Visualizing Smad1/4 signaling response to bone morphogenetic Protein-4 activation by FRET biosensors

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Received 11 April 2007; received in revised form 11 September 2007; accepted 20 September 2007

Available online 9 October 2007

Abstract

Smad proteins are the major signal transducers for the Transforming Growth Factor superfamily of cytokines and their serine/threonine kinase receptors. Smads mediate the signal from the membrane into the nucleus. Bone Morphogenetic Protein-4 stimulates phosphorylation of Smad1, which interacts with Smad4. This complex translocates into the nucleus and regulates transcription of target genes. Here, we report our development of cellular fluorescent biosensors for direct visualization of Smad signaling in live mammalian cells. Fluorescence resonance energy transfer between cyan and yellow fluorescent proteins fused to the Smad1 and Smad4 proteins was used to unravel the temporal aspects of BMP/Smad signaling. A rate-limiting delay of 2–5 min occurred between BMP activation and Smad1 activity. A similar delay was observed in the Smad1/Smad4 complexation. Further experimentation indicated that the delay is dependent on the MH1 domain and linker of Smad1. These results give new insights into the dynamics of the BMP receptor–Smad1/4 signaling process and provide a new tool for studying Smads.

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Keywords: BMP; Smad; GFP; FRET; Biosensor

1. Introduction

Bone morphogenetic proteins (BMPs) are members of the Transforming Growth Factor (TGF-β) superfamily of cytokines [1,2]. Smad proteins are intracellular mediators of the TGF-β family of cytokines and receptors, which transduce the signal from the cell surface into the nucleus and expressed in most, if not all, cell types. BMP-4 binds to the high-affinity bone morphogenetic receptor type 1 (BRI) [3] in preformed heterooligomeric complexes with bone morphogenetic receptor type 2 (BRII) [4] to transfer signals into the cell via Smad proteins [5,6]. By mediating these cytokine responses, Smad proteins modulate a variety of important biological processes such as control of cell growth, differentiation, adhesion, and migration as well as inhibition of proliferation apoptosis and immune responses [7]. There are three categories of Smads: receptor-regulated Smads (R-Smads), which form complexes with common-partner Smad (Co-Smad), and the third class, inhibitory Smads (I-Smads) which negatively regulate signaling by the R-Smads and Co-Smads [7,8]. All Smad proteins contain two structurally conserved domains. The N-terminal MH1 domain is responsible for binding to DNA, transcription factors, and also to the cytoskeletal scaffold [9]. The C-terminal MH2 domain is responsible for phosphorylation and complex formation between Smad proteins and for interaction with type I receptors, transcriptional activation, and degradation by ubiquitination [9–14].

R-Smads such as Smad1 mediate BMP-4 signaling events. Upon BMP-4 stimulation, BRII initiates the kinase activity of BRI leading to the phosphorylation of R-Smad (reviewed in [15–18]). R-Smads have the structurally important L3 loop in the MH2 domain which interacts with the type I receptor. Upon this interaction, the type I receptor kinases directly phosphorylate two distal serines in the C-terminus within the SSXS motif [19,20]. The current model suggests that two mechanisms control the specific phosphorylation of the R-Smads by the receptor kinase complexes. First, a loop structure in the receptor kinase domain, referred to as the L45 loop, specifically interacts with the L3 loop of an R-Smad protein [21–24]. Second, a receptor-
associated recruiting molecule, such as the SARA protein in the TGF-β/activin pathway, can recruit specific R-Smad proteins to receptor kinases for phosphorylation [25]. However, the interplay between receptor activation, the Smad conformation change upon phosphorylation, and specific Smad binding proteins is not clear.

The phosphorylated Smad1 dissociates from the type I receptor, presumably due to a conformational change [26], and forms a complex with the Co-Smad, Smad4. Smad4 does not have the C-terminal SSXS phosphorylation motif and, thus, is not phosphorylated by the receptor. Smad4 forms a complex with activated Smad1 [10,11,27] that translocates to and accumulates in the nucleus where it is involved in transcriptional regulation [5,27–29]. Smad proteins in the resting state realize passive nucleo-cytoplasmic shuttling [30], which is controlled by two opposing signals: the nuclear localization signal (NLS) and the nuclear export signal (NES). The regulation of this shuttling activity constitutes a critical cellular mechanism to modulate the activities of transcription factors [31–34].

During the signaling processes, Smad proteins can also interfere with other signaling pathways that can change the kinetics of Smad phosphorylation, complex formation, and the dynamics of nuclear translocation [9]. Previously the basic functions and rates of activation of Smad proteins were shown and investigated by means of standard biochemical techniques. Fluorescence microscopy techniques have led to an improved understanding of these processes. For example, the shuttling of the Smad4 was determined to be originally due to its basal level presence in both the cytoplasm and nucleus, which upon either stimulation [5,17,30,35–37] or by blocking the pore specific for its NES [37,38] caused nuclear accumulation. Direct evidence of the shuttling and the shuttling kinetics were determined by simple time-lapse fluorescence microscopy of translocation and by combined Fluorescence Loss In Photobleaching (FLIP) and Fluorescence Recovery After Photobleaching (FRAP) experiments. In these experiments, fluorescence labeled Smads were first observed to enter the nucleus upon stimulation and were then photobleached in the area of the nucleus to measure the diffusion rates in the nucleus and cytoplasm and rates between them during stimulation [39,40]. At present, the information from dynamic optical microscopic techniques indicates that Smad proteins are more mobile than as indicated from biochemical techniques. For example, it is not clear why activation and complexation of R-Smads is relatively slow [7] when they are apparently freely mobile in the cytoplasm [39]. The studies also indicate that the shuttling is reduced after Smads form complexes, Smads have their NES motif blocked after phosphorylation, and phosphorylated pools of Smads do not exist in the cytoplasm [39]. In these experiments and results, only fluorescence measurements were performed to indirectly indicate the detailed events up to Smad signaling, including the dynamics of phosphorylation and complex formation. Up to now, the direct imaging analogies to Western blot analyses of Smad phosphorylation and to immunoprecipitations of Smad complexes are lacking. Direct imaging could provide the evidence to find the rate-limiting steps in the process of phosphorylation and complex formation. In this work we created fluorescent biosensors of Smad1 and Smad4 proteins. With these biosensors we observed and monitored the activation of Smad1, the role of the Smad1 MH1 domain in this activation, and Smad1/Smad4 complex formation upon BMP-4 stimulation with detailed and unprecedented time resolution by Fluorescence Resonance Energy Transfer (FRET) microscopy.

2. Materials and methods

2.1. Plasmids, reagents and cells

HA-tagged BMP receptors type 1b and 2 were cloned in pcDNA3. BRib was used as a best partner of BRII for Smad activation [6]. The DNA fragments coding fluorescent proteins (CFP and YFP) were PCR amplified from pEYFP and pECFP (Clontech), respectively and inserted in-frame to the C- or N-terminus or at the stated position within of the DNA sequences coding for the full-length Smad protein or only part of Smad1 or Smad4. All fusion constructs were ligated into the pcDNA3 expression vector (Invitrogen) for transient expression in mammalian cells. HeLa, HEK 293, COS-1 and MDA-MB468 cell lines were grown in DMEM/10% FCS and were transiently transfected with FuGENE 6 (Roche Molecular Biochemicals), Effectene, or SuperFect (Qagen) transfection reagents.

2.2. Western blots

COS-1 cells were transiently transfected with Smad fusion protein and BMP receptor expression plasmids in a 6-well plate. Twenty-four hours after transfection, cells were starved in DMEM supplemented with 0.5% fetal calf serum for 6 h. After incubation with 20 nM of BMP-4 for 5, 15, 30, or 60 min cells were harvested and lysed in lysis buffer (Cell Signaling Technology). The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 8 or 12% gels. After electrophoreses, electrotransfer (PEQLAB Biotechnologie) and blocking (10 mM Tris, pH 7.9, 150 mM NaCl, 0.1% Tween 20, and 5% dry milk; 20 °C, 1 h), the blot was incubated with monoclonal rabbit antibodies against Phospho-Smad1 (Ser463/465), with anti-Smad1 and Smad4 vectors were co-transfected per well. Thirty-six hours after transfection, cells were starved in DMEM supplemented with 0.5% FCS. Cells were analyzed before or after treatment with 20 nM of BMP-4 for 20 h or 4 °C. Amounts of protein were also determined with a monoclonal rabbit antibody against β-actin (Cell Signaling Technology). Detection of adsorbed antibodies was performed by enhanced chemiluminescence (LumiGLO, Super-Signal WestPico, Upstate), using an HRP-conjugated secondary anti-rabbit polyclonal IgG antibody (Upstate) in blocking buffer.

2.3. Luciferase assays

Twenty-four hours before transfection, MDA-MB468 cells were seeded in triplicate at 2 × 10^5 cells per well in a 6-well plate. 0.2 μg each of pSBE-Luciferase construct (from C. H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden), 0.05 μg of BMP receptors constructs, and 0.4 μg of indicated fusion Smad1/Smad4 vectors were co-transfected per well. Thirty-six hours after transfection, cells were starved in DMEM (0.5% FCS) for 10 h and then incubated in DMEM (0.5% FCS) with 20 nM BMP-4 for 8 h. To control protein expression levels and normalize luciferase activity, all samples were transfected with a Renilla Luciferase coding vector (0.1 μg each). The cells were subsequently lysed with reporter lysis buffer, and luciferase activity was determined with a Dual-Luciferase assay kit (Promega) on a FLUOstar (BMG Labtech) luminometer. Each experiment was repeated at least three times.

2.4. Confocal microscopy

HeLa or COS-1 cells were grown to 50% confluence on 26-mm glass coverslips, transiently transfected, and incubated in complete DMEM medium for 24 h. After this, cells were incubated for 2 to 5 h with DMEM containing 0.5% FCS. Cells were analyzed before or after treatment with 20 nM BMP-4 or 20 nM leptomycin B (LMB). All live cell imaging was performed at 37 °C. The fluorescence images were recorded on either a modified confocal microscope (LSM-410, Carl Zeiss and LSM Tech) with a 100× NA 1.3 objective (Carl Zeiss)
or a Leica SP5 confocal microscope with a 63× NA 1.4 objective. The fluorescence emission resulted from excitation with 458 (for CFP) and 514 (for YFP) nm laser lines from an argon ion laser (Coherent Inova, I-308) from the modified Zeiss LSM 410 or Laos (DMI 600B, Germany) with the Leica SP5 LSM confocal with internal spectral parameter settings of 458 for CFP and 514 nm for YFP. The fluorescence was detected with either optical filter sets from the microscope manufacturer using a 475–495 band pass filter for CFP and a 530/55 band pass filter for YFP or with SP5 a 490–500 nm spectral band width setting for CFP and a 520–590 nm spectral band width setting for YFP.

2.5. FRET microscopy measurements

In this study, a fluorescence microscope was used to perform FRET measurements [41–43]. We quantified the FRET signal by calculating excess acceptor emission using FRET ratios from defined regions of interest in, for example, the cytoplasm. The setup for fluorescence resonance energy transfer between the cyan and yellow fluorescent proteins comprises an inverted microscope (Axiovert 200, Carl Zeiss) equipped with a high numerical aperture objective (Plan Apochromat 100×, 1.4 NA, Carl Zeiss). Samples were excited with wide-field light from a computer-driven monochromator Xenon lamp source (Polychrome IV, Till Photonics) at 436±5 nm (CFP) and 480±5 nm (YFP) that was connected to the microscope with an optical light guide and optical focusing system (Till Photonics) with the excitation light reflected by a dichroic beam splitter (for CFP and FRET measurements, DCLP 460 or for YFP measurements, DCLP 490, Chroma) into the microscope objective. The fluorescence CFP and YFP signals were detected in the emission light train of the microscope by avalanche photodiodes (Till Photonics) separated by a dichroic beam splitter at 505 nm (DCLP 505, Chroma) and with bandpass filters in front of each of the detectors, one at 480±20 nm (HQ480/40, Chroma) for CFP emission and the other at 535±15 nm (HQ535/30, Chroma.) for YFP emission, and were digitalized using an AD converter (Digitida1322A, Axon Instruments) and stored on a personal computer using Clampect 8.1 software (Axon Instruments) that also synchronized the excitation wavelength with the data acquisition. In a few cases fluorescence images and recordings were made by detection from a CCD camera (CoolSnap HQ, Photometrics) with the image on the CCD camera split spectrally in half and re-imaged on the CCD with a relay system with a dichroic beam splitter at 505 nm and two filtered split images bandpass filtered again with a 480±20 nm for CFP emission and the other at 535±15 nm for YFP emission (Dual View, Optical Insights). FRET (including the individual CFP and YFP) signals and images were acquired every 0.1 or 1 s.

FRET is calculated as the ratio of the corrected YFP and CFP emission intensities at 535±15 nm (\(I_{YFP}\)) and 480±20 nm (\(I_{CFP}\)) upon excitation of CFP at 436±5 nm (beam splitter DCLP 460 nm). The YFP (acceptor) direct excitation factor was determined with YFP transfected cells only. The YFP fluorescence was recorded first with 436±5 nm excitation (\(F_{YAbl}\)). Next the YFP emission was recorded with 480 nm excitation (\(F_{YAb0}\)). The direct excitation crossover was calculated by \(F_{YAbl}/F_{YAb0}\) and was equal to 0.06±0.01. The YFP only cells did not show any emission intensity in the CFP emission channel with 436±5 nm excitation. The bleed-through or spillover crosstalk of CFP (donor) into the 535-nm channel was determined first from cells expressing CFP with 436±5 nm excitation and also of pure recombinant CFP and YFP, respectively at 535±15 nm excitation, respectively, and \(\phi_{CFP}\) and \(\phi_{YFP}\) are the fluorescence quantum yields of CFP (0.36) and YFP (0.76) [45,46].

2.6. Fluorescence Lifetime Imaging (FLIM) and FLIM FRET determinations

HeLa or COS-1 cells were grown to 50% confluence on 26-mm glass cover slips, transiently transfected with the CFP-Smad1 and YFP-L-Smad4 plasmids (as individuals for control and co-transfected for the FRET experiments), and incubated in full DMEM medium for 24 h. After this, cells were incubated for 2 to 5 h with DMEM without serum. Cells were analyzed before and after treatment with 20 nM BMP-4 (Sigma) every 15 min after addition of BMP-4 up to 45 min. All imaging experiments were performed on a Leica SP5 confocal laser scanning microscope (Leica) using a DMI 6000 inverted microscope stand with a HCX PL APO lambda blue 63x/1.4 OIL UV objective, equipped with a “multi-function port” with a mechanically controlled beamsplitter that could be automatically placed in the excitation and imaging beam pathway for reflection of the laser excitation light and transmission of the fluorescence emission light and also with two built-in spectrally selective photon counting detectors (set at 460–500 nm for CFP and 520–590 nm for YFP, respectively for each channel) with a detector to show that the FRET change was induced by BMP-4 addition but not by buffer or media. The imaging data were analyzed with Origin (Microcal) software. Imaged and MetaMorph 5.0 (Universal Imaging) were also used in some cases. All live cell imaging was performed at 37 °C.

We proved FRET by photobleaching the acceptor and then observed the donor dequenching (donor signal increase) and also by FLIM FRET imaging (see below). The calculation of relative CFP:YFP concentrations for the intramolecular FRET were corrected by dividing by the brightness of the individual, initial CFP (\(I_{CFP436}\)) and YFP (\(I_{YFPcorr}\)) intensities:

\[
\text{I}_{\text{corr}(436)} = \left( \frac{\text{I}_{\text{YFP}}}{\text{I}_{\text{YFPcorr}}} \right) \text{I}_{\text{YFPcorr}}, \quad \text{I}_{\text{corr}(480)} = \left( \frac{\text{I}_{\text{CFP}}}{\text{I}_{\text{YFPcorr}}} \right) \text{I}_{\text{YFPcorr}}
\]

where \(t_{\text{CFP}}\) and \(t_{\text{YFP}}\) are the optical transmissions for CFP and YFP in the respective CFP (0.35) and YFP (0.60) detection channels, \(I_{\text{YFPcorr}}\) and \(I_{\text{YFPsum}}\) are the molar extinction coefficients of CFP (28,000 M\(^{-1}\) cm\(^{-1}\)) and YFP (7,000 M\(^{-1}\) cm\(^{-1}\)) at 436±5 nm excitation, respectively, and \(\phi_{CFP}\) and \(\phi_{YFP}\) are the fluorescence quantum yields of CFP (0.36) and YFP (0.76) [45,46].

Fig. 1. Construction of Smad1 FRET biosensor. (A) The Smad1YC fusion protein was constructed by inserting YFP between the MH1 and MH2 domains of human Smad1. CFP was attached with specific short linker sequence (L) to the C-terminus. (B) COS1 cells transfected with the Smad1YC expression plasmid were stimulated with BMP-4. Cells were harvested at the indicated times after BMP-4 stimulation and analyzed by Western blot for the presence of phosphorylated Smad1 (pSmad1). The pSmad1 antibody recognizes both endogenous pSmad1 and pSmad1YC. Total Smad1YC and α actin are shown as controls. The identity of the 110-kDa protein as Smad1YC was verified using an anti-fluorescent protein (αFP) antibody.
response time of less than 200 ps (Leica “Spectral FLIM” internal detectors). The FLIM excitation source was a Ti:Sapphire femtosecond pulsed laser (Maitai HP, Spectra-Physics). The laser was tuned to provide a wavelength of 860 nm. A second harmonic generation (SHG) crystal (LBO-Crystal for SHG wavelength: 800–1100 nm, LINOS Photonics) was used to frequency double the wavelength from 860 nm to 430 nm which efficiently excites CFP and causes minimal excitation of YFP. All remaining 860 nm light was filtered out by a 510 dcxr dichroic (Chroma) which reflected the 430-nm pulsed excitation beam into the “multifunction port” and, thus, the Leica SP5 scanning and imaging system. The 430-nm laser power for all experiments was measured at the objective to be 5 μW. Live cells on glass coverslips (26 mm) were imaged using a homebuilt incubation chamber kept at 37 °C via an objective heater (PeCon).

The FLIM measurements were carried out by time-correlated single photon counting (TCSPC) recording and used the ‘reversed start-stop’ approach, with accurate laser synchronization from a Becker & Hickl SPC-830 card (Becker & Hickl) together with a PHD-400-N reference photodiode recording the 80-MHz pulse frequency of the frequency excitation light. The Leica SP5 software recorded images and split the amplified signal between the Leica imaging software (LASAF, Leica) and the SPC-830 TPSPC card controlled by the SPC-830 software on a separate computer with the imaging synchronized via the external output of the frame, line, and pixel clock from the Leica SP5 into the SPC-830.

FLIM (TCSPC) recordings were acquired routinely for between 120 s and 150 s. The mean photon counts were between $10^4$ and $10^5$ counts per second to

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**Fig. 2. Intramolecular FRET kinetics measurement.** (A) Measurement and (B) computerized analysis of FRET (black line of fit in the gray calculated measurement points) represented by the ratio $F_{\text{YFP}}/F_{\text{CFP}}$ from (C), the individual $F_{\text{YFP}}$ and $F_{\text{CFP}}$ corrected measurements in single COS-1 cell transiently co-transfected with Smad1YC and BMP receptors. Stimulation with BMP-4 began with nearly no response in the FRET ratio until after about 300 s, when a FRET change with an exponential time constant of about 300 s was observed with corresponding decrease in the YFP channel and increase in the CFP channel. (D) Measurement and computerized analysis of FRET (represented by the ratio $F_{\text{YFP}}/F_{\text{CFP}}$) in single COS-1 cells transiently transfected with Smad1(SA)YC and BMP receptors. Stimulation with BMP-4 in a minority of cases began with a slow decrease in the FRET channel of about 880 s (right panel) or in the majority of cases with no significant change or decrease in FRET (left panel).
avoid pulse pileup. Images were recorded with 256 × 256 Pixel at 400 Hz line scanning speed.

2.7. FLIM data analysis and FRET calculations

Off-line FLIM data analysis used pixel-based fitting software (SPCImage 2.8, Becker & Hickl), able to import the binary data generated with the FLIM module. The fluorescence usually fit best to and, thus, was always assumed to follow a two-exponential decay which was used to calculate a weighted mean lifetime per pixel for complete images. The fitting procedure also included an adaptive offset correction and also convolution procedures to remove timing jitter occurring from both the detectors and the electronics for accuracy (see User’s Manual for SPC-830 Software). ImageJ 1.37 (NIH) was used for calculation of mean lifetime in cell areas.

Although both the CFP and YFP intensity signals were checked for appropriate levels (data not shown), we focused our attention to the CFP lifetime in the purely expressed case of the CFP-fusion protein \( \tau_{\text{CFP}} = 2.40 \pm 0.04 \) ns for CFP-Smad1 or in the case of FRET (with co-transfection of the YFP fusion protein, \( \tau_{\text{CFP, FRET}} \)). The FRET efficiency from these data can be calculated from the equation

\[
\text{FRET} = \left( 1 - \frac{\tau_{\text{CFP, FRET}}}{\tau_{\text{CFP}}} \right)
\]

The difference in calculated FRET before and after BMP-4 ligand addition was multiplied by 100 and reported for pixel (and thus entire FRET images) and also whole cell regions in % FRET change.

The calculation of relative CFP/YFP concentrations for the intermolecular FRET-FLIM were performed by the same procedure listed above FRET Microscopy section but with CFP (\( I_{\text{CFP}}(430) \)) and YFP (\( I_{\text{YFP}}(430) \)) photon counting intensities from cellular regions with 430 nm excitation. The YFP spillover or bleed-through correction into the CFP emission channel at 430 nm excitation was 0.02. The YFP direct excitation crosstalk was determined to be 0.02. The CFP bleed-through or spillover crosstalk was determined to be 0.31. The calculation of relative CFP/YFP ratios was also corrected by dividing by the brightness of the individual, initial CFP (\( I_{\text{CFP}}(430) \)) and YFP (\( I_{\text{YFP, corr}} \)) intensities:

\[
\frac{I_{\text{CFP}}(430)}{I_{\text{YFP, corr}}} = \frac{\epsilon_{\text{CFP}} \phi_{\text{CFP}}}{\Phi_{\text{CFP}}} \quad \text{and} \quad \frac{I_{\text{YFP}}}{I_{\text{YFP, corr}}} = \frac{\epsilon_{\text{YFP}} \phi_{\text{YFP}}}{\Phi_{\text{YFP}}}
\]

where \( \epsilon \) and \( \phi \) are the optical transmissions for CFP and YFP in the respective CFP

Fig. 3. Construction of Smad1 MH2 domain-based FRET biosensor. (A) The YSmad1C fusion protein was constructed by flanking the human Smad1 MH2 domain with YFP and CFP. CFP was attached with the specific short linker sequence (L) to the C-terminus. The construct does not contain the MH1 domain and the MH1 linker. (B) COS1 cells transfected with the YSmad1C expression plasmid were stimulated with BMP-4. Cells were harvested at the indicated times after BMP-4 stimulation and analyzed by Western blot for the presence of phosphorylated Smad1 (pSmad1). Total YSmad1C was detected using an anti-fluorescent protein (αFP) antibody.

Fig. 4. Intramolecular FRET kinetics measurement. (A) Measurement and (B) computerized analysis of FRET (black line of fit in the gray calculated measurement points) represented by the ratio \( F_{\text{YFP}}/F_{\text{CFP}} \) from panel C, the individual \( F_{\text{YFP}} \) and \( F_{\text{CFP}} \) corrected measurements in single COS-1 cell transiently transfected with YSmad1C and BMP receptors. Stimulation of the YSmad1C transfected cells with BMP-4 leads to a rapid decrease of FRET in about 300 s and a slow recovery phase of about 600 s with corresponding and decreases in YFP channel and increases in CFP. (D) Measurement of FRET (represented by the ratio \( F_{\text{YFP}}/F_{\text{CFP}} \)) in single COS-1 cells transiently transfected with YSmad1(SA)C and BMP receptors. Stimulation with BMP-4 showed no response in FRET channel.
(0.53) and YFP (0.80) detection channels, \( \varepsilon_{\text{CFP},430} \) and \( \varepsilon_{\text{YFP},430} \) are the molar extinction coefficients of CFP (28,000 M\(^{-1}\) cm\(^{-1}\)) and YFP (2000 M\(^{-1}\) cm\(^{-1}\)) at 430 nm excitation and \( \varphi_{\text{CFP}} \) and \( \varphi_{\text{YFP}} \) are the fluorescence quantum yields of CFP (0.36) and YFP (0.76) [45,46].

The level of CFP photobleaching was initially tested to be insignificant according to the levels reported by Tramier et al. [54]. First the CFP lifetimes were measured multiple times in CFP only transfected control cells and showed no photobleaching under the experimental conditions and no significant difference in lifetime. Second CSmad1 and YLSmad4 transfected cells, 5 min before and immediately after BMP addition showed less than 10% photobleaching and less than 2% change in fluorescence lifetime between the two successive images. Furthermore, we calculated the “apparent photobleaching ratio” (as defined by Tramier et al. [54]) for the CFP (\( I_{\text{CFP}}(430) \)) in the CSmad1 and YLSmad4 transfected cells from the intensity with images recorded at 15, 30, and 45 min after BMP addition and used the \( t=0 \) min (i.e. immediately after BMP addition) as the initial reference in every case.

3. Results

3.1. Construction of a full-length Smad1 FRET biosensor

To better understand the kinetics of activation of Smad1, we created a FRET sensor for Smad1 activation by fusing a cyan fluorescent protein (CFP) to the C-terminus and a yellow fluorescent protein (YFP) between the MH2 and the MH1 domains with a linker. This FRET sensor was named Smad1YC (Fig. 1A). As Smad1 is known to switch in its conformation upon phosphorylation [26,47] we hypothesized that it might lead to a change in the distance between the fluorophores upon activation by the BMP receptors. The linker between the C-terminus of Smad1 and CFP was inserted in order to avoid impairing phosphorylation of Smad1 at its C-terminal SSXS motif. Various linkers were tested, and a GSTSGSGK peptide linker proved to be most effective (data not shown). However, the Smad1 MH2 domain conformational change has also been shown to occur with homo- and heterocomplex formation after stimulation with a ligand [48]. First, we tested whether the fusion protein retained the phosphorylation properties of wild type Smad1. To do this, COS-1 cells were transfected with the Smad1YC expression plasmid for Western blot analysis. The fusion protein was phosphorylated at a similar time as the endogenous Smad1 in response to BMP-4 (Fig. 1B). We conducted kinetic experiments by FRET microscopy in living cells in order to test whether the Smad1YC fusion protein functions as a biosensor reflecting Smad1 phosphorylation. COS-1 cells were transiently transfected with plasmids encoding Smad1YC and BMP receptors, and FRET in single cells (\( n=10 \)) between CFP and YFP was measured showing a signal decrease in the YFP channel and an increase in the CFP channel (Fig. 2A and C). Computerized analysis of the calculated FRET data were used to determine the kinetics of the FRET change (Fig. 2B).

In cells stimulated with BMP-4, the FRET signal decreased slowly with an average time of 315±70 s but only after an initial delay of 284±45 s with no FRET change (Fig. 2B).

To demonstrate that the FRET signal change reflects Smad1 phosphorylation, we mutated the Smad1 phosphorylation motif (SSXS) by exchanging the three serines for alanines. The fusion was named Smad1(SA)YC. COS-1 cells were transiently transfected with plasmids encoding Smad1(SA)YC and BMP receptors and analyzed by FRET. The Smad1(SA)YC did not produce the same FRET change after BMP-4 addition as the Smad1YC biosensor did (Fig. 2E). The signals in some cases (\( n=3 \)) rather showed an immediate slow decay with a time constant of 800±180 s (Fig. 2D, right), but in most cases (\( n=5 \)) no FRET change or decay was observed at all (Fig. 2D, left). The smaller and slower FRET decrease occurring in the minority of cases of the non-phosphorylating mutant might be due to interactions with other proteins (as indicated previously) that could form a complex and thus slightly alter the conformational changes in the of non-phosphorylated mutated Smad1YC constructions. In summary, the approximate 300-s delay and 300-s decay are shown to be linked to the active, phosphorylating Smad1YC biosensor.

3.2. Construction of a Smad1 MH2 domain-based FRET biosensor

To analyze the role of the MH1 domain in more detail and improve the understanding of the kinetics of Smad1 activation by phosphorylation, we created a Smad1-MH2 biosensor without the MH1 domain and linker. We also intended to create a biosensor, with a faster response upon BMP-4 activation as compared to the full Smad1 biosensor. To construct the biosensor, the sequence of the human Smad1 MH2 domain was flanked by sequences coding for YFP and CFP as shown in Fig. 3A. This sensor was named YSmad1C. The same linker as for Smad1YC was inserted between the C terminus of Smad1 and the CFP. COS-1 cells were transfected with plasmids encoding YSmad1C and BMP receptors for Western blot analysis of the phosphorylation properties. The Western blots indicated that the YSmad1C showed a notably faster phosphorylation rate than wild-type Smad1. The band reflecting the phosphorylated fusion protein could be detected already without BMP-4 stimulation, and the signal increased within the first minute after BMP-4 addition (Fig. 3B). By contrast, phosphorylation of the endogenous Smad1 protein (as a control in the same cells) showed no significant phosphorylation within the first 5 min after BMP-4 addition (Fig. 3B). Next, COS-1 cells were transiently transfected with YSmad1C for FRET observa-
tions \((n=9)\) between CFP and YFP (Fig. 4A and C). The FRET data were subjected to computerized analysis to determine the kinetics of the FRET change. After stimulation of the cells with BMP-4, a fast decrease with subsequent slow recovery of FRET ratios was recorded with time constants of the decrease of 300 ± 40 s and of the slow increase of 600 ± 140 s (Fig. 4B). We also at times had observations of the same rate of FRET decrease without any recovery (data not shown). To demonstrate that the FRET signal changes reflected Smad1 phosphorylation we also created the same YSmad1C fusion protein with an exchange of the three distal serines to alanines. This fusion protein was named YSmad1(SA)C. COS-1 cells were transiently transfected with plasmids encoding YSmad1(SA)C and BMP receptors and analyzed by FRET microscopy \((n=6)\). As shown in Fig. 4D, the serine-to-alanine mutations abolished the FRET change, indicating that phosphorylation of the SSXS motif is required for the FRET change.

In summary, the Western blot (Fig. 3B) and FRET experiments (Fig. 4A) demonstrated that the YSmad1C sensor lacking an MH1 domain was phosphorylated almost immediately after addition of BMP-4.

3.3. BMP4-induced nuclear translocation and transcriptional activity of fluorescent Smad fusion proteins

To measure the interaction of Smad1 and Smad4 by FRET, we constructed expression plasmids encoding fusion proteins of human Smad1 with CFP (CSmad1) and Smad4 with YFP and linker (YLSmad4) as depicted in Fig. 5A. We used a linker between YFP and Smad4 to preserve the functional activity of Smad4. A linker was not necessary for the CSmad1 fusion protein. Nuclear translocation of CSmad1 and YLS-mad4 upon BMP-4 response would be an indicator of physiologically normal behavior, as has been shown for endogenous Smads [37]. To test this, we transfected COS-1 or HEK 293 cells with the fluorescent Smad fusion expression plasmids and analyzed the behavior of the fusion proteins in the absence or presence of BMP-4 (Fig. 5B and C). To ensure that the CFP/YFPSmads perform nucleocytoplasmic shuttling in the absence of BMP-4 stimulation, cells were pretreated with leptomycin B (LMB), which inhibits the nuclear export by CRM1/exportin-1. CRM1 has previously been identified as the nuclear transporter for NES-dependent export [49]. CSmad1 and YLSmad4 were predominantly cytoplasmic in untreated cells and predominantly nuclear in cells treated with LMB (Fig. 5B and C). Similarly, the fusion proteins accumulated in the nucleus after 60 min of induction with BMP-4 (Fig. 5B and C).

To test whether co-expression of the two Smad fusion proteins, CSmad1 and YLSmad4, impairs nuclear translocation, COS-1 cells were co-transfected with both CSmad1 and YLSmad4 expression plasmids to observe in parallel nuclear translocation of each fusion protein over time upon BMP-4 or LMB addition. With confocal microscopy, we clearly observed that co-expression does not prevent nuclear translocation of both fusion proteins upon LMB addition (Fig. 5D). The parallel observation of nuclear translocation of each fusion protein over time upon BMP-4 addition was more difficult. Previously published results of others indicated that nuclear translocation in complexes is less favored in comparison to the translocation of...
Fig. 7. Intermolecular FRET kinetics of CSmad1 and YSmad4 in single (A) COS-1 and (B) HeLa, (C) MDA-MB-468 cells. Cells were co-transfected with plasmids encoding the Smad fusion proteins and the BMP receptor. Stimulation with BMP-4 caused very little response in the FRET channel for a dormant period of about 300 s, after which a FRET increase occurred with a time constant of about 600 s with a corresponding increase in the YFP and a decrease in the CFP channel. (D) MDA-MB468 cell transiently expressing BMP receptors and YLSmad4 and CSmad1 fusion proteins were analyzed by a technique called Donor Dequenching after Acceptor Photobleaching. Emission Intensities of YFP (535 nm, yellow) and CFP (480 nm, cyan) were recorded before and after the acceptor fluorophore (YFP) was completely photobleached with 500 nm excitation light. (In all figures, the CFP:YFP concentration ratio was reported. See Materials and methods and also Results for the calculation and interpretation of the ratios.)
endogenous Smads [40] because fluorescent proteins fused to Smads have a lower mobility and apparently lower nuclear translocation probabilities spatially in heterocomplex. Nonetheless, nuclear translocation of the fusion proteins upon BMP addition, was observed by means of confocal fluorescence lifetime imaging (FLIM), a technique which can determine FRET efficiency by changes in the fluorescence lifetime of the donor in the presence of the acceptor on a pixel-by-pixel basis for time-lapsed images on the minute time-scale (see Materials and methods). We could indeed observe nuclear translocation of CSmad1/YLSmad4 upon BMP-4 addition with FLIM and describe it in the next section.

The transcriptional activity of CSmad1 and YLSmad4 was subsequently tested in established assays to ensure that they retained the activity of wild-type Smads. COS-1 cells were transfected with these expression plasmids for Western blot analysis. The Western blots indicated that CSmad1 was phosphorylated efficiently in response to BMP-4, as was endogenous Smad1 (Fig. 6A). We also tested whether complexes of Smad1 and Smad4 fusion proteins were transcriptionally active using a luciferase reporter assay similar to the one previously used by Nicolas et al. [40]. To do this, the Smad fusion proteins were expressed in MDA-MB468 cells which lack endogenous Smad4, but contain R-Smads. The results of the luciferase assays were difficult to obtain without long periods of starvation (see Materials and methods) due to over-expression of endogenous R-Smads mixed with normal cell signaling activities creating unfavorable basal activities. The starvation apparently reduces the endogenous Smad1 levels so that co-transfection of both Smad1 and Smad4 was necessary to observe the difference between the active and non-active cells. A Smad1/Smad4-dependent luciferase reporter plasmid, pSBELuciferase, containing the Smad binding element [50] was transiently transfected together with the CSmad1 (Fig. 6B) or YLSmad4 (Fig. 6C) expression plasmid. As shown in Fig. 6B, luciferase expression was enhanced by co-transfecting wtSmad4 with CSmad1. YLSmad4 co-transfected with wtSmad4 also increased the luciferase activity (Fig. 6C).

From the data presented in this part we concluded that CSmad1 and YLSmad4 retain the normal function as a transcription factor.

### 3.4. Kinetic studies of Smad1/Smad4 fusion proteins complex formation

We wanted to use the Smad fusion proteins to measure the complex formation rate between CSmad1 and YLSmad4 by FRET. COS-1 (n=3), HeLa (n=3) and MDA-MB468 (n=3) cells were transiently transfected with plasmid constructs expressing CSmad1, YLSmad4, and BMP receptors. Then we observed the kinetics of heteromeric complex formation of these fusion proteins before and after stimulation with BMP-4. The experiments in Fig. 7A and B demonstrated that FRET occurred between these constructs in COS-1 and HeLa cell after addition of BMP-4 with corresponding increases in the YFP channel and decreases in the CFP channel. Computer-assisted analysis of the FRET data revealed an increase of the FRET signal in the cells with an average time constant of 517 ± 160 s, but only after a 260 ± 48 s delay with no FRET change (Fig. 7A and B). A two times higher amplitude of FRET increase was observed in MDA-MB468 breast cancer cells. This could be explained by the absence of the endogenous Smad4 that likely serves as a competitor of the Smad4 fusion protein (Fig. 7C). We also performed another control in which we reliably determined FRET in the MDA-MB468 cell line (n=3) (20 min after BMP-4 addition) by acceptor photobleaching. We selectively photobleached the acceptor and, as expected, this maneuver significantly decreased the fluorescence of YFP accompanied by successive increase in the fluorescence of CFP by 15% (Fig. 7D). The donor to acceptor ratios ranges measured for all of the CSmad1/YLSmad4 intermolecular FRET cells from Fig. 7 (and further measured cells for statistics with data not shown) were between 0.7 and 2.1. Between these values, no major trends of FRET change and CFP/YFP ratio were observed. Although a few trials were attempted well above and well below these CFP/YFP ratio ranges, a stable FRET was never observed. These observations are in accordance with the predicted donor to acceptor concentration range of 0.1 to 10 by Berney et al. [51] for a stable, detectable FRET to occur.

As an additional method for proving intermolecular FRET we used fluorescence lifetime imaging (FLIM). We transiently co-transfected HeLa cells with plasmid constructs expressing CSmad1, YLSmad4, and BMP receptors. We observed the kinetics of FRET of these fusion proteins before and after addition of BMP-4 with corresponding increases in the YFP channel and decreases in the CFP channel. Computer-assisted analysis of the FRET data revealed an increase of the FRET signal in the cells with an average time constant of 517 ± 160 s, but only after a 260 ± 48 s delay with no FRET change (Fig. 7A and B). A two times higher amplitude of FRET increase was observed in MDA-MB468 breast cancer cells. This could be explained by the absence of the endogenous Smad4 that likely serves as a competitor of the Smad4 fusion protein (Fig. 7C). We also performed another control in which we reliably determined FRET in the MDA-MB468 cell line (n=3) (20 min after BMP-4 addition) by acceptor photobleaching. We selectively photobleached the acceptor and, as expected, this maneuver significantly decreased the fluorescence of YFP accompanied by successive increase in the fluorescence of CFP by 15% (Fig. 7D). The donor to acceptor ratios ranges measured for all of the CSmad1/YLSmad4 intermolecular FRET cells from Fig. 7 (and further measured cells for statistics with data not shown) were between 0.7 and 2.1. Between these values, no major trends of FRET change and CFP/YFP ratio were observed. Although a few trials were attempted well above and well below these CFP/YFP ratio ranges, a stable FRET was never observed. These observations are in accordance with the predicted donor to acceptor concentration range of 0.1 to 10 by Berney et al. [51] for a stable, detectable FRET to occur.

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stimulation with BMP-4 for 45 min (Fig. 8A (upper panels)) and measured every 15 min.

Analysis of the CFP lifetime of the 17 HeLa cells in Fig. 8A (upper panels) revealed a significant decrease in fitted and averaged decay time constants from 2.27±0.06 ns before addition to 1.87±0.03 ns 45 min after addition of BMP-4.

Shorter fluorescence lifetimes in comparison to the pure CFP lifetime (in this case of singly expressed CSmad1 in HeLa cells) are due to the interaction with the acceptor molecule (YLSmad4) causing the FRET [45,52,53]. Furthermore, the ratio of the CFP (FRET) lifetime to the pure CFP lifetime can be used to calculate the FRET efficiency (see Materials and methods). Before BMP addition of the co-transfected CSmad1/YLSmad4 HeLa cells (n=17), the CFP lifetime indicated a baseline FRET efficiency of 5±2% in contrast to 45 min after addition revealing a FRET efficiency of 22±2% giving rise to a total FRET change (ΔFRET) of 17±4%.

The ΔFRET was calculated on a pixel-by-pixel basis for all of the 17 cells showing a normal distribution (see Fig. 8B for 15 min and 45 min after BMP addition) to verify mean and standard error distribution of the FRET change (6±1% for 15 min and 11±1% for 45 min after BMP addition). The slight differences in FRET change calculation here (in comparison to the 17% calculated above) are almost entirely due to pixel shifts in time during image acquisition mostly due to the slow migration of the cells.

The ΔFRET was also determined for the 17 individual cells at 15, 30, and 45 min after BMP addition to show the dynamics and statistics contributing to this FRET (Fig. 8C). A majority of cells (11 of 17 and 12 of 17) show a significant ΔFRET increase of >6% in 30 and 45 min after addition, respectively, with nearly half (7 of 17) showing a >6% ΔFRET in 15 min after addition.

The range of CFP/ YFP ratios where ΔFRET occurred was found to be between 0.3 and 3.1 agreeing to the acceptable range as reported by Berney et al. However, due to the possibility of photobleaching (discussed below), and removal of data from photobleaching, the dynamic range would be reduced to 0.7 to 3.1, overlapping the values reported above.

Photobleaching of CFP has also been shown to affect fluorescence lifetime measurements and, thus, could cause a falsely reported FRET or ΔFRET [54]. Care was taken to use experimental conditions that did not allow photobleaching (see Materials and methods). However, upon application of the “apparent photobleaching ratio” (as defined by Tramier et al. [54]) for the CFP (see Materials and methods for calculation and Fig. 8C for individual cell values), 7 cells showed a significant “apparent photobleaching” (>0.3) and two cells showed a slight “apparent photobleaching” (<0.3 but >0.1) causing greater than 20% shorter CFP fluorescence lifetimes in the significant case and less than 10% shorter lifetimes in the slight case. Because the possibility exists that further CFP fluorescence intensity decreases, indicated by a lower “apparent photobleaching” value, could occur to either reorganization (by nuclear localization or cell shifting) or to photobleaching, we have further categorized all of the form from CFP for measurements into four possible cases: no photobleaching and no reorganization, no photobleaching and reorganization, photobleaching and reorganization, and photobleaching and no reorganization. The ΔFRET remains to be highly significant after rejection for any possibility of photobleaching with 5 of the 8 cells >0.4. The ΔFRET improves even further in dynamics and statistics if the reorganization of CFP rules out the photobleaching rejection with 12 of 15 cells >0.04 and 9 of 15 cells >0.06.

As mentioned above, we show in Fig. 8A (upper panels) FLIM images of CSmad1 before and after BMP-4 addition that have significantly decreasing donor (CFP) lifetimes in the majority of cases in the cytosol (12 of 17 cells) and that also show in some cases the FRET change slowly traveling in to the nucleus of HeLa cells (4 of 17 cells). Only one cell showed a comparatively clear CSmad1 and YLSmad4 nuclear translocation to complement the nuclear translocating FRET (Fig. 8A (encircled cell in upper panels) and (bottom panels)).

4. Discussion

In this study we report the development of fluorescent biosensors for direct visualization of Smad signaling. With these Smad biosensors we have investigated the kinetics of Smad1 activation which is defined by phosphorylation-induced concerted structural changes of the MH2 domain. In addition, the kinetics of the Smad1/Smad4 complex formation was analyzed by FRET using CFP and YFP fusions of Smad1 and Smad4, respectively. We have also demonstrated that these Smad fusion proteins closely mimic the behavior of endogenous Smad1 and Smad4 in terms of their activation and their formation of active Smad transcription factor complexes on Smad-responsive elements.

The kinetics of activation of Smad1 is a paradox in that it diffuses quickly through the cell but phosphorylates slowly. Using FRET we first addressed the question, what is the rate-limiting step of the BMP signaling cascade. BMP-4 signals via two types of receptors (BRI and BRII) that are expressed at the cell surface as homomeric or heteromeric complexes. The ligand, in this case BMP-4, has two options for binding to the receptors. It can bind to the high-affinity receptor BRI and then recruit BRII into a hetero-oligomeric complex. This process leads to activation of the Smad independent p38-MAPK pathway. The other alternative is to bind simultaneously to the preformed hetero-oligomeric complexes. These complexes then activate the Smad signaling pathway [55]. In previous publications [6,55,56] and own unpublished data, a high concentration of preformed complexes of BMP receptors which signal towards the Smad pathway was observed on the cell surface. Thus, we made the presumption that upon BMP-4 binding, there should be instantaneously activated receptor available to phosphorylate Smad1. Therefore, the BMP receptor activation should not be a limiting step of the Smad signaling pathway. Recently, the cytosolic diffusion constants were reported for Smad2 before activation. The diffusion was so fast that there should nearly always be Smad2 available for phosphorylation [39]. A similarly fast diffusion can be assumed also for Smad1. However, the kinetics of Smad2 phosphorylation and Smads deduced from Western blot analyses showed that the phosphorylated Smads were observed after 10–15 min incubation with
ligand [39]. Thus, we wanted to address the question: is Smad1 phosphorylation the rate-limiting step of BMP-4 signaling? To answer this question we have developed novel fluorescent biosensors of Smad1 that reflect the kinetics of Smad1 phosphorylation. One sensor was created as a fusion of the full-length Smad1 with YFP and CFP (Smad1YC). By Western blot we demonstrated that this biosensor had phosphorylation kinetics similar to wild-type Smad1. Upon activation by phosphorylation, Smad1 undergoes a conformational switch [26,47,48] that should correspond with a FRET change with our engineered Smad biosensor. However, several processes could occur that could slow the process such as diffusion driven processes or protein binding. In any case, the changes in the FRET level should be an indicator of the progression for cytosolic Smads to become phosphorylated. We suggest the following possibilities for the non-instantaneous FRET response: the endocytosis of the BMP receptors [57], binding before phosphorylation with other proteins, R-Smad stays bound to receptor after phosphorylation and not allowing more to be bound or that the event is complex formation. The bottom line is that all these processes can cause retardation in Smad signaling. After phosphorylation the Smad1 MH2 domain undergoes a conformation change and interacts with the C-terminal tails of other Smad1 MH2 domains forming a homotrimer [48]. Thus, it is possible that both, phosphorylation and homotrimerization could contribute to the FRET changes.

In the FRET experiments, high initial FRET ratio levels were observed. After BMP-4 stimulation of COS-1 cells, FRET signals from Smad1YC displayed an initial “non-response” period of 315±70 s with a subsequent slow decrease with an average time constant of 284±45 s indicating there are dynamic events occurring up to the phosphorylation process. In C2C12 and HEK 293 cells, a slightly shorter “non-response” period of 100–300 s followed by a slow signal decrease with an average time 300 s was observed (data not shown). This indicates that the kinetics of Smad1 activation may to some extent be cell type-specific leading to the hypothesis that the delay originates from the endocytosis rate of the BMP receptors on the plasma membrane as the corresponding delays reflect cell-type internalization rates [58]. For the period of 100–300 s after BMP-4 addition when we could not observe any change in FRET, we suggest that a slow, gated type of diffusion-controlled reaction is naturally occurring in the cells. Our interpretation of the “dead” response time after stimulation with BMP-4 is that it is an important kinetic factor of the Smad signaling process and perhaps even a major bottleneck to the kinetics of Smad signaling. What could cause the delayed response upon BMP-4 stimulation? Previous studies have shown that the MH1 domain of Smad1 is responsible for binding not only to specific DNA sequences, but also to cytoskeletal proteins [9]. Moreover, the MH1 domain has a regulatory role in inhibition of the Smad molecule in the cytoplasm prior to activation. This inhibition is mediated through the binding of the MH1 domain to an opposing MH2 domain of R-Smad, and Smad phosphorylation upon activation is thought to relieve it [5,7,9,59,60]. However, there has been no direct evidence that the MH1 domain could be responsible for regulation of Smad1 phosphorylation. It has also been shown that R-Smads are anchored at the cell membrane by interacting with various cytoplasmic proteins, including SARA which recruits and stabilizes the monomeric form of Smad2/3. This protein could also be responsible for the delay in Smad1 activation [25]. Smad1 was also observed to interact with the cytoplasmic domain of CD44, which anchors Smad1 at the plasma membrane [61] and could have a role in delaying Smad1 activation. Furthermore, the full number of Smad interacting proteins still remains to be determined [9,62]. All these factors could cause the delayed FRET response after BMP-4 stimulation. In fact, the regulatory process of R-Smad activation could be one of the rate limiting steps of the BMP receptor signaling pathway in general.

We addressed the question whether the MH1 domain could be responsible for the delay in Smad1 phosphorylation by developing a sensor based solely on the MH2 domain. The MH2-only Smad1 biosensor lacking the MH1 domain (YSmad1C) demonstrated how the MH1 domain influences the kinetics of R-Smad activation. Both, FRET measurements and Western blot analysis clearly showed that the YSmad1C was activated without the delay that wild-type Smad1 and the Smad1YC displayed. After stimulation with BMP-4 we observed that the FRET ratio quickly dropped (without the delay observed with the full sensor) with a time constant of 300 ± 40 s, suggesting that this reflects the dynamics without the delay observed in the full Smad biosensor up to the conformational changes in MH2 domain. A second phase of the FRET change was a slow recovery with a time constant of 600±140 s but was not always observed. These FRET experiments and those with the mutated sensor, YSmad1(SA)C, indicated that the FRET decrease reflected phosphorylation of the MH1-deficient Smad1, which apparently starts immediately without delay after BMP-4 addition. The data also demonstrate the important role of MH1 domain in regulating Smad1 phosphorylation. However, homotrimerization of the MH2 domain of Smad1 upon BMP stimulation could also contribute in the FRET changes as for Smad1YC biosensor. Thus, YSmad1C FRET signals can be robustly and more quickly detected than with the full sensor and potentially many times faster than with Western blot or luciferase assays. The usefulness of the YSmad1C biosensor was also further shown in that it is reversible in a low number of cases (~10%) tested by first observing the FRET decrease upon BMP-4 addition, removal of the BMP-4, waiting for a long period (>30 min) for recovery, and observation of the FRET decrease again in the same cell upon a second addition of BMP-4 (data not shown).

We have gone on to use FRET experiments to study the kinetics of Smad1/Smad4 complex formation in more detail. The results demonstrated that CSmad1 and YLSmad4 form a heteromeric complex in BMP-4 stimulated cells after a delay of 260±48 s. The time constant fit from the FRET ratio increase after stimulation with BMP-4 is on the order of 517±160 s and indicates how slow the complex formation process is between CSmad1 and YLSmad4. With the addition of FLIM, the statistics of positive recordings of the CSmad1/YLSmad4 complexation upon BMP addition in individual cells is quite high (70%). We also show by confocal microscopy and FLIM
that the activated CSmad1 and YLSmad4 complexes can enter the nucleus to further add to the usefulness of the biosensors.

Recent studies have shown an important role of TGF-β signaling in the regulation of cancer [63,64]. For example, specific mutations and deletions of the genes for R-Smad proteins occur in different human carcinomas [65]. Therefore, we believe that the fluorescent Smad biosensors could be used as a new fast tool for the screening of drugs against certain cancers.

The work presented here directly demonstrates the kinetics of Smad1 activation upon phosphorylation and heteromeric complex formation in real time and in living cells. With the advent of the Smad biosensors, the inception of Smad activation and complex formation can be observed in single living cells. In the future, this method could be used to build a complete kinetic model of BMP signaling that can help to calculate the behavior of the processes in the cell under different physiological conditions. Our results suggest that Smad1 phosphorylation is a rate-limiting step of the BMP signaling pathway. We have also created a sensor, the YSmad1C, with the MH1 domain deleted that has optimized the speed by removing the rate-limiting delay and the dynamic range of the FRET response by closer proximity of the fluorophores. Such physiological detectors allow us to further study the kinetics of BMP signaling pathways upon addition of different ligands. Furthermore, these sensors could give new insights into the BMP receptor–Smad1/4 signaling process and potentially provide a powerful tool for studying Smads, their physiological role and spatio-temporal regulation of Smad-dependent processes.

Acknowledgments

We thank members of our laboratory for discussions and assistance: Geoffrey Lambright, Ralf Steinmeyer, Isabell Weber and Wiebke Buck. We also thank Petra Knaus (Department of Biochemistry, FU Berlin, Germany), Caroline S. Hill (London Research Institute, London, UK) and C. H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) for graciously providing us plasmids with Smad sequences (P.K. and C.S.H.) and with BMP receptor sequences (P.K.), and Walter Se bald, Thomas Müller (Department of Physiological Chemistry, Univ. of Würzburg, Germany), Thorsten Stiewe, Martin J. Lohse, Viacheslav O. Nikolaev (RVZ and Department of Pharmacology, Univ. of Würzburg, Germany) for valuable suggestions and critical reading of the manuscript. This work was supported by funding from the German Science Foundation (DFG) for Forschungszentren, Grant FZ-82.

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