Importin \(\alpha\) Protein Acts as a Negative Regulator for Snail Protein Nuclear Import

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Snail, a zinc finger-containing transcriptional regulator, migrates into the nucleus where it controls gene expression. We demonstrated previously that importin \(\beta1\) directly recognizes the zinc finger domain of Snail and transports it into the nucleus. Here, using in vitro and in vivo assays, we show that importin \(\alpha\), an adaptor protein for importin \(\beta1\), negatively regulates the nuclear import of Snail mediated by importin \(\beta1\). In vitro binding assays indicated that importin \(\alpha\) interacted with the zinc finger domain of Snail to compete with the binding of importin \(\beta1\) and that Snail did not form a ternary complex with importin \(\alpha\)/importin \(\beta1\). Overexpression of importin \(\alpha\) in A549 cells reduced the endogenous Snail protein level, which was restored by inhibitors of the proteasome and glycogen synthase kinase 3\(\beta\). Furthermore, knockdown of importin \(\alpha\) by siRNA treatment increased the endogenous Snail protein level in several cancer cell lines. This study provides a novel regulatory mechanism of the nuclear protein import process by importin \(\alpha\) and gives an implication to control Snail activity by inhibiting its nuclear localization.

The epithelial mesenchymal transition (EMT) functions as a developmental switch that changes the morphology and behavior of epithelial cells to the highly motile mesenchymal phenotype. The hallmark of EMT is a decrease in E-cadherin expression (1). E-cadherin is a cell-cell adhesion molecule that forms stable epithelial adherent junctions and maintains the epithelial phenotype. Down-regulation of E-cadherin is mediated by several transcription factors such as Snail, Slug, ZEB, or Twist which repress E-cadherin transcription via interaction with the E-cadherin promoter (2). The loss of epithelial characteristics and the acquisition of migratory properties in EMT have been proposed to be the initial step of tumor metastasis. Indeed, the E-cadherin expression level is often inversely correlated to tumor grade (3). Although the loss of E-cadherin is one of the important markers of EMT, it is noteworthy that additional genes should be regulated to diminish the epithelial characteristics and to provoke the mesenchymal transition (4).

Snail is one of the prominent transcriptional regulators that contribute to EMT by suppressing E-cadherin expression (5, 6) and participates in EMT concerning the mesoderm and neural crest formation during embryonic development. In fact, Snail knockout mice represent early embryonic lethality (7). Emerging evidence indicates that Snail is also a key factor in the tumor progression and metastasis in melanoma, bladder, colorectal, and pancreatic carcinomas (5, 6, 8, 9). In these tumors, the down-regulation of E-cadherin was observed to correlate with the high expression of Snail. Signaling pathways such as TGF\(\beta\) and \(Wnt\) regulate Snail gene expression at the transcriptional level (10).

On the other hand, it has been shown that Snail is a fragile protein and that phosphorylation of Snail is critical for its post-translational regulation, which induces the degradation of the protein by the ubiquitin-proteasome system (11). In addition, the subcellular localization of Snail is regulated by serine phosphorylation, which induces its nuclear export by exportin1 (XPO1/CRM1) (12). Glycogen synthase kinase 3\(\beta\) and p21-activated kinase (PAK1) have been identified as kinases for Snail (11, 13). It has been shown that serine phosphorylation induces Snail localization in the cytoplasm, where it is rapidly degraded by the proteasome pathway (11, 14), whereas overexpression of small C-terminal domain phosphatase dephosphorylates Snail to enhance its activity (15). Furthermore, it has been reported that loss of the zinc transporter LIV1 causes the cytoplasmic localization of Snail in zebrafish (16). Taken together, the control of the nucleocytoplasmic localization of Snail is crucial for its activity.

The nuclear envelope restricts nuclear access of cellular molecules. Although the nuclear pore complex present in the nuclear envelope allows small molecules (40–60 kDa) to diffuse passively, an active transport system is required for large macromolecules to cross. Specific transport factors called importins and exportins facilitate translocation of macromolecules through the nuclear pore complex. Importin \(\beta\) family members play a central role in nucleocytoplasmic protein transport and recognize a broad class of nuclear localization signals (NLS) directly or indirectly to transport macromolecules into the nucleus through the nuclear pore complex via transient interactions with phenylalanine-glycine (FG) repeat-containing nucleoporins (17, 18). For classical basic-type NLS-containing proteins, importin \(\beta\) uses importin \(\alpha\) family members as NLS receptors and forms a ternary complex, cargo/
importin α/importin β1, to translocate through the nuclear pore complex into the nucleus. After the translocation, the import complex is dissociated in the nucleus by the binding of GTP-bound form of Ran (RanGTP) to importin β family members (19). Then, importins are exported from the nucleus to the cytoplasm for the next round of transport.

We have demonstrated previously that importin β1 mediates the nuclear import of Snail through direct binding with its zinc finger domains (20). On the other hand, it has recently been shown that other importin β family members, importin 7 and transportin, are also involved in the nuclear transport of Snail (21). Although it has been reported that the nuclear export of Snail is controlled in a phosphorylation-dependent manner, the regulation of its nuclear import remains to be elucidated.

In this study, we show that importin α, an adaptor protein between karyophilic proteins and importin β1, functions as an inhibitor of the nuclear import of Snail, indicating a novel regulatory mechanism of nuclear protein transport by importin α. Furthermore, we demonstrated that the suppression of Snail nuclear accumulation by importin α leads to a decrease in its cellular protein level through degradation by the proteasome system. This has implications for the prevention of tumor cell invasion by inhibiting Snail nuclear localization.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) (for HeLa, A172, A549, and A2058), L-15 medium (Gibco) (for MDA-MB 231, MDA-MB 435, SW480, and SW620) or RPMI 1640 (Gibco) (for DLD1) supplemented with 10% fetal bovine serum. For microinjection, HeLa, SW480, and SW620 (for DLD1) or RPMI 1640 (Gibco) (for MDA-MB 231, MDA-MB 435, L-15 medium (Gibco) (for MDA-MB 231, MDA-MB 435, SW480, and SW620) were seeded on the day before injection at 1.5×10⁵ cells/100-mm dishes for 24–48 h before the assay.

Antibodies—Antibodies used in this study were anti-Snail (Cell Signaling Technology), anti-GST (Santa Cruz Biotechnology), anti-GAPDH (Zymed Laboratories, Inc.), anti-GFP (Invitrogen), anti-importin α1 (Santa Cruz Biotechnology), anti-importin α5 (Abnova), anti-E-cadherin, anti-importin β1, and anti-Ran (BD Biosciences).

Plasmids and Recombinant Proteins—cDNA of full-length human Snail was inserted into pGEX-GFP (22) to produce the GST-Snail-GFP fusion protein in bacteria. The Escherichia coli BL21 (DE3) strain containing the Snail expression vector was cultured at 18 °C for 16–20 h in the presence of 0.1 mM IPTG, BL21 (DE3) strain containing the Snail expression vector was cultured at 18 °C for 16–20 h in the presence of 0.1 mM IPTG. Then, GST-Snail-GFP fusion protein in bacteria. The anti-Ran (BD Biosciences).

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Snail protein was retained in the cytoplasm when importin α was coinjected (Fig. 1C), although nuclear import of GST-SV40 NLS-GFP, a well known classical NLS-containing control substrate, was not affected by the coinjection of importin α (Fig. 1C). These results indicate that importin α can act as an inhibitor of the nuclear import of Snail.

Importin α Competes with the Binding of Importin β1 to Snail—To clarify the inhibitory effect of importin α on Snail nuclear import, first, the importin α-binding region of Snail was determined using full-length and three truncated mutants of Snail. As shown in Fig. 2A, the Snail C-terminal fragment (153–264), which contains four zinc finger domains, was directly recognized by importin α5. Previous studies showed that the zinc finger domains of Snail act as an NLS and that several importin β family members directly recognize this same domain to translocate the cargo protein (25, 26). Thus, we tested whether the importin α/β1 heterodimer forms a complex with Snail to suppress its nuclear import. When the preformed importin α5-β1 complex was incubated with increasing amounts of Snail, the complex dissociated and the importin α5-Snail complex was formed (Fig. 2C). This indicates that importin α disrupts the Snail-importin β1 transport complex and does not form a ternary complex comprising importin α-β1-Snail.

The importin α family consists of two functional domains, a short N-terminal importin β binding (IBB) domain and a large NLS binding domain comprising 10 Armadillo (Arm) repeats. Because the Snail binding region in importin α would overlap with the importin β binding site, the Snail binding region of importin α was determined using various deletion mutants of importin α5. Interestingly, Snail bound to a broad region of importin α5, including the IBB domain (Fig. 3A). To address the effects of the IBB domain of importin α on Snail nuclear localization, in vitro transport assays using importin α mutants were performed. SREBP2 is a well characterized substrate for the importin β1-dependent nuclear import pathway (27, 28). Although importin α does not associate directly with SREBP2, importin α disrupts the SREBP2-importin β1 complex by binding to importin β1 through the IBB domain of importin α (27). As shown in Fig. 3B, an importin α5 ΔIBB mutant suppressed the nuclear import of Snail, whereas it failed to inhibit SREBP2 nuclear accumulation. On the other hand, maltose binding protein (MBP)-IBB significantly inhibited the nuclear accumulation of both Snail and SREBP2 (Fig. 3B), consistent with the result that Snail bound to broad regions of importin α5 (Fig. 3A). However, the Arm repeat region showed the most efficient binding activity (Fig. 3A). Thus, the Snail nuclear import is inhibited in part by the IBB domain but mainly by Arm repeats.
in importin α, indicating that the inhibitory effect of importin α on Snail nuclear import is distinct from that on SREBP2 import.

Overexpression of Importin α Affects Snail Protein Levels—To confirm that importin α actually inhibits the nuclear import of endogenous Snail in living cells, we overexpressed importin α5 and detected the subcellular localization of endogenous Snail. When A549 cells were transfected with GFP-importin α5, the fluorescence intensity of Snail was greatly decreased compared with that of GFP-expressing control cells (Fig. 4A). It has been reported that Snail is a fragile protein that is rapidly degraded by the proteasome system (11). In A549 cells, we found that the half-life of endogenous Snail was ~50 min and that treatment with MG132 and LiCl, a proteasome inhibitor and a GSK3β inhibitor, respectively, effectively stabilized the Snail protein (supplemental Fig. S1). These results raised the idea that inhibition of Snail nuclear import by importin α enhances its degradation in the cytoplasm by the proteasome.

To test this, we treated transfected cells with MG132 and LiCl to prevent the degradation of Snail. We found that inhibition of proteasome function increased the nuclear fluorescence intensity of Snail (Fig. 4A). These results suggest that importin α can negatively regulate Snail nuclear import to facilitate its degradation in the cytoplasm, whereas a proportion of the Snail proteins escape from the importin α-mediated suppression of nuclear import and degradation by the proteasome to enter the nucleus.

Next, we concentrated the transfected cells and analyzed protein levels. Consistent with the results in Fig. 4A, although Snail protein levels were decreased in GFP-importin α5 expressing cells, MG132 and LiCl treatment restored them (Fig. 4B, a). Furthermore, we confirmed that the mRNA level of Snail was not affected under these conditions (Fig. 4B, b) and that the protein levels of importin β1 and Ran were not altered by GFP-importin α5 overexpression (B, a). From these findings, we conclude that the function of Snail is negatively regulated by importin α-mediated suppression of its nuclear import as well as by proteasome-dependent degradation in living cells.
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Knockdown of Importin α Increased Snail Protein Levels—To address the biological significance of the negative regulation of Snail nuclear import by importin α, we analyzed the protein expression level in several human cancer cell lines. As shown in Fig. 5A, low Snail expression correlated with a high E-cadherin protein level in DLD1, SW480, and SW620 cells; however, no correlation between importin α and the Snail protein level was found. MG132 and LiCl treatment increased the Snail protein level in HeLa and SW480 cells (Fig. 5B), indicating that Snail stability was regulated by the proteasome system in these cells. It is likely that decreased importin α expression would stabilize the Snail protein. We introduced a siRNA duplex into these cells to knock down importin α1 or importin α5. Fig. 5C indicated that knockdown of importin α1 increased the Snail protein in HeLa and SW480 cells and decreased E-cadherin expression in SW480 cells. Knockdown of importin α5 showed a weaker effect than that of importin α1 on the Snail protein level (Fig. 5C). These results suggested that the protein level of importin α affects Snail protein stability, and, thus, importin α controls both Snail nuclear import and its stability in living cells.

DISCUSSION

Proper subcellular localization of proteins is essential for their function. Snail is a transcriptional regulator and should be transported into the nucleus to function. In this study, we demonstrated the inhibitory effects of importin α on Snail nuclear import in vitro and in vivo. Our findings have two major implications for the regulatory mechanisms of nuclear protein transport and for Snail activity in EMT and tumor metastasis.

Importin α is an adaptor for importin β1 in the protein nuclear import pathway. In addition to its adaptor function, it directly recognizes and transports CAMKIV (29) and HIV1 Vpr (30) into the nucleus without using importin β1. In contrast, it has been shown that importin α sometimes inhibits the nuclear import of proteins. For example, PThRP and TRF1 form a complex with importin αβ1, but this ternary complex is not active and might bind to cytoplasmic components, leading to their cytoplasmic retention (23, 24). The formation of similar non-active complexes containing importin α was reported in importin 7/β1-mediated histone H1 nuclear transport (31). Furthermore, although the effects of importin α on nuclear import are not clear, the zinc finger-(like) proteins EKLF (32) and DMRT1 (33) were recognized by both importin α and importin β. Thus, the inhibition of Snail nuclear import may be one of the characteristic features of importin α. This implies that importin α-mediated control of nuclear protein import might be more common than expected, although its biological significance is still unclear in most cases.

Importin αβ1-mediated nuclear import is thought to be widely used in cells. For most cargo proteins containing the classical NLS, the Arm repeat region of importin α recognizes their NLS, and the IBB domain of importin α associates with the C-terminal half of importin β1 to generate a ternary complex. Why does Snail-bound importin α fail to associate with importin β1 to form a ternary complex? Using importin α5 deletion mutants, we found that broad regions of importin α5 associated with Snail but that the Arm repeat was predominant in the recognition of Snail (Fig. 3A), consistent with a previous report showing that the zinc finger-type transcription factor, SP1, has a similar binding property (34). Furthermore, the N-terminal half of importin β1, which contains the Ran binding domain, was mapped as the Snail-binding region (supplemental Fig. S2). Mingot et al. (21) identified that the basic residues are important for Snail nuclear localization and that these residues are distributed among the three zinc finger domains rather than in a single cluster. The characteristic binding of Snail to importin α and importin β1 may be due to such non-consensus NLS of Snail, and the ability of importin α to bind to Snail across a broad region may interfere spatially with ternary complex formation. Crystallographic studies of the Snail/importin complexes would provide answers as to why importin α disrupts the Snail-importin β1 complex and why Snail does not form a ternary complex with importin αβ1.

A recent study revealed that, in addition to importin β1, additional import factors, such as importin 7 and transportin, contribute to the nuclear import of Snail and that both import...
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factors recognized the zinc finger domain of Snail (21), suggesting that importin α may inhibit their transport pathway. However, this study also showed that the importin α/β1 system worked on the nuclear accumulation of Snail (21). This discrepancy with our data may arise because of the different constructs used: GST-Snail-GFP in our study and GST-GFP-Snail in that of Mingot et al. (21). To test this, we prepared GST-GFP-Snail and used it in our experimental conditions. We found that GST-GFP-Snail also associated directly with the importin α or β1 in vitro and that, similar to GST-Snail-GFP, its nuclear import was inhibited by the presence of importin α (supplemental Fig. S3). Further studies will be required to resolve this difference.

The EMT-related genes are thought to be critical for tumor metastasis. Recent studies have indicated that high levels of Snail expression are closely correlated with low E-cadherin expression in several tumors (5, 6, 8). Consistent with these studies, knockdown of Snail affects tumor growth and invasive-metastasis (35). However, E-cadherin and Snail mRNA have been detected in several types of tumors (12, 36). It is likely that highly expressed importin α would suppress nuclear import of Snail to facilitate its degradation, resulting in the down-regulation of Snail function and high expression of E-cadherin without a decrease in Snail mRNA levels. Consistently, Snail mRNA levels were not affected in importin α-overexpressing cells (Fig. 4B, b).

Furthermore, the expression level of importin α affected the Snail protein level in living cells (Fig. 5C). Although the sensitivity to importin α might vary with tumor cells, some cells would be affected by importin α, resulting in low Snail protein and high E-cadherin expression. Thus, it is possible that importin α contributes to a poor metastatic potential of tumors that express high levels of Snail mRNA. Collectively, elucidating the precise mechanism of inhibition of Snail nuclear import by importin α will suggest novel approaches for the suppression of tumor metastasis.

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