

# Effect of the distribution and clustering of the type I A BMP receptor (ALK3) with the type II BMP receptor on the activation of signalling pathways

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## Summary

Bone morphogenetic proteins (BMPs) play an important role during embryonic development, especially in chondrogenesis, osteogenesis, neurogenesis and hematopoiesis. There are over 19 BMPs known in mammals, but only three BMP-type-I receptors and three BMP-type-II receptors are known so far to mediate these responses. Previous reports provide evidence to support that oligomerisation of BMP receptors influences the activation of the downstream BMP signalling pathways, the Smad or the p38 MAPK pathway. To further explore the importance of BMP receptor clustering in signalling, image correlation spectroscopy has been used to investigate the clustering and distribution of BMP receptors at the surface of the cell membrane. Here we demonstrate that the co-expression of the BMP-type-II receptor (BRII) influences the aggregation and the distribution of the BMP-type-Ia receptor (BRIa) in COS7 cells and in A431 cells.

We also demonstrate that BMP-2 stimulation of the cells leads to a rearrangement of receptor complexes at the cell surface. Using A431 cells and limb bud-derived mesenchymal cells, we show that co-expression of the BRII and a constitutive active BRIa-ca is necessary for the activation of the Smad pathway. Importantly using a kinase-inactive BRII the rearrangement of BRIa is blocked. Together, these findings suggest that rearrangement of the receptors at the cell surface prior to forming preformed ligand independent complexes plays a critical role in activation of the Smad pathway. It also suggests further that the kinase activity of BRII is needed for signalling beyond the activation of BRIa at the GS domain.

Key words: Image correlation spectroscopy, Fluorescence, BMP receptors, Receptor clusters, Smad pathway

## Introduction

Bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) belong to the TGF- $\beta$  superfamily (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). For the BMPs and GDFs only three type-I receptors and three type-II receptors have been identified, all of which are serine threonine kinases. For activation of the signalling pathways, BMPs or GDFs bind to the receptors within distinct oligomeric structures (Nohe et al., 2002). The type-II receptor, which is constitutively active, phosphorylates the type-I receptor, which then phosphorylates Smad1, 5 or 8. Receptors of both types are needed to form a functional complex in order to initiate further signalling events. Phosphorylated Smads dissociate from the receptors and bind Smad4, a common mediator, leading to nuclear translocation and regulation of specific genes (Kretschmar et al., 1997; Massague, 1998; Rosenzweig et al., 1995; Attisano and Wrana, 2000; Hoodless et al., 1996; Kawabata et al., 1995; Liu et al., 1995; Nohno et al., 1995). Another pathway that can be activated is the p38 pathway, but the mechanism for the direct activation of this pathway is poorly understood (Kimura et al., 2000; Shirakabe et al., 1997; Nakamura et al., 1999; Nohe et al., 2002).

BMP receptors are expressed on the cell surface either as hetero-oligomeric preformed complexes (PFCs) or as homo-oligomeric complexes, which upon BMP-2 stimulation form hetero-oligomeric BMP-induced signalling complexes (BISCs) (Gilboa et al., 2000; Nohe et al., 2002). Binding of BMP-2 to PFCs activates the Smad signalling pathway, whereas BMP-2 induced recruitment of receptors into BISCs activates a different Smad-independent pathway resulting in the induction of alkaline phosphatase activity via p38 MAP kinase. These observations imply that the specific structural organisation of the BMP receptors prior to BMP-2 binding is a key prerequisite for activation of distinct signalling pathways at the cell surface. In order to understand the details of this process we need to study the quantitative distribution of the BMP receptors and the changes in their distribution following BMP-2 stimulation. Image correlation spectroscopy (ICS) provides a convenient and quantitative tool to measure the density of receptor clusters on cell surfaces (Brown and Petersen 1998; Brown et al., 1999; Srivastava and Petersen 1998; Wiseman and Petersen 1999; Wiseman et al., 1997).

To investigate BMP receptor clustering, COS7 cells were transfected with plasmids encoding various combinations of BMP receptors. After fixation we labelled the BMP receptors

using fluorescent antibodies and collected high magnification images of the distribution of the BMP receptors on the cell membrane. Applying ICS we calculated the average number of receptor clusters per unit area (cluster density, CD). Our data indicate that the co-expression of the BMP-type-II receptor (BRII) disperses aggregates of the BMP-type-I receptor (BRIa). Stimulation with BMP-2 leads again to a rearrangement of the receptors on the cell surface. Using the method described above we also observed a similar redistribution of the endogenous receptors in A431 cells. Upon serum starvation of these cells, BRII is upregulated and BRIa clusters are rearranged. To investigate whether the activation status of BRIa affects the clustering, we transfected COS7 cells with a constitutively active mutant BRIa-ca. We observed that BRIa-ca clusters are rearranged by BRII in the same manner as BRIa in the presence of BRII and BMP-2 stimulation. Importantly co-transfection of a kinase-inactive BRII (BRII-KR) with BRIa or BRIa-ca did not result in a change in BRIa distribution. We further performed reporter gene assays in A431 cells or primary limb mesenchymal cells either using a Col2-luc reporter construct or using pSBE, a construct specific for the activation of the Smad pathway. Co-transfection of BRIa-ca and BRII in these cells leads to an increase in luciferase activity, compared with cells transfected with BRIa-ca only. Further co-transfection of BRIa-ca and BRII-KR did not lead to an increase in luciferase activity. Our data suggest that the rearrangement of the BRIa by BRII on the cell surface is dependent on the kinase activity of BRII and important for the activation of the signalling pathways.

## Materials and Methods

### Materials

Recombinant BMP-2 was obtained from Wyeth (Boston, NY). Biotinylated monoclonal mouse antibody 9E10, directed against the myc tag [anti-myc (Evan et al., 1985)] was purchased from Babco (Berkeley, CA). Fluorescein isothiocyanate (FITC) conjugated monoclonal mouse antibody 12CA5 against the influenza hemagglutinin (HA) tag (Wilson et al., 1984), the polyclonal goat antisera against the BMP receptors BRIa and BRII, and the rhodamine red X conjugated donkey anti-goat antibody were from Santa Cruz (Santa Cruz, CA). Rhodamine red X (RRX) conjugated mouse monoclonal anti-biotin antibody was from Jackson Immuno Research Laboratories (Baltimore, PA). The cell lines COS7 (CRL 1651) and A431 (CRL 1555) were purchased from American Type Culture Collection (Manassas, VA). As shown in previous publications the myc epitope tag and the HA epitope tag do not affect the activity of the BMP receptors (Gilboa et al., 2000).

### RNA isolation and cDNA preparation

RNA was prepared from  $1 \times 10^7$  epidermal carcinoma A431 cells using the Qiagen (Mississauga, Canada) RNeasy MidiKit. Reverse transcription was performed using 1.5  $\mu$ g total RNA and Superscript II RNase-H reverse transcriptase (GibcoBRL, Burlington, Canada) according to the manufacturer's protocol. Random hexamers were purchased from Boehringer (Mannheim, Germany) and dNTPs from Pharmacia (Mississauga, Canada). The following cDNA amplification reactions were done by PCR using PFU-Polymerase (Stratagene, LaJolla, CA) and specific oligonucleotides for BRIa: BRIa-9 5'CAGAATCTGGATAGTATGC3' and BRIa-15 5'TGAGTCCAGG-AACCTGTACCTTTA3'; and specific oligonucleotides for BRII: BRII-15 5'ACATAATAGGCGTGTGCCA3' and BRII-25 5'CTT-

GATCTTTTCACCTG3'. The PCR products were separated on an agarose gel and visualised by UV.

### Transfection of COS7 and A431 cells

COS7 and A431 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Gibco BRL, Burlington, Canada) and transfected by the DEAE-dextran method (Aruffo and Seed, 1987) using 5  $\mu$ g of DNA/plasmid. In case of the reporter gene 1  $\mu$ g of the pSBE or Col2-luc was used.

### Immunofluorescence labelling of cell surface receptors

To measure the distribution of the BMP receptors on the cell surface, we employed confocal fluorescence imaging measurements. Co-transfected COS7 cells as well as normal or serum starved A431 cells were grown on 22 mm cover slips. A full 48 hours after transfecting COS7 cells or 72 hours after serum-starvation of A431 cells, cells were stimulated or mock stimulated with BMP-2 for 2.5 hours and fixed using acetone/methanol fixation (Brown et al., 1999). After blocking for 30 minutes with 5% BSA, cells were incubated with biotin or FITC conjugated monoclonal mouse antibodies against the epitope tags of the receptors or with polyclonal goat antisera recognising either BRII or BRIa according to the manufacturer's protocol. Cells were washed three times with PBS for 5 minutes. Afterwards, cells were incubated with the corresponding secondary monoclonal mouse anti-biotin or donkey anti-goat antibody at a concentration of 3.2  $\mu$ g/ml anti-biotin antibody or 20  $\mu$ g/ml in the case of donkey anti-goat IgG. Cells were washed again three times for 5 minutes with PBS and the cover slips were mounted in airvol and dried overnight. When monoclonal mouse antibodies against the epitope tags were used, cells were incubated with goat IgG (200  $\mu$ g/ml) for 30 minutes prior to addition of the primary antibody. The specificity of the polyclonal antisera was tested by transfecting COS7 cells with HA-BRI and HA-BRII. Cells were then fluorescently labelled using the HA-FITC antibody or the polyclonal antisera followed by a secondary donkey anti-goat antibody. The collected images showed a 100% co-localisation with the receptors as well as background fluorescence both on and off the cells.

### Preparation of cultures from limb mesenchyme

Cultures were prepared from murine fore and hind limb buds of E11.5 embryos as previously described (Weston et al., 2000) with the following modifications. After dispase digestion, cells were filtered through a Cell Strainer (40  $\mu$ m; Falcon) to obtain a single cell suspension. Culture media (40% Dulbecco's modified Eagle's medium and 60% F12 supplemented with fetal bovine serum to 10%; Gibco BRL) was changed daily.

### Luciferase reporter assay

Limb mesenchymal cells (isolated from E11.5 mouse embryos) were transfected with pSBE (Jonk et al., 1998) or a Col2-luc reporter expression vector containing either of the two receptors and pRLSV40 (for normalisation of transfection efficiency) using Fugene6 (Boehringer) as previously described (Weston et al., 2002). Cells were plated at high density in the wells of a 24-well tissue culture dish, 48 hours after transfection the cells were lysed and luciferase activity was measured using a dual luciferase assay system (Promega, Madison, WI).

A431 cells, grown in 60 mm dishes, were transfected with the pSBE and the BMP receptor constructs using the DEAE dextran method and stimulated or mock stimulated with BMP-2. After transfection, cells were stimulated or not stimulated for 12 hours with 20 nM BMP-2. then the cells were lysed and luciferase activity was measured using a dual luciferase assay system (Promega).

### Confocal microscopy

Labelled cells were visualised using a Biorad MRC 600 confocal microscope equipped with an Ar/Kr mixed gas laser and using the appropriate filter sets for dual fluorophore imaging. Cells expressing the receptors were selected under mercury lamp illumination using a 60× (1.4 NA) objective and an inverted Nikon microscope. An area on the cell, removed from the nucleus, was enlarged and visualised. For measuring FITC fluorescence the filter wheel 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 photon counting mode (to ensure linear scaling of the intensity). For measuring RRX fluorescence the filter wheel 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 10. After collection of each set of 20 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) is the technique used to study the distribution and localisation of the BMP receptors. ICS involves autocorrelation analysis of the intensity fluctuations within confocal images collected in this case from transfected cells that contain immunofluorescent labelled proteins (Petersen, 2001).

Let the fluorescent intensity in a pixel located at position  $x, y$  in the image be  $i(x,y)$ , then the corresponding normalised fluorescence intensity fluctuation,  $\delta i(x,y)$ , is given by:

$$\delta i(x,y) = \frac{i(x,y) - \langle i(x,y) \rangle}{\langle i(x,y) \rangle}$$

The normalised spatial autocorrelation function,  $g(\xi, \eta)$ , is then given by:

$$g(\xi, \eta) = \langle \delta i(x,y) \delta i(x + \xi, y + \eta) \rangle,$$

where the angular brackets indicate the average over all spatial coordinates, and  $\xi$  and  $\eta$  are position lag coordinates for the  $x$  and  $y$  axis. It is known that the limit of the autocorrelation function as  $\xi$  and  $\eta$  approach zero,  $g(0,0)$ , is equal to the variance of the normalised intensity fluctuations (Berne et al., 1976).

It is also known that for homogenous, non-interacting species, where the intensity is a true representation of the concentration, the variance of the normalised intensity fluctuations is equal to the variance of the concentration fluctuations, which in turn is equal to the inverse of the number of particles in the

$$g(0,0) = \text{var}[\delta i(x,y)] = \text{var}[\delta c(x,y)] = \frac{1}{\bar{N}_p}$$

observation area,  $\bar{N}_p$  (Wiseman et al., 1997). Once the  $g(0,0)$  is known, an important parameter, called the cluster density (CD), can be calculated. The CD value gives the average number of receptor clusters per unit area:

$$\text{CD} = \frac{1}{g(0,0)\pi w^2} = \frac{\bar{N}_p}{\pi w^2}$$

The average CD values were normalised to the average CD value for transfected cells solely with HA-BRIa or myc-BRII. In case of the A431 cells the average CD values were normalised to BRIa in normal A431 cells and BRII in serum starved A431 cells. Standard error of the mean (s.e.m.) values were calculated from the raw data at the 95% confidence level.

## Results

### Redistribution of BRIa by BRII in COS cells

To examine the influence of the BRII expression on the distribution of BRIa we transfected COS7 cells with plasmids encoding either HA- or myc-tagged BRIa alone or in combination with HA- or myc-tagged BRII. Cells were fluorescently labelled for the HA- or myc-BRIa and HA- or myc-BRII receptor expression and confocal images were collected (Fig. 1). To determine the specificity of the antibodies, mock-transfected cells were incubated with the primary and secondary antibodies after fixation. Confocal images collected from the mock-transfected fluorescently labelled cells showed no significant intensity (Fig. 1A,B). High magnification confocal images were collected from flat regions of the membrane of transfected cells fluorescently labelled for myc- or HA-BRIa (Fig. 1C) and HA- or myc-BRII (Fig. 1D). The cluster density, CD, which estimates the average number of clusters per unit area, was calculated using ICS (see materials and methods). The average CD values were normalised to the average CD value for cells transfected with myc- or HA-BRIa, or HA- or myc-BRII. As shown in Fig. 1E and F, a co-transfection in COS7 cells of BRIa and BRII leads to a four to six fold increase in the average CD, which can be interpreted as a dispersion of the myc- or HA-BRIa aggregates on the cell surface in the presence of BRII. In contrast, Fig. 1G shows that the myc-BRII distribution is unaffected by HA-BRIa, since the average CD value is unchanged.

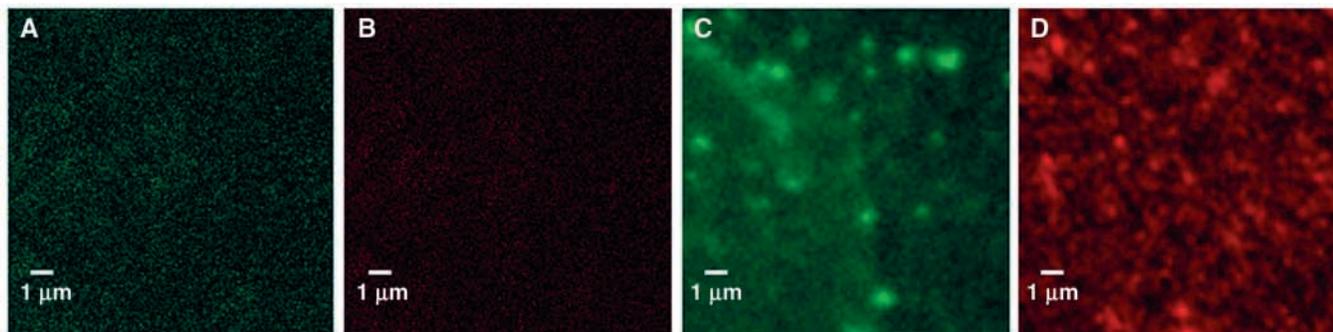
It should be noted that the average CD values are independent of the transfection level of the receptors since there is no correlation between the average intensity of the labelled receptors and the CD (data not shown). Thus the total density of receptors does not affect the average number of receptor clusters but rather the average number of receptors per cluster. The expression level of BRII can affect the distribution and therefore the CD of BRIa, as can be seen in the range of four to six fold increase of BRIa by co-transfection of myc- or HA-tagged BRII.

### Redistribution of BRIa and BRII by stimulation with BMP-2 in COS7 cells

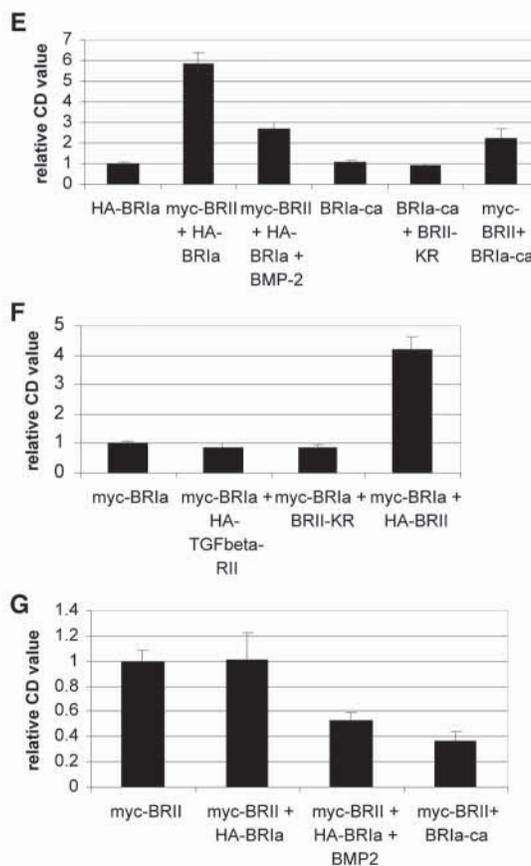
Fig. 1E,G shows that BMP-2 stimulation of cells co-transfected with both receptors decreases the average CD value by a factor of two, suggesting that BMP-2 directly affects the clustering of both myc-BRII and HA-BRIa on the cell surface. Stimulation for 2.5 hours with 20 nM BMP-2 causes both HA-BRIa and myc-BRII to redistribute into fewer but larger aggregates, since the average receptor density as estimated from the intensity is the same (data not shown).

### BRIa-ca shows the same CD as BRIa in COS7 cells

Fig. 1E also shows that the average CD of a constitutive active form of BRIa, BRIa-ca, is comparable to the native form. Interestingly co-transfection with BRII yields average CD values for BRIa-ca and BRII comparable to the average CD values obtained in co-transfected cells with BRIa and BRII after stimulation with BMP-2. The implication is that the state of aggregation of BRIa-BRII complexes depends on the phosphorylation state of BRIa, with the non-phosphorylated state being in fewer but larger clusters.



**Fig. 1.** Co-expression of BRII and BRIa leads to rearrangement of BRIa on the cell surface. Stimulation of cells expressing BRII and BRIa with BMP-2 causes an aggregation of the receptors. BRIa-ca, which is constitutively active, needs the co-expression of BRII for redistribution. COS-7 cells were transiently transfected with myc-tagged BRII and HA-tagged versions of BRIa or BRIa-ca. After 48 hours, cells stimulated with BMP-2 or nonstimulated cells were fixed and the receptors labelled with fluorescent antibodies against the epitope tags. Cell membrane expression of the receptors was visualised by confocal microscopy. Forty images were collected at the highest magnification (Zoom10). From these images the CD value of the BMP receptors was calculated using ICS. (A) COS7 cells were mock transfected, fixed and incubated with the fluorescent HA-antibody. The mock-transfected cells showed no significant fluorescence. (B) COS7 cells were mock transfected, fixed and incubated with the biotin conjugated myc antibody followed by the secondary anti-biotin RRX antibody. The mock-transfected cells show no significant fluorescence. (C) COS7 cells were transfected with a plasmid encoding HA-BRIa, fixed and fluorescently labelled using the FITC conjugated HA-antibody. (D) COS7 cells were transfected with a plasmid encoding myc-BRII. Cells were fixed and fluorescently labelled using the biotin-conjugated myc-antibody followed by a RRX conjugated anti-biotin antibody. (E) Calculated average CD values for BRIa or BRIa-ca. Transfected COS7 cells were fluorescently labelled with a fluorescent antibody against the HA-epitope. Confocal images were taken and the CD values for BRIa and BRIa-ca were calculated by applying ICS. (F) Calculated average CD values for myc-BRIa. After transfection cells were fixed and labelled using a biotin-conjugated antibody against the myc-epitope followed by a fluorescent secondary antibody. Confocal images were taken and the CD values for myc-BRIa were calculated by applying ICS. (G) Calculated average CD values for myc-BRII. Transfected COS7 cells were fluorescently labelled with a biotin-conjugated antibody against the myc-epitope followed by a fluorescent anti-biotin antibody. Confocal images were taken and the CD values for BRII were calculated by applying ICS.



### The redistribution of BRIa by BRII is dependent on the kinase activity of BRII

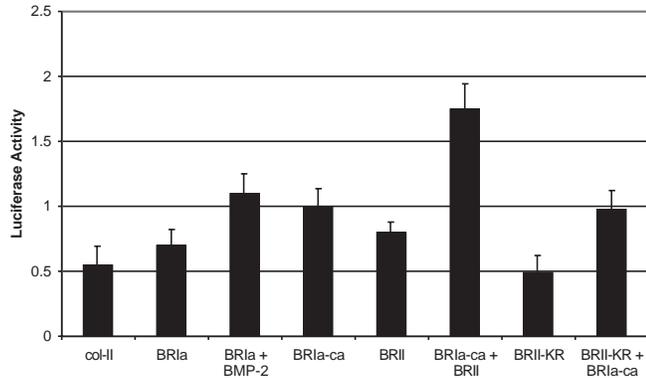
Fig. 1E,F indicates that co-transfection of kinase-inactive BRII-KR does not lead to a redistribution of BRIa or BRIa-ca. This indicates that the kinase activity of BRII is important for the reorganisation of BRIa on the cell surface. Also the co-transfection of another serine threonine type-II receptor, TGF $\beta$ -RII, which is involved in TGF- $\beta$  signalling, shows no effect on the CD of BRIa (Fig. 1F). This suggests that the dispersion of BRIa by BRII is regulated through the kinase activity of BRII and that it is specific for BRII.

### Effect of BRII expression on the activation of downstream signalling pathways in limb mesenchymal cells

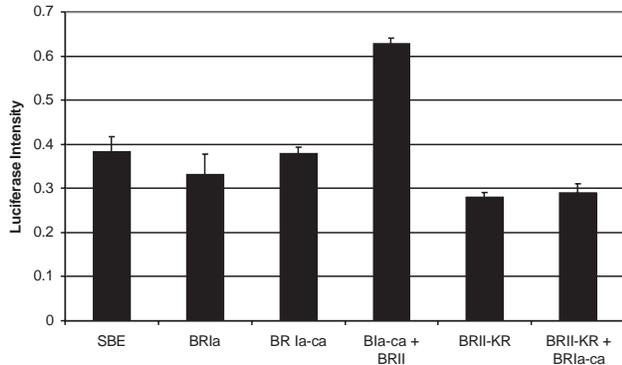
Limb mesenchymal cells were transfected with plasmids

encoding HA-BRIa-ca and myc-BRII together with a Col2-luc reporter and pRLSV40. The Col2-luc reporter is derived from the promoter and enhancer region of the type 2 collagen gene, and provides a readout for the status of chondroblast differentiation (Lefebvre et al., 1997; Weston et al., 2002). Fig. 2 shows that luciferase activity is increased by about a factor of two when BRIa-ca is induced in the system, irrespective of whether BRII is present or not. Myc-BRII causes some increase in the luciferase activity but co-transfection of myc-BRII and HA-BRIa-ca clearly leads to a strong increase in luciferase activity. Importantly, co-transfection of the kinase-inactive BRII-KR and BRIa-ca does not lead to an increase in luciferase activity beyond that observed for BRIa alone.

To investigate the effect of myc-BRII expression on the activation of the Smad signalling pathway, we co-transfected the limb mesenchymal cells with plasmids encoding HA-BRIa-

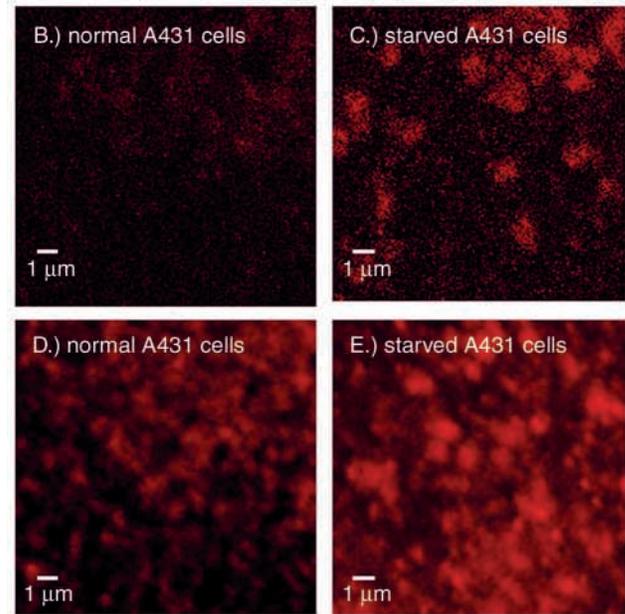
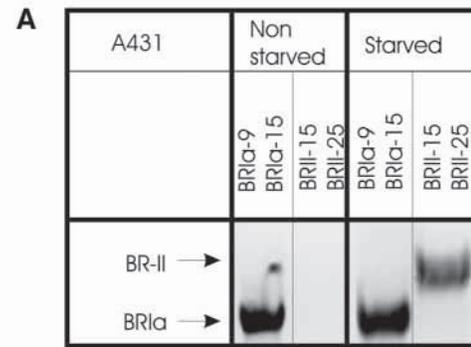


**Fig. 2.** Co-expression of BRll-myc and HA-BRla-ca leads to an increase in stimulation of the Col2-luc reporter gene activity compared with the single transfection of BRla-ca or BRll. Co-transfection of BRla-ca with BRll-KR does not lead to an increase in luciferase activity indicating that the kinase activity of BRll is required for signalling. Limb mesenchymal cells (isolated from day 11.5 mouse embryos) were transfected with a Col2-luc reporter, and expression constructs for BRll, BRla-ca and pRLSV40 for normalisation. Cells were plated at high density in the wells of a 24-well tissue culture dish, and 48 hours following transfection, cells were lysed and luciferase activity measured. The Col2-luc reporter is derived from the promoter and enhancer regions of the type 2 collagen gene and provides a readout on the status of chondroblast differentiation. BMPs as well as BRla-ca are known to promote chondrogenesis in limb mesenchymal cells.



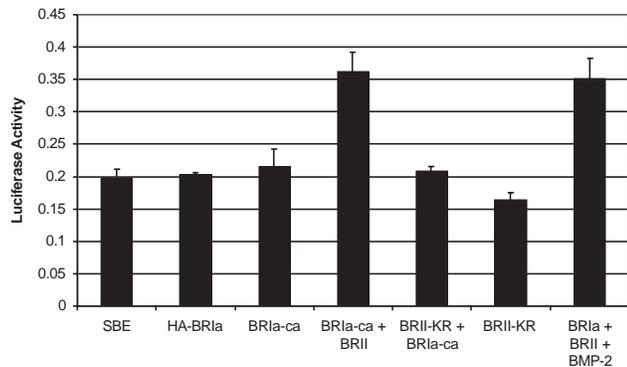
**Fig. 3.** Co-expression of BRll-myc and HA-BRla-ca leads to an increase in stimulation of the pSBE-Luc reporter gene activity compared with the single transfection of BRla-ca. BRla-ca and BRll-KR do not increase the luciferase activity of the pSBE-Luc. Also co-expression of BRla-ca and BRll-KR does not result in an increase in luciferase activity, indicating that the kinase activity of BRll is required. Limb mesenchymal cells (isolated from day 11.5 mouse embryos) were transfected with a pSBE-Luc reporter, expression vector containing either of the two receptors and pRLSV40 (normalisation). Cells were plated at high density in the wells of a 24-well tissue culture dish, and 48 hours following transfection cells were lysed and luciferase activity measured.

ca, myc-BRll, BRll-KR and the pSBE-luc reporter. The pSBE-luc provides a readout specific for the activation of the Smad signalling pathway (Jonk et al., 1998; Nohe et al., 2002). As shown in Fig. 3, HA-BRla-ca alone fails to activate the pSBE-luc. Only co-transfection of BRla-ca with myc-BRll leads to an



**Fig. 4.** Expression of BMP receptors in A431 cells. (A) A431 cells upregulate BRll mRNA expression upon serum starvation. RNA was prepared from starved and nonstarved A431 cells and reverse transcribed. Using specific primer combinations for amplification of BRla (primer BRla-9 and BRla-15) or BRll (primer BRll-15 and BRll-25) by PCR the presence of the receptors in the cells was tested. A431 cells express the mRNA for BRla in both starved and nonstarved conditions, whereas BRll mRNA is upregulated in starved cells. (B,C) Serum starvation of A431 cells leads to upregulation of BRll at the cell surface. Normal (B) or serum starved A431 cells (C) were fixed and fluorescently labelled for the expression of BRll using a polyclonal antiserum against BRll and a secondary donkey anti-goat RRX antibody. Cells were fixed and images collected using a confocal microscope. (D) Normal A431 cells were fixed and fluorescently labelled for BRla using a polyclonal antibody against BRla followed by RRX-conjugated secondary antibody. (E) Serum starved A431 cells were fixed and fluorescently labelled for BRla using a polyclonal antibody against BRla followed by RRX-conjugated secondary antibody.

increase in pSBE-luc reporter activity, suggesting that BRll is needed with BRla-ca to activate the Smad signalling pathway. Co-transfection of BRll-KR and BRla-ca fails to increase the luciferase activity suggesting again that the kinase activity of BRll is needed for activation of BMP receptor signalling beyond its activation of BRla via the Smad signalling pathway.



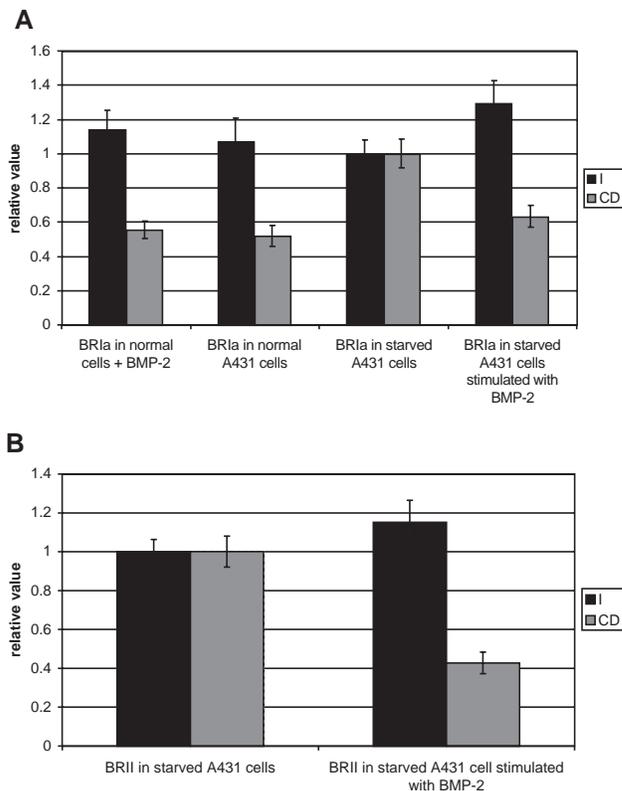
**Fig. 5.** BRII and BRIa-ca are both necessary for the activation of the Smad pathway. A431 cells were transfected with various combinations of BRIa, BRII, BRIa-ca and BRII-KR, and the pSBE-Luc. For normalisation, the renilla luciferase construct pRLSV40 was co-transfected and the emission measured. Only co-transfection of BRIa-ca with BRII or co-transfection of BRIa and BRII with addition of BMP-2 leads to activation of the reporter. The data indicate that BRII is necessary for the activation of the Smad pathway.

#### BRII mRNA is upregulated upon serum starvation of A431 cells

The distribution and aggregation studies described so far were performed by transfecting COS7 cells. We wanted to compare the results obtained from cells overexpressing the receptors of interest with a cell line that endogenously expresses these receptors. A431 cells were used as a model system to study the redistribution of the BMP receptors on the cell surface in untransfected cells. Normal A431 cells are known to overexpress the EGF receptor. Using RT-PCR and primers specific for BRIa and BRII, we examined the BMP receptor expression in this cell line. As shown in Fig. 4A, serum starvation of A431 cells for 72 hours leads to upregulation of BRII mRNA. We also labelled serum starved and normal A431 cells using a polyclonal antiserum against BRII and a fluorescently labelled secondary antibody. Serum starvation of A431 cells for 72 hours leads to increased BRII expression on the cell surface (Fig. 4B,C). BRIa is expressed in A431 cells cultured in 10% FBS and in A431 cells serum starved for 72 hours as shown by RT-PCR (Fig. 4A) and appears on the cell surface as seen by immunofluorescence using a polyclonal antiserum against BRIa and a secondary fluorescent antibody (Fig. 4D,E). Clearly, serum starvation of A431 cells provides a means of studying the effect of BRII upregulation and expression on the distribution of BRIa without the need of transfections.

#### Effect of BRII expression on the activation of the Smad signalling pathway in A431 cells

Normal A431 cells were transfected with the pSBE-Luc, pRLSV40 and various combinations of BRIa, BRII and BRIa-ca. As shown in Fig. 5, neither the transfection of BRIa-ca nor BRIa and BRII affected reporter gene activity. Only co-transfection of BRIa-ca with BRII or co-transfection of BRIa and BRII with addition of BMP-2 leads to activation of the reporter. These results are comparable to those seen for primary



**Fig. 6.** CD and intensity (I) of BMP receptors in A431 cells. A431 cells were cultured either in DMEM with 10% FBS or under serum starved conditions. Cells were grown in DMEM without FBS for 72 hours, then stimulated with BMP-2 or not stimulated. The cells were then fixed and the receptors labelled using polyclonal antisera against (A) BRIa and (B) BRII, and a secondary fluorescently labelled antibody. Cell membrane expression of receptors was visualised by confocal microscopy. Forty images were collected at the highest magnification. From these images the CD value of the receptors was calculated using ICS. The CD value of the background fluorescence caused by nonspecific labelling of the antibody was subtracted.

mesenchymal cells (Fig. 3). Clearly an activated assembly of BRIa and BRII is required to activate the Smad signalling pathway in either system.

#### Redistribution of BRIa by BRII in A431 cells

A431 cells were cultured for 72 hours in DMEM containing 10% FBS or in DMEM containing no FBS. The cells were either stimulated or not stimulated with BMP-2, fixed and the endogenous receptors BRIa and BRII were fluorescently labelled using appropriate polyclonal antisera and a secondary fluorescently labelled antibody. Confocal images were collected and ICS analysis applied. Because the polyclonal antisera showed some background fluorescence, corresponding images from the glass coverslip were collected and were used to correct the calculation of the CD of BRIa and BRII for the cells (Wiseman et al., 1999). The average CD values were then normalised to the average CD value for normal A431 cells for BRIa and serum starved A431 cells for BRII. As indicated in Fig. 6A, upregulation of BRII by starvation of A431 cells leads to a two fold higher CD value of BRIa, even though the

expression level of BRIa in normal and serum starved cells is unchanged [the average intensity (I) stays the same]. This indicates that BRIa clusters are dispersed, because of upregulation of BRII expression, into twice as many clusters with half the number of receptors in each.

#### Redistribution of BRIa and BRII upon BMP-2 stimulation in A431 cells

To investigate whether BMP-2 stimulation affects the distribution of BRIa and BRII in cells expressing the BMP receptors, we grew A431 cells for 72 hours in DMEM without FBS and stimulated them with 20 nM BMP-2 for 2.5 hours or left them untreated. Stimulation with BMP-2 leads to a two fold decrease in the CD value of BRIa (Fig. 6A) and a three fold decrease in the CD value of BRII (Fig. 6B), suggesting an aggregation of both BRIa and BRII. As Fig. 6 shows, the total expression level of neither BRIa nor BRII changes significantly by stimulation with BMP-2, since the average intensity (I) of the collected images is nearly constant.

#### Discussion

The results obtained by ICS show that BRIa is located in aggregates at the cell surface of A431 and COS7 cells. Upon co-expression of BRII, the aggregates of BRIa disperse into smaller clusters on the cell surface, which is indicated by an increase in the CD value without a change in the fluorescence intensity. The aggregates of BRII themselves are not influenced by the co-expression of BRIa, since their CD value does not change. Stimulation with BMP-2 leads to a redistribution of BRIa and BRII into fewer and larger aggregates. This result is shown by co-transfecting COS7 cells with plasmids encoding BRIa or BRII, and in a more natural cell system, A431 cells, where BRII can be upregulated upon serum starvation of the cells. These experiments suggest that BRII can control the redistribution of BRIa on the cell surface of these cells. The BRIa receptor distribution on the cell surface is therefore very flexible and sensitive to the co-expression of BRII. Moreover a kinase inactive mutant of BRII, BRII-KR, shows no influence on the redistribution of BRIa indicating that the kinase activity of BRII is important for the redistribution in these cells. Previous data show that BRIa and BRII can be present as hetero-oligomeric preformed complexes (PFC) and as homo-oligomeric complexes on the cell surface (Gilboa et al., 2000; Nohe et al., 2002). Our results suggest that BRII can influence the distribution of BRIa confirming the high flexibility of the receptor system. Since BRII-KR still can form a preformed complex with BRIa but does not lead to a redistribution of BRIa, our results indicate that the redistribution of the BRIa on the cell surface in A431 or co-transfected COS7 cells is not solely as a result of the formation of PFCs (Gilboa et al., 2000; Nohe et al., 2002). This gives evidence that the reshuffling is independent of the formation of PFCs and needed for the activation of the signalling pathway.

The aggregation of BRII is not influenced by BRIa. It is only influenced by the stimulation of cells with BMP-2, which also leads of a change in the distribution pattern of BRIa. As described previously the BMP-2 ligand has two options to initiate signalling: the first needs the formation of PFCs, and the second is mediated by the binding of the BMP-2 to the high

affinity binding BRIa receptor, then recruitment of BRII into the ligand-mediated signalling complex (BISC) (Gilboa et al., 2000; Nohe et al., 2002). The ICS studies performed here indicate a very dynamic receptor-ligand relationship. They also suggest that the receptors are rearranged on the cell surface into different domains and that this reshuffling is necessary for signalling. The data also indicate that the redistribution of the receptors is not solely due to the formation of PFCs or BISCs.

The reporter gene assay in A431 cells and in primary mesenchymal limb cells, suggests that the activity of BRIa-ca, which is able to stimulate chondrogenesis on its own as indicated by its ability to increase Col2-luc reporter activity, can be increased by co-expression with BRII. In contrast BRII-KR co-transfected with BRIa-ca fails to increase the luciferase activity. Here we show that the role of BRII in BMP signalling is not only to phosphorylate and activate BRIa, but also to enhance BMP signalling, as demonstrated in primary cells. The kinase activity of BRII is needed for this reshuffling and is crucial for signalling despite the phosphorylation of BRIa. The pSBE-Luc is activated by Smads (Jonk et al., 1998; Nohe et al., 2002). Here we give evidence that PFCs, which may be formed as a result of the recruitment of BRIa by BRII into special regions at the cell surface, are necessary for activation. But our data further suggest that the kinase activity of BRII is still needed for the reshuffling and is one critical step in activating the BMP-Smad pathway. A431 cells overexpress the EGFR, which leads to continuous activation of the EGFR pathway. It is known that activation of ERK by the EGFR pathway can lead to a phosphorylation of Smad1 at the linker region, resulting in the inhibition of the BMP signalling pathway (Kretschmar et al., 1997). Because of this negative feedback mechanism, the BMP signalling in these cells still needs careful exploration.

In conclusion we show that the BMP receptor distribution on the cell surface is very flexible and dynamic during activation of signalling pathways. We provide strong evidence to support an additional role of BRII in recruiting BRIa into special regions of the cell surface. We further showed that the kinase activity of BRII is required for this reshuffling of BRIa. We demonstrate that ICS is a powerful tool to determine the CD value of receptors at the cell surface and is sensitive to subtle changes in the receptor distribution patterns.

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