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IL-6-induced stimulation of c-myc translation in multiple myeloma cells is mediated by myc IRES function and the RNA-binding protein, hnRNP A1¹

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Abstract

Prior work indicates that c-myc translation is upregulated in multiple myeloma cells. To test a role for IL-6 in myc translation, we studied the IL-6-responsive ANBL-6 and IL-6-autocrine U266 cell lines as well as primary patient samples. IL-6 increased c-myc translation which was resistant to rapamycin, indicating a mechanism independent of mTOR and cap-dependent translation. In contrast, the cytokine enhanced cap-independent translation via a stimulatory effect on the myc internal ribosome entry site (IRES). As known IRES-trans-activating factors (ITAFs) were unaffected by IL-6, we used a yeast-three-hybrid screen to identify novel ITAFs and identified hnRNP A1 (A1) as a mediator of the IL-6 effect. A1 specifically interacted with the myc IRES in filter binding assays as well as EMSAs. Treatment of myeloma cells with IL-6 induced serine phosphorylation of A1 and increased its binding to the myc IRES in vivo in myeloma cells. Primary patient samples also demonstrated binding between A1 and the IRES. RNA interference to knock down hnRNP A1 prevented an IL-6 increase in myc protein expression, myc IRES activity and cell growth. These data point to hnRNP A1 as a critical regulator of c-myc translation and a potential therapeutic target in multiple myeloma.

Keywords

multiple myeloma; interleukin-6; c-myc; hnRNP A1; IRES

INTRODUCTION

The c-myc gene encodes a transcription factor which is a key regulator of proliferation (1). Dysregulated myc expression plays a role in Burkitt's lymphoma, murine plasmacytoma and human multiple myeloma (MM) (2-4). In myeloma, dysregulation of myc can result from a gene translocation with juxtaposition of myc to the Ig enhancer (4). Another potential mechanism of dysregulated transcription is from stimulation with the myeloma growth factor IL-6 via a STAT-3-dependent mechanism. However, this cytokine effect is cell-type specific and IL-6 treatment of some cells actually results in decreased myc transcription (5,6). Myc over-expression in MM cells can also be due to upregulated translation through cap-

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independent mechanisms (7,8). Cap-independent translation is the fail-safe mechanism of protein expression when cap-dependent translation is prevented or when leaders contain structural elements which are inhibitory to scanning ribosomes (9-11). Translation initiation is then achieved via internal ribosome entry sites (IRES) in the mRNA's 5'UTR. The three dimensional IRES structure directly or indirectly recruits the 40S ribosomal subunit to the mRNA for translation initiation. Several IRES-associated trans-acting factors (ITAFs) are required for IRES activity. These factors bind to RNA at sites in the 5'UTR and induce conformational changes which facilitate recruitment of the ribosome to the IRES. Several publications (7,8) document a myeloma-specific upregulation of myc IRES function.

In this study, we tested whether the upregulated myc translation in MM cells could be further enhanced by the myeloma growth factor, IL-6. As IL-6 is an important growth-promoting cytokine for MM cells (12) and in MM patients (13), its ability to affect myc expression could support a pathophysiologic relevance of myc translation in this disease. In several MM cell line models as well as primary patient samples, the tumor growth factor IL-6 was shown to enhance IRES-dependent myc translation. In addition, this effect on the myc IRES was mediated by a novel myc ITAF, hnRNP A1. IL-6 induced serine phosphorylation of hnRNP A1 and significantly enhanced its binding to the myc IRES. Thus, RNP A1 is an important regulator of MM myc expression and MM tumor cell expansion.

MATERIALS AND METHODS

Cell lines, constructs, reagents

The ANBL-6 wild type, K-ras-transfected ANBL-6 and U266 cell lines were gifts from Dr. Brian Van Ness, University of Minnesota. The pRF construct was a kind gift of Dr. A. Willis, Univ. of Leicester, UK. The c-myc, and p27 IRESes were amplified from IMAGE clones 4667496 and 4298338 and cloned into the intercistronic region of pRF to generate pRmF and pRp27F. These have been previously described in detail (14). The pR-V-F, pSP65-R-F and pSP65-R-VEGF-F plasmids were gifts from Dr. Greg Goodall (Univ of Adelaide, Australia). They have also been previously described (15).

Primary myeloma samples

Primary patient myeloma cells were isolated from bone marrow by positive selection for CD38 as previously described (16). The purity was >99% plasma cells. These cells were then treated with or without IL-6 and subsequently studied for myc expression by Western blot and for myc IRES-dependent reporter expression by the dicistronic assay (see below). In addition, four primary myeloma samples that had been previously purified and cryopreserved, were thawed and washed, followed by immunoprecipitation of hnRNP A1 and assessment of bound myc IRES by IP-RT-PCR assay (see below).

Western and Northern blot

Western and Northern blot was performed as previously described (12,14,15).

Polysome analysis of translational state

As previously described (17), cells treated with or without IL-6 and with or without rapamycin were lysed in ice-cold lysis buffer supplemented with 100 ug/ml cycloheximide at 4°C. Following removal of nuclei and mitochondria, supernatants were layered onto 15-50% sucrose gradients and spun at 38,000 rpm for 2 hr at 4°C in a SW-40 rotor. Eleven 1 ml fractions were collected using an ISCO Density Gradient Fractionator at a flow rate of 3 ml/min. The UV absorbance at 254 nm of each fraction was measured to generate a polysome profile of the sucrose gradient that was used to differentiate between the monosome (fractions 1-3) and

polysome (fractions 4-11) fractions. The RNA from individual fractions was extracted using phenol/chloroform and precipitated in ETOH. The RNA of fractions 1-3 (monosome) were pooled as were the RNAs from fractions 4-11 (polysome-associated RNA). Northern blot analysis was performed on these pooled RNA fractions for myc or actin. The percent of total mRNA present (measured by densitometry of equally exposed autoradiographs) in the polysomal fractions versus the monosomal fractions were analyzed.

Dicistronic reporter assay

The reporter constructs were transfected into cell lines or primary MM cells using Lipofectamine Plus (Invitrogen) and normalized for transfection efficiency by co-transfection with pSVbetaGal (Promega). Transfection efficiency was generally 5-10%. A transfection efficiency of at least 5% was required for carrying out a dicistronic reporter assay. After 12-14 hrs, they were treated with or without IL-6 for varying durations. Cells were then harvested and Renilla luciferase, Firefly luciferase and beta-galactosidase activities were determined as previously described (14). To test IRES-dependent reporter expression from transfected RNA, polyadenylated dicistronic mRNAs were first generated in vitro (as previously described (15)) and 500 ng RNA were transfected by electroporation (230V for 25 ms). Transfection efficiency was again controlled by assaying beta-galactosidase activity.

Yeast methods and screening

All constructs used for the yeast three-hybrid assay were based on the RNA-Protein Hybrid Hunter System (Invitrogen, Carlsbad, CA). cDNA encoding full-length hnRNP A1 was inserted into pYesTrp3. A 233 nucleotide segment of the *c-myc* IRES was inserted into the hybrid RNA plasmid pRH5'. These plasmids were transformed into the strain L40uraMS2 (*MATa*, *ura3-52*, *leu2-3112*, *his3* Δ 200, *trp1* Δ 1, *ade2*, *LYS2::*(LexA_{op})₄-*HIS3*, *ura3::*(LexA_{op})₈-*LacZ* carrying pLexA/MS2/Zeo as previously described (18).

Filter binding assay

Indicated amounts of GST-hnRNP A1 were added to in vitro transcribed ³²P-labeled RNAs corresponding to either the *c-myc* IRES or the p27 IRES in separate reactions in a volume of 10 ml in buffer containing 5mM HEPES (pH 7.6), 30mM KCL, 2mM MgCl₂, 200mM DTT, 4% glycerol and 10 ng of yeast tRNA for 10 mins at room temperature. Eight ul of each binding reaction was applied to nitrocellulose membranes on a slot blot apparatus (Minifold II, Schleicher & Schuell). Membranes were washed, dried and signals quantified using a phosphorimager. Binding curves of three independent experiments were fitted by using SigmaPlot to determine the apparent dissociation constants.

Evaluation of in vivo binding of hnRNP A1 to myc IRES

The IP-RT-PCR assay was performed as previously described (19). Briefly, myeloma cell lines were treated with or without IL-6 for varying durations, treated with 1% formaldehyde and then hnRNP A1 was immunoprecipitated and bound RNA was evaluated by RT-PCR. Real time PCR for myc RNA and GAPDH RNA was performed on total extracted RNA from the immunoprecipitates. Gene amplifications for real time PCR were performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, California, USA). Each 20 μ L reaction in a 96-well plate comprised 9 μ L of cDNA template, 1 μ L of 20x primer mixtures for *c-myc* or GAPDH (containing two unlabelled primers for amplifying the *c-myc* or GAPDH sequence respectively and one 6-FAM dye labeled Taqman MGB Probe) and 10 μ L 2xTaqman Universal PCR Master mixed with AmpErase® UNG. Plates were sealed with adhesive optical film. After an initial 2 minute at 50 °C to activate ampErase® and a denaturation step of 10 min at 95°C, 60 cycles of amplification were performed with denaturation for 15 s at 95°C and annealing/extension for 1 minute at 60°C. All samples were

run in triplicate and no template controls were included in all plates for both c-myc and GAPDH. Comparative C_T method with c-myc and GAPDH in separate tubes was used according to manufacture's manual. C_T corresponded to the number of cycles at which the fluorescence signal can be detected above a threshold value, which is automatically set in our case.

siRNA analysis

siRNA transfections targeting human hnRNP A1 were performed using synthetic oligonucleotides (ON-TARGETplus SMARTpool™, Dharmacon, Lafayette, CO) directed at sequences within the coding region and 3'UTR. An siRNA with a scrambled sequence was used as a negative-targeting control. siRNAs were introduced into cells by electroporation (230V for 25 ms).

c-myc protein turnover assay

c-myc protein stability was determined as previously described (20). Briefly, cells were stimulated with or without IL-6 (100U/ml) overnight and then preincubated (15 min in DMEM without methionine and cysteine plus 5% dialyzed FCS) followed by a pulse with Tran³⁵S-label (ICN;300uCi/ml) for 1 hr. The cells were then chased (DMEM/10%FCS plus 400 mg/liter methionine) as indicated in fig 1. Lysates were immunoprecipitated with anti-myc antibody (Santa Cruz, #SC-42).

EMSA

The c-myc IRES, Apaf-1 IRES or GAPDH RNA were in vitro transcribed (mMessage T7 transcription kit, Ambion, Austin, TX) as previously described (14) and 5' end labeled with (³²P) gamma-ATP (GE Healthcare) using T4 polynucleotide kinase. Radiolabeled RNA was incubated with or without purified GST-hnRNP A1 in binding buffer containing 10 mM HEPES (pH7.5), 90 mM potassium acetate, 1.5 mM magnesium acetate, 2.5 mM DTT and 40U SUPERase-IN (Ambion) for 30 mins at 30°C. Subsequent EMSA procedures were performed as described previously (21).

Statistics

The t test was used to determine significance of differences between groups

RESULTS

Effects of IL-6 on myc expression and myc IRES activity in MM cells

We initially studied the ANBL-6 MM cell line, which expresses functional IL-6 receptors. ANBL-6 wild type cells are IL-6-dependent in that their growth ceases after approximately 1-2 weeks of IL-6-depletion. In contrast, "K-RAS" cells are ANBL-6 MM cells stably transfected with an activated K-Ras allele (22). These cells become IL-6-independent in that they proliferate in the absence of IL-6 although they still respond to exogenous IL-6 with enhanced growth. Activating mutations of K-RAS are frequently found in primary MM specimens and correlate with aggressive disease (23). The ANBL-6 cell line does not contain a myc-Ig translocation.

Both cell lines (wild type (WT) empty vector-transfected and K-RAS-transfected (K-RAS) ANBL-6 cells) were initially exposed to 100 U/ml of recombinant IL-6. Each cell line demonstrated a significant increase in growth as previously shown (22,24). This growth was accompanied by upregulation of c-myc protein expression in both lines (fig 1A, top). In contrast, fig 1A (bottom) shows that induction of c-myc RNA only occurred in wild type (WT) ANBL-6 cells. Two additional experiments confirmed these results and also demonstrated no

significant RNA induction in K-RAS mutated cells at earlier time points (30-120 mins). Northern data from all three experiments are summarized as bar graphs in fig 1A. The latter K-RAS-mutated cells may express heightened constitutive myc RNA expression which results from the RAS mutation with little room for further stimulation when exposed to IL-6. Nevertheless, these data demonstrate that the IL-6-induced upregulation of c-myc protein expression in mutant K-RAS-containing MM cells is not accompanied by increased RNA expression.

Additional studies, using the pulse-chase technique, demonstrated that the increased myc protein expression in IL-6-treated K-RAS cells was not due to increased protein stability. As shown in fig 1B, the opposite result was detected, namely a slight decrease in protein stability due to IL-6 exposure. A second possible explanation for enhanced protein expression could be an IL-6-induced stimulation of cap-dependent translation. We had previously demonstrated that IL-6 could activate mTOR in MM cells (24,25) which could achieve this effect. We, thus, addressed effects of IL-6 on myc translation using the methodology of Zong (26) which is based on the observation that well translated transcripts are associated with polysomes while poorly translated mRNAs are monosomal. ANBL-6 cells were treated with or without IL-6, polysomes were separated from monosomes on a sucrose gradient, and the associated RNA from these two groups was assessed by Northern analysis. Densitometric analysis of the signals obtained from monosomal vs polysomal fractions confirmed an enhancement in c-myc translation induced by IL-6. IL-6 induced an increase in polysome c-myc RNA from 28% to 44% in wild type ANBL-6 cells and from 25% to 52% in K-RAS mutant cells (fig 1C). Interestingly, however, the mTOR inhibitor rapamycin (at 1 and 10 nM) had no effect on enhanced translation although both concentrations inhibited mTOR mediated phosphorylation of p70S6kinase with complete ablation at 10 nM (fig 1D). This indicated that the increase in c-myc translation induced by IL-6 was not mediated by mTOR and cap-dependent translation and suggested a role for cap-independent translation.

To test whether IL-6 enhanced IRES-dependent cap-independent translation, we utilized the myc discistronic vector in which the myc 5'UTR, containing its IRES, was subcloned into the intracistronic space between the Renilla and Firefly luciferase ORFs in the parental pRF vector ((27), fig 2A). This generated the pRmF vector whose firefly luciferase translation would be driven by the 5'UTR. As a control, we also subcloned the p27 IRES into pRF, generating the pRp27F vector. Following transfection of wild type or K-RAS cells with the vectors, the mRNAs were tested for their ability to direct cap-independent translation (Firefly luciferase). Results (fig 2B) are normalized for transfection efficiency by co-transfection with a beta-galactosidase construct. The presence of the myc 5'UTR IRES in pRmF markedly enhanced Firefly luciferase expression (vs pRF) in both wild type and K-RAS lines while having no effect on Renilla luciferase expression, a monitor of cap-dependent translation. When exposed to 100 U/ml IL-6 a time-dependent increase in Firefly luciferase was demonstrated in both cell lines. As a negative control, figure 2B demonstrates that similar IL-6 treatment of K-RAS cells did not increase firefly luciferase driven by the p27 IRES (right panel). It is difficult to see in figure 2 because of the range of luciferase activity assayed (Y axis) but there was a very modest but significant increase in Renilla luciferase expression also following IL-6 exposure (to 150-175% of control values (no IL-6)). This is consistent with the positive effect of IL-6 on global cap-dependent translation secondary to activation of mTOR. A representative experiment with raw data is shown in supplemental figure 1.

To further support an effect of IL-6 on myc IRES activity in MM cells, we studied a third cell line, the U266 MM line, in which an autocrine IL-6 growth pathway exists. This cell line secretes IL-6 which, subsequent to its binding to IL-6 receptors, results in activation of many pathways and cell growth. As shown in fig 2C, U266 cells exhibit a huge amount of myc IRES activity, shown by the marked increase in Firefly luciferase expression in pRmF compared to

pRF. When the cells are exposed to neutralizing anti-IL-6 and blocking anti-IL-6R antibodies (+anti-IL-6 AB), Firefly luciferase is greatly inhibited while identical concentrations of isotype-specific control antibodies have no effect. To the right of fig 2C is shown an immunoblot assay for STAT-3 phosphorylation (STAT-3-P) used as a monitor for IL-6 signal transduction. It shows that the anti-IL-6/anti-IL6R antibodies interrupted the IL-6 autocrine loop while the control antibodies had no effect. Thus, these results in three IL-6-responsive MM cell lines demonstrate that this growth factor enhanced RAP-resistant myc translation and myc IRES activity. This upregulation of IRES activity completely explains the enhanced myc protein expression in IL-6-treated mutant RAS-containing cells and works together with enhanced RNA expression in wild type cells.

To rule out the possibility that IL-6 increased myc firefly luciferase expression because of effects on a cryptic myc promoter located in the 5'UTR, we exploited RNA transfection using dicistronic RNAs to avoid cryptic promoter activity. To achieve this, the control pRF dicistronic reporter construct or the corresponding construct containing the myc 5'UTR in the dicistronic space was inserted into the pSP65-20B vector which contains the SP6 RNA polymerase promoter and a 38 nucleotide poly(A) tail (gift of Dr. Greg Goodall). Polyadenylated dicistronic RNAs were generated (fig 3A) and transfected into ANBL-6 WT cells. As an additional control, we also used the dicistronic RNA which contains the VEGF 5' UTR in the intra-cistronic space (pR-V-F, generated as previously described (15)). The Renilla and Firefly luciferase activity was then measured and the relative luciferase activity of pRmF RNA was compared to the pRF control RNA in MM cells treated with or without IL-6. As shown in fig 3B, IL-6 significantly enhanced firefly luciferase expression in pRmF RNA-transfected cells by 2.5 x fold while having no effect on expression in cells transfected with control pRF RNA or in the additional control cells transfected with the VEGF 5'UTR-containing RNA (pR-V-F). This ruled out the possibility of a cryptic promoter in the myc IRES DNA that could have responded to IL-6 stimulation.

Identification of hnRNP A1 as a potential IL-6-responsive c-myc ITAF

We initially hypothesized that an IL-6-induced increase in enhancing ITAFs or a decrease in inhibitory ITAFs could explain an increased IRES activity. However, no significant effects of IL-6 were found on protein levels of known myc ITAFs (including PTB, hnRNP K, hnRNP E1/E2, hnRNP C1/C2 and unr) or their electrophoretic mobility (not shown). We, thus, utilized a yeast three-hybrid screen (supplemental fig 2) to identify novel proteins which specifically bound to the smallest construct containing sequences from the myc 5'UTR which retained full IRES activity, a nucleotide segment spanning -396 to -165 upstream of the initiation codon. From this screen we identified several clones encoding hnRNP A1 which specifically bound to the c-myc IRES sequence and was dependent on both the IRES sequence and the hnRNP A1 activation domain fusion for reporter expression.

Filter binding assays (fig 4A) and EMSA analysis (fig 4B) demonstrated specific binding of hnRNP A1 to the myc IRES. The *k*D for A1 binding to the myc IRES was approximately 200 nM while binding to the p27 IRES (darkened circles in fig 4A) was undetectable. In EMSAs, addition of recombinant hnRNP A1 retarded the migration of radiolabeled c-myc IRES but had no effect on migration of the Apaf-1 IRES or GAPDH RNA (fig 4B). In addition, when added as recombinant protein to extracts prepared from U87 cells in which endogenous hnRNP A1 had been silenced, hnRNP A1 markedly enhanced myc IRES-dependent reporter expression (28). These data indicate hnRNP A1 is an ITAF for the myc IRES.

Although IL-6 had no effect on the expression level of hnRNP A1 in MM cells (fig 4C), it increased the binding of A1 to the myc IRES *in vivo* as determined in an immunoprecipitation-RT-PCR assay (fig 4D). In this experiment, we treated both ANBL-6 wild type and "K-RAS" cell lines with IL-6 and, after varying incubation periods, immunoprecipitated hnRNP A1 and

performed PCR analysis on the precipitate for detection of myc IRES sequences. As shown in figure 4D, myc IRES RNA binding to hnRNP A1 was undetected in resting wild type ANBL-6 MM cells while a modest amount was bound to A1 in K-RAS cells. As a control, immunoprecipitation with non-specific IgG could not bring down any myc IRES sequences. Exposure to IL-6 induced an increase in binding of hnRNP A1 to the myc IRES in both cell lines in a time-dependent fashion with optimal binding at 0.5-3 hrs. To further confirm these data, we performed real time PCR for quantification of myc IRES sequences bound to hnRNP A1. As shown in figure 4E, the real time PCR data are consistent with a maximal induced binding after 3 hr of IL-6 exposure up to 7× fold over control (no IL-6). Thus, IL-6 increased the amount of hnRNP A1 bound to the myc IRES inside MM cells and this was not due to any increase in A1 expression.

Although IL-6 did not alter total expression of hnRNP A1, A1 became serine phosphorylated as shown in fig 4F. In this experiment, ANBL-6 MM cells were treated with or without IL-6 or, as a positive control for A1 phosphorylation, sorbitol (29). HN-RNP A1 was then immunoprecipitated and immunoblotted for phospho-serine or total A1. As shown, A1 serine phosphorylation due to IL-6 exposure was comparable to that of sorbitol treatment. In a time course experiment, serine phosphorylation of A1 in MM cells was identified by 2 hrs of exposure to IL-6 (fig 4F).

Effect of hnRNP A1 knockdown

To directly test a role for hnRNP A1 in the IL-6-induced stimulation of myc IRES function, A1 was silenced by siRNA, using a pool of three siRNA sequences. As shown in fig 5A (top gel), effective knockdown of A1 was accomplished in K-RAS cells with a pool of scrambled sequences having no effect. These cell lines were then treated with or without IL-6 and the IRES-dependent discistronic reporter assay was performed. As shown in fig 5B, A1 knockdown prevented the ability of IL-6 to stimulate myc IRES activity. In addition, there was a corresponding absence of myc protein upregulated expression in these IL-6-treated cells (fig 5A, bottom gel). Finally, the A1 siRNA silencing also prevented the ability of IL-6 to stimulate cell growth in vitro (fig 5C).

Studies in primary MM specimens

To test whether IL-6 could enhance myc IRES function in primary MM specimens, three patient bone marrow samples were processed (see Materials & Methods) to isolate pure myeloma tumor cell populations (>99% pure). The cells were treated with or without IL-6 (100 u/ml for 6 hrs) and then transiently transfected with the dicistronic reporters to test IRES activity. As shown in fig 6A and 6B, the patient samples demonstrated a significant enhancement of firefly luciferase expression (IRES activity) and myc protein expression following exposure to IL-6.

We were also able to investigate if hnRNP A1 binds the myc IRES in primary MM cells. Cryopreserved patient isolated myeloma cell specimens (>99% pure tumor cells, n=4) were thawed, RNA-protein binding was secured by treatment with 1% formaldehyde, and A1 was immunoprecipitated with anti-A1 antibody or control IgG. The immunoprecipitates were then analyzed for bound myc IRES RNA by RT-PCR. As shown in fig 6C, the myc IRES was present in the anti-A1 immunoprecipitates obtained from all the myeloma patient samples. Thus, in vivo binding between hnRNP A1 and the myc IRES is present in primary MM cells.

DISCUSSION

The results of this study indicate that part of the upregulation of myc protein expression in MM cells stimulated with IL-6 is due to enhanced IRES-dependent translation. This was shown in the ANBL-6 MM cell model, the U266 cell line and in primary MM specimens. Furthermore,

hnRNP A1 is an important protein which mediates IL-6-induced enhancement of myc translation. Importantly, siRNA knockdown of A1 expression in RAS-mutated MM cells prevented the stimulation of myc IRES activity, curtailed upregulation of myc protein expression and abrogated IL-6-enhanced cell growth.

HnRNP A1 has recently been identified as an ITAF for the FGF-2 and XIAP IRESes (30,31) and our data (28) support its role as a myc ITAF. The ability of IL-6 to increase the binding between A1 and the myc IRES suggests a possible mechanism that could explain how A1 mediates the IL-6 effect on IRES activity. This increased binding may facilitate the ability of A1 to induce the proper conformational change in the IRES required for recruitment to the 40S ribosomal subunit. Whether or not the detected IL-6-induced serine phosphorylation of hnRNP A1 is critical for the enhanced A1-IRES binding remains to be examined.

An additional potential mechanism that must be considered is that IL-6 enhances A1 cytoplasmic localization. Initial binding between A1 and the myc IRES presumably occurs in the nucleus where hnRNP A1 is primarily localized. However, A1 is a nuclear-cytoplasmic shuttling protein and is likely involved in the nuclear export of mRNAs like myc to the cytoplasm, the translationally competent compartment. Cytoplasmic shuttling is regulated by phosphorylation of A1 on 4 serine residues in the F peptide, an 18 amino acid segment in the C-terminus of the protein (32,33). Such phosphorylation is mediated by MNK kinases (34) which are activated downstream of p38 MAPK (35). Although IL-6 does not activate the p38MAPK cascade, it is well accepted that it can activate the RAS-RAF-MEK-ERK MAPK cascade (12). Interestingly, MNKs can also be activated by ERK (36). Thus, an additional potential mechanism of IL-6 stimulation of A1-IRES function is via stimulation of ERK with subsequent activation of MNKs, MNK-dependent phosphorylation of A1 at the F peptide and enhanced shuttling of A1/Myc RNA to the cytoplasmic translational compartment. Experiments are underway to determine which residues of A1 become serine phosphorylated by IL-6 and whether there is an IL-6-dependent increase in A1 cytoplasmic localization.

Recent work (37) supports the hypothesis that dysregulation of c-myc expression is key to the progression from the pre-malignant MGUS syndrome to overt myeloma. Gene set enrichment analysis identified a myc activation gene set signature that was significantly associated with MM as compared to MGUS. Since myc translocations to the Ig enhancer element only occur in approximately 15% of newly diagnosed patients (38), it is possible that a heightened myc translation is also important in progression and this may be facilitated by hnRNP A1 following IL-6 stimulation.

In summary, our data underscore the potential importance of a myc RNA-binding protein, hnRNP A1, in the enhanced myc translation that occurs in myeloma cells whose growth is stimulated by IL-6. A further understanding of the regulation of A1 and myc IRES function may allow future manipulation of myeloma tumor growth by targeting A1 therapeutically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

In **A**, ANBL-6 wild type (WT) or mutant K-RAS-transfected (K-RAS) ANBL-6 MM cells were exposed to IL-6 (100U/ml) for varying durations (0(control=C), 3, 6, 12 or 24 hrs) and Western blot performed for c-myc or actin protein (top gel) and Northern blot for c-myc or GAPDH RNA. Bottom graph shows fold increase in c-myc/GAPDH RNA (mean of 3 separate experiments with SDs <5% of the mean in all groups). In **B**, myc protein stability assayed (as described in Materials & Methods) in K-RAS cells. Cells were treated with or without IL-6 overnight, pulsed for 1 hr and chased for 0, 30, 60 or 120 minutes. Autoradiography performed on immunoprecipitated c-myc (top). Lane 5 is a control where non-specific Ig antibody was used in immunoprecipitation. Densitometric analysis is graphed below (x=+IL-6, o=control). The experiment was repeated once with identical results. In **C**, myc translation polysome profile assay (see Materials & Methods) in WT or K-RAS cells treated with or without IL-6 (100u/ml)+/- rapamycin (RAP) used at 1 or 10 nM. Results are mean+/-SD of 3 experiments. Significant ($p<0.05$) increases in translational state (% myc RNA attached to polysomes) was induced by IL-6 in both cell lines in presence or absence of rapamycin. In **D**, Western blot shown for expression of total p70 or phosphorylated p70 (P70-P) in the WT cells treated with or without rapamycin at 1 and 10 nM.

Fig 2.

In **A**, pRF, pRmF and pRp27F reporter plasmids shown. In **B**, Firefly (dark bars) or Renilla (open bars) luciferase expression in pRF vs pRmF in WT or K-RAS cells, treated with IL-6 for 0, 3, 5 or 7 hrs. All luciferase activity are normalized to the luciferase values (both Renilla and Firefly) obtained for pRF in the absence of IL-6 stimulation which is designated as a value of "1". In right panel, lack of effect of IL-6 on p27 IRES-dependent expression is shown. In **C**, myc IRES activity (firefly luciferase) in IL-6-autocrine U266 MM cells treated with anti-IL-6 neutralizing or control antibodies. Immunoblot to right shows ability of anti-IL-6 antibodies to inhibit constitutive STAT-3 phosphorylation in U266 cells.

Fig 3.

In **A** is shown denaturing gel electrophoresis of in vitro transcribed pRF, pR-V-F or pRmF mRNA (M=markers). In **B**, polyadenylated dicistronic pRF, pRmF or pR-V-F RNAs were transfected into WT cells treated with or without IL-6 and firefly (dark bars) or Renilla (open bars) luciferase expression assessed. Data are mean $\pm$ SD of quadruplicate samples. A significant ($p<0.05$) increase in firefly luciferase expression induced by IL-6 was only seen in pRmF-transfected cells.

Fig 4.

In **A**, filter binding assay between GST-hnRNP A1 and 2 separate IRESes. The open circle represents the myc IRES and the darkened circles represent the p27 IRES as a control. In **B**, EMSA analysis performed to demonstrate binding of hnRNP A1 to the myc IRES (top panel) but not to the Apaf-1 IRES (middle panel) or to GAPDH RNA (lower panel). In **C**, Western blot for hnRNP A1 protein expression in ANBL-6 cells treated with IL-6 for 0, 2 or 4 hrs. In **D**, wild type (WT) or K-RAS-mutated (K-RAS) ANBL-6 cells were treated with IL-6 for 0-12 hrs and, at varying time points, hnRNP A1 was immunoprecipitated with anti-A1 antibody (hnRNP A1) or control antibody (IgG). RT-PCR was then performed on the immunoprecipitates for presence of the myc IRES. In **E**, hnRNP A1 was immunoprecipitated as in **B** after varying durations of IL-6 exposure and real time PCR performed for quantification of the attached myc IRES. Open bars are WT ANBL-6 cells and dark bars are K-RAS cells. In **F**, ANBL-6 cells treated with IL-6 (100u/ml) or sorbitol (S, 600 mM) for 3 hrs (upper gels) or IL-6 for varying time points (lower gels) after which A1 was immunoprecipitated and the precipitate was immunoblotted with an antibody against total A1 or phosphoserine.

Fig 5.

In **A)**, K-RAS cells were electroporated with siRNA for hnRNP A1 (siRNA) or control scrambled sequences (SCR). Sixteen hrs later, viable cells were immunoblotted for expression of hnRNP A1 (A1) or actin (top panel of blots) or treated with or without IL-6 (100U/ml, 24 hrs), after which immunoblot assay was performed for expression of c-myc or actin (bottom panel of blots). In **B)**, K-RAS cells were electroporated and treated with or without IL-6 as in **A)**. Cells were transiently transfected with the dicistronic pRF or pRmF reporter plasmids and luciferase expression was assayed. Results are Firefly/Renilla ratios, mean $\pm$ SDs of 3 experiments. Cells knocked down for A1 expression (siA1) showed no IL-6-induced increase in Firefly/Renilla ratios while cells exposed to scrambled control (SCR) did show a significant increase ($p < 0.05$). In **C)**, K-RAS cells were electroporated with SCR or siRNA and treated with or without IL-6 as in **B)** and fold increase in cell growth assayed 72 hrs later. Results are fold increase over control (no IL-6), mean $\pm$ SD of 3 experiments.

Fig 6.

In **A) and B)**, primary myeloma cells were isolated from 3 separate patients and treated with or without IL-6. Eighteen hrs later, immunoblot assay was performed on an aliquot of cells for c-myc or actin expression (in A) and the dicistronic reporter assay performed on another aliquot (in B). Results in B are presented as Firefly (FF)/Renilla (R) luciferase ratios for each patient where 1 is arbitrarily identified as the pRF values in absence of IL-6. In **C)**, cryopreserved primary isolated myeloma cells from four patients were thawed and protein extracts immunoprecipitated with anti-hnRNP A1 antibody (A1) or control IgG. RT-PCR analysis was then performed for identification of hnRNP A1 IRES bound to the immunoprecipitated hnRNP A1.