Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial–mesenchymal transition

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We report that the activity of glycogen synthase kinase-3 (GSK-3) is necessary for the maintenance of the epithelial architecture. Pharmacological inhibition of its activity or reducing its expression using small interfering RNAs in normal breast and skin epithelial cells results in a reduction of E-cadherin expression and a more mesenchymal morphology, both of which are features associated with an epithelial–mesenchymal transition (EMT). Importantly, GSK-3 inhibition also stimulates the transcription of Snail, a repressor of E-cadherin and an inducer of the EMT. We identify NFκB as a transcription factor inhibited by GSK-3 in epithelial cells that is relevant for Snail expression. These findings indicate that epithelial cells must sustain activation of a specific kinase to impede a mesenchymal transition.

Introduction

The distinct architecture of epithelia is derived from the interactions of epithelial cells with each other, with the underlying basement membrane, and, less directly, with mesenchymal cells in the stroma. Within this complex set of interactions, a prime determinant of epithelial structure is E-cadherin, a transmembrane protein that mediates Ca^{++}-dependent, homophilic intercellular adhesion (Thiery, 2002; Nelson and Nusse, 2004). The central role of E-cadherin in epithelia is evidenced by the fact that loss of either its expression or function results in the dissolution of the epithelial architecture and the acquisition of a mesenchymal phenotype. This process, referred to as the epithelial–mesenchymal transition (EMT), occurs in the contexts of development and tumor progression (Thiery, 2002).

The central importance of E-cadherin for epithelial architecture leads to the novel hypothesis that epithelial cells may support signaling pathways that preserve E-cadherin expression as a means of preventing an EMT. In this connection, we were intrigued by reports that glycogen synthase kinase-3 (GSK-3), a ubiquitously expressed protein serine kinase, is active in resting epithelial cells (Papkoff and Aikawa, 1998; Murray et al., 1999), but that its function in epithelial biology had not been defined. Our assessment of GSK-3 function in epithelial cells revealed that its activity is essential for maintaining epithelial structure because it maintains the expression of E-cadherin. Inhibition of GSK-3 activity or expression results in a bona fide EMT. Moreover, we report that one mechanism by which GSK-3 maintains E-cadherin expression is by inhibiting the transcription of Snail, a zinc finger transcriptional repressor of E-cadherin that is absent in epithelial cells but expressed in tumors (Batlle et al., 2000; Cano et al., 2000; Blanco et al., 2002).

Results and discussion

Initially, we assessed the effects of inhibiting GSK-3 activity in normal breast epithelial cells (MCF10A) using SB415286, a highly specific, small molecule inhibitor of GSK-3 (Coghlan et al., 2000). The ability of SB415286 to inhibit GSK-3 activity was evidenced by the increased levels of cyclin D1, a protein subject to GSK-3–dependent proteolysis (Diehl et al., 1998), in SB415286-treated MCF10A cells, relative to those treated with DMSO (Fig. 1 A). Inhibiting GSK-3 activity also disrupted the epithelial morphology of these cells, as evidenced by the loss of cell–cell contacts (Fig. 1 A).

Loss of E-cadherin and expression of mesenchymal proteins are defining steps in the EMT. Based on our observation that inhibition of GSK-3 activity reduced cell–cell contacts in MCF10A epithelial cells, we hypothesized that GSK-3 may be a regulator of E-cadherin expression and an inhibitor of the EMT. Supporting these hypotheses, the treatment of MCF10A

Figure 1. **GSK-3 maintains the epithelial phenotype.** (A) MCF10A cells were incubated with either DMSO or 25 μM SB415286 (Biosource International) in 0.5% FBS-containing medium. Cell morphology was assessed after 72 h by phase contrast microscopy. Expression of Cyclin D1, Stat-1, E-cadherin, and vimentin was assessed by immunoblotting. (B) HaCaT cells were incubated with DMSO or SB415286, and E-cadherin, Stat-1, and cyclin D1 expression was assessed as indicated in A. Similar results were obtained in four independent experiments. (C) E-cadherin and GAPDH mRNA levels were determined after 48 h of drug treatment by RT-PCR. (D) MCF10A cells were cotransfected with GSK-3α– and GSK-3β–specific inhibitory RNA pools (α+β siRNA) or a nonspecific control pool (Scr). RNA was extracted from these cells 48 h after transfection, and E-cadherin and GAPDH mRNA levels were determined by RT-PCR. GSK-3 and Stat-1 expression was assessed by immunoblotting.
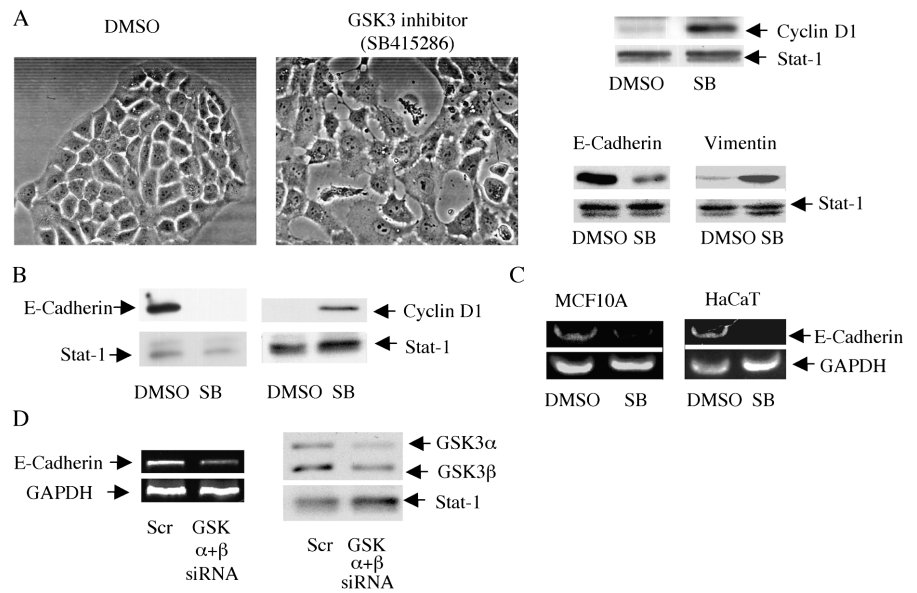
cells with SB415286 reduced the expression of total cellular E-cadherin protein significantly and induced the expression of the mesenchymal protein vimentin (Fig. 1 A) without influencing cell viability (not depicted). Together, these data indicate that epithelial cells in which GSK-3 activity has been inhibited manifest changes characteristic of an EMT.

The effects of GSK-3 inhibition can be generalized to other epithelial cells, as demonstrated by the significantly reduced levels of E-cadherin protein in HaCaT skin cells that had been incubated with the GSK-3 inhibitor (Fig. 1 B). This treatment also increased cyclin D1 expression, indicating the efficacy of SB415286 in HaCaT cells (Fig. 1 B). GSK-3 inhibition also reduced the levels of E-cadherin mRNA in GSK-3 inhibitor–treated HaCaT and MCF10A cells, relative to controls (Fig. 1 C). We implemented a small interfering RNA (siRNA) strategy to reduce expression of both the α and β isoforms of GSK-3 in MCF10A cells. Cells were transfected with a pool of eight siRNAs, targeting unique regions of GSK-3α and GSK-3β genes, or with a pool of nonspecific sequences (Fig. 1 D, Scr). Relative to the control pool, the GSK-3α and GSK-3β siRNA pool significantly decreased expression of GSK-3α and GSK-3β proteins (Fig. 1 D). Similar to the effect of the GSK-3 inhibitor, GSK-3–specific siRNAs markedly reduced E-cadherin mRNA (Fig. 1 D).

We next sought to determine the mechanism by which GSK-3 regulates E-cadherin expression. Snail, a member of the zinc finger family of transcriptional repressors, is an established suppressor of E-cadherin transcription, and its activity is an important determinant of the EMT, in the contexts of both mesoderm development (Carver et al., 2001) and tumor progression (Batlle et al., 2000; Cano et al., 2000). Snail is absent in epithelial cells but expressed in tumors, and its expression has been shown to correlate inversely with tumor grade (Blanco et al., 2002). We investigated whether GSK-3 regulates E-cadherin expression by repressing Snail expression. Inhibition of GSK-3 activity in either MCF10A or HaCaT cells significantly increased Snail mRNA levels (Fig. 2 A).

Snail mRNA levels were also elevated in epithelial cells transfected with GSK-3–specific siRNAs (Fig. 2 A). As evidence that GSK-3 inhibition alters Snail transcription, we observed increased activity of a Snail promoter–driven reporter gene in HaCaT cells treated with SB415286, relative to that measured in DMSO-treated cells (Fig. 2 B). Increased levels of Snail protein were also detected in cells treated with the GSK-3 inhibitor (Fig. 2 C).

Because of our recent finding that NFκB drives Snail expression (Barbera et al., 2004), we explored whether GSK-3 inhibits Snail transcription by repressing NFκB activity. Indeed, inhibition of GSK-3 activity in HaCaT cells stimulated NFκB-dependent reporter gene expression (Fig. 3 A). In addition, we observed significantly decreased levels of IκB, an inhibitor of NFκB, in SB415286-treated MCF10A cells (Fig. 3 B). Finally, an NFκB inhibitor (SN50) suppressed the ability of SB415286 to induce Snail expression (Fig. 3 C). Together, these data indicate that the NFκB pathway is inhibited by GSK-3 in epithelial cells, which results in the silencing of Snail expression.

The data presented here indicate that GSK-3, a kinase that is active in resting epithelial cells (Papkoff and Aikawa, 1998; Murray et al., 1999), is a critical determinant of epithelial structure and a suppressor of the EMT. This finding implies that epithelial cells must sustain activation of a specific kinase to prevent an EMT, a mechanism distinct from that suggested by previous studies, which have shown that activation of kinases such as Akt (Grille et al., 2003) and ILK (Oloumi et al., 2004) can promote an EMT. An important implication of our findings is that endogenous suppressors of GSK-3, such as Wnt and PI3-kinase, which are frequently activated in carcinoma cells (Woodgett, 2001), may also inhibit E-cadherin transcription and promote an EMT. Our observation that inhibition of both GSK-3α and GSK-3β isoforms increased Snail expression in epithelial cells (Fig. 2 A), whereas inhibition of either isoform alone had no effect on Snail levels (not depicted), indicates that these isoforms may serve redundant functions in maintaining epithelial architecture. Thus, we hypothesize that
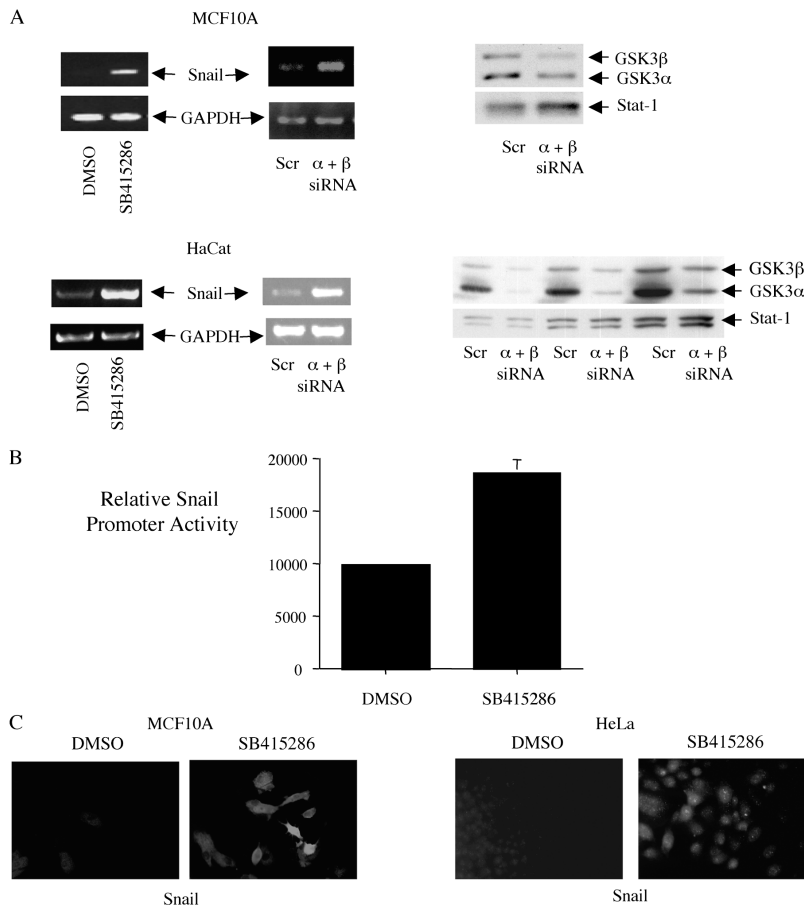
A

MCF10A

Snail→

GAPDH→

DMSO SB415286

Scr α + β
siRNA

← GSK3β
← GSK3α

← Stat-1

Scr α + β
siRNA

HaCat

Snail→

GAPDH→

DMSO SB415286

Scr α + β
siRNA

← GSK3β
← GSK3α

← Stat-1

Scr α + β Scr α + β Scr α + β
siRNA   siRNA   siRNA

B

Relative Snail
Promoter Activity

20000

15000

10000

5000

0

DMSO   SB415286

C

MCF10A
DMSO   SB415286

Snail

HeLa
DMSO   SB415286

Snail

Figure 2. **GSK-3 inhibits Snail transcription in epithelial cells.** (A) MCF10A and HaCaT cells were either incubated with 25 μM of the GSK-3 inhibitor SB415286 or with DMSO in 0.5% FBS-containing medium. Alternatively, these cells were transfected with GSK-3α– and GSK-3β–specific inhibitory RNA pools (α+β siRNA) or a nonspecific control pool (Scr). Snail and GAPDH mRNA levels were assessed by reverse transcription, followed by PCR. GSK-3 and Stat-1 expression was assessed by immunoblotting. (B) HaCaT cells were cotransfected with a Snail promoter–driven and a control β-galactosidase reporter construct. These transfectants were then incubated with DMSO or SB415286, as described in A, for 24 h. Data are reported as relative luciferase activity (normalized to β-galactosidase activity), ± SD, and are representative of three independent experiments. (C) MCF10A and HeLa cells were treated with SB415286 as described in A, and Snail expression was assessed by immunofluorescence.

GSK-3α and GSK-3β isoforms serve redundant functions in epithelial cells, and that mice deficient for both GSK-3α and GSK-3β expression will exhibit prominent defects in epithelial structure and function.

The identification of NFκB as a novel target of GSK-3 activity that is relevant to the EMT is of considerable interest in light of a recent report that established NFκB as a central mediator of the EMT (Huber et al., 2004). However, this study did not define transcriptional targets of NFκB that are important for the EMT. Clearly, Snail is a prime candidate for such an NFκB target.

Although we identify NFκB and Snail as novel targets of GSK-3 activity that are relevant to the EMT, it is likely that multiple GSK-3 substrates participate in the regulation of epithelial structure. Upon its phosphorylation by GSK-3, β-catenin, a protein that has been implicated in mesenchymal transitions (Kim et al., 2002; Liebner et al., 2004), is degraded by the proteosome pathway, resulting in its inability to stimulate TCF/LEF transcription factors (Beals et al., 1997). TCF/LEF can induce the expression of genes that influence the EMT, such as vimentin (Gilles et al., 2003), the levels of which are elevated in epithelial cells treated with the GSK-3 inhibitor (Fig. 1 A). Thus, multiple signaling pathways that control the EMT are likely to be regulated by GSK-3, substantiating our hypothesis that this kinase is a central regulator of epithelial structure and function.

It is important to mention that Snail was recently identified as a direct target of GSK-3 kinase activity (Zhou et al.,

2004), and the two GSK-3 phosphorylation sites identified were shown to influence Snail protein stability and localization in tumor cells. In contrast, the present study indicates that, in epithelial cells, GSK-3 impedes Snail transcription. The data presented here provide one explanation for why epithelial cells, which are characterized by high basal GSK-3 activity (Papkoff and Aikawa, 1998; Murray et al., 1999), express negligible levels of Snail mRNA (Fig. 2; Domínguez et al., 2003; Peinado et al., 2003). The combined abilities of GSK-3 to block Snail transcription, promote Snail degradation, and prevent Snail nuclear localization indicate that this kinase plays a central role in regulating Snail expression. Future studies addressing the importance of posttranslational control of Snail by GSK-3 in epithelial cells will be crucial for establishing the relevance of this mode of regulation for the EMT.

GSK-3 is a central target for the development of therapeutics because it has been implicated in numerous pathologies, including diabetes and neurologic disorders (Woodgett, 2001). Our findings suggest that this kinase is active in epithelial cells for a very important reason, namely to prevent the acquisition of a mesenchymal phenotype. Because reduced E-cadherin expression has been reported to be characteristic of some malignant tumors (Bringuier et al., 1993; Hirohashi, 1998; Kowalski et al., 2003), our findings indicate that inhibition of GSK-3, an enzyme that maintains E-cadherin expression, will not prove to be a reasonable therapeutic strategy because it has the potential to alter epithelial function significantly.
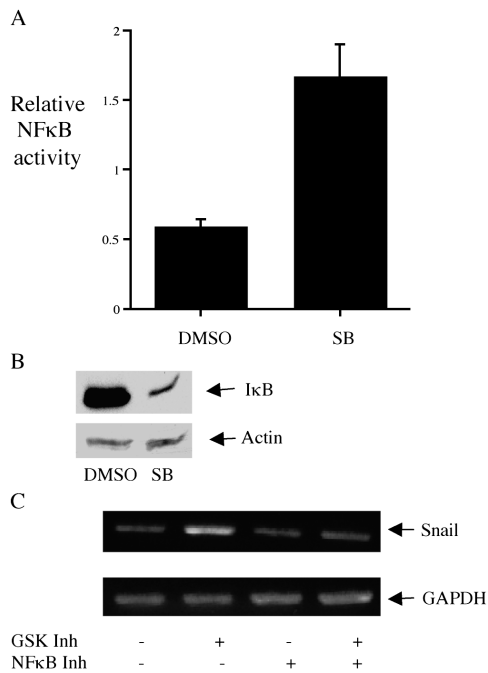
A

Relative
NFκB
activity

2
1.5
1
0.5
0

DMSO   SB

B

← IκB

← Actin

DMSO SB

C

← Snail

← GAPDH

| GSK Inh | - | + | - | + |
| NFκB Inh | - | - | + | + |

Figure 3. **GSK-3 inhibits NFκB, an activator of Snail transcription.**
(A) HaCaT cells were transfected transiently with a luciferase construct driven by NFκB binding sites in addition to a control renilla luciferase reporter gene. These transfectants were then incubated with DMSO or SB415286, as described for Fig. 2, for 15 h. Data are reported as relative luciferase activity (normalized to renilla luciferase activity), ± SD, and are representative of two independent experiments. (B) MCF10A cells were incubated with DMSO or SB415286 as described for Fig. 2. Equivalent amounts of total cellular protein were extracted from these cells, subjected to SDS-PAGE, and immunoblotted with an IκB- or actin-specific antibody. Results are representative of those obtained in two independent experiments. (C) MCF10A cells were incubated for 2 h with an NFκB-specific inhibitor (SN50; Calbiochem) or control peptide (SN50M; Calbiochem). These cells were then incubated with DMSO or 25 μM SB415286. RNA was isolated after 15 h, and the levels of Snail and GAPDH were determined by RT-PCR. Similar results were obtained in three independent experiments.

Materials and methods

Cells
HaCat and HeLa cells were provided by A. Toker (Beth Israel Deaconess Medical Center, Boston, MA). MCF10A cells were provided by J. Brugge (Harvard Medical School, Boston, MA).

Analysis of protein expression
Cells were extracted in RIPA buffer. Equivalent amounts of total cellular protein extracted from these cells were subjected to SDS-PAGE and transferred to nitrocellulose. The antibodies used for immunoblotting were as follows: GSK-3 monoclonal antibody (Upstate Biotechnology), rabbit anti–cyclin D1 (Santa Cruz Biotechnology, Inc.), rabbit anti–Stat-1 (Santa Cruz Biotechnology, Inc.), mouse anti–E-cadherin (Santa Cruz Biotechnology, Inc.), rabbit antivimentin (Santa Cruz Biotechnology, Inc.), mouse anti—IκB α (Cell Signaling), and HRP-conjugated secondary antibodies (Pierce Chemical Co.).

Luciferase assays
HaCaT cells were cotransfected transiently using Lipofectin (Life Technologies) with a Snail promoter luciferase reporter construct (composed of human snail promoter sequences −869 to 59) and a control β-galactosidase reporter construct (pCS2-(n)-βgal; Promega). For NFκB studies, HaCaT cells were transfected with a luciferase construct controlled by sequential NFκB binding sites (pNF-κB-Luc; CLONTECH Laboratories, Inc.) and a control renilla luciferase construct (pRL-TK renilla luciferase; Promega). After incubation for 12 h, cells were treated with the indicated drugs (Fig. 3

A) for an additional 15 h. Luciferase and β-galactosidase activities were measured according to the manufacturer's instructions (Promega) using a luminometer and a UV/Vis spectrophotometer, respectively. Data are reported (Fig. 3 A) as the mean (± SD) relative promoter activity obtained from triplicate wells.

Analysis of mRNA expression
mRNA was purified from the indicated cell lines using the RNEasy kit (QIAGEN) according to the manufacturer's recommended protocol. 2 μg RNA was added to RT-PCR reactions containing the indicated primers at a concentration of 0.6 μM. After a 55°C/30-min reverse transcription step, Snail mRNA was PCR amplified in 32 cycles for 1 min at each of the following temperatures: 94°, 62°, and 72°C. Likewise, E-cadherin mRNA was PCR amplified in 25 cycles for 1 min at each of the following temperatures: 94°, 55°, and 72°C. PCR products were analyzed on 1% agarose gels. The sequences of amplification primers were as follows: Snail forward, GGGCAGGTATGGAGAGGAAGA; Snail reverse, TTCTTCTG-CGCTACTGCTGCG; E-cadherin forward, CAGCACGTACACAGCCC-TAA; E-cadherin reverse, GCTGGCTCAAGTCAAAGTCC; GAPDH forward, CCTGGCCAAGGTCATCCATGAC; and GAPDH reverse, CAT-GTAGGCCATGAGGTCCACCAC.

siRNA experiments
GSK-3α, GSK-3β, and control siRNA pools were designed and synthesized by Upstate Biotechnology. Cells at 60% confluence were transfected in penicillin/streptomycin-free medium with 20 μM of the indicated siRNA using TKO lipid (Mirus) according to the manufacturer's recommended protocol. Cells were harvested 48 h after transfection.

Immunofluorescence
Cells were fixed in 2% PFA, permeabilized in 0.2% Triton X-100 and 1 mM EGTA, blocked for 30 min in 1% albumin/5% donkey serum, and incubated overnight at 4°C with a Snail-specific (Domínguez et al., 2003) or control rabbit serum in blocking buffer (at a 1:20 dilution). The cells were then incubated with a fluorescein-conjugated donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories) in blocking buffer (at a 1:150 dilution) for 30 min. Snail expression was analyzed using an inverted fluorescent microscope (Diaphot 300; Nikon).

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