TGFβ induces SIK to negatively regulate type I receptor kinase signaling

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Signal transduction by transforming growth factor β (TGFβ) coordinates physiological responses in diverse cell types. TGFβ signals via type I and type II receptor serine/threonine kinases and intracellular Smad proteins that regulate transcription. Strength and duration of TGFβ signaling is largely dependent on a negative-feedback program initiated during signal progression. We have identified an inducible gene target of TGFβ/Smad signaling, the salt-inducible kinase (SIK), which negatively regulates signaling together with Smad7. SIK and Smad7 form a complex and cooperate to down-regulate the activated type I receptor ALK5. We further show that both the kinase and ubiquitin-associated domain of SIK are required for proper ALK5 degradation, with ubiquitin functioning to enhance SIK-mediated receptor degradation. Loss of endogenous SIK results in enhanced gene responses of the fibrotic and cytostatic programs of TGFβ. We thus identify in SIK a negative regulator that controls TGFβ receptor turnover and physiological signaling.

Introduction

The profound roles that TGFβ plays during embryogenesis, adult tissue homeostasis, and disease pathogenesis necessitate deeper understanding of mechanisms that regulate this signaling pathway. Signaling occurs via the TGFβ type II receptor (TβRII) that trans-phosphorylates TβRI, also known as activin receptor-like kinase (ALK) 5. Activated ALK5 phosphorylates receptor-regulated Smads (Smad2 and Smad3), promotes their association with Smad4, and leads to regulation of transcription (Feng and Derynck, 2005).

Smad7, a negative regulator of TGFβ/Smad signaling, is an immediate early gene target of the pathway (for review see Itoh and ten Dijke, 2007). Smad7 binds to ALK5, competing with Smad2/3 phosphorylation and mediating receptor ubiquitination, mechanisms which link to the process of TGFβ receptor internalization and lysosomal degradation (Di Guglielmo et al., 2005).

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We have identified gene targets of Smad signaling (Kowanetz et al., 2004). A highly regulated gene, SNF1LK (sucrose nonfermented 1-like kinase) or salt-inducible kinase 1 (SIK), hereby abbreviated as SIK, encodes a serine/threonine kinase. SIK is one of 14 AMP-activated protein kinases that can be phosphorylated by the tumor suppressor LKB1 (Alessi et al., 2006). SIK expression is induced during cardiogenesis and skeletal muscle differentiation (Ruiz et al., 1994) or in adrenal glands, leading to steroidogenesis (Okamoto et al., 2004).

We uncover a new functional role of SIK in the TGFβ pathway. SIK induction is necessary for physiological TGFβ signaling. Ubiquitin promotes association of SIK to Smad7, which down-regulates ALK5. Thus, SIK defines a new negative-feedback loop initiated by incoming Smad signals.

Results and discussion

TGFβ induces SIK in a Smad-dependent manner

We have reported Smad4-dependant gene profiles in response to TGFβ1 or bone morphogenetic protein (BMP) 7 using human

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Abbreviations used in this paper: ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; CA, constitutively active; GAPDH, glyceraldehyde-3’-phosphate dehydrogenase; PAI-1, plasminogen activator inhibitor 1; SIK, salt-inducible kinase; TβRII, TGFβ type II receptor; TCL, total cell lysate; UBA, ubiquitin associated.

The online version of this paper contains supplemental material.

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Caenorhabditis elegans orthologue of SIK, Kin-29, regulates chemosensory neuronal signaling and body size, a process which is dependent on TGFβ/Smad (Lanjuin and Sengupta, 2002; Maduzia et al., 2005), which is in agreement with our data.

SIK down-regulates activated ALK5

Gain-of-function experiments with transfected SIK showed specific down-regulation of ALK5 after TGFβ1 stimulation, whereas minor effects were scored without stimulation (Fig. 2 A). SIK did not down-regulate Smad7 or GFP (Fig. S1 B), excluding translational inhibition or induction of proteolysis.

SIK affected ALK5 turnover as it reduced the half-life of a constitutively active (CA) ALK5 from 5.5 to 2.8 h without affecting a KR- (kinase-dead) ALK5 or an unrelated adaptor protein CIN85 (Fig. 2 B). Proteasomal (LLnL) and lysosomal (chloroquine) inhibitors stabilized the ALK5 levels in the presence of SIK (Fig. 2 C), suggesting both proteasomal and lysosomal mechanisms in ALK5 down-regulation by SIK.

SIK has an N-terminal kinase domain with lysine 56 binding to ATP and a central ubiquitin-associated (UBA) domain, which regulates conformation and kinase activity (Jaleel et al., 2006). A catalytically inactive (K56R) SIK or a deletion mutant lacking the UBA domain failed to down-regulate CA-ALK5 (Fig. 2 D). This suggests that both catalytic activity and UBA domain of SIK affect ALK5 turnover.

RNAi against SIK significantly enhanced endogenous ALK5 levels (Fig. 2 E). Increased presence of endogenous cell surface receptors measured with chemically cross-linked radioligand upon depletion of SIK further strengthened this evidence (Fig. 2 F, 0 h). Thus, endogenous SIK must regulate total ALK5 levels, which also affects cell surface receptor numbers, available for signaling. Receptor down-regulation was also slower after SIK RNAi. Significantly higher ligand-bound receptor levels were observed for up to 1 h of internalization compared with control (Fig. 2 F).

Because proteasomal and lysosomal inhibitors block ALK5 degradation (Fig. 2 C), we suggest that ALK5 turnover takes place in lysosomes. Proteasomes might promote trafficking to the lysosome, as has already been established for the EGF receptor (Longva et al., 2002; Alwan et al., 2003). How proteasomes regulate TGF-β receptor internalization and degradation remains unclear.

SIK associates and cooperates with Smad7 for ALK5 down-regulation

Down-regulation of ALK5 is mediated by Smad7 (for review see Itoh and ten Dijke, 2007). We reasoned that SIK could associate directly with ALK5 or Smad7. Coimmunoprecipitation assays showed that SIK formed complexes with both ALK5 and Smad7 (Fig. 3, A and B). To detect SIK–ALK5 complexes, we had to achieve low SIK expression and high CA-ALK5 levels (Fig. 1 A and B). In human HaCaT keratinocytes, TGFβ1 induced and sustained SIK mRNA and protein levels (Fig. 1 C and D; and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200804107/DC1). Endogenous TGFβ1-induced SIK protein showed punctuate nuclear, cytoplasmic, and peripheral localization (Fig. 1 E). SIK represents a new gene target of TGFβ/BMP7 SIK signaling. Interestingly, the

breast carcinoma MDA-MB-468 cells that lack both Smad4 gene copies (Kowanetz et al., 2004). After reconstitution with Smad4, TGFβ1 or BMP7 rapidly induced SIK mRNA, followed by slow decrease (Fig. 1, A and B). In human HaCaT keratinocytes, TGFβ1 induced and sustained SIK mRNA and protein levels (Fig. 1, C and D; and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200804107/DC1). Endogenous TGFβ1-induced SIK protein showed punctate nuclear, cytoplasmic, and peripheral localization (Fig. 1 E). SIK represents a new gene target of TGFβ/BMP7 SIK signaling. Interestingly, the
ALK5 (Fig. 3 C), confirming the evidence with transfected proteins. Thus, protein complexes between endogenous SIK, Smad7, and ALK5 occur in vivo.

Smad7 resides in the nucleus and moves to the cytoplasm in response to TGF-β (for review see Itoh and ten Dijke, 2007). SIK also resides in both nucleus and cytoplasm, and its localization is regulated by hormones in adrenal cells or by the 14–3–3 adaptor (Okamoto et al., 2004; Al-Hakim et al., 2005). In transfected TGF-β-sensitive Mv1Lu cells, SIK localized in nuclei and cytoplasm with pronounced punctate distribution proximal to the plasma membrane (Fig. 3 D), which is similar to endogenous SIK (Fig. 1 E). Colocalization of SIK, CA-ALK5, and Smad7 was primarily observed in peripheral clusters (Fig. 3 D, insets). Thus, SIK, Smad7, and ALK5 must form complexes in cytoplasmic regions proximal to the plasma membrane.

To verify the cooperation of Smad7 and SIK in negatively regulating TGF-β signaling, we measured the effects of SIK and Smad7 on the transcriptional induction of a Smad3/Smad4-specific promoter-reporter, CAGA12, by TGF-β1 (Fig. 3 E). SIK enhanced the inhibitory effect of Smad7 by more than twofold. Knockdown of endogenous Smad7 stabilized and rescued CA-ALK5 down-regulation by SIK, even though Smad7 depletion led to increased SIK levels (Fig. 3 F). Thus, SIK mediates down-regulation of signaling ALK5 receptor in a Smad7-dependent manner.
SIK readily coprecipitated ubiquitinated proteins of diverse sizes, confirming that they were distinct from ubiquitinated SIK. Wild-type and SIK-K56R bound to ubiquitinated proteins, whereas SIK-K56R/UBA or a mutant lacking both kinase and UBA domains (SIK-N) did not, suggesting that the SIK UBA domain is needed for binding to polyubiquitinated proteins (Fig. 4 C). C-terminal deletion of SIK, after its UBA domain, had no effect on binding ubiquitinated proteins (Fig. 4 C). Thus, SIK can associate with ubiquitinated proteins in cells.

Obvious ubiquitinated protein candidates to which SIK could bind were ALK5 and Smad7. Accordingly, the SIK–Smad7 complex was enhanced by coexpressing ubiquitin, whereas deletion of the UBA domain of SIK prohibited such enhancement (Fig. 4 D). A slow migrating SIK form was obvious in complex with Smad7 (Fig. 4 D, asterisk), which may represent ubiquitinated SIK. In contrast, neither ubiquitin nor UBA domain deletion had any effect on SIK–ALK5 complexes (Fig. S2 B). Ubiquitin therefore enhances association between SIK and Smad7, and the SIK UBA domain mediates formation of this ternary complex.

The SIK UBA domain was recently shown to not bind ubiquitin but to modulate SIK kinase activity in vitro (Jaleel et al., 2006).
We also failed in demonstrating in vitro binding of recombinant mono- or oligoubiquitin chains to SIK (unpublished data). SIK immunopurified from mammalian cells clearly bound to ubiquitinated proteins via its UBA domain (Fig. 4 C and Fig. S2 A). This could reflect an in vivo modification or the requirement for another binding protein. Further studies will reveal the mechanism by which ubiquitin and the SIK UBA domain regulate its own function.

**SIK negatively regulates TGFβ signaling**

Stimulation of cells after endogenous SIK depletion enhanced the potency of TGF-β signaling (Fig. 5, A and B). Signaling was assessed by measuring mRNA levels of well characterized TGFβ gene targets, representing three distinct physiological programs: the fibrotic program (*plasminogen activator inhibitor 1* [PAI-1], TGFβ inducible), the cytostatic program (cell cycle inhibitors p21 and p15, TGFβ inducible; transcriptional regulators c-myc and Id2, TGFβ repressible), and the negative-feedback program (*Smad7*, TGFβ-inducible; Fig. 5 B and Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200804107/DC1). We also confirmed that SIK knockdown enhanced the magnitude of endogenous PAI-1 and p21 protein accumulation in response to TGFβ1 (Fig. 5 C). Finally, protein levels and pericellular localization of fibronectin, another TGFβ gene target from the fibrotic program, augmented significantly after depleting SIK (Fig. 5, D and E). Thus, induction of SIK by TGFβ represents a central step in negative regulation of TGFβ signaling.

The identification of SIK as an immediate early gene induced by the Smad pathway led to important new insights into how TGFβ receptor signaling is negatively regulated (Fig. 5 F). SIK joins Smad7 in the negative-feedback loop of TGFβ signaling (for review see Itoh and ten Dijke, 2007). SIK and Smad7 interact, and ubiquitin enhances their complex. SIK mutant proteins were immunoprecipitated (anti-Flag) and ubiquitinated proteins are detected (anti-HA immunoblot). Total SIK levels (bottom) and immunogoldulin heavy chain (lg) are shown. (D) Commonprecipitation of wild-type Smad7 with wild-type myc-SIK or UBA deletion mutant in the absence or presence of ubiquitin. The asterisk indicates a slow migrating SIK species, possibly representing ubiquitinated SIK. TCL controls of SIK variants, the immunogoldulin heavy chain (lg), and protein sizes in kilodaltons are shown. Black lines indicate that intervening lanes have been spliced out.

**Figure 4.** Role of ubiquitin and the UBA domain of SIK in ALK5 down-regulation. (A) Immuno blot of CA-ALK5 in the presence of increasing doses of WT GFP-SIK and coexpression of WT or K48R mutant ubiquitin. (B) Confocal immunofluorescence analysis of wild-type or mutant SIK proteins cotransfected with HA-ubiquitin in Mv1Lu cells. Inserts show higher magnifications of peripheral clusters below the plasma membrane (bar, 10 μm). (C) SIK lysate was mixed briefly with lysate for ubiquitinated proteins before immunoprecipitation. SIK mutant proteins were immunoprecipitated (anti-Flag) and ubiquitinated proteins are detected (anti-HA immunoblot). Total SIK levels (bottom) and immunogoldulin heavy chain (lg) are shown. (D) Commonprecipitation of wild-type Smad7 with wild-type myc-SIK or UBA deletion mutant in the absence or presence of ubiquitin. The asterisk indicates a slow migrating SIK species, possibly representing ubiquitinated SIK. TCL controls of SIK variants, the immunogoldulin heavy chain (lg), and protein sizes in kilodaltons are shown. Black lines indicate that intervening lanes have been spliced out.
pathways guided by TGFβ signaling opens new territories for exploration of such pathways across species. Characterizing the role of the Drosophila melanogaster SIK orthologue will greatly benefit this cause.

Because SIK is activated by the tumor suppressor kinase LKB1 (Alessi et al., 2006), our study suggests a role of LKB1 in processes of TGFβ signaling and receptor down-regulation. Thus, SIK being a component of the TβR–Smad7 complex provides a nodal point for crosstalk of the TGFβ pathway with LKB1 or AMP-activated protein kinase pathways. SIK therefore opens a new window for the elucidation of the process of down-regulation of serine/threonine kinase receptors.
A PCR-based strategy and were subcloned in pcDNA3-Flag and pEGFP. 6myc-hSIK, and pEGFP-hSIK. The SIK ATP binding site mutant K56R was fused to a Flag, 6myc, and GFP creating pcDNA3-Flag-hSIK, pcDNA3-6myc-hSIK, and pcDNA3-6myc-Smad7, pcDNA3-6myc-Smad7 deletion mutants (N-, Linker, C), pcDNA3-HA-ubiquitin, and pcDNA3-HA-ubiquitin[Flag(K48R)] and gene reporters pGL3-CAGA 12-luc and pCMV-deletion mutants (N-, Linker, C)-gal. pcDNA3-Flag-Smad7, pcDNA3-6myc-Smad7, pcDNA3-6myc-Smad7, pcDNA3-ALK5(KR)-HA (kinase dead ATP-binding site mutant receptor), pcDNA3-CIN85 was previously described (Mörner et al., 2005); pcDNA3-Tubulin (Tubulin (TU-02) from Santa Cruz Biotechnology, Inc.; rabbit polyclonal anti-human SIK (988, raised against peptide CEEQDTQESLPSSTGRR mapping C-terminally from the UBA domain), anti-PAI-1 and anti-p21 (Cip1/WAF1) from BD Biosciences; anti-HA antibodies were obtained from Sigma-Aldrich; anti-GFP (A11122) from Invitrogen; and AMCA7 (7-amino-4-methylcoumarin-3-acetic acid) from Jackson ImmunoResearch Laboratories.

Materials and methods

Reagents

HaCaT keratinocytes, MDA-MB-468, HEK293T and HepG2 carcinoma, COS1 and Mv1 Lu cells, and culture conditions were as previously described (Kowanetz et al., 2004; Mörner et al., 2005). Recombinant TGF-β1 was obtained from PeproTech. Anti-β-tubulin [T8535] and anti-Flag [M5] antibodies were obtained from Sigma-Aldrich, anti-GFP [A11122] from Invitrogen; anti-PAI-1 and anti-p21 [Cip1/WAF1] from BD Biosciences; anti-HA antibody [Y-11], anti-Tbx21 (V-22), and anti-ubiquitin [TU-02] from Santa Cruz Biotechnology, Inc.; rabbit polyclonal anti-human SIK [988, raised against peptide CEEQDTQESLPSSTGRR mapping C-terminally from the UBA domain], anti-PAI-1 and anti-p21 (Cip1/WAF1) were made in house; rabbit polyclonal anti-c-Myc [9E10] was obtained from I. Dikic (Goethe University, Frankfurt, Germany); secondary antibodies coupled to FITC and TRITC from Dako; Alexa Fluor 546 from Molecular Probes, Inc.; and secondary antibodies coupled to FITC and TRITC from DAKO, Alexa Fluor 546 from DAKO, Roche. Anti-GFP (A11122) from Invitrogen; and AMCA7 (7-amino-4-methylcoumarin-3-acetic acid) from Jackson ImmunoResearch Laboratories.

Human SIK-specific siRNA, ONTARGET plus SMARTpool reagent L-003959-00, and control siRNA against the luciferase reporter vector pGL2 (X65324) were from Thermo Fisher Scientific. An EST clone BG719047 of human SIK complete cDNA was obtained from H. Takemori and M. Oka- moto [National Institute of Biomedical Innovation, Osaka, Japan]. The cDNA was fused to a Flag, 6myc, and GFP creating pcDNA3-Flag-hSIK, pcDNA3-6myc-hSIK, and pEGFP-hSIK. The SIK ATP binding site mutant K56R was created by the Quick-Change mutagenesis kit (Stratagene). Deletion mutants SIK-SJUBA, SIK (K56R)-SJUBA, SIK-ΔC, and SIK-ΔN were created using a PCR-based strategy and were subcloned in pcDNA3-Flag and pEGFP. DNA constructs were sequence verified. Vectors pcDNA3-ALK5[w]-HA [wild-type receptor with C-terminal hemagglutinin tag], pcDNA3-ALK5[CA]-HA [CA 1204D mutant receptor], pcDNA3-ALK5[KR]-HA [kinase dead ATP-binding site mutant receptor], pcDNA3-Smad7, pcDNA3-6myc-Smad7, pcDNA3-6myc-Smad7 deletion mutants (N-, Linker, C), pcDNA3-HA-ubiquitin, and pcDNA3-HA-ubiquitin[Flag(K48R)] and gene reporters pGL3-CAGA 12-gal were previously described (Mörner et al., 2005); pcDNA3-CIN85 was from I. Dikic (Goethe University, Frankfurt, Germany); pcDNA3-Tubulin (Tubulin (TU-02) from Santa Cruz Biotechnology, Inc.; rabbit polyclonal anti-human SIK (988, raised against peptide CEEQDTQESLPSSTGRR mapping C-terminally from the UBA domain), anti-PAI-1, and anti-p21 (Cip1/WAF1) were made in house; rabbit polyclonal anti-c-Myc [9E10] was obtained from I. Dikic (Goethe University, Frankfurt, Germany). Secondary antibodies coupled to FITC and TRITC from DAKO, Alexa Fluor 546 from Invitrogen; and AMCA7 (7-amino-4-methylcoumarin-3-acetic acid) from Jackson ImmunoResearch Laboratories.

A previously published reference or the NCBI database accession number is indicated.

Table I. Human oligonucleotide primers used for RT-PCR

| Gene     | Primer Sequence  | Strand | Product size | Temperature | PCR cycle | Reference       |
|----------|------------------|--------|--------------|-------------|-----------|----------------|
| SIK      | 5'-GTCTGGGCGGCACTTTAGCA-3' | +      | 387 bp       | 56 °C       | 27        | NM_173354      |
|          | 5'-CAGCTTTCGCCACCCCGCTT-3' | -      |              |             |           |                |
|          | 5'-AGGGGCCGACGGTGACACC-3' | +      | 213 bp       | 55 °C       | 24        | Kowanetz et al. (2004) |
|          | 5'-AAGGGCTCTGGACAGATCGT-3' | -      |              |             |           |                |
|          | 5'-CAGGACCACCTCTTGGTCTC-3' | +      | 123 bp       | 57 °C       | 28        | Kowanetz et al. (2004) |
|          | 5'-ATCACTGGCAGGCTCCGGTC-3' | -      |              |             |           |                |
| ID2      | 5'-TCTGGCACTTTGGGCTTTC-3' | +      | 681 bp       | 63 °C       | 23        | Kowanetz et al. (2004) |
|          | 5'-GGGGCTGTGCTGTCGCTG-3' | -      |              |             |           |                |
|          | 5'-GGGATGCTACCTTCCGCC-3' | +      | 533 bp       | 53 °C       | 27        | Kowanetz et al. (2004) |
|          | 5'-GGGTTCGTTGACATTGTCGCT-3' | -      |              |             |           |                |
| CDKN1A   | 5'-TGACAGTCCACCTTCTTCTC-3' | +      | 443 bp       | 57 °C       | 22        | Kowanetz et al. (2004) |
|          | 5'-ATGAGTCCACACCCCTGCCTG-3' | -      |              |             |           |                |

A previously published reference or the NCBI database accession number is indicated.

Table II. Human oligonucleotide primers used for quantitative real-time RT-PCR

| Gene     | Primer Sequence  | Strand | Product size | Temperature | PCR cycle | Reference       |
|----------|------------------|--------|--------------|-------------|-----------|----------------|
| SIK      | 5'-CAACTTGCGTGCGTCCCTCA-3' | +      | 167 bp       | 60 °C       | 24        | NM_173354      |
|          | 5'-GCGGCTTGGCGGCAGTTCACT-3' | -      |              |             |           |                |
|          | 5'-GGGCGCAGCCTGCGGATCTCC-3' | +      | 130 bp       | 60 °C       | 24        | Niimi et al. (2007) |
|          | 5'-AGGGCCCTTAGGTTGCTACA-3' | +      | 123 bp       | 60 °C       | 24        | Niimi et al. (2007) |
|          | 5'-AAACCCATGCCACCTTCC-3' | +      | 101 bp       | 60 °C       | 24        | ENST00000223095 |
|          | 5'-GAGTTCAAGCTGACGGCTTC-3' | +      | 85 bp        | 60 °C       | 24        | ENST00000262158 |
|          | 5'-GGGACAGTGATCTGCTGTA-3' | +      | 78 bp        | 60 °C       | 24        | Niimi et al. (2007) |
|          | 5'-GGCACAAATCCCTTACCTCGAGT-3' | -      |              |             |           |                |
levels after control or SIK-specific knockdown in triplicate (PAI-1, p21, and p15) or quadruplicate (Smad7) determinations.

**Promoter-reporter assays**

HepG2 cells transfected with the Smad3-responsive reporter pGL3-CAGAö12-luc for 36 h before stimulation with TGF-β1 for 6 h were analyzed with the enhanced luciferase assay kit (BD Biosciences). Normalized promoter activity is plotted as mean values from triplicate determinations with standard deviations.

**Immunoblotting, immunoprecipitation, and ubiquitination assays**

SDS-PAGE, immunoblot, coimmunoprecipitation, pulldown, and ubiquitination analysis was as previously described (Kowanetz et al., 2004; Morén et al., 2005). For efficient detection of endogenous SIK, HaCaT cells were pretreated with 50 μM MG132 (EMD) for 6 h at 37°C. For pulse-chase analysis, a 30 min pulse with 35S-methionine/cysteine was followed by a chase, as indicated in the Figures and as previously described (Morén et al., 2005). Cells treated with proteasomal inhibitor, calpain inhibitor I (N-Acetyl-L-leucyl-leucyl-norleucinal, LNL, Roche) or lysosomal inhibitor chloroquine (Sigma-Aldrich) were also analyzed. To measure association between SIK and ubiquitinated proteins, HEK293T cells were transfected with Flag-SIK in one plate and HA-ubiquitin in another and treated with proteasomal inhibitors, MG132, or LNL. Individual cell lysates were combined before immunoprecipitation.

**Confocal microscopy**

Approximately 70% confluent transfected MvI1Lu monolayers were analyzed by immunofluorescence 24 h after transfection as previously described (Kowanetz et al., 2004). Nuclei were counterstained with DAPI or propidium iodide. A confocal microscope [Axiovert 200 M; Carl Zeiss, Inc.] equipped with LSM 510 laser was used with the 63×/0.75 NA objective lens (Carl Zeiss, Inc.) and photographing at ambient temperature in the presence of immersion oil. Images acquired with a charge-coupled device camera [C4742-95; Hamamatsu Photonics] and the acquisition software QED Camera Plug-in v.1.1.6 (QED Imaging Inc.) were reduced in memory content using Photoshop 6.0 (Adobe), without additional processing.

**Receptor affinity cross-linking**

MvI1Lu cells incubated on ice with iodinated TGF-β1 were cross-linked, as previously described (Moustakas et al., 1993), and shifted to 37°C, as indicated in the figure, before SDS-PAGE and autoradiography.

**Online supplemental material**

Fig. S1 shows endogenous SIK protein levels in response to TGFβ1 and the lack of effect of SIK expression on GFP or Smad7 levels. Fig. S2 shows interaction of SIK with ubiquitinated proteins and interaction between SIK and AIK5 in presence of ubiquitin. Fig. S3 shows mRNA profiles of Smad7, p21, c-myc, and Id2 during a time course in response to TGFβ1 in control cells or after SIK knockdown. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200804107/DC1.

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