Analysis of Smad nucleocytoplasmic shuttling in living cells

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Summary

Transforming growth factor β (TGF-β) signalling leads to phosphorylation and activation of receptor-regulated Smad2 and Smad3, which form complexes with Smad4 and accumulate in the nucleus. The Smads, however, do not seem to reside statically in the cytoplasm in the absence of signalling or in the nucleus upon TGF-β stimulation, but have been suggested to shuttle continuously between these cellular compartments in both the absence and presence of TGF-β. Here we investigate this nucleocytoplasmic shuttling in detail in living cells using fusions of Smad2 and Smad4 with enhanced GFP. We first establish that the GFPSmad fusions behave like wild-type Smads in a variety of cellular assays. We go on to demonstrate directly, using photobleaching experiments, that Smad2 and Smad4 shuttle between the cytoplasm and nucleus in both TGF-β-induced cells and in uninduced cells. In uninduced cells, GFPSmad2 is less mobile in the cytoplasm than is GFPSmad4, suggesting that it may be tethered there. In addition, we show that both GFPSmad2 and GFPSmad4 undergo a substantial decrease in mobility in the nucleus upon TGF-β stimulation, suggesting that active complexes of Smads are tethered in the nucleus, whereas unactivated Smads are more freely diffusible. We propose that regulated cytoplasmic and nuclear retention may play a role in determining the distribution of Smads between the cytoplasm and the nucleus in both uninduced cells and upon TGF-β induction.

Key words: TGF-β, Smad, Nucleocytoplasmic shuttling, GFP, Nuclear import, Nuclear export, Photobleaching

Introduction

Signals from receptors for Transforming growth factor β (TGF-β) superfamily members are transduced to the nucleus by the Smads (Shi and Massagué, 2003). In the case of TGF-β itself, the prototype of the family, receptor activation leads to phosphorylation of the receptor-regulated Smads (R-Smads) Smad2 and Smad3 at two serines in an SSXS motif at their extreme C termini. This results in activation of the R-Smads that then form complexes with the common mediator Smad, Smad4, which accumulate in the nucleus where they are directly involved in the regulation of transcription of target genes (Shi and Massagué, 2003). Recent work has suggested that both the strength and duration of signalling, reflected in the levels of active nuclear Smads and their residence time in the nucleus, are important for determining the biological response to a signal, and that mechanisms exist in the cell for continuously monitoring receptor activity and levels of active nuclear Smad (ten Dijke and Hill, 2004).

It is becoming clear that the distributions of Smads in uninduced or in TGF-β-induced cells are not static, but rather, the Smads appear to shuttle continuously between these two compartments under both conditions (Inman et al., 2002b; Reguly and Wrana, 2003; Xu et al., 2003; Xu et al., 2002). The first evidence for shuttling in uninduced cells came from studies of nucleocytoplasmic transport of Smad4. Smad4 was shown to contain a leucine-rich nuclear export signal (NES) that is recognised by the nuclear exporter CRM1 (Pierreux et al., 2000; Watanabe et al., 2000). In the absence of TGF-β, treatment of cells with an inhibitor of CRM1, leptomycin B (LMB), led to the rapid accumulation of Smad4 in the nucleus (Pierreux et al., 2000; Watanabe et al., 2000). This indicated that under basal conditions, Smad4 must be rapidly shuttling between the cytoplasm and nucleus. Thus, if nuclear export is inhibited, Smad4 accumulates in the nucleus. This result suggested the presence of a constitutively active nuclear localisation signal (NLS) in Smad4, and such a signal was indeed identified in the N-terminal so-called Mad homology (MH) 1 domain (Pierreux et al., 2000). Further characterisation revealed that this sequence is a basic bipartite NLS that binds importin α (Xiao et al., 2003), which can then bind importin β for nuclear import (Görlich and Kutay, 1999). Recently it has been proposed, based on in vitro transport assays, that Smad4 import is not driven by a transport receptor, but rather by direct interaction with the nucleoporins that are components of the nuclear pore (Xu et al., 2003).

Like Smad4, Smad2 and Smad3 also appear to shuttle between the cytoplasm and nucleus in the absence of TGF-β. This nuclear transport has also been proposed to be transport receptor independent and mediated by direct contact between the C-terminal (MH2) domains of the R-Smads and nucleoporins, in particular, CAN/Nup214 and Nup153 (Xu et al., 2003; Xu et al., 2002). However, an NLS has additionally been identified in the MH1 domain of Smad3, which is thought to bind directly to importin-β, and mutation of this NLS

prevents Smad3 accumulating in the nucleus upon TGF-β stimulation (Kurisaki et al., 2001; Xiao et al., 2000a; Xiao et al., 2000b). This NLS does not seem to be functional in Smad2 although its sequence is conserved. Its function is thought to be inhibited by the presence of adjacent residues encoded by Smad2 exon 3 (Kurisaki et al., 2001). Smad2 and 3 export is insensitive to LMB treatment, indicating that CRM1 is not involved, but has been shown to be ATP-dependent, suggesting that Smad2 and 3 are actively exported in a transport receptor-dependent manner (Inman et al., 2002b).

Recent work has suggested that the Smads also shuttle during TGF-β signalling, and this acts as a mechanism whereby the Smads continuously monitor receptor activity (Inman et al., 2002b). The data demonstrate that continuous TGF-β receptor activity is required for the R-Smads to remain phosphorylated, and for R-Smad/Smad4 complexes to persist in the nucleus. In addition, it has been shown that the R-Smads exported from the nucleus are dephosphorylated (Inman et al., 2002b; Xu et al., 2002) and that the R-Smads and Smad4 are exported independently of each other (Inman et al., 2002b). The interpretation of this data is that in the nucleus the R-Smads are being continuously dephosphorylated and dissociate from complexes with Smad4. The monomeric inactivated Smads are then exported to the cytoplasm, where the R-Smads are rapidly rephosphorylated by the receptors, if they are still active. These activated R-Smads form complexes with Smad4 and relocalise to the nucleus. If the receptors are no longer active however, then the Smads accumulate back in the cytoplasm (Inman et al., 2002b). This continuous nucleocytoplasmic shuttling provides a mechanism whereby the time that the Smad complexes remain in the nucleus will directly reflect the time that the receptors remain active.

Although the concept of Smad shuttling has been inferred from several studies, it has never been directly demonstrated. In addition, the constant Smad shuttling between the cytoplasm and the nucleus in both the absence and presence of TGF-β signalling raises important questions as to what determines the distribution of Smads between the cytoplasm and nucleus in uninduced cells and after TGF-β stimulation. In unstimulated cells, the R-Smads are predominantly cytoplasmic and Smad4 is distributed throughout the cytoplasm and nucleus. After TGF-β stimulation the Smads are all predominantly nuclear (Pierreux et al., 2000; Reguly and Wrana, 2003). Are these distributions dictated by the presence of cytoplasmic and nuclear retention factors that have different affinities for monomeric versus activated Smads? Alternatively, are they dictated by the relative rates of import and export of monomeric Smads versus activated complexed Smads?

To begin to answer these questions we have studied Smad nucleocytoplasmic shuttling in real time using fusions of Smads with enhanced GFP. Focusing on Smad2 and Smad4, we have demonstrated that these GFP fusions faithfully mimic the activity of wild-type Smads in both uninduced cells and in response to TGF-β. We have used Fluorescence Loss In Photobleaching (FLIP) experiments (reviewed by Lippincott-Schwartz et al., 2003) to demonstrate nucleocytoplasmic shuttling and also to show that whereas Smad4 is relatively mobile in the cytoplasm in uninduced cells, Smad2 is significantly less mobile, suggesting at least some degree of cytoplasmic tethering. We have then used Fluorescence Recovery After Photobleaching (FRAP) experiments (reviewed by Lippincott-Schwartz et al., 2003; Pederson, 2001) in combination with FLIP to investigate the mobility of the Smads in the nucleus. We demonstrate that both Smad2 and Smad4 undergo a significant decrease in their mobility after TGF-β stimulation, suggesting that active Smad complexes may be actively retained in the nucleus.

Materials and Methods

Plasmid and reagents

The following plasmids have been previously described: ARE_3-Luciferase, FLAG-XSmad2, XFoxH1a (formerly XFast-1) in an EF-FLAG expression vector (Germain et al., 2000), FLAG-hSmad3 and HA-hSmad4 (Inman and Hill, 2002), $CAGA_{12}$-Luciferase (Dennler et al., 1998), EF-*lacZ* (Bardwell and Treisman, 1994) and plasmids expressing GFP, GFPNLS and GFPPKCα (Lillemeier et al., 2001). The plasmid expressing GFPRanBP1ΔNES was a gift from Paul Clarke and that expressing GFPβ-galactosidaseNLS was a gift from Ray Truant. The plasmids expressing enhanced GFPSmads were generated by amplifying the human Smad2, Smad3 and Smad4 coding sequences by PCR and subcloning them into pGFP-C1 (Clontech), such that the GFP was at the N-terminus in each case.

TGF-β1 (PeproTech) was dissolved in 4 mM HCl, 1 mg/ml BSA and used at a final concentration of 2 ng/ml. Leptomycin B (Sigma) in 70% methanol was used at a final concentration of 20 ng/ml. 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) in DMSO was used at a final concentration of 400 nM. SB-431542 in DMSO was used at final concentrations as indicated in the Figure legends. Cycloheximide was used at 20 μg/ml 20 minutes prior to TGF-β addition. This concentration is sufficient to inhibit protein synthesis by >90% (Pierreux et al., 2000).

Cell culture, generation of cell lines, transient transfections, bandshift assays, western blotting and reporter assays

All cell lines were grown in DMEM/10%FCS. HeLa thymidine kinase$^-$ (TK$^-$) cells (Angel et al., 1987) were transfected with LipofectAMINE (Invitrogen), MDA-MB468 cells (Schutte et al., 1996) were transfected with Superfect Reagent (Qiagen) and HaCaT cells were transfected with FuGENE 6 (Roche), all according to the manufacturers' instructions. The HaCaT cell lines stably expressing GFPSmads were generated by transiently transfecting HaCaT cells with the appropriate plasmids, then selecting transfected cells using 1 mg/ml G418. Pools of GFP-positive cells were selected by FACS sorting.

Whole-cell and nuclear extracts were prepared as described (Germain et al., 2000; Wong et al., 1999). Western blotting was performed using standard techniques. The following antibodies were used: anti-Smad2/3 (BD Biosciences), anti-Smad4 (B8; Santa Cruz), anti-phosphorylated Smad2 (Faure et al., 2000), anti-phosphorylated Smad3 (Wilkes et al., 2003) and anti-Smad3 (Zymed). Bandshift assays using nuclear extracts and the probe corresponding to the Smad binding region (SBR) from the c-*jun* 5′UTR were as described (Inman and Hill, 2002), and those using whole-cell extracts and the probe corresponding to the activin responsive element (ARE) were as described (Germain et al., 2000).

Luciferase assays were performed as described previously (Pierreux et al., 2000). β-galactosidase assays were performed using Galactostar (Applied Biosystems) and analysed in a luminometer as for luciferase.

Confocal microscopy

For transiently transfected HeLa TK$^-$ cells, 2 to 5 hours after transfection, cells were trypsinized and seeded in glass-bottom microwell dishes (MatTek, Ashland, MA). Fourteen to eighteen hours

later cells were washed twice with PBS and incubated with DMEM containing 25 mM HEPES pH 7.4 and 10% FCS, low bicarbonate (2.2 g/l $NaHCO_3$), and no phenol red or fluorescent agents. An LSM 510 confocal laser scanning microscope equipped with an argon laser (Zeiss, Germany) was used for analysis. GFP was detected at $\lambda>505$ nm after excitation at $\lambda=488$ nm. For the HaCaT cell lines, cells were seeded in the glass-bottom microwell dishes (MatTek), and analysed 48 hours later. They were treated as for the HeLa TK^- cells. All live cell imaging was performed at 37°C and for the experiments shown in Fig. 2, a humidified CO_2 chamber was used.

FLIP experiments and analysis

To photobleach GFP-tagged proteins in living cells, a small region (20×20 pixels corresponding to an area of 4.8 μm^2) of the cytoplasm was scanned with maximum laser power for the times indicated in the Figure legends. Confocal sections of the cells were taken at the times after photobleaching indicated in the Figure legends. Fluorescence was quantified at the bleaching point and at other areas of interest. The resulting intensities of fluorescence or relative fluorescence were plotted against the accumulated time of bleaching.

FLIP/FRAP experiments and analysis

Before photobleaching, eight measurements of fluorescence were taken over a period of 2 seconds. A region in the nucleus of 6×6 pixels (corresponding to an area of 2.5 μm^2) was then photobleached for 11 seconds using maximum laser power. A series of images of the sample were taken every 250 milliseconds for up to 70 seconds. The fluorescence was quantitated at the bleach point and at a reporting point in the nucleus diametrically opposite the bleach point. Fluorescence levels were normalised to the average levels of fluorescence prior to photobleaching.

Results

The GFPSmads are activated in response to TGF-β and form transcriptionally active DNA-bound complexes

We constructed plasmids expressing enhanced GFP fusions of human Smad2, Smad3 and Smad4, and then tested their activity in a variety of assays to ensure that they retained the properties of wild-type Smads. A Smad3/Smad4-containing complex forms on the c-*jun* SBR upon TGF-β stimulation (Inman and Hill, 2002). In HeLa TK^- cells, which contain relatively low levels of Smad3, this is readily detected by bandshift assay when Smad3 is overexpressed (Fig. 1A, lanes 1-4). Both GFPSmad4 and GFPSmad3 were incorporated into this complex, as demonstrated by expressing them in HeLa TK^- cells and observing the change in mobility of the TGF-β-induced complex formed, as a result of the large size of the GFP. Thus, expression of GFPSmad4 and FLAG-Smad3 resulted in a complex that migrated slightly more slowly than that resulting from expression of FLAG-Smad3 alone (Fig. 1A, lanes 4, 6). Expression of HA-Smad4 and GFPSmad3 resulted in a complex with a strikingly lower mobility than that observed with HA-Smad4 alone (Fig. 1A, lanes 8, 10). It is not clear why the presence of GFP on Smad3 has a greater effect than the presence of GFP on Smad4. The western blots in Fig. 1A show that all tagged Smads were equivalently expressed. In addition, GFPSmad3 was phosphorylated efficiently in response to TGF-β, as was FLAG-tagged Smad3.

Similarly, we demonstrated by bandshift assay that GFPSmad2 and GFPSmad4 were incorporated into an Activin responsive factor (ARF) complex on the ARE probe with the transcription factor XFoxH1a in response to TGF-β (Fig. 1B). When XFoxH1a was expressed in HeLa TK^- cells, a TGF-β inducible complex was detected (Fig. 1B, lanes 1-4) that contained XFoxH1a, Smad2 and Smad4 (data not shown) (Germain et al., 2000). The migration of this complex was decreased slightly when either GFPSmad2 or GFPSmad4 was expressed, and was markedly decreased when both were expressed, demonstrating that they were both incorporated into the complex (Fig. 1B, lanes 4,6,8,10). The control western blots demonstrate that all the Smads were well expressed, and that the GFPSmad2 was phosphorylated in response to TGF-β, as was endogenous Smad2.

We also demonstrated that these GFPSmad-containing complexes were transcriptionally active by transfecting the GFPSmads into MDA-MB468 cells which lack endogenous Smad4, but contain R-Smads (Schutte et al., 1996), and measuring their activity on the Smad3/Smad4-dependent reporter $CAGA_{12}$-luciferase (Dennler et al., 1998), or on the Smad2/Smad4-dependent reporter, ARE_3-luciferase, together with XFoxH1a (Germain et al., 2000). GFPSmad4 rescued the deletion of Smad4 in these cells, and this activity was enhanced by addition of either GFPSmad3 (for the $CAGA_{12}$-luciferase) or GFPSmad2 (for ARE_3-luciferase) (Fig. 1A,B, right panels). Thus all three of these GFPSmads are functional in these assays, and behave similarly to wild-type Smads.

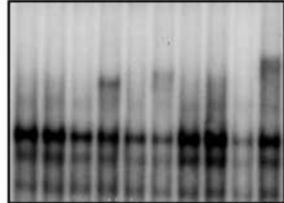
TGF-β-induced nuclear translocation of GFPSmads is dependent upon continuous receptor signalling

We next investigated whether these GFPSmads translocated to the nucleus in response to TGF-β, as has been shown for endogenous Smads (Pierreux et al., 2000). We made stable cell lines of HaCaT cells expressing the GFPSmads, and analysed the behaviour of the GFPSmads in the absence or presence of TGF-β in pools of expressing cells (Fig. 2A). These cell lines express GFPSmads on average at levels comparable to endogenous Smads (data not shown). When performing these experiments, cells were pretreated with the protein synthesis inhibitor, cycloheximide. This was to ensure that during the experiment, the same pool of GFPSmads was observed. In control experiments, the kinetics of GFPSmad nucleocytoplasmic shuttling were not affected by the presence of cycloheximide (data not shown).

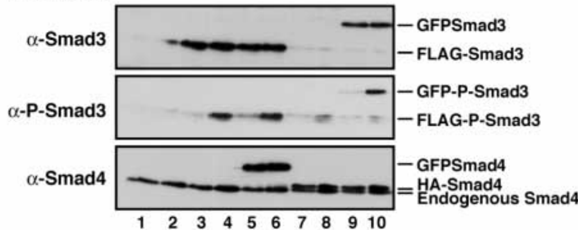
GFPSmad2 was predominantly cytoplasmic in uninduced cells, and predominantly nuclear after 60 minutes of induction with TGF-β (Fig. 2A, top panels, quantitated in Fig. 2A left-hand graph). In the nucleus it was excluded from the nucleoli, as was endogenous Smad2 (Pierreux et al., 2000). If the ALK5 inhibitor, SB-431542 (Inman et al., 2002a; Laping et al., 2002) was added at the 60-minute time-point, GFPSmad2 accumulated back out in the cytoplasm by the 220-minute time-point (Fig. 2A, top panels, quantitated in Fig. 2A middle graph). If, however, no receptor inhibitor was added, the GFPSmad2 remained nuclear during this time (data not shown). The GFPSmad2 protein behaved in an identical fashion when transiently transfected into HeLa TK^- cells (Fig. 2B, upper panels). The same behaviour of GFPSmad2 was observed over a wide range of expression levels. This activity and its kinetics faithfully mirrors that of

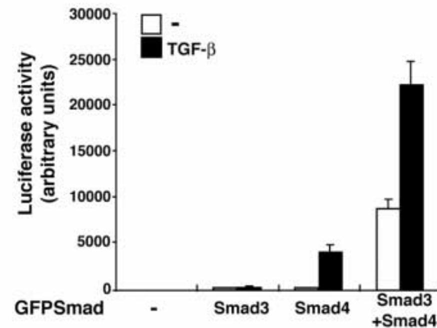
A

Bandshift



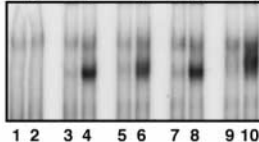
Western Blots



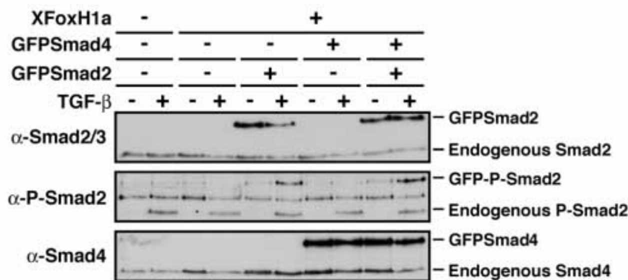


B

Bandshift



Western Blots



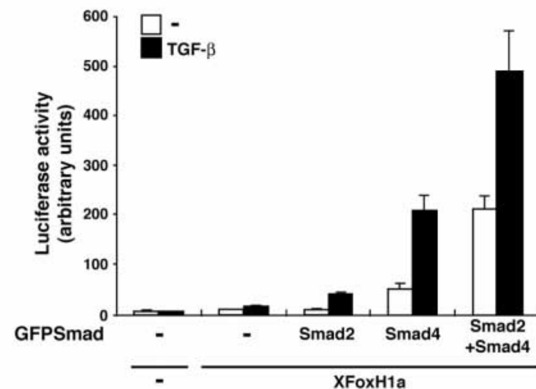


Fig. 1. GFPSmad2, GFPSmad3 and GFPSmad4 fusion proteins form DNA-bound complexes that are transcriptionally active in response to TGF-β. (A) HeLa TK$^-$ cells expressing either GFPSmad3, GFPSmad4, HA-Smad4 or FLAG-Smad3 as indicated were either untreated or treated with TGF-β1 for 1 hour. Cell extracts were assayed by bandshift analysis using the c-*jun* SBR as a probe. The positions of migration of complexes containing different combinations of tagged Smad3 and Smad4 are indicated. The same extracts were western blotted with antibodies recognising Smad3, phosphorylated Smad3 (P-Smad3) or Smad4 (below). The right-hand panel shows transcription assays in which MDA-MB468 cells were transfected with the $CAGA_{12}$-luciferase reporter gene, plasmids expressing the different GFPSmad fusion proteins and pEF-*lacZ* as an internal control for transfection efficiency. Cells were treated with or without TGF-β1 for 8 hours. Luciferase was quantitated relative to β-galactosidase from the pEF-*lacZ* internal control. The data are means and standard deviations of a representative experiment performed in triplicate. (B) HeLa TK$^-$ cells were transfected with plasmids expressing XFoxH1a, GFPSmad2 or GFPSmad4 as indicated. Total cell extracts from cells either left untreated or treated with TGF-β1 for 1 hour were assayed by bandshift analysis using the activin response element (ARE) as probe. DNA-bound complexes containing either XFoxH1a and endogenous Smad2 and Smad4 or XFoxH1a and GFPSmad2 and GFPSmad4 are indicated. The same extracts were western blotted with antibodies recognising Smad2/3, phosphorylated Smad2 (P-Smad2) or Smad4 (below). The right-hand panel shows transcription assays performed as above, where MDA-MB468 cells were transfected with the ARE_3-luciferase reporter gene, plasmids expressing XFoxH1a, the different GFPSmad fusion proteins and pEF-*lacZ*. The data are means and standard deviations of a representative experiment performed in triplicate.

endogenous Smad2 (Inman et al., 2002b). For endogenous Smad2 this observation has been interpreted as an indication that Smad2 is constantly shuttling between the nucleus and cytoplasm during active TGF-β signalling, undergoing cycles of phosphorylation by the receptors and dephosphorylation by a phosphatase in the nucleus (Inman et al., 2002b). If the TGF-β receptors are turned off by SB-431542, Smad2 is no longer activated in the cytoplasm and thus accumulates there.

Similarly, the behaviour of GFPSmad4 mimicked that of

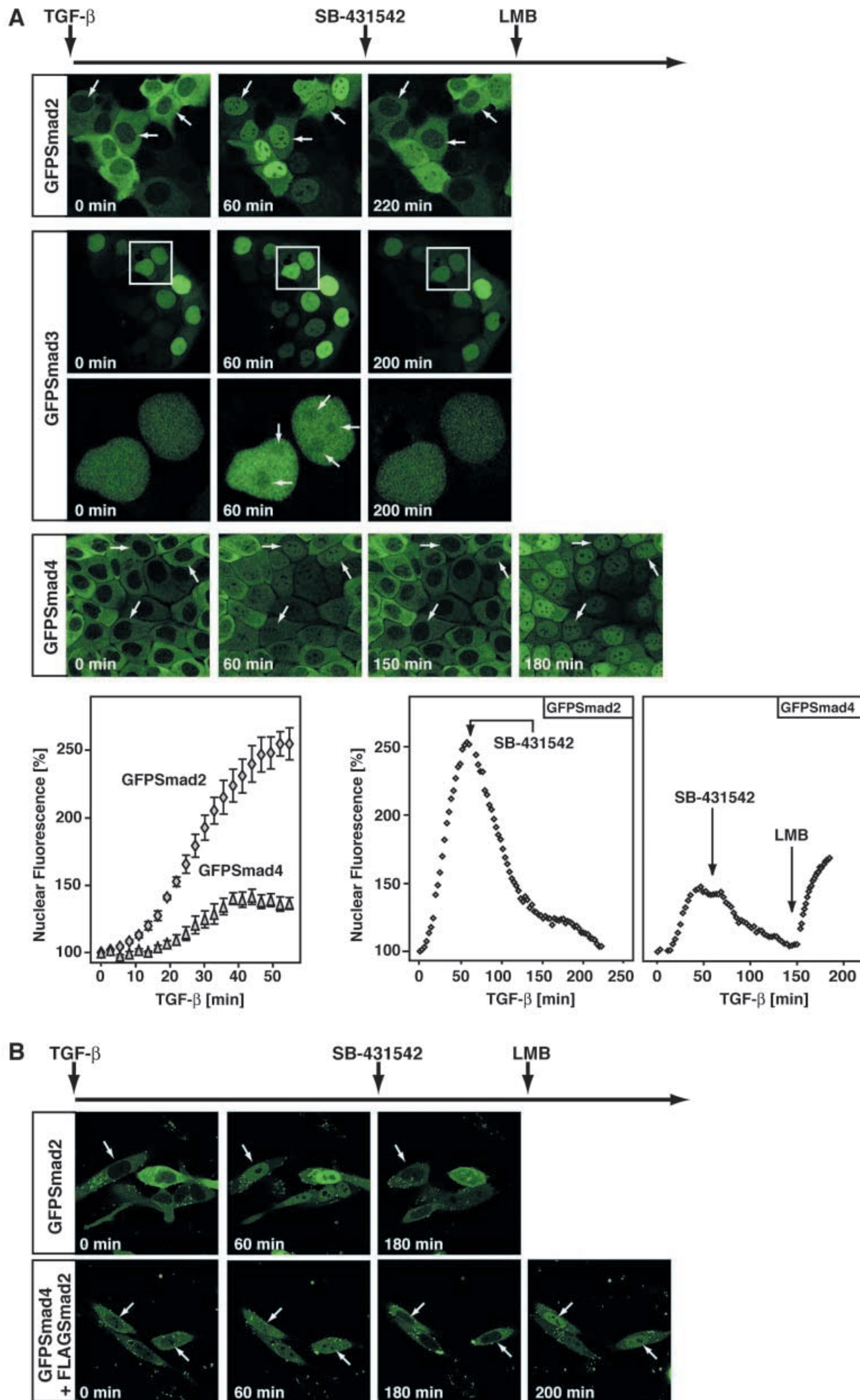


Fig. 2. TGF-β-induced nuclear translocation of GFPSmads is dependent upon continuous receptor signalling. (A) HaCaT cell lines stably expressing GFPSmad2, GFPSmad3 or GFPSmad4 were pretreated with cycloheximide and were then incubated with TGF-β1 for 1 hour, followed by SB-431542 (7.5 μM) for up to 160 minutes. In the case of the GFPSmad4 cells, leptomycin B (LMB) was added 90 minutes after SB-431542 addition (150-minute time-point). Fluorescence images are shown at different time-points after initial TGF-β treatment. Arrows indicate representative examples of cells demonstrating nucleocytoplasmic shuttling. For GFPSmad3 cells, the boxed region is shown magnified below to demonstrate that GFPSmad3 is partially excluded from the nucleoli upon TGF-β treatment. Below are graphs showing quantitation of nuclear fluorescence, with fluorescence images collected every 3 minutes. The left-hand graph shows the average of the TGF-β-induced nuclear fluorescence of the GFPSmad2 and GFPSmad4 cells marked with an arrow. Means and standard deviations are shown. The right-hand graphs show quantifications of the nuclear fluorescence for GFPSmad2 and GFPSmad4 throughout the whole experiment for one of the indicated cells in each case. (B) HeLa TK$^-$ cells were transiently transfected with plasmids expressing GFPSmad2 or GFPSmad4 together with FLAG-Smad2 and treated with cycloheximide, TGF-β1, SB-431542 and LMB as in A. Fluorescence images are shown at different time-points after initial TGF-β treatment. Arrows indicate representative examples of cells demonstrating nucleocytoplasmic shuttling. The punctate fluorescence observed in the cytoplasm of transiently transfected HeLa TK$^-$ cells is not seen in the stable HaCaT cell lines and thus seems to be a consequence of transient transfection. The experiments shown are representatives from at least three independent experiments.

endogenous Smad4. GFPSmad4 accumulated in the nucleus upon TGF-β signalling and then accumulated back in the cytoplasm after addition of SB-431542 (Fig. 2A, lower panels, quantitated Fig. 2A, graphs). In the nucleus GFPSmad4 was excluded from the nucleoli as was endogenous Smad4 (Pierreux et al., 2000). It is striking that the TGF-β-induced nuclear accumulation of GFPSmad4 is much less complete than that of GFPSmad2, and also plateaus earlier. This is probably because of the fact that in response to TGF-β, nuclear accumulation of Smad4 requires complex formation with activated R-Smads, whereas accumulation of activated homomeric complexes of Smad2 can occur in the absence of Smad4 (De Bosscher et al., 2004; Nicolás and Hill, 2003). Expression of GFPSmad4 in the cell line at approximately endogenous levels results in excess Smad4 over endogenous R-Smads, and thus only a proportion of the GFPSmad4 can accumulate in the nucleus in response to TGF-β. The GFPSmad4 protein behaved in a similar fashion when transiently transfected into HeLa TK$^-$ cells (Fig. 2B, lower panels). In this case, to see any accumulation of GFPSmad4 in the nucleus we had to also overexpress FLAG-Smad2, possibly because of lower levels of R-Smads in these cells. The same behaviour of GFPSmad4 was observed over a wide range of expression levels. Smad4 export from the nucleus is mediated via the nuclear exporter CRM1 (Pierreux et al., 2000; Watanabe et al., 2000). When the inhibitor of CRM1 (LMB) was added to the cells after prolonged incubation with SB-431542 (at the 150-minute time-point), the GFPSmad4 rapidly accumulated in the nucleus (Fig. 2A, lower panels, quantitated in Fig. 2A, right-hand graph; Fig. 2B). This indicates that even in the absence of activated TGF-β receptors, GFPSmad4 (presumably monomeric) is constitutively imported into the nucleus and is exported by CRM1. If CRM1 activity is inhibited by LMB, GFPSmad4 accumulates in the nucleus.

In contrast to GFPSmad2 and GFPSmad4, GFPSmad3 did not behave as endogenous Smad3 in translocation experiments, although in the biochemical assays it appeared to function normally (Fig. 1). In the absence of TGF-β, GFPSmad3 was predominantly nuclear, even though we have shown that it is unphosphorylated (Fig. 1A, Fig. 2A middle panels). It was also detected in the nucleoli, which was not the case for endogenous Smad3 (Pierreux et al., 2000) (Fig. 2A, middle panels). GFPSmad3 was, however, sensitive to TGF-β treatment, as this nuclear fluorescence intensified upon TGF-β stimulation, and some partial exclusion of fluorescence from the nucleoli was detected, suggesting that monomeric GFPSmad3 is not excluded from the nucleoli, but complexed activated GFPSmad3 is (Fig. 2A, middle panels). The GFPSmad3 also responded to SB-431542 treatment, as upon addition of this receptor inhibitor, the nuclear fluorescence decreased again to the levels seen in uninduced cells. Because GFPSmad3 did not faithfully mimic the behaviour of endogenous Smad3, we have not studied this protein further.

From the data presented in this section we conclude that GFPSmad2 and GFPSmad4 closely mimic endogenous Smad2 and Smad4, respectively, and can be used to investigate the nucleocytoplasmic shuttling behaviour of these Smads in both HaCaT and HeLa TK$^-$ cells.

Shuttling of GFPSmad2 and GFPSmad4 between the cytoplasm and nucleus occurs in both unstimulated and TGF-β-induced cells

FLIP experiments can be used to investigate whether a protein shuttles between two compartments of the cell, and also to indicate how mobile a protein is in a given compartment of the cell. A prolonged bleaching is applied to a defined area of the cell and the fluorescence at the bleaching point and at a distant reporting point is quantitated over time (reviewed by Lippincott-Schwartz et al., 2003). If the GFP-labelled molecules are shuttling between the bleaching and reporting points, then the fluorescence will decrease at both points. Relatively immobile proteins in contrast will be bleached effectively at the bleaching point, but not at the reporting point.

We first performed a series of controls to validate the FLIP experiments. GFP is a small protein that diffuses throughout the cell. When HeLa TK$^-$ cells expressing GFP alone were bleached in the cytoplasm, the nuclear GFP also rapidly bleached (Fig. 3Ai), as expected for a protein freely diffusing through the nucleus and cytoplasm. GFPRanBP1ΔNES is a GFP fusion of RanBP1 that is trapped in the nucleus because it is imported efficiently via its non-classical Ran-dependent NLS, but cannot be exported because its NES has been deleted (F.J.N. and P. R. Clarke, unpublished data) (Plafker and Macara, 2000). In this case, when the bleaching occurred in the cytoplasm, virtually no nuclear bleaching was detected (Fig. 3Aii). This is the behaviour expected of a protein that does not shuttle between the cytoplasm and nucleus. As a control for the use of this technique to determine the mobility of a protein in a given compartment of the cell, we investigated the behaviour of a fusion protein of GFP with PKCα. In uninduced cells this protein was distributed throughout the cytoplasm (Fig. 3Aiii) (Lillemeier et al., 2001; Ng et al., 1999). It was completely mobile in this compartment, as seen by the rapid photobleaching at a point in the cytoplasm distant from the bleach point (Fig. 3Aiii) (Lillemeier et al., 2001). However, upon stimulation with the phorbol ester TPA for 10 minutes, GFPPKCα accumulated at the plasma membrane (Fig. 3B). In this compartment it was not very mobile, and hence virtually no photobleaching was detected at a reporting point on the plasma membrane distant from the bleach point (Fig. 3Aiv) (Lillemeier et al., 2001).

Having demonstrated that FLIP can be used to detect shuttling of GFP-tagged molecules between the cytoplasm and the nucleus, we investigated the shuttling behaviour of GFPSmad2 and GFPSmad4 in both the absence and presence of TGF-β signalling, using the HaCaT cell lines stably expressing these fusion proteins. In these experiments, the cytoplasm was bleached. If nuclear bleaching is observed in the same cell, this indicates that the GFPSmad is in dynamic equilibrium between these two compartments. Thus this experiment provides a direct demonstration of nucleocytoplasmic shuttling.

When GFPSmad2 was bleached in the cytoplasm of an uninduced cell, it also bleached in the nucleus of the same cell. In contrast, nuclei of adjacent cells did not bleach, indicating that this nuclear bleaching is specific (Fig. 4i). Similarly, when the same experiment was performed using TGF-β-induced cells, cytoplasmic bleaching resulted in nuclear bleaching in the same cell (Fig. 4ii). Note that in the

TGF-β-induced cell, the proportion of nuclear molecules bleached was not as high as in an uninduced cell (compare graphs in Fig. 4i and Fig. 4ii). This suggests that after TGF-β stimulation, the proportion of nuclear Smad that is mobile is lower than in an uninduced cell (see below and Discussion). Exactly the same behaviour was observed for GFPSmad4 (Fig. 4iii,iv). These data directly demonstrate that in both uninduced cells and in TGF-β-induced cells, GFPSmad2 and

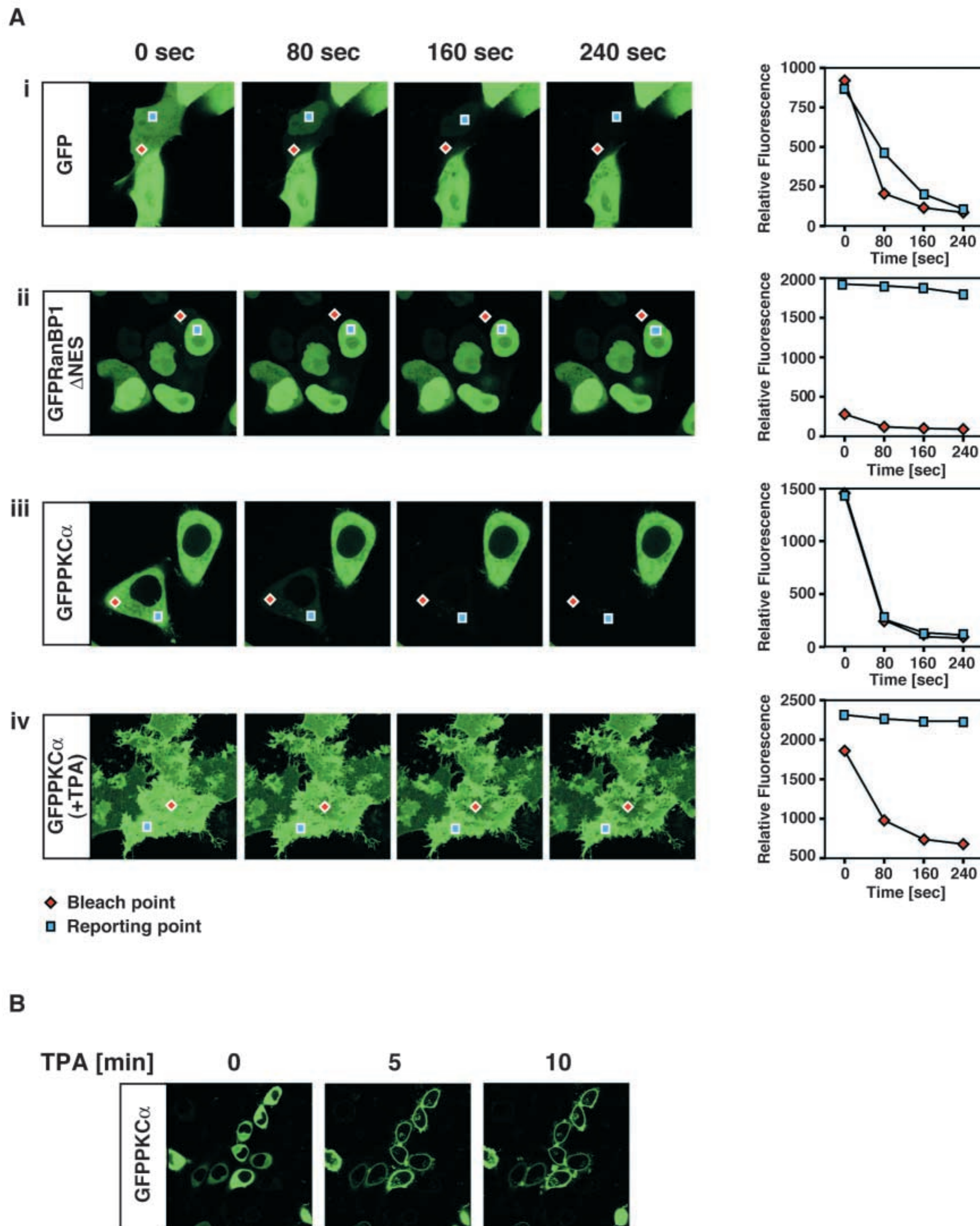


Fig. 3. Controls for FLIP analysis. (A) HeLa TK⁻ cells were transiently transfected with plasmids expressing GFP, GFPRanBP1ΔNES or GFPPKCα. Cells were unstimulated except for those expressing GFPPKCα which were stimulated with TPA. Cells were photobleached in the cytoplasm as indicated by the red diamond. The fluorescence images shown prior to photobleaching (time=0 seconds) and then after three consecutive 80-second bleaching periods. In all cases, the plane of focus was a cross-section through the cell, except for the TPA-induced cells expressing GFPPKCα where the plane of focus was in the plasma membrane. The right-hand graphs show the FLIP analysis. The fluorescence was quantitated at the bleach point and at a reporting point indicated by the blue square. The intensity of fluorescence is represented in arbitrary units. (B) Stimulation of cells with TPA induces the translocation of GFPPKCα to the membrane. HeLa TK⁻ cells expressing GFPPKCα were either untreated or treated with TPA for up to 10 minutes. Fluorescence images are shown. Here, the plane of focus is again a cross-section through the cell. The experiments shown are representatives from at least three independent experiments.

GFPSmad4 are constantly shuttling between the cytoplasm and the nucleus.

FLIP analysis reveals that GFPSmad4 is more mobile in the cytoplasm than is GFPSmad2

We noticed in the experiments shown in Fig. 4 that in uninduced cells GFPSmad2 bleached less readily in cytoplasmic regions distant from the cytoplasmic bleach point than did GFPSmad4. This suggested that GFPSmad2 might be less mobile in the cytoplasm than is GFPSmad4. We investigated this in more detail, using both transiently transfected HeLa TK$^-$ cells and the stably transfected HaCaT cell lines. In both cell lines GFPSmad2 bleached more slowly at the reporting point in the cytoplasm than does GFPSmad4 (Fig. 5). The graphs demonstrate that the rate of bleaching at the cytoplasmic reporting point for GFPSmad4 was very similar to the rate of bleaching at the bleach point. For GFPSmad2 however, the rate of bleaching at the reporting point was significantly slower than the rate of bleaching at the bleach point itself. These data strongly suggest that GFPSmad2 is substantially less mobile in the cytoplasm than is GFPSmad4.

FLIP/FRAP analysis reveals a TGF-β-dependent change in mobility of GFPSmad2 and GFPSmad4 in the nucleus

Finally we investigated the mobility of GFPSmads in the nucleus in uninduced or TGF-β-induced cells. For this we used FRAP in combination with FLIP. In these experiments a photobleaching pulse (11 seconds) was applied to a particular area of the cell, and then the recovery of fluorescence in that

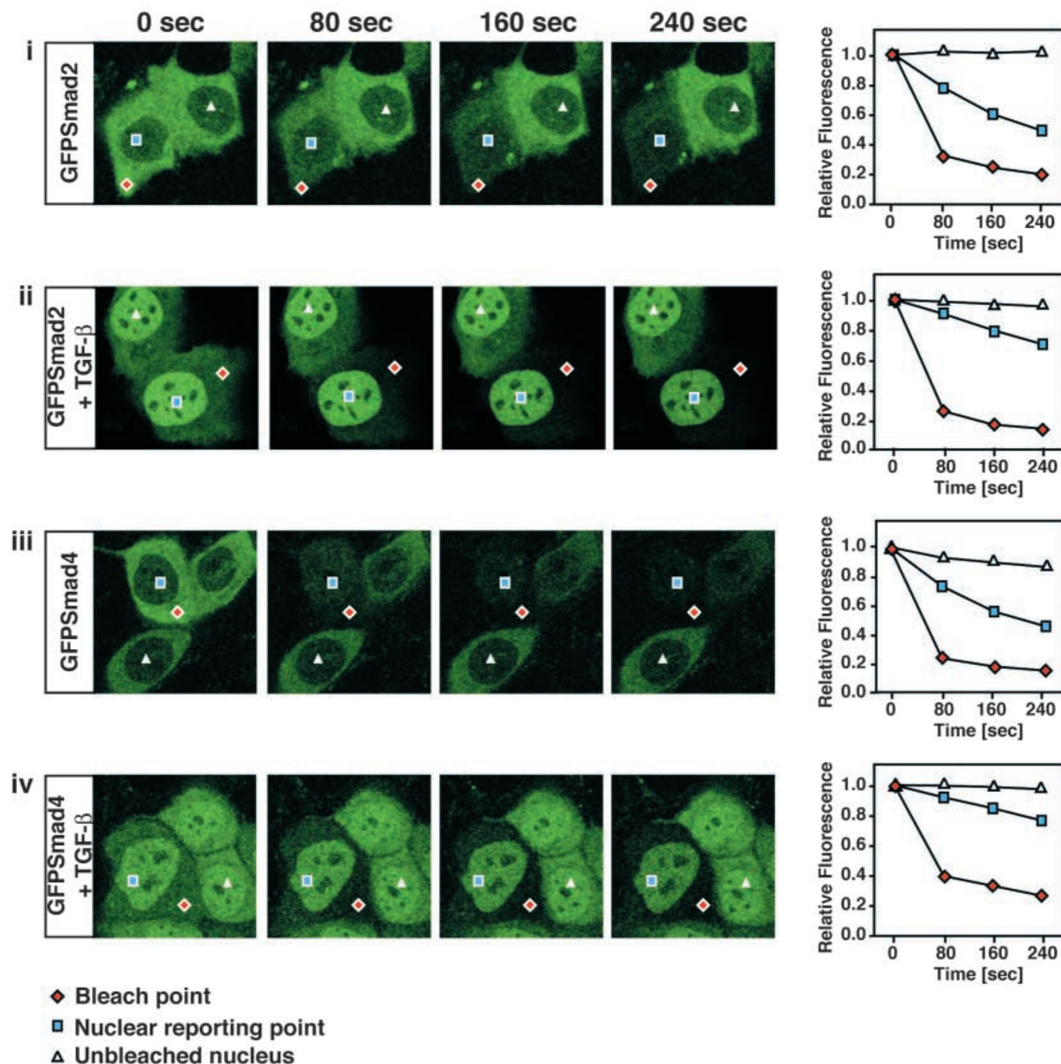


Fig. 4. FLIP analysis of GFPSmad2 and GFPSmad4 proves nucleocytoplasmic shuttling. (A) HaCaT cell lines stably expressing GFPSmad2 or GFPSmad4 remained either unstimulated (i,iii) or were treated for 1 hour with TGF-β1 (ii,iv). The bleach region in the cytoplasm is indicated (red diamond). Each row shows the fluorescence image prior to bleaching (time=0 seconds) and after three consecutive 80-second bleaching periods. The fluorescence was quantitated at the bleach point, at a reporting point in the nucleus of the same cell (blue square) and at a reporting point in the nucleus of an adjacent cell (white triangle). In each case the fluorescence was normalised to the initial fluorescence prior to photobleaching, and the relative fluorescence was plotted. Note that in unstimulated cells expressing GFPSmad4, the cell to the right of the bleached cell also bleaches because the bleach point also contacts the cytoplasm of that cell. The data are representatives from at least three different experiments.

area was monitored together with fluorescence loss at a distant point, also in the nucleus. Again we performed controls to validate the approach. For nuclear localised GFP (GFPNLS), it was evident that recovery at the bleach point was extremely fast, as was the rate of bleaching at the reporting point (Fig. 6i). When the same experiment was performed using a much larger nuclear GFP fusion protein (GFPβ-galactosidaseNLS) which has a native molecular weight as a tetramer of over 400 kDa (Jacobson et al., 1994), the recovery after photobleaching was slower, as was the rate of bleaching at the reporting point (Fig. 6ii). This indicates that, as expected, the mobility of the much larger GFPβ-galactosidaseNLS protein in the nucleus is lower than that of GFP alone. We performed the same experiment on a protein that we have shown to be relatively immobile (GFPPKCα at the plasma membrane after TPA treatment). In this case the recovery after photobleaching was very slow and no detectable bleaching was observed at the reporting point (Fig. 6iii).

Having demonstrated that the FLIP/FRAP experiment can give an indication as to the mobility of a protein in a given

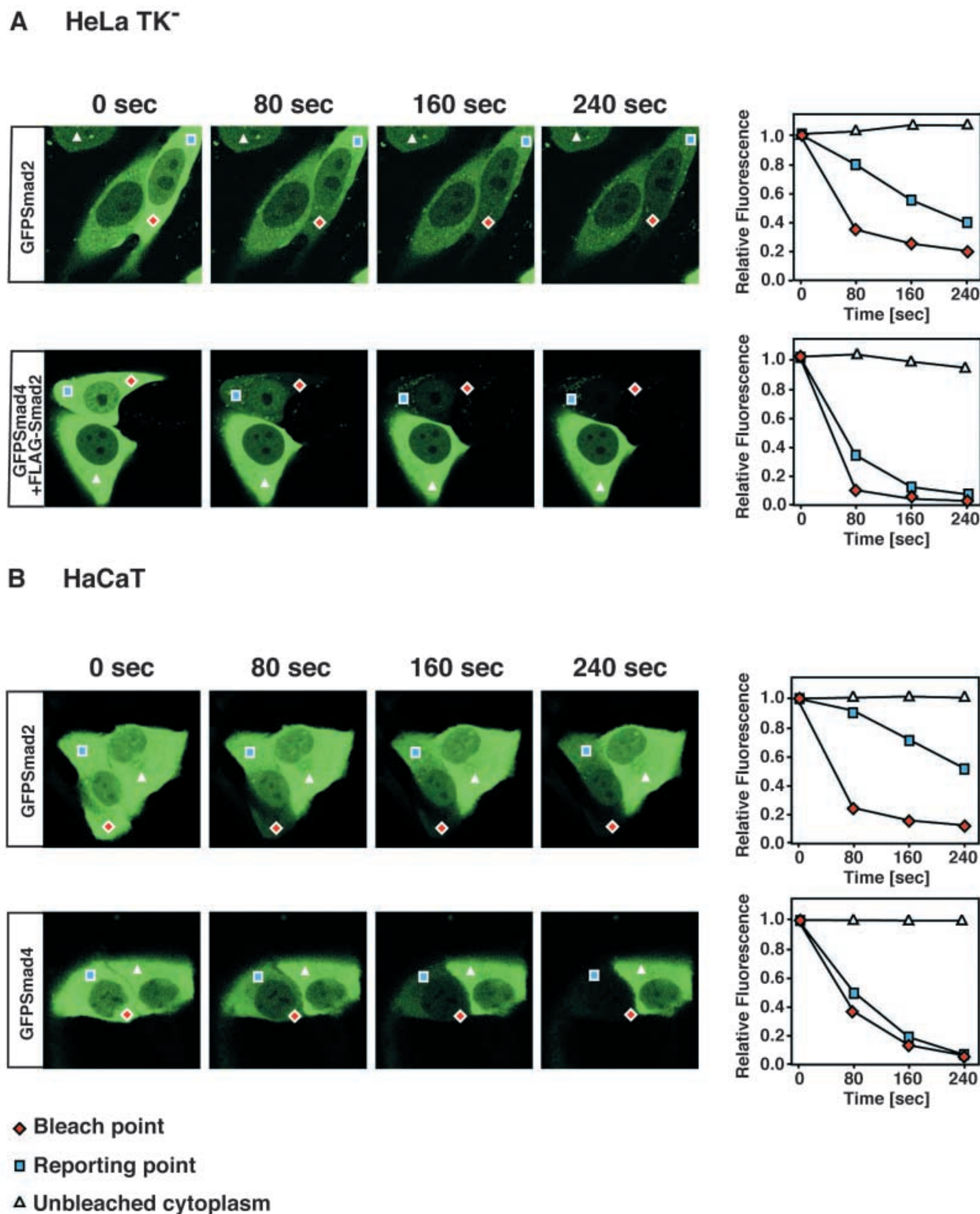


Fig. 5. FLIP analysis reveals that GFPSmad4 is more mobile in the cytoplasm than is GFPSmad2. FLIP analysis was performed on either HeLa TK⁻ cells transiently transfected with plasmids expressing GFPSmad2 or GFPSmad4 and FLAG-Smad2 (A) or HaCaT cell lines stably expressing GFPSmad2 or GFPSmad4 (B). Each row shows the fluorescence image prior to bleaching (time=0 seconds) and then after three consecutive 80-second bleaching periods. The fluorescence was quantitated at the bleach point (red diamond) and at a distant reporting point in the cytoplasm of the same cell (blue square) and at a reporting point in the cytoplasm of an adjacent cell (white triangle). In each case the fluorescence was normalised to the initial fluorescence prior to photobleaching, and the relative fluorescence was plotted. The data are representatives from at least three different experiments.

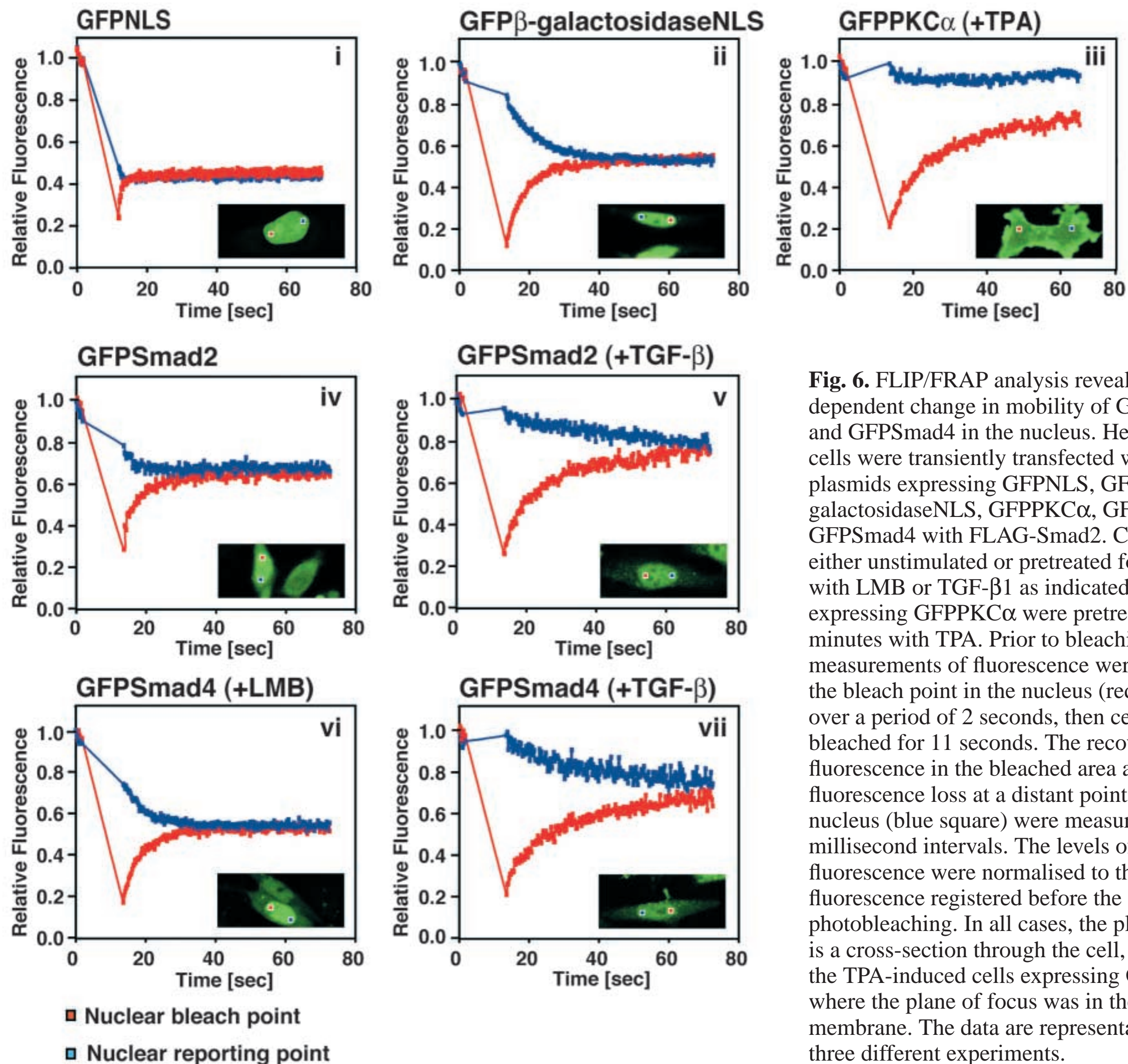


Fig. 6. FLIP/FRAP analysis reveals a TGF-β-dependent change in mobility of GFPSmad2 and GFPSmad4 in the nucleus. HeLa TK$^-$ cells were transiently transfected with plasmids expressing GFPNLS, GFPβ-galactosidaseNLS, GFPPKCα, GFPSmad2 or GFPSmad4 with FLAG-Smad2. Cells were either unstimulated or pretreated for 1 hour with LMB or TGF-β1 as indicated. The cells expressing GFPPKCα were pretreated for 10 minutes with TPA. Prior to bleaching, eight measurements of fluorescence were taken at the bleach point in the nucleus (red square) over a period of 2 seconds, then cells were bleached for 11 seconds. The recovery of fluorescence in the bleached area and fluorescence loss at a distant point also in the nucleus (blue square) were measured at 250-millisecond intervals. The levels of fluorescence were normalised to the mean of fluorescence registered before the photobleaching. In all cases, the plane of focus is a cross-section through the cell, except for the TPA-induced cells expressing GFPPKCα where the plane of focus was in the plasma membrane. The data are representatives from three different experiments.

cellular compartment, we investigated the mobility of the GFPSmads in the nucleus. In uninduced cells expressing GFPSmad2 at relatively low levels the protein is predominantly cytoplasmic, but if highly expressed a substantial amount is nuclear, although it is not phosphorylated and thus likely to be monomeric. Thus to investigate the behaviour of unactivated GFPSmad2 in the nucleus, we used highly expressing cells. The behaviour of unactivated GFPSmad2 in the FLIP/FRAP assay was intermediate between that of GFPNLS and GFPβ-galactosidaseNLS (Fig. 6iv). The FLIP/FRAP kinetics were not dependent on the level of unactivated monomeric GFPSmad2 in the nucleus, because we could show that the FLIP/FRAP curves were the same for cells expressing very different levels of nuclear GFPSmad2 (data not shown). Upon TGF-β stimulation however, the behaviour of GFPSmad2 in this assay changed to indicate that it became much less mobile (Fig. 6v). In this case the recovery at the bleach point was substantially slower, as was the rate of bleaching at the reporting point. This indicates that active complexes of GFPSmad2 (probably a mixture of homomeric GFPSmad2 complexes and heteromeric complexes with endogenous Smad4) are less mobile in the nucleus than unactivated GFPSmad2. GFPSmad4 behaved in a similar manner. LMB treatment was used to allow accumulation of unactivated monomeric Smad4 in the nucleus in the absence of TGF-β. In this case, the kinetics of FLIP/FRAP were similar to those observed with GFPSmad2 in uninduced cells (Fig. 6iv,vi). Upon TGF-β stimulation, the FLIP/FRAP experiment indicated that, like GFPSmad2, GFPSmad4 became substantially less mobile (Fig. 6vii). Again, the kinetics of FLIP/FRAP were similar to those observed with GFPSmad2 in the same conditions.

Thus we find a similar TGF-β-induced decrease in the mobility of GFPSmad2 and GFPSmad4 in the nucleus. We therefore propose that activated Smad complexes are actively retained in the nucleus through a tethering mechanism.

Discussion

Here we have investigated nucleocytoplasmic shuttling of Smad2 and Smad4 using enhanced GFP fusions. We have demonstrated that these Smad fusions behave as wild-type Smads in terms of their activation in response to TGF-β and their formation of transcriptionally active Smad-transcription

factor complexes on TGF-β-responsive elements. We have then used these Smad fusions to investigate Smad nucleocytoplasmic shuttling in detail in living cells. Two different cell lines (HaCaTs and HeLa TK^{-}) have been used and we have also compared both stably transfected cell lines and transiently transfected cells. The results obtained are similar in the different systems, suggesting that neither the cell type nor the particular levels of expression of GFPSmad are critical.

For Smad4, we demonstrate here in a simple experiment that even though GFPSmad4 is predominantly localised in the cytoplasm in the absence of TGF-β signalling, it is actually rapidly shuttling between the cytoplasm and the nucleus. We have shown that after prolonged treatment of GFPSmad4-expressing cells with SB-431542, treatment of the cells with the CRM1 inhibitor LMB leads to rapid accumulation of GFPSmad4 in the nucleus. This is because GFPSmad4 is constitutively imported into the nucleus and under these conditions cannot be exported.

We have gone on to use photobleaching experiments (FLIP and FRAP) to study the Smad nucleocytoplasmic shuttling in more detail. Indirect methods have previously suggested that the Smads undergo nucleocytoplasmic shuttling (Inman et al., 2002b; Pierreux et al., 2000; Watanabe et al., 2000; Xu et al., 2002), but here for the first time we have directly demonstrated that both GFPSmad2 and GFPSmad4 shuttle between the cytoplasm and nucleus in both uninduced cells and in TGF-β-stimulated cells. There is a striking difference between the proportion of nuclear GFPSmad that bleaches during the 240 seconds of cytoplasmic photobleaching in the uninduced cells versus that in the TGF-β-induced cells, suggesting that the proportion of nuclear Smad that is mobile in TGF-β-induced cells is substantially lower than that in unstimulated cells. In contrast to uninduced cells, the nuclear GFPSmad2 and GFPSmad4 in TGF-β-induced cells are not completely bleached even after prolonged cytoplasmic photobleaching (data not shown). The FLIP/FRAP experiments confirm that TGF-β induction results in a decrease in Smad mobility in the nucleus. The data obtained with endogenous Smads suggest that only monomeric Smads are exported from the nucleus (Inman et al., 2002b). Thus in TGF-β-induced cells, dissociation of Smads from active Smad complexes after dephosphorylation of the R-Smads will be a prerequisite for nuclear export. The rate of R-Smad dephosphorylation and complex dissociation will thus dictate the amount of nuclear Smad that is mobile, and can be exported to the cytoplasm.

We have also used photobleaching experiments to investigate the mobility of the GFPSmads in both the cytoplasm and the nucleus. This issue is important as it impinges on the mechanism underlying the distribution of the Smads between the cytoplasm and nucleus in uninduced cells and in TGF-β-induced cells. Two different models (not mutually exclusive) can be envisaged. One model proposes the existence of cytoplasmic and nuclear retention factors (Reguly and Wrana, 2003; Xu et al., 2000; Xu et al., 2002). Monomeric Smads would have a higher affinity for cytoplasmic retention factors and complexed Smads would have a higher affinity for nuclear retention factors. This could explain the cytoplasmic localisation of the Smads in unstimulated cells, and their accumulation in the nucleus upon TGF-β treatment. An alternative view is that the distribution of the Smads between the cytoplasm and nucleus is determined by the relative rates of import and export. Monomeric Smads would be preferentially exported from the nucleus and complexed Smads, preferentially imported. Complex formation might either stimulate import or inhibit export (De Bosscher et al., 2004; Reguly and Wrana, 2003; Watanabe et al., 2000).

Our results demonstrate that in uninduced cells GFPSmad2 is less mobile in the cytoplasm than is GFPSmad4. This suggests that monomeric inactivated GFPSmad2 is likely to be associated with cytoplasmic retention factors. Two different proteins have been suggested to act in such a way, and in both cases monomeric Smad2 has been shown to preferentially bind the factor compared with active complexed Smads. First, the microtubule network may play this role. Smads 2, 3 and 4 have been shown to bind tubulin and long-term (18 hour) treatment of cells with microtubule disrupting agents, such as nocodazole, results in increased TGF-β-induced Smad2 phosphorylation and increased TGF-β-induced transcription (Dong et al., 2000). We think, however, that the microtubule network is unlikely to be the cytoplasmic retention factor predicted by our experiments, because in HaCaT cells we find that short-term treatment with nocodazole, which was sufficient to depolymerise the microtubules, had no effect on the localisation of endogenous Smad2 (data not shown). Another candidate for the cytoplasmic retention factor for Smad2 is Smad Anchor for Receptor Activation (SARA) (Xu et al., 2000; Xu et al., 2002). In vitro, the purified Smad binding region of SARA inhibits nuclear import of Smad2 (Xu et al., 2000). However, SARA is unlikely to be the major endogenous Smad2 retention factor, because it is predominantly localised on early endosomes (Di Guglielmo et al., 2003; Hayes et al., 2002; Itoh et al., 2002), whereas Smad2 is distributed equally throughout the cytoplasm (Inman et al., 2002b; Pierreux et al., 2000). Moreover, there appears to be substantially more Smad2 in a cell than SARA because overexpression of SARA has been shown to relocalise Smad2 to the early endosomes (Tsukazaki et al., 1998). More work is obviously required to confirm the identity of the putative cytoplasmic retention factor for Smad2.

In the nucleus we have used FRAP in combination with FLIP to investigate the mobility of GFPSmad2 and GFPSmad4 in both uninduced cells and after 1 hour of TGF-β stimulation. GFPSmad2 and 4 behaved similarly in this assay, and in both cases ligand-induced activation led to a substantial decrease in mobility. This strongly suggests that unactivated monomeric Smads in the nucleus are not actively retained, but activated Smad complexes are. Similar results have recently been demonstrated for a subset of hormone receptors. In these experiments ligand binding has been shown to decrease the mobility of GFP fusions of estrogen receptor and glucocorticoid receptor and to increase the fraction of receptors unable to diffuse (Maruvada et al., 2003; Schaaf and Cidlowski, 2003). What could be the nuclear retention factors for the active Smad complexes? Smads are known to associate with transcription activators and repressors as well as co-activators and co-repressors in the nucleus, and these could act as nuclear retention factors (Shi and Massagué, 2003; Xu et al., 2002). If this were the case, the complexes would have to be extremely large, because the active Smad complexes are substantially less mobile than GFPβ-galactosidaseNLS which has a native molecular weight as a tetramer of over 400 kDa. The other possibility is that DNA acts as a nuclear retention factor for the Smads. The Smads bind DNA both directly and

indirectly through interactions with other transcription factors. We have demonstrated that here the GFPSmads, like endogenous Smads, are located uniformly throughout the interphase nucleus, excluded only from the nucleoli (see also Pierreux et al., 2000). Thus it is unlikely that the association with DNA represents Smad complexes actively engaged in transcription at specific promoter sites, because these sites would be relatively rare (Hager et al., 2002). It is most likely that an association with DNA reflects Smad complexes weakly bound to DNA engaged in a 'scanning process' to locate specific binding sites (Lillemeier et al., 2001; Pederson, 2001). These weakly bound Smads may be in dynamic equilibrium with those bound to specific promoter elements, as has been recently demonstrated for hormone-bound glucocorticoid receptor and its interacting protein GRIP-1 (Becker et al., 2002; Hager et al., 2002; McNally et al., 2000).

The work presented here provides evidence for cytoplasmic retention of Smad2 in uninduced cells and nuclear retention for both Smad2 and Smad4 in TGF-β-induced cells. Because the Smads are constantly shuttling between the cytoplasm and nucleus in both uninduced and in TGF-β-induced cells, the binding to the putative retention factors must be readily reversible. Based on these findings, our previous work and the work of others, we propose the following model for regulation of Smad nucleocytoplasmic shuttling. Unphosphorylated Smad2 may be retained in the cytoplasm through interaction with retention factors, whereas Smad4 may be retained there because its export from the nucleus is dominant over import. Activation of Smad2 leads to dissociation of Smad2 from cytoplasmic tethering, and to complex formation with Smad4. These complexes accumulate in the nucleus at least partly because they are actively retained there. In addition, it remains possible that nuclear import of the Smad complexes dominates over export, either because import is potentiated or export is inhibited by Smad complex formation (Kurisaki et al., 2001; Watanabe et al., 2000). The R-Smads in the nucleus are continuously being dephosphorylated, leading to dissociation of Smad complexes, release from nuclear tethering and export of the Smads from the nucleus. For the duration of signalling, this cycle of phosphorylation and activation in the cytoplasm and dephosphorylation and deactivation in the nucleus continues. When the receptors are downregulated (Ebisawa et al., 2001; Kavsak et al., 2000), and signalling is terminated, the Smads accumulate back in the cytoplasm.

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