Dysregulating IRES-Dependent Translation Contributes to Overexpression of Oncogenic Aurora A Kinase

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Abstract

Overexpression of the oncoprotein Aurora A kinase occurs in multiple types of cancer, often early during cell transformation. To identify the mechanism(s) contributing to enhanced Aurora A protein expression, a comparison between normal human lung fibroblast and breast epithelial cells to nontumorigenic breast (MCF10A and MCF12A) and tumorigenic breast (MCF-7) and cervical cell lines (HeLa S3) was performed. A subset of these immortalized lines (MCF10A, MCF12A, and HeLa S3) exhibited increased levels of Aurora A protein, independent of tumorigenicity. The increase in Aurora A protein in these immortalized cells was not due to increased transcription/RNA stability, protein half-life, or cap-dependent translation. Assays utilizing monocistronic and dicistronic RNA constructs revealed that the 5'-leader sequence of Aurora A contains an internal ribosomal entry site (IRES), which is regulated in a cell cycle–dependent manner, peaking in G2/M phase. Moreover, IRES activity was increased in the immortalized cell lines in which Aurora A protein expression was also enhanced. Additional studies indicated that the increased internal initiation is specific to the IRES of Aurora A and may be an early event during cancer progression. These results identify a novel mechanism contributing to Aurora A kinase overexpression.

Implications: The current study indicates that Aurora A kinase contributes to immortalization and tumorigenesis. Mol Cancer Res; 11(8); 887–900. ©2013 AACR.

Introduction

Aurora A is a serine/threonine kinase that plays a crucial regulatory role during mitotic events including centrosome duplication, separation, and maturation as well as mitotic spindle stabilization (1–3). Regulation of Aurora A expression is tightly controlled with both the mRNA and protein detected in late S and early G2 phase, peaking in G2–M, and rapidly degrading before G1-phase (4, 5). Aberrant expression of this kinase is detrimental to the cell. Overexpression contributes to centrosome amplification and a failure in cytokinesis creating aneuploidy, setting the stage for carcinogenesis (6, 7). Numerous tumor cell lines and human tumors exhibit elevated levels of Aurora A kinase, suggesting it may play a role in tumorigenesis (8–11). Indeed, ectopic Aurora A overexpression leads to cell transformation (12). Alternatively, loss of Aurora A leads to centrosomal separation defects resulting in a monopolar spindle, which in turn activates the G2–M checkpoint and eventually apoptosis (13). For this reason, the Aurora A kinase is considered a target for the development of anticancer drugs.

Enhanced expression of Aurora A protein in tumors is reportedly due to a concomitant increase in Aurora A mRNA owing to gene amplification and/or increased transcription (7, 8, 14–16). However, there are examples in many cancers whereby increased Aurora A protein expression is not accompanied by changes in mRNA levels (9, 10, 17). These results suggest that posttranscriptional processes including enhanced protein synthesis and/or protein stability are also contributing to the increased Aurora A kinase levels.

The major regulatory step in protein synthesis occurs at the initiation of translation (18). Most eukaryotic mRNAs are thought to initiate translation in a cap-dependent manner. This mechanism involves the binding of the preinitiation complex to the methyl-7-guansine (m7G) cap structure at the 5' end of the mRNA and scanning of the 40S ribosome to the first initiator codon in a proper context (ref. 19; reviewed in refs. 20, 21). In a subset of cellular mRNAs, an alternative mechanism to initiate translation occurs in which the preinitiation complex internally binds the 5' leader or untranslated region (UTR; ref. 22, 23). The binding site is referred to as an internal ribosome entry site (IRES). During periods in which cap-dependent translation is decreased, including in response to cellular stress or throughout the G2–M phase of the cell cycle, IRES-dependent translation is proposed to be maintained or elevated (24–27). Indeed,
many mRNAs translated during mitosis contain IRESes. For example, IRES-dependent translation of the ornithine decarboxylase (26) and PITSRLE p58 (28) mRNA occurs exclusively during mitosis (25). In addition, most of the small subset of eukaryotic IRESes identified to date are located in mRNAs encoding proteins that affect tumorigenesis. These proteins include oncogenes (c-myc; ref. 29), growth factors [fibroblast growth factor 2 (FGF2); refs. 30–32], growth factor receptors (TrkB; ref. 33), pro- and anti-apoptotic factors [X-linked inhibitor of apoptosis protein (XIAP) and APAF-1, respectively; refs. 34, 35], and angiogenic factors (VEGF; ref. 32). Deregulating IRES-dependent translation of these mRNAs could be a mechanism to promote cell survival and uncontrolled cellular proliferation during carcinogenesis.

In the present report, we chose multiple cell lines that differentially express Aurora A protein to identify mechanisms contributing to its overexpression. Transcription, mRNA stability, cap-dependent translation, and protein stability could not account for the increased Aurora A protein expression in a subset of cell lines. However, an IRES was identified in the 5′ leader of the Aurora A mRNA. Aurora A IRES activity positively correlated with Aurora A protein levels. Moreover, Aurora A expression in these cells was unaffected when cap-dependent translation was reduced. We propose there is a switch from cap- to IRES-dependent translation of the Aurora A mRNA that contributes to overexpression of the protein. In turn, this enhanced IRES activity may be a key determinant in generating genomic instability that may eventually result in cellular immortalization.

Materials and Methods

Constructs

The Aurora A (Supplementary Fig. S1) and β-globin (GenBank: V00497.1) 5′ leaders were PCR-amplified from a human fetal brain cDNA library (Clontech) and inserted into the dual luciferase vector-RP (ref. 36, 37; a generous gift from Dr. Anne Willis, University of Leicester, Leicester, United Kingdom) with EcoRI and NcoI endonuclease restriction sites. The dicistronic construct for in vitro transcription was created by digesting the RP vector with EcoRV and BamHI releasing the Renilla and Photinus luciferase genes and the SV40 3′-UTR. The 2 luciferase genes were inserted into the multiple cloning site of the SK Bluescript vector (Stratagene) downstream of the T7 promoter. The monocistronic construct for in vitro transcription was created by digesting the RP vector with EcoRI and BamHI. The digest released the 5′ leader, the Photinus luciferase gene and the SV40 3′-UTR, which were inserted into the EcoRI and BamHI sites of the SK′ Bluescript vector (Stratagene) downstream of a T7 promoter.

In experiments using a hypophosphorylated form of 4E-binding protein 1 (4E-BP1; containing Thr-37-Ala/Thr-46-Ala/Ser-65-Ala/Thr-70-Ala/Ser-83-Ala mutations), HeLa cells were transfected with 4 μg of a plasmid expressing hypophosphorylated 4E-BP1 or a control vector (Paltag) using Fugene 6 transfection reagent (Roche). After 48 hours, expression was analyzed via Western blotting. The 4E-BP1 mutant was generously provided by Dr. Davide Ruggero (University of California, San Francisco, San Francisco, CA).

In vitro transcription

The dicistronic and monocistronic SK′ Bluescript vectors were linearized with BamHI and used as templates for in vitro transcription. For the in vitro translation assay, monocistronic templates were transcribed using mMessage mMachine T7 Ultra (Ambion) producing capped mRNA. For the RNA transfection assays, dicistronic and monocistronic templates were transcribed using MEGAscript T7 (Ambion) producing either A capped (New England BioLabs) or uncapped RNA. The m7G cap was added using Script Cap m7G capping system (CELLSCRIPT, Inc.) and transcripts were poly(A) tailed using poly(A) polymerase tailing kit (Epicycle) as per manufacturer’s instructions. All mRNA was extracted with phenol/chloroform and run on an agarose gel to ensure RNA integrity.

In vitro translation

An aliquot of 0.5 μg of in vitro transcribed mRNA, cap analog (Ambion), and 1.6 mmol/L methionine was added to rabbit reticulocyte lysate (Speed Read; Novagen) and incubated for 1 hour at 30°C. The sample was subsequently assayed for Photinus and Renilla luciferase activity.

siRNA plasmid transfections

Ten mmol/L of nonsense siRNA (Dharmacon; D-001206-10-20) or human elf-4E siRNA (Dharmacon; M-003884-03, J-003884-08, or J-003884-10) were incubated in 35-mm plate wells with 12 μL of INTERFERin transfection reagent (PolyPlus-Transfection) at 37°C for 10 minutes. HeLa cells were plated at 1.0 × 10^4 in wells already containing siRNA complexes and serum-free growth media. Complete growth media was then added to a final volume of 2 mL. After 48 hours, cells were harvested in cell lysis buffer (Promega) with protease (Roche) and phosphatase inhibitors (Pierce) and analyzed via Western blot analysis.

Cell culture/luciferase assays

WI-38 cells (CCL-75) were obtained by American Type Culture Collection (ATCC) and cultured in minimum essential medium (MEM) plus 10% FBS and 5% penicillin/streptomycin. MCF-7 (HTB-22), HeLa (CCL-2), and HeLa S3 cells (CCL-2.2) were also obtained by ATCC and cultured in Dulbecco’s modified Eagle medium (DMEM) plus 10% FBS and 5% penicillin/streptomycin. Human mammary epithelial cells (HMEC; A10565) or uncapped RNA. The m7G cap was added using Script Cap m7G capping system (CELLSCRIPT, Inc.) and transcripts were poly(A) tailed using poly(A) polymerase tailing kit (Epicycle) as per manufacturer’s instructions. All mRNA was extracted with phenol/chloroform and run on an agarose gel to ensure RNA integrity.

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fingerprinting using the AmpF/STR Identifier Kit according to the manufacturer’s instructions (Applied Biosystems, cat 4322288). The STR profiles were compared with known ATCC fingerprints (ATCC.org), with the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://bioinformatics.istie.it/clima/; Nucleic Acids Research 37:D925-D932 PMCID: PMC2668656), and with the MD Anderson fingerprint database. The STR profiles matched known DNA fingerprints or were unique.

Cells were transfected with 2 μg of mRNA using TransMessenger transfect kit (Qiagen; RNA/TransMessenger lipid 1:8) as per manufacturer’s instruction. After 4 hours, the cells were lysed with 500 μL of lysis buffer (Promega). Forty microliter of the supernatant were used for the luciferase assays using the Dual-Luciferase Reporter Assay System (Promega) and analyzed in a Luminoskan luminometer.

**Western blot analysis**

Cells were harvested in cell lysis buffer (Promega) with protease (Roche) and phosphatase inhibitors (Pierce). The cell lysate was analyzed by Western blot analysis by separating the proteins on a 12% SDS-PAGE, with subsequent transfer onto nitrocellulose. The membranes were blocked and probed with an antibody directed against Aurora A (35C1; Calbiochem), eukaryotic elongation factor 2 kinase (eEF2K; Cell Signaling Technology), eIF4E (BD Transduction), glyceraldehyde-3-phosphate dehydrogenase (Gapdh; Promega), eukaryotic elongation factor 2 kinase (eEF2K; Cell Signaling Technology), eIF-4G (C45A4; Cell Signaling Technology), phospho-4E-BP1 (Thr37/46; Cell Signaling Technology), 4E-BP1 (53H11; Cell Signaling Technology), glyceraldehyde-3-phosphate dehydrogenase (Gapdh; Promega), and probed with an antibody directed against Aurora A (35C1; Calbiochem). The blots were then incubated with horseradish peroxidase–conjugated secondary antibodies (Promega). Immunoreactive bands were detected using ImageQuant version 5.2 (Molecular Dynamics) or ImageJ software (NIH http://rsweb.nih.gov/ij/).

**Polyosome analysis**

Cells were treated with cycloheximide (50 ng/mL) for 30 minutes, then harvested on ice in PBS containing 50 ng/mL of cycloheximide, and finally lysed with 400 μL of 100 mmol/L KCL, 50 mmol/L Tris–Cl, 1.5 mmol/L MgCl2, 1 mmol/L dithiothreitol, 1.5% NP-40, protease inhibitors (Roche), 100 μg/mL cycloheximide, and 100 U RNasin plus RNase inhibitor (Promega). Then 300 μL of lysate was loaded on a 20% to 60% sucrose gradient created using a BIOMICRO Gradient Station and centrifuged at 39,000 rpm for 2 hours at 4°C using a SW40Ti rotor (Beckman Coulter). The gradient was fractionated and RNA was extracted from each fraction using TRIzol Reagent (Sigma) followed by PureLink RNA Mini Kit (Invitrogen) and analyzed by quantitative real-time PCR (qRT-PCR).

**RNA extraction/qRT-PCR**

Total RNA was extracted using TRIzol Reagent (Sigma) followed by PureLink RNA Mini Kit (Invitrogen). cDNA libraries were synthesized using iScript cDNA Synthesis Kit (Bio-Rad), including a (-)RT control. The primer pairs used for the qRT-PCR were as follows: 5'-TCTTACAGAGGGCAATCCA-3' (forward) and 5'-AATAGTTACACACTCTACTACCTA-3' (reverse) for Aurora A mRNA, 5'-ACAGTCAGCGCATCTTCTT-3' (forward) and 5'-GTCAAAGGAGCGGCTGTTGA-3' (reverse) for Gapdh mRNA, and 5'-AAAGCTCCTCAATCTCCTAAA-3' (forward) and 5'-GAGATGTCACACGTGTTGT-3' (reverse) for Pho61 luciferase mRNA. qRT-PCR was conducted using a Roche Lightcycler 480 with either LightCycler 480 SYBR Green I Master (Roche) or dsoAdvanced SYBR Green Supermix (Bio-Rad) as per manufacturer’s instructions.

**Results**

Enhanced protein synthesis contributes to overexpression of Aurora A kinase

Increased Aurora A kinase expression is proposed to contribute to cellular immortalization and to the epithelial–mesenchymal transition (EMT; refs. 36, 37). To identify mechanisms contributing to this enhancement, Aurora A protein expression was examined in a variety of cells, focusing on breast epithelial cell lines. Aurora A protein levels were quantified in 2 finite lines, normal human lung fibroblasts (WI-38) and HMEC. Aurora A protein expression in these cells was chosen to represent basal protein levels and compared with the following immortalized cell lines: non-tumorigenic breast epithelial cell lines (MCF10A and MCF12A), and tumorigenic epithelial cell lines (MCF-7 and HeLa S3) from breast and cervix, respectively. Lysates were analyzed via Western blotting for Aurora A and Gapdh (as a loading control). Expression of Aurora A kinase in the control cell lines, WI-38 and HMEC, were similar and comparable with that observed in MCF-7 cells (Fig. 1A). However, Aurora A kinase expression was 2- to 4.2-fold higher in MCF10A, MCF12A, and HeLa S3 cells (Fig. 1A). Three of 4 immortalized cell lines showed Aurora A protein levels similar to those observed in breast and cervical cancer (36).

The Aurora A gene is transcribed and the mRNA is translated during late S-phase and peaks during G2–M phase of the cell cycle (4, 5). Therefore, it is possible that differences in the number of cells in these phases from an asynchronous population could contribute to the observed differences in Aurora A protein levels. However, fluorescence-activated cell sorting (FACS) analysis of the cell lines did not show a correlation with the percentage of cells in G2–M and Aurora A protein levels (Supplementary Fig. S2). Therefore, alterations in transcription, translation, and/or protein stability are potentially contributing to increased Aurora A kinase expression in MCF10A, MCF12A, and HeLa S3 cells.

To quantify Aurora A mRNA levels, cDNA libraries were constructed from total RNA isolated from the individual cell lines. Aurora A transcript levels from each cell line were measured by qRT-PCR and showed that mRNA levels were...
similar between each cell line except for a modest increase in MCF-7 cells (Fig. 1B). The ratio of Aurora A protein to mRNA within each cell line was significantly elevated (ranging from approximately 2–5-fold higher) in MCF10A, MCF12A, and HeLa S3 cells compared with WI-38 and HMEC cells, and even higher when compared MCF-7 cells (Fig. 1C). These results indicate that increased Aurora A protein expression in the MCF10A, MCF12A, and HeLa S3 cells is not due to enhanced transcription of the Aurora A gene or Aurora A mRNA stability, but the result of increased protein synthesis and/or protein stability.

To identify if alterations in protein stability contributed to the differential Aurora A expression, the half-life of the Aurora A protein was determined. Cells were harvested at 2 to 12 hours after being treated with cycloheximide. The Aurora A protein levels were then analyzed via Western blotting (Supplementary Fig. S3) with Gapdh as a loading control as it has a half-life of 90 to 120 hours (40). The half-life of the Aurora A protein was similar between the cell lines, ranging between 2.2 to 2.9 hours (Fig. 1D). This range is consistent with a previous study that found the Aurora A protein half-life to be approximately 2.5 hours (5). In addition, this result indicates that differences in protein stability do not contribute to the differential expression of Aurora A protein.

By order of elimination, the previous results implicated protein synthesis as one of the remaining mechanisms contributing to the variable Aurora A protein levels. To confirm this hypothesis, a polysome gradient analysis was conducted. Aurora A mRNA levels were quantified in nonpolysomal, low-molecular weight (LMW) polysome, and high-molecular weight (HMW) polysome fractions between the low Aurora A protein–expressing MCF-7 cells and high Aurora A protein–expressing MCF12A cells. Association with HMW fractions suggests increased translation initiation and/or reinitiation as the result of more efficient loading of the ribosomes onto the mRNA (41). Aurora A mRNA was most concentrated in the HMW
fractions from MCF12A cells. In contrast, the majority of Aurora A mRNA levels from MCF-7 cells resided with the LMW fractions (Fig. 1E). These results indicated translational upregulation as a contributing mechanism to enhanced protein expression in MCF12A cells compared with MCF-7 cells.

**Cap-dependent translation initiation is increased in the immortalized cell lines**

The major mechanism by which translation is regulated is at the step of initiation. And the primary mode by which translation is initiated is through recognition of the m7G cap structure (42, 43). Misregulating cap-dependent translation can lead to elevated levels of protein synthesis and tumorigenesis. For example, increased expression of the rate-limiting canonical factor eIF-4E (which binds the cap structure) is found in many tumors (44–46). Furthermore, ectopically induced overexpression of eIF-4E has transforming capabilities leading to malignant phenotypes (47–49). To determine if elevated eIF-4E levels were contributing to the overexpression of Aurora A, Western blot analyses of lysates were analyzed. eIF-4E levels were similar in all 6 cell lines (Supplementary Fig. S4A). This observation suggests that a global increase in cap-dependent translation may not be occurring. On the other hand, the eIF-4E that is present may be differentially restricted in its ability to bind the cap structure.

steady-state expression of 4E-BP1, a negative regulator of eIF-4E, is often decreased in cancer cells (50). Analysis of 4E-BP1 protein levels via Western blotting showed there was no difference between 4E-BP1 levels in WI-38, MCF12A, or MCF-7 cells (Supplementary Fig. S4B). However, there was actually a 1.5- to 2.2-fold increase in the expression of 4E-BP1 in HMEC, MCF10A, and HeLa S3 cells. The phosphorylation state of 4E-BP1 is integral to its activity with the hypophosphorylated 4E-BP1 able to bind eIF-4E and inhibit cap-dependent translation. Interestingly, there was a 1.5- to 3-fold increase of hypophosphorylated 4E-BP1 in all the cell lines compared with WI-38 cells (Supplementary Fig. S4B, right). Taken together, these results do not support the hypothesis that cap structure accessibility is altered in the different cell lines.

The scaffolding protein eIF-4G is the key structural component in the preinitiation complex. Its expression level is proposed to contribute to the rate of translation (51). However, eIF-4G levels did not correlate with Aurora A protein levels as shown by the increased expression of eIF-4G in MCF-7 cells compared with WI-38 and HMEC cells (Supplementary Fig. S4C). In addition, eIF-4G protein expression was low in MCF-12A cells.

The expression patterns of the critical cap-dependent regulatory proteins eIF-4E, 4EBP, and eIF-4G did not yield any candidates that may be contributing to the overexpression of Aurora A kinase. To definitively compare cap-dependent translation initiation between these cell lines, we used a reporter assay. In vitro transcribed mRNA from monocistronic DNA containing the Photinus luciferase open reading frame (ORF), the β-globin 5′ leader, and the SV40 poly-adenylation (A) site was created. The β-globin 5′ leader was chosen because it is short (53 nt), unstructured, without an upstream ORF (uORF), and the β-globin mRNA is exclusively translated in a cap-dependent manner (43). The transcripts were capped with a m7G cap, poly(A) tailed, and transfected into each cell line. After 4 hours, the cells were harvested and assayed for Photinus luciferase activity. The luciferase activity from each cell line was compared with transcript levels quantitated using qRT-PCR to normalize for transfection efficiency as well as mRNA stability. Translation of the reporter was enhanced by 1.9- to 2.6-fold in all of the immortalized cell lines compared with the normal cells (Fig. 2A). Interestingly, MCF-7 cells, which do not overexpress the Aurora A protein, elicited the second largest increase of cap-dependent translation compared with WI-38 and HMEC cells (Fig. 2A). This result indicates that other mechanisms aside from eIF-4E, 4E-BP, and eIF-4G expression are contributing to enhanced cap-dependent translation. However, increased cap-dependent translation did not correlate with increased expression of Aurora A protein, thereby suggesting that an alternate mechanism may be regulating translation of the Aurora A mRNA.

**Aurora A protein expression level is unaffected by inhibiting cap-dependent translation initiation**

To further determine the role of cap-dependent translation initiation in the synthesis of the Aurora A protein, cap-dependent translation was inhibited using 2 different approaches. Initially, eIF-4E expression was knocked down. HeLa cells were transfected with siRNA (Dharmacon) targeting eIF-4E mRNA or nonsense siRNA for 48 hours. Quantification of Western blot analyses showed that eIF-4E protein expression was reduced by nearly 70% (Fig. 2B). eEF2K expression in the eIF-4E siRNA-treated cells decreased by 57%, similar to previous observations (52). In contrast, the level of Aurora A protein was equivalent in the eIF-4E and nonsense siRNA conditions (Fig. 2B).

As a second approach to modulate cap-dependent translation, HeLa cells were transfected with a DNA plasmid encoding a hypophosphorylated mutant of 4E-BP1 in which the main 5 phosphorylation sites are mutated rendering the protein nonphosphorylated (generously provided by Dr. Davide Ruggero, University of California, San Francisco) or a control vector (Paltag). The constitutively expressed hypophosphorylated 4E-BP1 should sequester eIF-4E, thereby disrupting eIF-4F formation and inhibit cap-dependent translation (53). Forty-eight hours after transfection, cells were harvested and protein levels were analyzed by Western blotting. eEF2K expression decreased by 75% in the presence of hypophosphorylated 4E-BP1 (Fig. 2C). eIF-4E expression decreased by 53%, similar to previous results (52), indicating that the eIF-4E mRNA is translated via a cap-dependent mechanism and a decrease in cap-dependent translation has occurred. On the other hand, the Aurora A protein level decreased by only 16% (Fig. 2C). Taken together, these results show that synthesis of Aurora A kinase protein is relatively unaffected when cap-dependent translation is inhibited in HeLa cells indicating that an alternative
A mechanism may be contributing to the translation initiation of the Aurora A mRNA.

To determine if cap-dependent translation regulates expression of the Aurora A protein in other cells, 2 siRNAs targeting eIF-4E were individually transfected into WI-38, HMEC, MCF10A, MCF12A, and MCF-7 cells. eIF-4E was significantly knocked down by each siRNA in all cell lines (Fig. 2D and E). Aurora A protein expression was...
significantly decreased in MCF-7 cells, indicating cap-dependent initiation is the primary mechanism for translation of Aurora A mRNA in these cells. However, Aurora A protein levels were unaffected when eIF-4E levels were reduced in WI-38, HMEC, MCF10A, and MCF12A cells (Fig. 2D and E). Taken together, these results suggest an alternate mechanism may be used to initiate translation of the Aurora A mRNA.

The Aurora A 5' leader contains an IRES

Cap-dependent translation did not seem to be a significant mechanism for the synthesis of Aurora A protein in the HMEC cells and in the cell lines in which Aurora A kinase was overexpressed. This result indicated it may be translated in a cap-independent manner. To determine if the Aurora A 5' leader has an IRES, it was inserted into the intercistronic region of a dicistronic luciferase construct coding for Renilla and Photinus luciferase in the first and second cistrons, respectively (29). The β-globin 5' leader, which does not contain an IRES, was used as a negative control. Two viral IRESes, the encephalomyocarditis virus (EMCV) IRES and the IRES in the intergenic region of the cricket paralysis virus (CrPV) were chosen as positive controls (54, 55). The constructs were transfected into HeLa cells with decreasing concentrations of cap analog. The initial level of Photinus luciferase activity from each monocistronic mRNA was normalized to 100. The Photinus luciferase activity from each monocistronic mRNA was normalized to 100. n = 3 in triplicate; *P < 0.0001; Student t test.
lysate. Cap-dependent translation was inhibited by adding increasing amounts of cap analog to compete with the m7G cap. Luciferase activity measured from the β-globin construct decreased by 70% in the presence of 100 μmol/L of cap analog, whereas luciferase activity from the Aurora A construct was relatively unchanged (Fig. 3A, right). This result showed that the Aurora A 5’ leader initiates translation independently of the cap structure and supports the hypothesis that it contains an IRES.

Expression levels of both Aurora A mRNA and Aurora A protein peak during the G2–M phase of the cell cycle (4, 5). This temporal expression coincides with reports indicating that IRES activity is regulated through the cell cycle and peaks at G2–M (58, 59). To determine if the Aurora A IRES is regulated by the cell cycle, HeLa cells were synchronized by a double thymidine block. During the last thymidine block the cells were transfected with in vitro transcribed dicistronic mRNA containing either the β-globin or Aurora A 5’ leader in the intercistronic region. Cells were harvested between 0 and 8 hours after release from the double thymidine block into normal growth medium with the total transfection time being 11 hours for all cells. FACS analysis showed that the majority of cells were synchronized in G1 phase of the cell cycle in which translation of the endogenous Aurora A mRNA occurs—G2–M.

**Aurora A IRES activity is increased in the cell lines that overexpress Aurora A protein**

To determine if Aurora A IRES activity parallels Aurora A protein expression, dicistronic mRNA containing the Aurora A or β-globin 5’ leaders was transfected into all 6 cell lines. After 7 hours, the cells were harvested and assayed for Renilla and Photinus luciferase activity. The P:R ratio for the mRNA containing the Aurora A 5’ leader was at least 2-fold higher than the control in the 6 cell lines indicating the Aurora A IRES is operative in all cells (Fig. 4A). Moreover, the Aurora A P:R ratio was highest in the cell lines that also overexpressed the Aurora A protein (Fig. 4A). These results show a positive correlation between Aurora A protein expression and Aurora A IRES activity. This correlation supports the hypothesis that IRES-dependent translation is contributing to the overexpression of the Aurora A protein observed in the subset of cell lines.

All eukaryotic transcripts contain a m7G cap. Accordingly, translation of the Aurora A mRNA may be initiated through the cap structure and/or the IRES. To quantitate the contribution of the Aurora A IRES in a monocistronic mRNA, the β-globin or Aurora A 5’ leader were placed upstream of the Photinus luciferase ORF and in vitro transcribed. The transcripts were capped with an m7G or ApppG cap (A cap) and poly(A) tailing (Fig. 4B). The A cap is not recognized by eIF-4E preventing cap-dependent translation initiation of the mRNA. In addition, the poly(–A)–specific 3’ exonuclease PARN does not recognize the A cap, thereby inhibiting deadenylation and preventing both 5’ and 3’ exonucleolytic mRNA decay (60, 61). The mRNA was cotransfected into each cell line with a m7G-capped and poly(A) tailed mRNA containing the humanized Renilla luciferase ORF to normalize for transfection efficiency. For each cell line, the P:R ratio obtained from the m7G cap Photinus constructs was set to 100. The P:R ratio obtained from the A cap Photinus luciferase mRNA was compared with the P:R ratio obtained from the corresponding m7G cap Photinus luciferase constructs. The A cap reporter mRNA containing the β-globin leader was poorly translated (5%) to 16% of the m7G cap P:R ratio; Fig. 4C, left). This result showed that the translation of the mRNA containing the β-globin 5’ leader was dependent on the presence of the m7G cap structure. In the WI-38 and HMEC cells, translation of the A cap mRNA containing the Aurora A 5’ leader was higher than the mRNA with the β-globin leader. This result indicates that the Aurora A 5’ leader was initiating cap-independent translation, although it was only approximately 25% of that obtained by a m7G cap mRNA containing the Aurora A 5’ leader (Fig. 4C, right). On the other hand, the A cap P:R ratio from the Aurora A construct was considerably higher in the cell lines that overexpress the Aurora A protein (MCF10A, MCF12A, and HeLa S3). Remarkably, in the MCF12A cells, Photinus luciferase activity from the A cap mRNA was 95% of that obtained from the m7G cap Aurora A mRNA (Fig. 4C). These results suggest cap-dependent translation is most likely a contributing mechanism for Aurora A protein synthesis in the low-expressing lines, but IRES-dependent translation is the predominant mechanism in the Aurora A overexpressing lines.

To determine if IRES activity in general was upregulated in the Aurora A overexpressing cell lines, the experiment was repeated using IRESes from the PITSLRE kinase, EMCV, and FMR1 mRNAs. Although all 3 IRESes are used in proliferating cells, PITSLRE and EMCV IRES activity is upregulated during G2–M phase of the cell cycle, whereas the FMR1 IRES is predominantly used in postmitotic neurons (52, 62). Internal initiation mediated by the FMR1 IRES varied between the cell lines but the activity did not correlate with that observed from the Aurora A IRES (Supplementary Fig. S5A). FMR1 IRES activity was similar in HMEC, MCF12A and MCF-7 cell lines, significantly elevated in MCF10A, slightly decreased in WI-38 cells, and significantly decreased in HeLa S3 cells. Interestingly, activity from the EMCV and PITSLRE IRESes also did not correlate with that observed from the Aurora A IRES (Supplementary Fig. S5B and S5C, respectively). EMCV IRES activity was significantly lower in MCF10A and MCF-7 cells compared with HMEC cells. There was no difference in activity between WI-38, HMEC, MCF12A, and HeLa S3 cells. On the other hand, activity from the PITSLRE IRESes
was significantly elevated in MCF12A and HeLa S3 cells yet significantly decreased in MCF-7 cells compared with the normal cells, similar to Aurora A IRES activity. However, unlike the Aurora A IRES, the PITSLRE IRES was less active in MCF10A cells. In addition, PITSLRE IRES activity was higher in the WI-38 and HMEC cells compared with Aurora A IRES activity. These results indicate that not all IRESes are upregulated in the Aurora A overexpressing cell lines. Furthermore, they suggest possible variations in the regulation of IRESes that are upregulated under similar circumstances, such as the G2–M phase of the cell cycle.

To determine if the correlation between Aurora A IRES activity and protein expression extends to other cell lines, we conducted the same assay with 3 lines from the 21T cell line series (21PT, 21NT, and 21MT2). These cell lines were derived from a single patient diagnosed with infiltrating and intraductal mammary carcinoma and are often used as an in vitro model for cancer progression. The nontumorigenic 21PT and the tumorigenic 21NT cell lines were derived from a primary tumor. The 21MT2 line was derived from a metastatic tumor (63–65). All 3 cell lines overexpress Aurora A protein (Fig. 5A), yet mRNA levels and protein half-life (2.8–3.1 hours) were equivalent to the normal cell lines (Fig. 5B and D). The elevated Aurora A protein/mRNA ratio indicated an increase in Aurora A translation (Fig. 5C). And the A/m7G cap monocistronic transfection experiment showed a high level of Aurora A IRES usage. The percentage of A cap–mediated translation to that obtained from the m7G cap with the Aurora A 5′ leader ranged from 60% (21PT) to 83%–85% (21MT2 and 21NTm respectively; Fig. 5E, bottom). These results are similar to what was observed with the other Aurora-A overexpressing cell lines.

To resolve whether increased proliferative activity contributed to alterations in Aurora A IRES activity, this assay was conducted in a HMEC cell line that ectopically expresses telomerase (HMEC-t); a cellular process that prevents replicative cellular aging. Overexpressing telomerase did not alter Aurora A protein levels, mRNA levels, or protein stability (Fig. 5A–D). In addition, the A cap $P_R$ ratio exhibited by these cells was similar to that obtained from WI-38 and HMEC cells (Fig. 5E).
In summary, these results show that IRES-dependent translation initiation mediated by the Aurora A 5′ leader is used to a greater extent in cells overexpressing the Aurora A protein. Simply extending proliferative activity does not alter Aurora A IRES activity or protein levels. These results support the hypothesis that enhanced IRES-dependent translation of the Aurora A mRNA contributes to the increase of Aurora A kinase expression, which predisposes the cell to immortalization and tumorigenesis.

Discussion

Overexpression of the Aurora A kinase is observed in a broad range of malignancies including breast, brain, and pancreatic tumors. Misregulation of its expression is proposed to be both an early event in the immortalization of the cell as well as a contributor to the EMT. In the present report, the goal was to identify mechanism(s) contributing to Aurora A protein overexpression. To this end, we chose 6 cell lines, which included primary, immortalized, and tumorigenic cell types. They were categorized as either high or low Aurora A protein–expressing cells. Transcription/mRNA stability, protein stability, nor cap-dependent translation could account for enhanced levels of Aurora A protein in the high-expressing lines. On the other hand, we identified an IRES in the Aurora A 5′ leader and IRES activity strongly correlated with Aurora A protein expression.

Using RNA dicistronic luciferase constructs, Aurora A IRES activity was shown to correlate with Aurora A protein expression. However, this assay does not measure the contribution of the IRES in an m7G-capped monocistronic mRNA, the state in which the Aurora A mRNA is present. It has been suggested that eukaryotic IRESes initiate translation at a considerably reduced rate compared with m7G cap-dependent initiation. Thus, even if IRES activity is enhanced in a subset of cell lines, the overall increase may not...
be physiologically significant. To address this issue, we created monocistronic mRNA containing the Aurora A IRES with an A or m7G cap. These RNAs would permit comparison between translation initiated in a cap-independent manner to translation mediated by an m7G cap and an IRES (60). The resulting A cap/m7G cap ratio we termed "IRES usage." In WI-38 and HMEC cells, A-capped translation of the RNA containing the Aurora A leader was less than 25% of that obtained from an m7G-capped mRNA (Fig. 4C). This result indicated that cap-dependent translation is likely the predominant mechanism. However, transfection into the other cell lines yielded dramatically different results. In MCF12A cells, translation of the mRNA was similar irrespective of the cap structure (Fig. 4C). This is one of the first examples whereby a eukaryotic IRES initiates translation at a rate similar to an m7G-capped transcript. We interpret this result as showing that IRES-dependent translation is elevated and is the principal mechanism used to initiate translation of the m7G-capped mRNAs. Alternatively, reduced cap-dependent translation without any concomitant alteration in IRES activity could yield a similar result. To differentiate between these 2 explanations, we examined cap-dependent translation of a monocistronic mRNA and found that it was relatively similar between the 4 immortalized lines and actually higher than the 2 primary cell lines (Fig. 2A). Accordingly, these results indicate an apparent switch from low level cap-dependent translation of the Aurora A mRNA in normal, finite lifespan cells to increased IRES-dependent translation in immortalized cells before malignant transformation. Identifying the mechanism(s) that play a role in this switch could be crucial to understanding oncogenesis mediated by Aurora A kinase overexpression and possibly of other oncoproteins, which are translated in an IRES-dependent manner. For example, a subset of breast tumors exhibiting overexpression of HER2 and EGFR has been implicated in affecting Aurora A kinase expression (69).

Examination of additional cell lines reinforced the positive correlation between IRES usage and Aurora A protein expression. The 21T series exhibited both elevated Aurora A protein levels and IRES activity; whereas Aurora A mRNA and protein half-life was similar to the finite cell lines (see Fig. 5). This group of cells included immortalized, but nontumorigenic (21PT), tumorigenic (21NT), and metastatic (21MT2). These results are consistent with previous reports suggesting enhanced Aurora A expression contributes to immortalization and continues through the EMT (37, 69).

Internal initiation of translation has been invoked as a mechanism involved in carcinogenesis (reviewed in ref. 71). Multiple mRNAs encoding proteins contributing to cell proliferation [FGF2 and platelet-derived growth factor 2 (PDGF2)], cell cycle (PITSLRE p58), cell death/survival (Bcl-X and Apaf1), and tumor development (p53, c-myc, and c-jun) contain IRESes (28, 29, 32, 72–75). Misregulating IRES-dependent translation contributes to cancer progression through multiple means. For example, in the disease X-linked dyskeratosis congenita, mutations in the dyskerin gene reduce pseudouridylation of rRNA, which inhibits IRES-dependent translation of tumor suppressors (p53 and p27) and apoptotic factors (Bcl-X and XIAP) predisposing individuals to cancer (76–78). Alternatively, the low oxygen environment in the center of solid tumors increases IRES-dependent translation of the VEGF mRNA, which in turn promotes angiogenesis and tumor growth (79). In the present study, we found that the Aurora A IRES is regulated through the cell cycle and by cell type. As a first step toward identifying the mechanism, other IRESes that are upregulated in G2–M were examined. However, there was no correlation of IRES usage between the PITSLRE, EMCV, and the Aurora A IRES. It would be of interest to determine if there is a subset of IRESes, perhaps those encoding other oncoproteins, which exhibit IRES usage patterns similar to Aurora A.

The mechanism regulating the Aurora A IRES is unknown. Identifying cis-elements that control IRES activity would be informative. Viral IRESes generally rely on extensive secondary structure, a feature that is often conserved phylogenetically, although the sequences can vary (80–82). The presence of uORFs is observed as well (23, 83). Alternatively, eukaryotic IRESes are quite variable; sequence and/or secondary structure does not imply the presence of an IRES. Previous studies have reported eukaryotic IRES motifs as short as 9 to 50 nt (84, 85), but they can extend to over 1,000 nt (86). Viral IRESes exhibit a variable requirement for trans-factors. Some IRESes recruit the ribosome directly, whereas others use both canonical factors (including eIF-4E in certain situations) and IRES-transacting factors (ITAF) such as polyuridyline-tract—binding (PTB) protein or La protein (54, 87–89). The rate-limiting step for eukaryotic IRESes is proposed to be the expression level of ITAFs (reviewed in ref. 90). These are RNA-binding proteins that aid in the recruitment of the translational machinery and/or alter the RNA secondary structure, which in turn promotes binding of the translation complex. ITAF mis-expression can alter translation of cancer-related mRNAs. For example, murine double minute (MDM2) is an oncoprotein that binds the IRES in the mRNA encoding the XIAP. Increased XIAP expression in the MDM2 overexpressing cells leads to a resistance to radiation-induced apoptosis (91). Alternatively, a loss of 2 other ITAFs, TCP80 and RHA diminishes p53 IRES activity and protein expression; promoting cell survival in response to DNA damage (92). Presumably, there are ITAFs whose increased expression is responsible for the enhanced Aurora A IRES activity. Because there is no in vivo assay to quantify Aurora A IRES activity in normal/immortalized cells, the level of these regulatory proteins would be a useful biomarker and provide a novel drug target to modulate Aurora A IRES activity in cancer cells. Two ITAFs known to be expressed in G2–M are PTB and upstream of ras (Unr). They both bind the PITSLRE p58 IRES and regulate the G2–M–specific expression of the PITSLRE p58 protein (59, 93). They can also regulate IRESes during other phases of the cell cycle, such as the Apaf-1 IRES during G1 (35). As noted earlier, there was no correlation between
PITSLRE and Aurora A IRES usage. Furthermore, knocking down Unr did not affect Aurora A IRES activity (Unpublished Data) and there does not seem to be any potential PTB-binding sites in the Aurora A 5′ leader. Consequently, there are likely to be additional ITAFs that regulate the Aurora A and possibly other mitotic IRESes. Indeed, it would be of interest to determine whether the Aurora A IRES represents another subset of G2–M functionally related IRESes.

Current anticancer drugs target Aurora A kinase activity as its inhibition leads to formation of a monopolar spindle and cell death (94). However, it has been difficult to design reagents that exhibit specificity for the ATP pocket of the Aurora A kinase (95, 96). Moreover, overexpressing a kinase dead mutant of Aurora A can still contribute to cellular immortalization (97).

Targeting the Aurora A mRNA could be an effective alternative approach to regulate expression of the kinase. HMEC (and WI-38 cells) yielded low Aurora A IRES activity, but surprisingly Aurora A protein expression was relatively unaffected when elf-4E levels were reduced. This result indicates that endogenous Aurora A mRNA in normal cells (e.g., HMEC) can be translated in both a cap- and IRES-dependent manner. We postulate that the HMEC cells use both translation initiation mechanisms to ensure expression of the Aurora A kinase. Inhibiting cap-dependent translation in these cells is compensated by the normal upregulation of IRES activity during G2–M. On the other hand, Aurora A expression was sensitive to the reduction of elf-4E in MCF-7 cells. Aurora A IRES activity (and protein expression) remains low and cannot compensate for the reduction in cap-dependent translation. This result suggests that reducing cap-dependent translation would decrease endogenous Aurora A protein in tumors that cannot use the Aurora A IRES. Finally, IRES-dependent translation was increased in the Aurora A protein overexpressing cells and is the principal translation initiation mechanism. Consequently, designing reagents targeting the IRES would inhibit Aurora A protein expression primarily in cancer cells that overexpress the kinase.

In summary, we have identified an IRES situated in the 5′ leader of the human Aurora A mRNA. In an examination of multiple cell lines, IRES activity was the only mechanism that correlated with Aurora A protein expression. Moreover, this mechanism seems to be upregulated early during cancer development and remains elevated as cell transformation advances indicating that targeting this mechanism may be beneficial for multiple stages of cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Development of methodology: T. Dobson, L.A. Krushel
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Dobson, J. Chen
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Dobson, J. Chen
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