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Mitochondrial localization of Smad5 in a human chondrogenic cell line☆

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Abstract

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily and regulate the formation of cartilage and bone tissues as well as other key events during development. TGF-β superfamily signaling is mediated intracellularly by Smad proteins, some of which can translocate into the cell nucleus and influence gene expression. Although much progress has been made in understanding how TGF-β superfamily signaling regulates expression of target genes, little formal proof has been presented regarding the intracellular distribution of the Smad proteins before their entry into the nucleus. In the literature, non-nuclear Smad proteins are generally referred to as cytoplasmic. Using confocal microscopy, we here show for the first time that immunofluorescent labeling of Smad5, one of the Smad proteins associated with BMP signaling, colocalizes with the mitochondrion-specific probe MitoTracker, demonstrating a mitochondrial distribution of Smad5 in non-stimulated chondroprogenitor cells.

Keywords: Smad5; Mitochondria; BMP signaling; Subcellular localization

The transforming growth factor-β (TGF-β) superfamily consists of more than 30 structurally related proteins, including TGF-βs, activins, and bone morphogenetic proteins (BMPs). These secreted polypeptide growth factors have crucial roles during development and homeostasis and regulate fundamentally important events such as cell proliferation, differentiation, and apoptosis [1,2].

The downstream events of TGF-β signaling have been thoroughly studied in recent years [3,4]. Although there are pathway-specific differences between signaling by TGF-βs, activins, and BMPs, the basic principles are similar. Binding of the growth factor to two kinds of transmembrane serine/threonine kinase receptors (type I and type II) leads to phosphorylation of intracellular proteins belonging to the Smad family [5,6]. To date, eight different Smads (Smad1–8) have been described. Five of these (Smad1, Smad2, Smad3, Smad5, and Smad8) are directly activated by ligand-bound TGF-β superfamily receptor complexes and are thus commonly referred to as *receptor-regulated* (*R*)-*Smads*. Activated R-Smads initially form homo-oligomers that are rapidly converted to hetero-oligomeres containing *common-partner* (*Co*)-*Smads* (Smad4) [7,8]. The resulting R/Co-Smad complexes migrate into the nucleus and influence expression of target genes by direct DNA binding or by association with nuclear transcription factors [3,9–14]. *Inhibitory* (*I*)-*Smads* (Smad6 and Smad7) can counteract signaling by competing with the R-Smads at the receptor, thus preventing the receptor from phosphorylating the R-Smads [15,16]. Receptor-Smads, Smad2 and Smad3, are associated with TGF-β and activin signaling, whereas Smad1, Smad5, and Smad8 primarily mediate BMP responses [12,17,18].

The intracellular events following stimulation with TGF-β and activin are relatively well understood compared to the BMP signaling pathway, which has proven harder to map out. Not only are there a multitude of BMP ligands, and at least three different BMP specific type I receptors [19,20], but there also seems to be variability across experimental systems as to which Smad or Smads are activated by which ligand/receptor

☆ *Abbreviations:* TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; R-Smads, receptor-regulated Smads; Co-Smads, common-partner Smads; I-Smads, inhibitory (I)-Smads; MAPK, mitogen activated protein kinase; SARA, Smad anchor for receptor activation; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; AIF, apoptosis inducing factor.

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combination. Nevertheless, different BMPs have different biological effects, and the issue of how specificity is achieved is one of the most challenging questions in the field of BMP signaling today. Furthermore, BMP activated Smads can establish cross-talk to other signaling pathways and BMP signals may also be transduced by alternative pathways, e.g., the MAPK pathway [21,22]. Thus, the full complexity of BMP signaling is far from elucidated.

Although R-Smads and Co-Smads have been shown to enter the nucleus in response to TGF-β, activin and BMPs, few reports deal with the subcellular localization of the Smads before stimulation with growth factors. Throughout the literature, the Smads are generally described as being cytoplasmic proteins before their entry into the nucleus, with the exception of *Xenopus* Smad4β that has been localized to the nucleus even in the absence of ligand stimulation [23]. It should be noted that the term *cytoplasmic* is a general term meaning *intracellular but not nuclear*, not to be confused with the more specific designation *cytosolic*, which excludes not only the nucleus but also all other cellular organelles. Since R- and Co-Smads exert their effects after they have relocated into the nucleus, it is perhaps not surprising that little effort has been focused on pinpointing more exactly their subcellular distribution in the absence of growth factor signaling.

An interesting feature of the TGF-β/activin pathway is the presence of a membrane associated protein Smad Anchor for Receptor Activation (SARA) that facilitates receptor mediated phosphorylation of Smad2 and Smad3 by binding simultaneously to non-activated Smads and the TGF-β receptor complex [24]. The non-phosphorylated forms of Smad2, Smad3, and Smad4 have also been shown to interact with tubulin of the microtubules [25]. Chemical destabilization of the microtubules results in release of the tubulin associated Smads and increased levels of phosphorylated R-Smads, demonstrating that the levels of Smads available for TGF-β/activin signal transduction are regulated by microtubules. Although no corresponding regulatory mechanisms have been described for BMP signal transduction, it is likely that the BMP-Smad response may also, at least in part, be regulated by differences in subcellular compartmentation of the Smads.

In the present study, computer analysis of the amino-acid sequences of the different Smads was performed to predict the most likely subcellular locations for the Smads. Unexpectedly, a mitochondrial distribution was suggested for Smad5, Smad6, and Smad7 and we focused our efforts on Smad5, one of the R-Smads implemented in BMP signaling. A BMP-responsive human chondrogenic cell line, T/C28a2, was chosen for immunofluorescent labeling of Smad5 in combination with the mitochondrial marker MitoTracker. Confocal microscopy demonstrated colocalization between Smad5 labeling and MitoTracker, strongly indicating that Smad5 is a mitochondrial protein.

Materials and methods

Cells, antibodies, and fluorescent probes. The human chondrogenic cell line T/C28a2 was provided by Dr. Mary Goldring, Division of Rheumatology, Harvard Medical School, Boston, USA. Cells were cultivated in a growth medium consisting of 50% (v/v) Dulbecco's modified Eagle's medium with GLUTAMAX I (Life Technologies), 50% (v/v) F12 Ham's mix (Life Technologies), 10% (v/v) fetal calf serum, and 1% antibiotic–antimycotic solution (Life Technologies). A goat anti-Smad5 antibody, raised against part of the carboxy terminus of the protein, was purchased from Santa Cruz and a rabbit anti-Smad5 antibody, directed towards a 15 amino-acid peptide from a central part of Smad5, was from Zymed Laboratories. Cross-reactivity with other Smad family members has not been observed for either of

Table 2
Probabilities of mitochondrial localization of human Smad1–7, according to the MitoProt analysis tool

	MitoProt score (%)
Smad1	87
Smad2	70
Smad3	63
Smad4	8
Smad5	92
Smad6	100
Smad7	99

Table 1
Computer prediction of the subcellular localizations of human Smad1–7 using the PSORT prediction tool

	PSORT prediction (%)						
	Cytosolic	Mitochondrial	Cytoskeletal	Golgi	Extracellular	Other[a]	Nuclear
Smad1	**43.5**[b]	8.7	8.7	—	—	—	39.1
Smad2	**34.8**	—	13.0	8.7	—	4.4	39.1
Smad3	**17.4**	4.3	8.7	4.3	—	8.8	56.5
Smad4	**26.1**	4.3	8.7	4.3	—	4.4	52.2
Smad5	**17.4**	**17.4**	—	—	13	4.4	47.8
Smad6	8.7	**52.2**	—	—	—	4.3	34.8
Smad7	8.7	**56.5**	4.3	—	—	0.1	30.4

[a] Includes vesicles of the secretory system, plasma membrane, and peroxisomes.
[b] Data shown in bold indicate the most likely subcellular compartment.

the two antibodies. Anti-goat and anti-rabbit Cy2 was from Jackson ImmunoResearch Laboratories. MitoTracker Red CM-H_2XRos was obtained from Molecular Probes, Leiden, The Netherlands. DAPI was from Sigma.

Prediction of subcellular location. The amino-acid sequences for human Smad1–7 were retrieved from the Swiss Prot database (http://tw.expasy.org/sprot/). Two independent web based services were used to analyze the amino-acid sequences of the Smads; MitoProt II 1.0a4 (http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter) [26] and PSORT II Prediction (http://psort.nibb.ac.jp/).

Mitochondrial and immunofluorescent labeling. All steps were carried out at room temperature unless otherwise indicated. As much as 1.4×10^5 cells were seeded per well in 8-well culture slides and grown to sub-confluency. For mitochondrial labeling, cells were incubated for

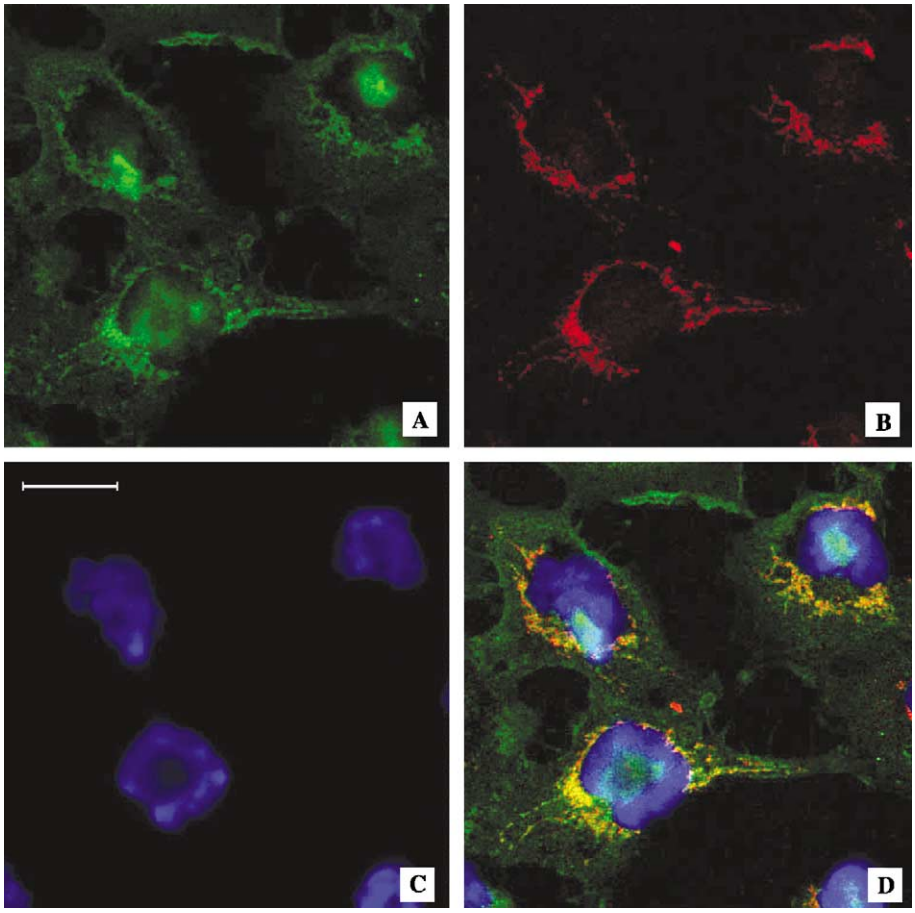

Fig. 1. Triple staining of T/C28a cells labeled with goat α-Smad5 and Cy2 linked α-goat IgG, DAPI, and MitoTracker. (A) Smad5, (B) MitoTracker, (C) DAPI, and (D) overlay. Scale bar: 80 μm.

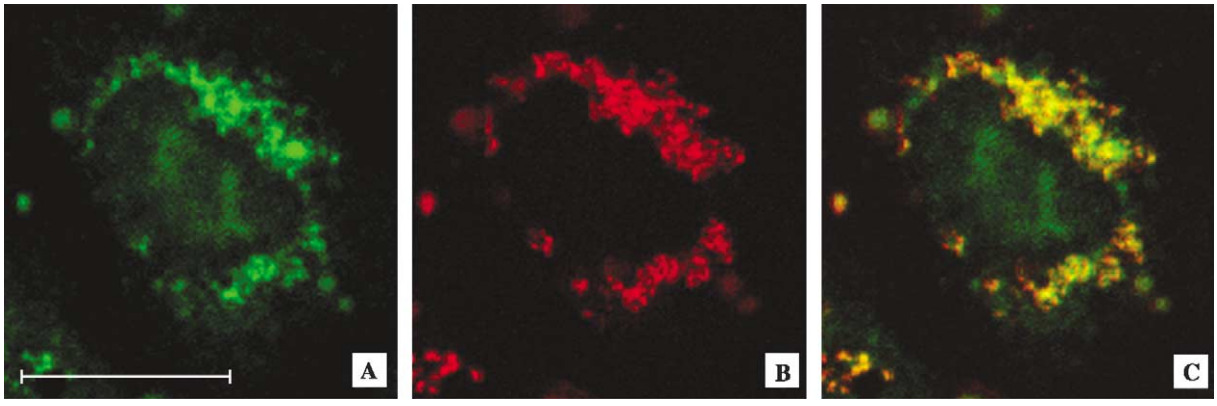

Fig. 2. Double staining of T/C28a cells labeled with rabbit α-Smad5 and Cy2 linked α-rabbit IgG, and MitoTracker (higher magnification). (A) Smad5, (B) Mito-Tracker, and (C) overlay. Scale bar: 80 μm.

45 min at +37 °C with growth medium containing 200 nM MitoTracker Red CM-H_2XRos. Following brief incubation with growth medium without MitoTracker, the cells were fixed for 3 min with methanol and blocked with 0.1 M L-lysine in PBS for 1.5 h. After washing with PBS, the cells were incubated overnight at +4 °C with either goat or rabbit antibodies directed against Smad5 diluted 1:300 and 1:125 in PBS, respectively. Following extensive washing with PBS, the cells were incubated for 2 h with Cy2 linked anti-goat or anti-rabbit IgG diluted 1:200 in PBS. After final washing steps and incubation with 1 μM DAPI for 10 s, the slides were mounted and sealed. The slides were analyzed in a confocal microscope (Leica SP2) fitted with the appropriate filters. The Leica confocal software was used to generate images of individual fluorescent markers as well as overlay pictures demonstrating the relative distribution of the different fluorescent markers from the same portion of the slide.

Results

Computer-based prediction of subcellular distributions

All human Smads for which amino-acid sequences were available were analyzed using the web-based tools PSORT Prediction (Table 1) and MitoProt (Table 2). When interpreting the data obtained from the PSORT Prediction, strong predictions of nuclear localizations were disregarded, since it is known that all human Smads are non-nuclear before stimulation by the appropriate growth factor. Most likely to be mitochondrial were Smad7 (56.5%), Smad6 (52.2%), and Smad5 (17.4%). PSORT II suggested a cytosolic distribution for Smad1 (43.5%), Smad2 (34.8%), Smad4 (26.1%), and Smad3 (17.4%). In accordance with these results, Smad7, Smad6, and Smad5 also showed the highest probability of export to the mitochondria when analyzed by MitoProt (Table 2).

Confocal microscopy

The majority of the cells probed with goat anti-Smad5 and Cy2-linked secondary antibodies showed strong fluorescence distributed in a distinctive perinuclear pattern (Fig. 1A). Mitochondria, visualized by the highly specific probe MitoTracker, exhibited a pattern almost identical to the Smad5 labeling (Fig. 1B). Computerized overlay of the images demonstrated a high degree of colocalization (yellow in Fig. 1D). The cells shown in Fig. 1 are representative for at least three independent experiments. Control samples incubated with secondary Cy2 linked antibody only displayed background staining below the detection level when using identical scanning settings as those used for Fig. 1A (not shown). Samples labeled only with MitoTracker emitted no detectable fluorescence at the wavelength used to scan for Cy2 fluorescence and cells probed with the anti-Smad5 antibody and the secondary Cy2-linked antibody gave no signal in the spectrum used to scan for MitoTracker (data not shown). To further rule out the possibility that the mitochondrial distribution of the Smad5 staining in these experiments was due to non-specific staining, we repeated the experiments using a rabbit antibody raised against Smad5. Similar results were obtained with strong fluorescence distributed in a perinuclear pattern (Fig. 2A) that colocalized with the distribution of MitoTracker (Figs. 2B and C).

Discussion

We here provide evidence for a mitochondrial localization of human Smad5 in sub-confluent T/C28a2 cells. Although the functional relevance of this novel finding is yet to be determined, the mitochondrial distribution of Smad5 raises several new questions. Mitochondria contain four sub-compartments: the mitochondrial *matrix*, surrounded by the *inner membrane*, an *outer membrane*, and the *intermembranous space*, situated between the two membranes. In order for mitochondrial Smad5 to access the cytosolic side of the plasma membrane to become phosphorylated in response to BMP signaling, the protein has to traverse at least one of the two mitochondrial membranes, depending on in which mitochondrial sub-compartment it resides. The concept of release of mitochondrial proteins into the cytosol is not new. During apoptosis, numerous pro-apoptotic proteins, e.g., cytochrome *c*, AIF (apoptosis inducing factor), and several caspases, translocate from the mitochondrial intermembranous space to partake in the apoptotic cascade in the cytosol [27,28], reviewed in [29]. Although most proteins that exit the mitochondria during the onset of apoptosis are located in the intermembranous space, there is at least one example of cytosolic redistribution of a mitochondrial matrix protein [30]. Several models have been proposed to explain how proteins can translocate from mitochondria and although some studies indicate that mitochondrial proteins may simply leak out into the cytosol after damage to the mitochondrial membranes, there is accumulating evidence suggesting that the release of mitochondrial proteins is a regulated event rather than a result of mitochondrial disintegration [31–33], reviewed in [29].

Interestingly, a link between Smad5 and apoptosis has been demonstrated. Targeted knock-out of Smad5 in mice resulted in increased apoptosis of mesenchymal cells, indicating that Smad5 may have anti-apoptotic properties [34]. A recent publication also reports up-regulation and potential pro-apoptotic effects of Smad5 in *Helicobacter pylori* infected cells [35]. Although little is known regarding the mechanism by which Smad5 could act to suppress or promote apoptosis, it is well established that mitochondria play a key role in the regulation of apoptosis. The mitochondrial distribution

of Smad5 shown here may indicate a direct involvement of Smad5 by influencing the mitochondrial response to apoptotic stimuli.

In the light of the microtubular association with the Smads of the TGF-β/activin pathway demonstrated by Dong et al. [25], it is also tempting to speculate that the mitochondrial distribution of Smad5 may serve to regulate cytosolic levels of the protein directly accessible for phosphorylation and subsequent BMP signal transduction.

In conclusion, the nature and biological significance of the mechanisms responsible for mitochondrial retention and release of Smad5 will be the subject for future investigation. We will also conduct further studies to evaluate the computer-predicted mitochondrial distributions of the two I-Smads, Smad6 and Smad7.

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