ARHI (DIRAS3), an imprinted tumour suppressor gene, binds to importins and blocks nuclear import of cargo proteins

Shaoyi HUANG*, In Soon CHANG†, Wenbo LIN§, Wenduo YE§, Robert Z. LUO*, Zhen LU*, Yiling LU‡, Ke ZHANG†, Warren S.-L. LIAO*, Tao TAO§, Robert C. BAST, Jr*, Xiaomin CHEN† and Yinhua YU*∥

*Department of Experimental Therapeutics, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A., †Department of Biochemistry and Molecular Biology, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A., §Department of Systems Biology, The University of Texas, M.D. Anderson Cancer Center, Houston, Texas 77030, U.S.A., ¶School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, People’s Republic of China, and ∥Obstetrics and Gynecology Hospital of Fudan University, Shanghai 200011, People’s Republic of China

Synopsis

ARHI (aplasia Ras homologue member I; also known as DIRAS3) is an imprinted tumour suppressor gene, the expression of which is lost in the majority of breast and ovarian cancers. Unlike its homologues Ras and Rap, ARHI functions as a tumour suppressor. Our previous study showed that ARHI can interact with the transcriptional activator STAT3 (signal transducer and activator of transcription 3) and inhibit its nuclear translocation in human breast- and ovarian-cancer cells. To identify proteins that interact with ARHI in nuclear translocation, in the present study, we performed proteomic analysis and identified several importins that can associate with ARHI. To further explore this novel finding, we purified 10 GST (glutathione transferase)–importin fusion proteins (importins 7, 8, 13, β1, α1, α3, α5, α6, α7 and mutant α1). Using a GST-pulldown assay, we found that ARHI can bind strongly to most importins; however, its binding is markedly reduced with an importin α1 mutant that contains an altered NLS (nuclear-localization signal) domain. In addition, an ARHI N-terminal deletion mutant exhibits greatly reduced binding to all importins compared with wild-type ARHI. In nuclear-import assays, the addition of ARHI blocked nuclear localization of phosphorylated STAT3. ARHI also inhibits the interaction of Ran–importin complexes with GFP (green fluorescent protein) fusion proteins that contain an NLS domain and a β-like import receptor-binding domain, thereby blocking their nuclear localization. By conducting GST-pulldown assays, we found that ARHI could compete for Ran-importin binding. Thus ARHI-induced disruption of importin-binding to cargo proteins, including STAT3, could serve as an important regulatory mechanism that contributes to the tumour-suppressor function of ARHI.

Key words: aplasia Ras homologue member I (ARHI), importin, nuclear import, nuclear translocation, Ran, signal transducer and activator of transcription 3 (STAT3)

INTRODUCTION

The transport of macromolecules between the nucleus and cytoplasm is critical for the normal function of eukaryotic cells. Two groups of karyopherins (importins and exportins) mediate RanGTPase-dependent transport through the nuclear pore [1]. During malignant transformation, aberrant nucleocytoplasmic transport of transcription factors [such as STAT3 (signal transducer and activator of transcription 3) and E2F1 (E2F transcription factor 1)] [2,3] and their regulatory kinases [such as SGK (serum- and glucocorticoid-induced protein kinase) and ERK [extracellular-signal-regulated kinase; also known as MAPK (mitogen-activated protein kinase)]] [4] occurs through impaired nuclear import, enhanced export, suppression of degradation and sequestration in protein aggregates. Conversely, secreted factors such as CYR61 (cysteine-rich protein 61), CTGF (connective tissue growth factor) and NOV (the protein encoded by nephroblastoma overexpressed gene; also known as CCN3), and EGF (epidermal growth factor), FGFs (fibroblast growth factors) and their receptors are often detected in the nuclei of cancer cells. Nuclear localization of these molecules has been correlated with tumour progression and poor prognosis for patient survival [5,6].

Abbreviations used: ARHI, aplasia Ras homologue member I; BIB, beta-like import receptor binding; E2F1, E2F transcription factor 1; GFP, green fluorescent protein; GST, glutathione transferase; IBB, importin β-binding; IL, interleukin; MAPK, mitogen-activated protein kinase; NLS, nuclear-localization signal; NTD, N-terminal deletion mutant; pSTAT, phosphorylated signal transducer and activator of transcription; RanGTPase; small GTP-binding protein Ran.

∥To whom correspondence should be addressed (email yinhuay@gmail.com).
The classical nuclear-import pathway consists of importin α and importin β. Whereas importin α interacts with NLS (nuclear-localization signal) in the cargo, importin β binds to the autoinhibitory domain on importin α and mediates the transport of the trimeric complex from the cytoplasm to the nucleus through the nuclear pores. Once inside the nucleus, RanGTP (small GTP-binding protein Ran) dissociates the complex by interacting with importin β. Importins α and β are shuttled separately back to the cytoplasm [7]. The importin α family includes importins α1, α3, α4, α5, α6, and α7 [8]. There are 20 members in the importin β superfamily, including importins β1, β7, β8, β9, and β13 [9–13]. Importin β proteins are composed of a flexible N-terminal IBB (importin β-binding) domain. The flexible IBB domain interacts either in trans with importin β or in cis with the cNLS (classical NLS)-binding groove [8]. Importin β proteins have in common an N-terminal Ran-binding domain. Importins direct the import of various cargos and may have different functions. For example, the importin β–importin 7 heterodimer is a functional nuclear-import receptor for histone H1 [10]; importin β, transportin, importin 7, and importin 9 promoted efficient import of c-Jun into the nucleus; by contrast, importin α inhibited nuclear import of c-Jun in vitro [11]. Importin 13, a recently identified importin β family member, regulates nuclear import of the glucocorticoid receptor in airway epithelial cells [12,13].

Ran is a small Ras-like GTP-binding protein that switches between a GTP-bound and a GDP-bound form by GTP hydrolysis and nucleotide exchange [14]. The GTase Ran plays a crucial role in nuclear-cytoplasmic transport of tumour suppressors, proto-oncogenes, signalling molecules and transcription factors. The RanGTase cycle provides directionality to nucleocytoplasmic transport, regulating interactions between cargos and nuclear-transport receptors of the importin β family. The common principle underlying these diverse functions throughout the cell cycle is thought to be anisotropy of the distribution of RanGTP (the RanGTP gradient), driven by the chromatin-associated guanine nucleotide-exchange factor RCC1 (regulator of chromatin condensation 1) [15].

ARHI (aplasia Ras homologue member I) is a maternally imprinted tumour-suppressor gene that encodes a 26 kD protein with 55–62% homology to Ras and Rap [16]. In contrast with Ras, ARHI contains a 34 amino acid N-terminal extension and inhibits the growth, motility and invasion of cancer cells [16,17]. Our recent work found that ARHI regulates autophagy and tumour dormancy in human ovarian-cancer cells by down-regulating PI3K (phosphoinositide 3-kinase) and Ras/MAPK signalling, thereby down-regulating mTOR (mammalian target of rapamycin) [18]. ARHI can also interact with the transcription activator STAT3 and inhibit its nuclear translocation and transcription activity in human breast-cancer and ovarian-cancer cells [19]. The ARHI NTD (N-terminal deletion mutant) has markedly reduced growth inhibitory activity, suggesting that this unique extension may contribute to the inhibitory effects of ARHI on STAT3-mediated transcriptional activities [19]. To identify additional ARHI-interacting proteins, we have performed proteomic analysis and found that ARHI is complexed with several importin proteins. We have explored the possibility that ARHI might displace cargo proteins, including STAT3, and inhibit their nuclear localization.

**MATERIALS AND METHODS**

**Cell lines and reagents**

The SKBr3 human breast-cancer cell line was maintained as described in [17]. HeLa cells were grown at 37°C to near-confluency in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum. Constructs of Ran and importin α1 mutant that contained an altered NLS domain, as well as anti-importin β1 antibody were a gift from Dr Karsten Weis (Department of Molecular and Cell Biology, University of California, Berkeley, CA, U.S.A.). Constructs of GST (glutathione transferase), GST–importin 7, GST–importin 8, GST–GFP (green fluorescent protein), GST–GFP–BIB (beta-like import receptor binding) and GST–GFP–NLS were a gift from Dr Keith Yamamoto (Department of Cellular and Molecular Pharmacology, University of California San Francisco, San Francisco, CA, U.S.A.) [20]. Anti-importin 7 and anti-importin 9 antibodies were a gift from Dr Dirk Görlich (Department of Molecular Biology, University of Heidelberg, Heidelberg, Germany). Anti-Ran and anti-GST antibodies were purchased from Upstate Biotechnology. Anti-ARHI antibody was produced by our group as previously described in [17]. Adenovirus constructs expressing LacZ, ARHI and NTD were prepared as described in [21]. The reagents for a nuclear-import assay (digitonin, ATP, GTP, creatine phosphate and creatine kinase) were purchased from Sigma.

**Proteomic analysis**

Wild-type ARHI and its NTD were re-expressed in the breast-cancer cell line SKBr3 using a dual adenovirus system [21]. LacZ adenovirus-infected cells were used as a control. ARHI-interacting proteins were immunoprecipitated using an anti-ARHI monoclonal antibody. In brief, cell lysates were incubated with anti-ARHI antibody at 4°C on a rocker for 90 min, then purified further with prewashed Protein G beads (Pierce) at 4°C on a rocker for 90 min. After washing three times with immunoprecipitation wash solution [0.5% (v/v) Triton X-100, 0.5% (v/v) Nonidet P40, 150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 1 mM PMSF and 10% glycerol], immunoprecipitates were separated by SDS/PAGE (15% gel) and analysed by MS at the University of Texas M.D. Anderson Cancer Center Proteomics Facility.

**Western-blot analysis**

Confluent SKBr3 cells were lysed with lysis buffer [20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5% (v/v) Nonidet P40, 0.5% (v/v) Triton X-100, 1 mM PMSF, 1 mM Na3VO4 and aprotinin 10 μg/ml], the lysates were incubated on ice for 20 min with occasional mixing and clarified by centrifugation in a microcentrifuge for 5 min at 16000 g at 4°C. Equal amounts of total cellular proteins were electrophoresed by SDS/15% PAGE and transferred to a PVDF membrane.
ARHI binds to importins and blocks nuclear import

(Figure 1 ARHI and importin form complexes

(A) Proteomic analysis. Wild-type ARHI and its NTD were expressed in SKBr3 breast-cancer cells using a dual adenovirus system. LacZ adenovirus-infected cells served as a control. Lane 1, ARHI complexes; lane 2, NTD complexes; lane 3, LacZ control. Arrows point to protein bands corresponding to the indicated novel binding proteins. kD, kDa. (B–D) Immunoprecipitation of ARHI–importin complexes. Protein lysates from (B) SKBr3 or (C) HeLa cells infected with ARHI, NTD or LacZ adenoviruses were incubated with anti-ARHI antibody. The immunoprecipitates were analysed by Western blotting with anti-importin (Imp) 7, 9 and β1 antibodies. Mouse IgG was used as a control. To confirm the efficiency of the assays, the immunoprecipitates were also analysed by Western blotting with anti-ARHI antibody. (D) Proteins immunoprecipitated with anti-importin 7 antibodies were analysed by Western blotting with anti-ARHI antibody. To confirm the efficiency of the assays, the immunoprecipitates were also analysed by Western blotting with anti-importin 7 antibody. The ARHI and NTD cDNA sequences were PCR amplified from pcDNA3-ARHI plasmid DNA and cloned into pQE30 vector (Qiagen). The importin 7 coding sequence was retrieved from the GST–importin 7 plasmid by restriction digestion and inserted into pQE30 vector. For the nuclear import substrates, GST–GFP–BIB and GST–GFP–NLS proteins were purified by glutathione–Sepharose beads (GE Healthcare), as described in the manufacturer’s instructions. pQE-Ran, pQE–importin 7, pQE–ARHI, and pQE–NTD were expressed in SG13009 competent cells and purified as described by Nachury and Weis [23]. Importin β1 was cleaved from the GST–β1 fusion protein by PreScission Protease (GE Healthcare) and importin α7 was cleaved from GST–α7 fusion using TEV Protease (Invitrogen), and the mixture was loaded on to a HiTrap Q cartridge (GE Healthcare) to separate GST from the importin proteins. All fusion proteins were concentrated using Centricon-30 concentrators (Millipore), divided into aliquots (50 μl), flash-frozen in liquid nitrogen and stored at −80°C.

Labelling of pSTAT3β

pSTAT3β proteins were FITC-labelled using an FITC Protein Labeling Kit (Pierce), as described in the manufacturer’s instructions.

GST-pulldown assay

GST–importin fusion proteins (2–4 μg) were mixed with 10–30 μg of cell lysates from SKBr3 cells in binding buffer [20 mM...
Hepes, pH 7.9, 150 mM NaCl, 0.5 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100 and 1 mM DTT]. The mixture was incubated with shaking at 4°C for 1 h. A 15 μl volume of glutathione–sepharose beads, pre-equilibrated in TEE buffer (50 mM Tris/HCl, pH 7.9, 1 mM EDTA and 1 mM EGTA) were added to the mixture and incubated with shaking at 4°C for another 1 h. The beads were collected and washed 4–5 times with washing buffer [20 mM Hepes, pH 7.5, 100 mM KCl, 2.5 mM MgCl2, 1% (v/v) glycerol and 1% (v/v) Triton X-100] and once with ice-cold PBS. The beads were resuspended in 20 μl of 2× SDS loading buffer and then boiled for 5 min. The soluble proteins were separated on SDS/15% PAGE gels for Coomassie Blue staining or Western-blot analysis.

Nuclear-import assay
HeLa cells were grown on glass coverslips for 40–42 h. Cells were then washed twice with ice-cold transport buffer (20 mM Hepes, pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM MgOAc, 2 mM DTT and 1 mM EGTA), incubated with 40 μg/ml digitonin in transport buffer for 6 min on ice, washed twice in cold transport buffer and kept on ice for 20 min. Coverslips with permeabilized cells were incubated for 30 min at 30°C with a 30 μl nuclear-import reaction mixture [2 μM GFP-tagged import substrate or 4 μM FITC-labelled p-STAT3 and an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, 10 units/ml creatine kinase and 0.5 mM GTP)]. Some experiments were conducted with 3 μM RanGDP, 3 μM importin α7, 2 μM importin β1, and 3 μM ARHI or 3 μM NTD. After the import reactions were completed, the cells were rinsed once in PBS, fixed in 4% (v/v) paraformaldehyde in PBS, and analysed by fluorescence microscopy using an Olympus microscope.

RESULTS

ARHI interacts with importins
Our previous studies have shown that when ARHI and STAT3 were both expressed in SKOv3 cells, ARHI formed a complex with STAT3 in the cytoplasm and prevented IL-6 (interleukin-6)-induced STAT3 accumulation in the nucleus [19]. To identify the protein(s) interacting with ARHI in the nuclear-import process, we performed proteomic analysis. Wild-type ARHI and its NTD were expressed in SKBr3 breast-cancer cells with a dual adenovirus system [21]. Cells infected with the LacZ adenovirus served as a control. ARHI or NTD complexes were immunoprecipitated using a specific ARHI antibody, and co-immunoprecipitated proteins were analysed by MS. Several novel interacting proteins were identified in complexes with wild-type ARHI and showed weaker binding to the NTD mutant. Three of these proteins (Figure 1A) belong to the family of importins: importin 7, importin 9 and importin α-re-exporter. These results were further confirmed by co-immunoprecipitation assays. As shown in Figures 1(B) and 1(C), in SKBr3 and HeLa cells, ARHI antibody could immunoprecipitate importins 7, 9 and β1, and anti-importin 7 antibody could also immunoprecipitate ARHI (Figure 1D). NTD–importin binding was greatly reduced compared with wild-type ARHI–importin interaction.

ARHI can bind to multiple importins
To investigate the ARHI–importin interaction, 10 GST–importin fusion proteins (importins α1, α1 mutant, α3, α5, α6, α7, β1, 7, 8 and 13) were purified. GST protein served as a negative control. ARHI bound strongly to most of the importins than does the NTD. Equal amounts of ARHI or NTD protein lysates were used in GST-pulldown assays. A total of nine GST–importin fusion proteins (upper panel: GST, Imp β1, Imp 1, Imp 7, Imp 8; lower panel: Imp α1, Imp α3, Imp α5, Imp α6, Imp α7) and anti-ARHI antibody were used to detect the binding. GST proteins in the complexes were detected by SDS/PAGE. DAPI, 4′,6-diamidino-2-phenylindole. kD, kDa.

The N-terminus of ARHI may mediate the binding with importins
Our previous studies demonstrated that deletion of the unique 34 amino acid N-terminal extension of ARHI nearly abolished its inhibitory effect on cancer-cell growth [17] and its ability to block the DNA-binding activity of STAT3 [19]. Thus the ARHI
ARHI binds to importins and blocks nuclear import

Figure 3 ARHI can block pSTAT3β nuclear translocation

(A) STAT3-importin binding analysed by GST-pulldown assays. SKBr3 cells were treated with or without IL-6 (10 ng/ml) for 30 min before cells were harvested for cell lysate preparation. A total of ten GST–importin (Imp) fusion proteins and STAT3/pSTAT3 antibodies were used to detect binding between STAT3 and importins. GST protein was used as a control. Loading was measured with anti-GST antibody. (B) Nuclear-import assay for pSTAT3β–GFP. Nuclear-import assays were performed as described in the Materials and methods section. Purified pSTAT3β and RanGDP importin α7 and importin β1 were incubated with permeabilized HeLa cells. Import assays were also performed by adding ARHI protein. DAPI, 4',6-diamidino-2-phenylindole.

N-terminal extension could be important for its biological function. In the proteomic analysis and immunoprecipitation assays (Figure 1), NTD protein exhibited greatly reduced binding to importins. To test further the possible role of the N-terminus of ARHI in binding importins, equal amounts of ARHI complexes and NTD complexes were tested in GST-pulldown assays. As presented in Figure 2(B), wild-type ARHI bound more strongly to most importins than did the NTD mutant, consistent with the possibility that the 34 amino acid N-terminal extension of ARHI mediates ARHI–importin binding.

ARHI can block the STAT3 protein nuclear-localization signal

Using a similar GST-pulldown assay, STAT3 protein was shown to bind to importins β1, α1, α3, α6 and α7, but not to the other importins and importin α1 mutant (Figure 3A). After IL-6 stimulation, the level of pSTAT3 greatly increased. Importins β1, α1, α3, α6, and α7 bound to both pSTAT3 (after IL-6 stimulation) and non-phosphorylated STAT3 (without IL-6 stimulation), but these bindings to pSTAT3 were much stronger. We also produced the pSTAT3β core fragment as described in [22]. Using native gel
Figure 4 Nuclear-import assay for NLS–GFP
The nuclear-import assay was performed as described in the Materials and methods section. NLS–GFP protein was mixed with RanGDP, importin α7 and/or importin β1 and incubated with permeabilized HeLa cells. Import assays were also performed by adding an equal amount of ARHI or NTD proteins. DAPI, 4′,6-diamidino-2-phenylindole.

electrophoresis, we confirmed that this core fragment can bind to a subset of the importins, namely α3, α6 and α7 (K. Zhang and X. Chen, unpublished work). To investigate if ARHI could block nuclear translocation of STAT3 protein, pSTAT3β was labelled with FITC and evaluated in the nuclear-import assay. As shown in Figure 3(B), pSTAT3β protein alone was concentrated at nuclear pores; when importins and Ran were added to the system, pSTAT3β was found in the nucleus. The addition of purified ARHI protein blocked Ran-dependent pSTAT3β nuclear translocation.

**ARHI can block the protein nuclear-localization signal**
Several studies have shown that interaction of importins with the Ran protein is required to facilitate the transport of cargo proteins into the nucleus [14]. Since ARHI and Ran belong to the same
family of small G-proteins, ARHI therefore might antagonize the interaction of importins with Ran. To test this hypothesis, we have purified ARHI and NTD proteins and assessed their effects on nuclear transport of cargo proteins in HeLa cells. In nuclear-import assays, an NLS–GFP fusion protein could be imported into the nucleus in association with importin α7, importin β1 and RanGDP (Figure 4), whereas the BIB–GFP fusion protein only needed help from importin 7 and RanGDP for nuclear localization (Figure 5). ARHI protein blocked the interaction of NLS with Ran–importin β and α complexes, and also the interaction of BIB with Ran–importin 7 complexes, preventing their nuclear translocation (Figures 4 and 5). In comparison, NTD protein did not block these interactions or nuclear transport (Figures 4 and 5).

**ARHI can compete for importin–Ran binding**
To determine whether ARHI binds to the same region of importins as Ran and could compete for importin–Ran binding, we performed GST-pulldown competition assays using GST–importin fusion proteins. pSTAT3, GFP–NLS and GFP–BIB proteins were bound to these GST proteins, and ARHI protein was added for competition. NTD protein served as a control. As

---

**Figure 5  Nuclear-import assay for BIB–GFP**
The nuclear-import assay was performed as described in the Materials and methods section. BIB–GFP protein was mixed with RanGDP and/or importin (Imp) 7 and incubated with permeabilized HeLa cells. Import assays were also performed by adding an equal amount of ARHI or NTD proteins. DAPI, 4',6-diamidino-2-phenylindole.
Figures 6(A) and 6(B) show, ARHI can significantly block the binding of NLS–importin α1, as well as partially block the binding of BIB–importin 7 and pSTAT3–importin α7.

Using GST–Ran fusion proteins, importins 13 and β1 were strongly bound to Ran, and ARHI protein could reduce these binding abilities by at least 50% (Figure 6C). NTD protein did not have competitive activity in these assays.

DISCUSSION

STAT3, a latent transcription factor, transduces signals from the cell surface to the nucleus and activates gene transcription, triggering proliferation, resistance to apoptosis, motility, invasion and angiogenesis [24,25]. Previous studies clearly demonstrate that STAT3 is required for both tumour initiation and promotion [24]. STAT3 is frequently phosphorylated and activated in the majority of breast and ovarian cancers [26], where cytokines and growth factors, such as IL-6, bind to specific receptors and activate JAK2 which, in turn, phosphorylates STAT3 and prompts translocation of pSTAT3 to the nucleus. The classic NLS–importin pathway has been reported to mediate the nuclear translocation of STAT3. Liu et al. [27] reported that STAT3 nuclear import is mediated by importin α3, whereas other studies have shown that the regulation of STAT3 nuclear import is through importins α5 and α7 [28]. Few studies investigated factors that regulate the translocation of pSTAT3 [29,30].

From our previous studies, re-expression of the putative tumour suppressor gene ARHI in cancer cells markedly inhibited binding of pSTAT3 to STAT response elements in target gene promoters and down-regulated STAT3-dependent promoter activity without significantly affecting STAT3 phosphorylation [19]. When ARHI and STAT3 were co-expressed in SKOV3 ovarian-cancer cells, ARHI formed a complex in the cytoplasm with STAT3 and prevented IL-6-induced STAT3 translocation to the nucleus [19]. The present study has elucidated the mechanism by which ARHI prevents nuclear translocation of pSTAT3. Nuclear-import assays have shown that pSTAT3 could be translocated to the nucleus in the presence of Ran and importins (Figure 3B), whereas non-phosphorylated STAT3 could not (results not shown). ARHI protein bound to importins (Figures 1 and 2) and blocked nuclear translocation of pSTAT3 (Figure 3B). ARHI also blocked nuclear translocation of NLS–GFP and BIB–GFP in the presence of Ran and appropriate importins (Figures 4 and 5). Thus ARHI might affect nuclear localization of STAT3, but might also affect transport of other proteins that are required for oncogenesis.

The precise mechanism by which ARHI disrupts the interaction of cargo proteins, Ran and importins remains to be elucidated. ARHI is a member of the Ras superfamily, but contains a unique 34 residue extension at the N-terminus. Like Ras, ARHI can bind to GTP with high affinity, but has low intrinsic GTPase activity [17]. ARHI associates with the cell membrane after prenylation at the C-terminal cysteine residue. Mutation of the conserved CAAX box at the C-terminus leads to a loss of its membrane-association ability and a modest decrease in its
ability to inhibit cell growth [16]. Most strikingly, deletion of the unique N-terminal extension of ARHI nearly abolishes its inhibitory effect on cell growth [17,19]. In the present study, the NTD lost much of the ability of the wild type protein to bind to importins and did not block the nuclear translocation of NLS–GFP or BIB–GFP. This suggests that interaction of the N-terminal extension of ARHI with importin may be required to prevent effective interaction of Ran–importin complexes with NLS and BIB. Interestingly, C-terminal deletion mutants of ARHI localize in the nucleus (results not shown), raising the possibility that accumulation of pSTAT3 in the cytoplasm in the presence of wild-type ARHI protein could result from direct binding of ARHI to STAT3, which we have demonstrated [19], or to lack of an effective nuclear-transport mechanism. As wild-type ARHI exhibits a nuclear localization signal on its N-terminal extension, the ARHI might compete with cargo for binding to importins in the presence of Ran. Once bound, ARHI could trap importins in the cytoplasm as the small G-protein is tethered to cell membranes through its prenylated C-terminus.

ARHI [also known as DIRAS3 (DIRAS family, GTP-binding RAS-like 3)] and Ran share a 22% sequence identity and are expected to have a similar overall structure. Although the crystal structure of ARHI is not yet available, those of the closest neighbours in the Ras family, DIRAS1 and DIRAS2, have been solved. We obtained a homology model for ARHI via the SWISS-MODEL server (http://swissmodel.expasy.org/). The model was generated using the DIRAS2 structure (PDB code: 2ERX) as the template and it covers residues 37–206 of the protein’s 229 residues. When the ARHI model is superimposed on to the crystal structure of Ran (PDB code: 1IRB, chain A) [31], the structural conservatism is clear. A DaliLite (http://www.ebi.ac.uk/DaliLite/) pairwise comparison resulted in a Z-score of 23.4 and a Cα RMSD (root mean square deviation) of 2.4 Å (1 Å = 0.1 nm), over 165 aligned residues. In the crystal structure of the importin β–Ran complex [31], Ran utilizes an extensive surface in binding to importin β. In the present study, by GST-pulldown competition assay, ARHI protein could significantly block the binding of NLS–importin and partially block the binding of BIB–importin and pSTAT3–importin. ARHI protein could also partially block importin–Ran binding (Figure 6), suggesting that ARHI binds to the same region of importins as does Ran, and ARHI-induced disruption of Ran–importin binding could serve as an important regulatory mechanism that contributes to the tumour-suppressor function of ARHI.

Acknowledgements

We gratefully thank Dr Walter N. Hittelman, M.D. Anderson Cancer Center, Houston, TX, U.S.A., for help with microscope observations.

Funding

This work was supported by grants from the National Cancer Institute [grant numbers CA 80957 (to Y.Y.), CA 64602 (to R.C.B.) and GM68556 (to X.C.)]; from the National Foundation for Cancer Research [grant number LF 2004-00009224HM (to R.C.B.)]; from the National Natural Science Foundation of China [grant numbers 3047085 and 90608007 (to T.T.)] and from the Ministry of Science and Technology of China [grant number 2006AA02A310 (to T.T.)].

References