCS provides quantitative information about the extent of colocalization of two different receptors or, here, receptor and binding proteins. If the interaction is transient, F will be smaller but still greater than zero.

Statistics

Standard error of the mean (s.e.m.) values were calculated from the raw data at the 95% confidence level.

Results

BRII expression is upregulated in starved A431 cells

We examined the BMP receptor expression in A431 cells by immunofluorescence, using polyclonal antisera against BRIa or BRII. For this, we collected high-magnification confocal images of flat regions within the cell membrane. The spots in Fig. 1A-D represent clusters of receptors on the cell surface of A431 cells. Fig. 1A-D shows a sample of high-magnification images of the flat regions of the cell membrane. As shown in panels A and B, starvation of the cells upregulates BRII expression on the plasma membrane. Panels C and D confirm that BRIa is expressed at comparable levels at the surface in both, normal and starved, A431 cells. Panel E and F summarize average fluorescence-intensity measurements from about 40 cells in each experiment. Within experimental error, the total number of BRIa receptors is unaffected by the starvation, whereas BRII expression is upregulated. We used the upregulation of BRII that naturally occurs in A431 cells, to study the effect of this upregulation on the colocalization of BRIa and BRII with different caveolin-1 isoforms.

Starvation of A431 cells followed by BMP-2 stimulation leads to the reorganization of caveolin-1 isoforms

Normally cultured and starved A431 cells were fixed and fluorescently labeled with antibodies recognizing only caveolin-1 α or both isoforms, caveolin-1 α and β. As shown in Fig. 2, panels A and B, the intensity of fluorescence detected for caveolin-1 α is only marginally increased upon starvation (Fig. 2B), whereas the fluorescence for caveolin-1 α and β almost doubled (Fig. 2A). This suggests that caveolin-1 β expression is upregulated upon starvation of A431 cells, whereas the expression of caveolin-1 α is not significantly changed. The CD value, the average number of clusters per unit area, detected for caveolin-1 α, and for caveolin-1 α and β is nearly the same in normally cultured and in starved A431 cells (Fig. 2C). This indicates that, when its expression is upregulated, caveolin-1 β is still expressed in the same areas on the cell surface. BMP-2 stimulation leads to a decrease in the CD value of caveolin-1 α and β, indicating that the caveolin-1 β isoform forms caveolae with the α isoform only.

Redistribution of BRIa by upregulation of BRII in A431 cells

A431 cells were cultured for 76 hours in DMEM with 10% FBS or without FBS. After 3 days, cells were fixed and BRIa and BRII were fluorescently labeled using polyclonal antisera. As a control, BRII was co-transfected in normally cultured A431 cells and cells were stained for the caveolin-1 isoforms,

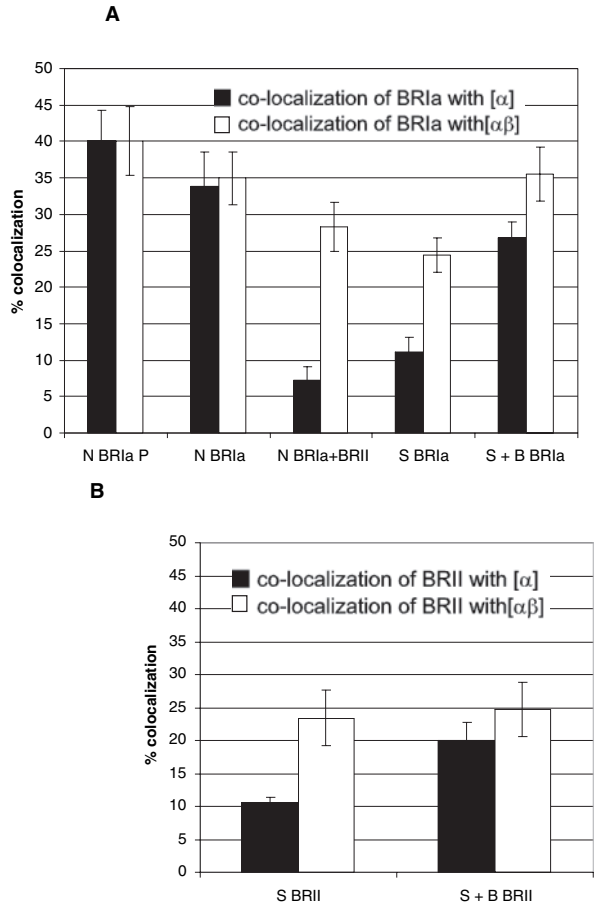

Fig. 3. Redistribution of BRII in A431 cells. Colocalization of BMP receptors with caveolin-1 isoforms. A431 cells were either cultured in DMEM with 10% FBS (N) or were serum-starved (S). A431 cells cultured with serum were transfected with BRII (N +BRII) or not. After 3 days starved cells were stimulated with BMP-2 (S+B)or not stimulated (S). Cells were than fixed with paraformaldehyde-saponin (P) or acetone-methanol; BMP-receptors were labeled using polyclonal antisera against BRIa or BRII and a secondary fluorescently labeled antibody. The same cells were labeled for caveolin-1 using an antibody specific for the α isoform [α] or for both α and β isoforms [αβ] and a secondary fluorescently labeled antibody. Cell membrane expression was visualized by confocal microscopy and 40 images were collected at the highest magnification from 40 different cells. From these images the fraction of BMP receptors colocalizing either with caveolin-1 α or with α and β were calculated using ICCS. (A) The percent colocalization of BRIa with both isoforms of caveolin-1 [α] (black bars) or with both isoforms (all the caveolae) as well as β alone [αβ] (white bars). (B) The percent colocalization of BRII with caveolae with both isoforms [α] (black bars) and those with both isoforms and those with mainly β [αβ] (white bars).

using antibody recognizing either caveolin-1 α or both isoforms. Images were collected as described and analyzed by ICCS to calculate the fraction of BRIa colocalizing with either caveolin-1 α, or caveolin-1 α and β (Fig. 3A). In the normally cultured A431 cells – where BRII is absent – about 35% of BRIa colocalizes with caveolin-1 α. When BRII is co-transfected in these cells, the fraction of BRIa that colocalizes is higher with the antibody that recognizes both isoforms. This

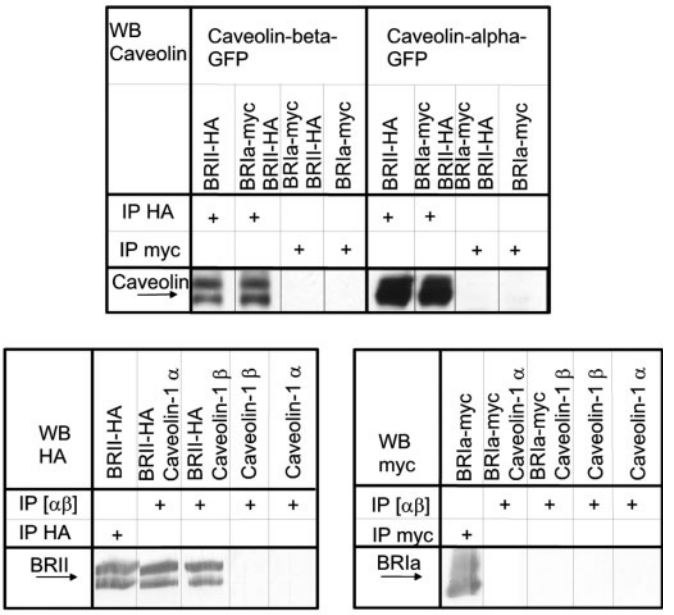

Fig. 4. BRII binds to caveolin-1 α and caveolin-1 β. COS-7 cells were co-transfected with plasmids encoding BRII-HA or BRIa-myc and one of the two caveolin isoforms, caveolin-1 α or β. After 48 hours, cells were lysed and proteins were immunoprecipitated as indicated. The immunoprecipitated proteins were subjected to SDS PAGE and transferred onto nitrocellulose. The nitrocellulose membrane was incubated with an antibody recognizing caveolin-1 α and β, the HA or myc- epitopes, and a secondary HRP conjugeated antibody. The top panel shows that the interactions with the caveolin isoforms is specific for BRII and that there are no direct interactions between BRIa and the caveolin-1. The two bottom panels show that BRIa is present in the transfected cells.

shows that BRIa associates only with caveolae that have both the α and β forms, or only the β isoform. The same result is obtained when A431 cells are starved. Then, the fraction of BRIa that colocalizes with the caveolin-1 α antibody is reduced to about 10%, whereas the fraction that colocalizes with the caveolin-1 α and β antibody is about 25%. This indicates that BRIa is dissociating from those caveolae that contain caveolin α and β together, and some of this is associating with caveolae containing predominantly caveolin-1 β. Correspondingly, Fig. 3B shows that the fraction of BRII colocalizing with caveolin-1 α antibodies is about 10% and the one colocalizing with caveolin-1 α and β antibodies is about 25%. This suggests that after the upregulation of BRII in A431 cells, BRIa and BRII are equally associated with the same caveolae, possibly together. However the majority of the BRIa and BRII are not present in any of the caveolae.

BRIa and BRII redistribute upon BMP-2 stimulation in A431 cells

To investigate whether BMP-2 stimulation affects the colocalization of BRIa and BRII with the caveolin-1 isoforms, we grew A431 cells for 3 days in DMEM without FBS and stimulated with 20 nM BMP-2 for 2.5 hours. The ICCS analysis was done to calculate the fraction of BRIa and BRII colocalizing with the caveolin-1 isoforms. Figs 3A and B both

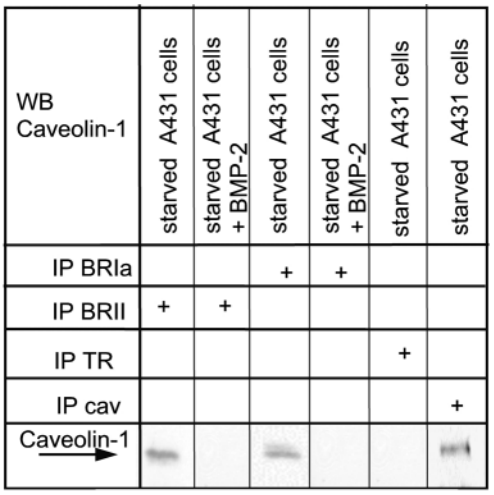

Fig. 5. Caveolin-1 interacts with BRIa and BRII in starved A431 cells. A431 cells were serum-starved for 72 hours were then stimulated with BMP-2 or left in medium for 2 hours as indicated. Cells were lysed and BRIa, BRII, the transferrin receptor (TR) and caveolin-1 (cav) were immunoprecipitated with specific antibodies against the proteins. The interaction with caveolin-1 was tested by western-blot. Caveolin-1 interacts with BRII and BRIa in starved A431 cells. After stimulation with BMP-2 these interactions are disrupted.

show that the fractions of BRIa and BRII that colocalize with caveolin-1 α antibodies and caveolin-1 α and β antibodies increase, and approach those observed for BRIa in the absence of BRII. It therefore appears that the stimulation causes a reassociation between the BMP-receptors and the caveolae.

BRII binds to caveolin-1 whereas BRIa fails to interact with caveolin-1

To examine whether colocalization of either BRIa or BRII with caveolin-1 isoforms is owing to direct binding or coexistence in similar domains, we transfected COS-7 cells with plasmids encoding BRII-HA, BRIa-myc, GFP-tagged caveolin-1 α or GFP-tagged caveolin-1 β. After cell lysis, using a lysis buffer containing octylglucoside to solubilize the caveolae, the BMP receptors were immunoprecipitated using antibodies against their respective epitope tags. The immunoprecipitates were subjected to SDS PAGE, transferred onto nitrocellulose and incubated with caveolin-1 antibodies to probe for presence of BMP receptor. As shown in Fig. 4, caveolin-1 is detected only when BRII is immunoprecipitated. This suggests that BRII directly binds both caveolin-1 isoforms, whereas BRIa binds neither. To confirm the interaction of BRII with caveolin-1, we lysed normal and serum-starved A431 cells, immunoprecipitated the lysate with antibodies against the BMP receptors and checked for caveolin-1 by western blotting. When BRII is immunoprecipitated, caveolin-1 is detected in serum-starved cells (Fig. 5). After stimulation of the serum-starved A431 cells with BMP-2, the interaction with caveolin-1 is no longer observed, suggesting that stimulation releases bound caveolin-1 from BRII and indicating further that, whereas BRII resides in caveolae (Fig. 3), there is no direct interaction with caveolin-1 following stimulation. Coimmunoprecipitation experiments with BRIa and caveolin-1 in COS-7 cells indicate that BRIa alone cannot bind to caveolin-1, whereas in A431 cells, BRIa can be immunoprecipitated with caveolin-1. Because BMP receptors exist as preformed complexes on the cell surface the data suggest that the preformed complex-formation in A431 cells is stronger than in COS-7 cells. This could be through a complex-stabilizing protein present in A431 cells, which endogenously express BMP receptors.

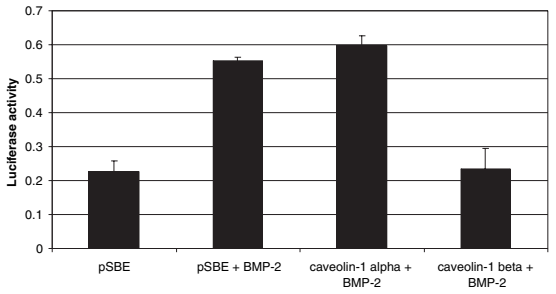

Fig. 6. Caveolin-1 β inhibits BMP signaling. Serum-starved A431 cells were transfected with the pSBE and caveolin-1 isoforms as indicated. Cells were stimulated or not with BMP-2 for 24 hours, lysed and reporter gene activity was measured.

Caveolin-1 β inhibits BMP signaling

A431 cells were transfected with the pSBE-Luc (Jonk et al., 1998), pRLSV40 and the caveolin-1 isoforms. The plasmid construct pSBE encodes a reporter gene, that specifically activates the Smad signaling pathway (Jonk et al., 1998; Nohe et al., 2003). Overexpression of caveolin-1 β in A431 cells leads to decreased reporter gene activity compared to A431 cells stimulated only with BMP-2 (Fig. 6). Interestingly, overexpression of the caveolin-1 α isoform does not result in a change of reporter gene activity in these cells. This clearly indicates that in these cells, the caveolin-1 α and β isoforms play different roles in BMP-signaling.

Discussion

There is mounting evidence that two different caveolar structures exist at the cell surface in fibroblasts: one enriched in caveolin-1 α and β, and one enriched in caveolin-1 β alone. Further evidence suggests that growth factors influence the distribution of caveolin-1 isoforms (Fujimoto et al., 2000; Nohe et al., 2004). Previous data provided evidence that the reshuffling of BRIa on the cell surface is important for the activation of BMP-signaling (Nohe et al., 2003). The results obtained here by using ICCS suggest that a significant number of BRIa receptors colocalize with caveolin-1 α, but not with the β isoform. Coexpression of BRII results in recruitment of BRIa into caveolae domains that mainly express the caveolin-1 β isoform. BRII and BRIa are known to form ligand-independent preformed complexes (PFCs) (Gilboa et al., 2000; Nohe et al., 2002). Because expression of BRII leads to the redistribution of BRIa into caveolae enriched in caveolin-1 β, this provides evidence that PFCs are located in these specific domains. The coimmunoprecipitation studies performed in A431 cells confirm this result because BRIa, which is not capable of binding caveolin-1, can be immunoprecipitated with

the complex. The observation that BRII and BRIa are found at similar percentages in caveolae that express the α or the β isoform, provides further evidence of their coexistence in that same structure. The data also suggest that the interaction between BRIa and caveolin-1 has to be stabilized by either another protein or by the cell-structure-organization in A431 cells, because binding of BRIa to caveolin-1 in the presence of BRII is not detectable in COS-7 cells under our conditions.

After stimulating the cells with BMP-2 receptors are redistributed into domains enriched in caveolin-1 α. At the same time, BMP-2 stimulation causes a redistribution of caveolin-1 β into domains that contain caveolin-1 α. The mechanism of the redistribution is not clear but it might be that BRII and BRIa move into the domains that contain both (α and β) isoforms, releasing caveolin-1 β in its domains. It is known that receptor tyrosine kinases are inhibited when bound to caveolin-1; stimulation with the respective ligand releases caveolin-1 and the signaling is initiated (Schlegel and Lisanti, 2001). Because BRII and BRIa can form a preformed complex, and BRII is a constitutive active kinase, binding of BRII to caveolin-1 might be one mechanism of inhibiting phosphorylation and activation of BRIa in the absense of a ligand; even though the two receptors are interacting in the PFCs. It has been shown that, in the case of the TGF β receptors, the type I receptor binds caveolin-1 (Razani et al., 2001), suggesting that this binding inhibits Smad2 phosphorylation and activation of the Smad signaling pathway. PFCs are necessary to activate the Smad signaling pathway in BMP signaling (Nohe et al., 2002).

Here, we also show that the caveolin-1 isoforms have a different effect on BMP signaling. Only the β isoform inhibits the signaling, the α isoform does not influence the BMP signaling pathway.

Taken together, our data suggest that, BMP-signaling is a complex but dynamic and flexible process at the cell membrane. Initiation of signaling might be regulated at the cell surface depending on the kind of interaction between receptors and caveolae. Based on the data presented here, we believe that there could be three steps that involve PFCs: (1) BRIa is recruited by BRII into a preformed complex and at the same time BRIa moves out of caveolae that are enriched in caveolin-1 α and β, into regions enriched with caveolin-1 β alone. (2) The ligand BMP-2 binds to its receptors in the PFCs, causing them to move from caveolae enriched in caveolin-1 β into the domains containing both caveolin-1 α and β. (3) Caveolin-1 is released from the BMP-2 so that activation of the Smad signaling pathway can proceed.

We thank the Wyeth Institute for providing us with BMP-2 and also thank D. Litchfield for providing lab space, techniques and helpful discussions. We further thank D. Lacey, C. Beaumier and V. Olds for their support. This research was supported by NSERC.

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