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Peptides modeled on the RGG domain of AUF1/hnRNP-D regulate 3'UTR-dependent gene expression

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Abstract

Messenger RNA binding proteins control post-transcriptional gene expression of targeted mRNA's. The RGG (arginine-glycine-glycine) domain of the AUF1/hnRNP-D mRNA binding protein is a regulatory region that is essential for protein function and is the focus of this report. Previously, we have established that AUF1-RGG peptides, modeled on the RGG domain of AUF1, repress expression of the macrophage cytokine, VEGF. This report expands studies on AUF1-RGG peptides and evaluates the role of post-translational modifications of the AUF1 protein. Tandem to the RGG domain in AUF1-RGG peptides are a poly-glutamine motif and a nuclear localization signal (NLS). This report shows that a minimal 31-amino acid AUF1-RGG peptide that lacks the poly-glutamine and NLS motifs retains activity on a VEGF-3'UTR reporter. In addition, studies have shown that arginines in RGG motifs of mRNA binding proteins may be methylated with resulting changes in protein function. To determine if the RGG motif in AUF1 is affected by cell activation, mass spectroscopy analysis was performed on AUF1 expressed in RAW-264.7 cells. In resting cells, arginines in the first and second RGG motifs are monomethylated. When RAW-264.7 cells are activated with lipopolysaccharide, the arginines are dimethylated. To evaluate if the arginine residues are essential for AUF1-RGG activity, the methylatable arginines in the AUF1-3RGG peptide were mutated to lysine or alanine. The R→K and R→A mutants lack activity. These results support the hypothesis that the RGG domain of AUF1 is a regulatory motif. We also demonstrate that PI3K/AKT inhibitors reduce VEGF gene expression. Although immunoscreening of AUF1 suggests that LPS and PI3K inhibitors alter the phosphorylation status of AUF1-p37, mass spectroscopy results show that the p37 AUF1 isoform is not phosphorylated with or without lipopolysaccharide stimulation. In summary, arginines in the

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RGG domain of AUF1 are methylated, and AUF1-RGG peptides modeled on the RGG domain may be novel reagents that reduce macrophage activation in inflammation.

Keywords

AUF1; hnRNP-D; mRNA binding protein; RGG domain; AURE; arginine methylation; VEGF

1. Introduction

In an inflammatory pathology like sepsis, invading pathogens multiply and cells of the immune system, including resident macrophages, respond by producing cytokines and initiating other defense mechanisms [1,2]. If the immune system is overwhelmed, the expanding pathogen population triggers a still greater immune response, leading to severe inflammation, hypotension and septic shock [1,2]. Organ failure, such as acute kidney injury, follows the inflammatory response [2]. Severe inflammation is characterized by a rapid increase in cytokines, often referred to as a “cytokine storm” [1,2]. Because the onset of severe inflammation is difficult to recognize and the disease progresses very rapidly, diagnosis is often too late. Sepsis is the leading cause of death in emergency medicine [3]. Rapid and powerful intervention is needed when severe inflammation is discovered, and treating multiple targets is critical.

Vascular endothelial growth factor (VEGF) has multiple biological functions, including action as a permeability factor, a growth factor in blood vessel growth, and as a cytokine in immune cell activation [4–6]. In the early stages of sepsis, VEGF promotes influx of immune cells and activates macrophages [2,4,7–10]. As inflammation increases, VEGF promotes hypotension, leading to septic shock. The VEGF protein recognizes a family of cellular receptors [11]. With macrophages, VEGF stimulates through VEGFR-1 (Flt-1) [4,11–13]. Growth factor activation of the PI3K/AKT signaling pathway stimulates transcription and translation of the VEGF gene [14–16]. Signaling pathways also affect mRNA regulatory proteins that act as cytoplasmic gatekeepers, controlling mRNA transport, storage, translation, and degradation [17]. In macrophages, our group showed that the mRNA regulatory protein AUF1/hnRNP D decreases VEGF gene expression by acting on the 3'UTR of VEGF mRNA [18]. Regulation of VEGF by AUF1 has also recently been reported in cancer cells [19].

There are four AUF1 isoforms that are created by alternative RNA splicing [reviewed in 37]. Isoforms are named for their apparent protein molecular weight (p37, p40, p42, p45). The two larger isoforms, p42 and p45, retain Exon 7. The Exon 7 insert has a nuclear localization signal that contributes to nuclear localization of p42 and p45 [37]. The Exon 2 insert is found in p40 and p45 and contains serine and threonine residues that are phosphorylated in human AUF1 [37]. The p37 and p40 isoforms are more cytoplasmic and have the most significant regulatory effect on mRNA translation [25]. This report focuses on the cytoplasmic isoform, AUF1-p37.

Genetic deletion of AUF1 in mice increases susceptibility to endotoxemia and activates macrophages [20], demonstrating the clinical relevance of AUF1. To control VEGF gene expression we sought to identify novel regulators of AUF1 activity based on regulatory regions of the AUF1 protein. An RGG domain in the C-terminal end of AUF1 contains three RGG motifs. Early reports showed that RGG domains may participate in RNA binding, but deletion of a large part of the RGG domain from AUF1 did not affect RNA binding affinity [21], suggesting that the RGG domain regulates AUF1 activity independent of direct RNA binding. The RGG domains are found in proteins associated with mRNA ribonuclear

particles [17], and an essential protein modification of RGG proteins is the enzymatic methylation of arginine residues in RGG motifs [22,23]. Blocking methylation of the RGG domain in hnRNP A2 affected cellular localization [24]. Although the RGG domain in AUF1 is involved in nuclear transport [25], blockade of arginine methylation did not affect localization of AUF1 [26]. To investigate the regulation of AUF1 activity by the RGG domain, we expressed peptides modeled on the AUF1 RGG domain in cultured cells. The C-terminal 55 and 43 amino acid peptides each contain the three RGG motifs of AUF1. Surprisingly, the AUF1-RGG peptides mimicked full-length AUF1, reducing VEGF protein expression and inhibiting the activity of the VEGF-3'UTR luciferase reporter [18]. These results suggest that AUF1-RGG peptides repress 3'UTR-dependent gene expression by activating AUF1, but the mechanism is not clear. This report establishes the importance of the RGG domain in cell activation and shows that the AUF1-RGG peptides may be an important new tool for treating diseases where blockade of mRNA translation is a rapid method to shut down expression of multiple 3'UTR-regulated disease genes. Development of rationally designed small molecule drugs based on the RGG motif will be a novel approach to control cytokine gene expression in complex diseases.

This report studies the mechanism of action by AUF1-RGG peptides to alter gene expression. To identify the active component of AUF1-RGG peptides, we have performed sequence analysis on peptide motifs tandem to the RGG domain. Arginine residues in RGG domains of other proteins are often methylated, and methylation can change with cell activation [22–24]. To determine if the RGG domain is methylated and if the methylation changes with cell activation, we perform mass spectrometry analysis on AUF1 in resting and activated macrophages. Mass spectroscopy results show that four arginines are methylated in AUF1. These arginine residues were mutated to create arginine→lysine and arginine→alanine mutant AUF1-RGG peptides. The effects of these mutations are examined to determine if they retain activity on the VEGF-3'UTR reporter. Together, these results support previous findings showing that the RGG domain is a regulatory module of AUF1. Finally, signaling pathways controls mRNA regulatory proteins, and we ask whether the PI3K signaling pathway affects VEGF expression or the phosphorylation status of AUF1.

2. Materials and methods

2.1 Reagents

Lipopolysaccharide (LPS) was from Sigma. Gentamycin sulfate (G418) was from Fisher. Penicillin/streptomycin was from Invitrogen. LY294002 was from Tocris.

2.2. Cell culture and stable cell lines

The RAW-264.7 monocyte-macrophage cell line was obtained from ATCC and maintained as previously described [4]. Briefly, medium was low-glucose (1 g/mL) DMEM supplemented with 10% heat-inactivated fetal bovine serum. RAW-264.7 cells were grown without antibiotics. Stable cell lines were created using parental RAW-264.7 cells. Kill curve studies showed that 500 µg/mL of G418 was the lowest dose that gave 100% killing. Cells were transfected with plasmids (pcDNA-3.1 parent plasmid, Invitrogen) that confer G418 resistance. Transfected cells were incubated in DMEM/10% FBS/G418 for 4–6 weeks in 10 cm tissue culture plates. Colonies were selected and grown in six-well plates in DMEM/10% FBS/G418/penicillin/streptomycin. After at least five passages the expression of AUF1-RGG peptide in clones was confirmed by RT-PCR.

2.3. Cell transfection and luciferase assay

For luciferase reporter transfection studies, cultured cells were plated 24 hours prior to transfection in clear bottom 96-well tissue culture plates with sufficient cells to give ~50% confluence at the time of transfection (~40,000 cells per well). Plasmid DNA was complexed with Lipofectamine-2000 as described by the manufacturer (Invitrogen). Cells were transfected with luciferase constructs at 50 ng of plasmid DNA per well. Twenty-four hr after transfection, cells were lysed with Cell Culture Lysis Reagent (Promega). Lysates were frozen at -80°C . The luciferase activity was determined following addition of luciferin substrate (Promega) using a Centro LB 960 (Berthold) luminometer. Transfections were performed in three or six replicate wells. Reporter activity of stable cell lines is expressed relative to reporter activity in the control cell line ("PC"). For transient transfection experiments, control cells were transfected with the parental plasmid, pcDNA-3.1. All experiments were performed two or more times. The efficiency of plasmid transfection was measured in separate experiments by co-transfection with a plasmid expressing firefly luciferase plus a plasmid expressing renilla sea pansy luciferase (pRL-SV40, Promega). Sea pansy luciferase was titrated to produce 2–10% of the firefly luciferase activity. Correction of firefly activity with renilla activity did not change the variation in the firefly data.

2.4. RNA isolation and Real-Time PCR

Cellular RNA was isolated using the RNeasy kit (Qiagen). The RNA concentration was quantified by absorption (NanoDrop, Thermofisher Scientific). Total RNA (2.5–5 μg) was treated with Turbo-DNA-free (Ambion) to remove contaminating DNA, and cDNA was synthesized using the Maxima First Strand cDNA kit with RevertAid reverse transcriptase (Fermentas). Real-time PCR was performed with Maxima SYBR Green 2X mix (Fermentas) on a BioRad CFX-96 thermocycler using standard conditions. Levels of mRNA (Ct) were normalized to the level of actin mRNA in the same sample. PCR products from different inserts displayed unique melting temperatures, and melting curves were determined on all reactions to confirm single PCR products. Primer pairs used to confirm AUF1-RGG peptide expression are: forward primer, ("T7") 5'-TAATACGACTCACTATAGGG-3'; reverse primer, ("BGH") 5'-CCTCGACTGTGCCTTCTA-3'. Following real-time amplification, PCR product size was determined by separation on 2% NuSieve DNA gels, staining with Syber Gold (Molecular Probes), and imaging on VersaDoc (BioRad).

2.5. Plasmid Construction

Luciferase Constructs—The parental VEGF-3'UTR (mouse VEGF-3'UTR, nt-209–1747) luciferase expression plasmids were constructed as previously described [4]. Briefly, the luciferase gene from the Promega pGL3 expression plasmid was PCR amplified and cloned into the mammalian expression plasmid, pcDNA-3.1 (Invitrogen). The VEGF 3'UTR was PCR amplified and cloned into the 3'UTR of the luciferase gene. The integrity of constructs was verified by DNA sequencing. The human TNF-AURE reporter contains nt 1306–1406 of the NCBI Reference sequence NM_000594. The human GLUT1-AURE reporter [28] contains nt 2231–2265 of the NCBI Reference sequence NM_006516.

AUF1-RGG expression constructs—Peptides of the AUF1 RGG domain were generated by PCR and cloned into pcDNA-3.1-V5-His (Invitrogen) by TA cloning. Amino acid sequences and numbering of AUF1-RGG peptides are shown in Figure 1. An initiating methionine codon was introduced into all peptides.

AUF1-RGG point mutants—Point mutants were created that convert the methylatable arginines in the RGG peptide to either lysine or alanine. The methylatable arginines mutated are: R-253, R-259, R-263, and R-277. Peptide sequences are:

AUF1-3RGG-WT	MGSRGGFAGRARGRGGDQQSGYGKVSRRGGHQ
AUF1-3RGG-R→K	MGSKGGFAGKARGKGGDQQSGYGKVSRRGGHQ
AUF1-3RGG-R→A	MGSAGGFAGAARGAGGDQQSGYGKVSRRGGHQ

The “AUF1-3RGG” was used as template to create the “AUF1-3RGG→K” and “AUF1-3RGG→A” plasmids in pcDNA-3.1 by PCR and TA cloning. Constructs and all mutations were confirmed by DNA sequencing. Equivalent expression levels of wild-type (AUF1-3RGG) and mutant constructs were confirmed by real-time PCR 24 hr after transient transfection.

2.6. Immunoblotting

Immunoblotting was performed as previously described [18]. Briefly, total protein was measured by Bradford (BioRad Protein Assay) and equal amounts of protein were separated on Invitrogen 4–12% Tris-glycine gels and transferred to PVDF membranes by wet blotting (BioRad). Blots were probed with antibodies to VEGF (clone 4G3, Sigma) and developed with HRP secondary antibodies (Sigma). Images were captured using X-Omat film (Kodak) or the Versa-Doc imaging system (BioRad). Protein levels were quantitated by scanning immunoblots or Versa-Doc digital images. The image intensities were measured using NIH ImageJ. Protein bands were normalized to α -tubulin (Sigma).

2.7. Immunopurification of AUF1

For immunopurification, 1.5×10^7 RAW-264.7 cells were plated in 10 cm plates and transfected after 18 hr with the plasmid expressing AUF1-p37 coupled to the V5 epitope tag. Some cells were treated with LPS (50 ng/mL) 4 hr after transfection. After 24 hr, cells were rinsed twice on ice for 5 min with cold PBS and lysed on ice with 0.75 ml cold lysis buffer (PBS, 2 mM MgCl₂, 0.5% Tween-20, 0.5 mM DTT, Roche “Complete” protease inhibitor mix). Lysates were frozen at –80°C, thawed and centrifuged cold at 15,000xg for 10 minutes and the supernatant saved. Agarose anti-V5 beads (50 μ L) (Sigma) were washed with PBST and incubated with lysate overnight at 4°C with continuous mixing. Beads were washed 4 times with PBST, suspended in SDS sample buffer, heated at 85°C for 10 min, and loaded on a 4–16% gel. Gels were stained with silver (SilverSnap for Mass Spectroscopy, Pierce). Separate gels were stained with Simply Blue (Invitrogen) to identify AUF1 proteins. Isolated AUF1 protein bands were excised for mass spectroscopy. Control immunopurifications were performed without adding antibody and brought down no AUF1 protein.

2.8. LC-MS/MS analysis of post-translational modifications of AUF1

Liquid chromatography mass spectrometry (LC-MS/MS) analysis was performed essentially as described [29]. Prior to treatment with trypsin (10 ng/ μ L), cysteines in the AUF1 were reduced and blocked by incubation with iodoacetamide under reducing conditions. The reactions were terminated with acetic acid, and then subjected to electrospray ionization (ESI) LC/MS, employing a fused-silica microcapillary C18 LC column (12 cm $\times$ 75 μ m id). The ESI was fitted onto a linear trap quadrupole (LTQ) Orbitrap mass spectrometer (Thermo Electron). Six μ L of tryptic peptide samples were injected and separated by applying a gradient of 3–60% acetonitrile in 0.1% formic acid at a flow rate of 250 nL/min for 45 min. Mass spectrometry data were acquired in a data-dependent acquisition mode, in which a full orbitrap-MS scan (from m/z 400–1700, resolution $r=30,000$ at m/z 400) was followed by 10 LTQ-MS/MS scans of the most abundant ions. Obtained MS spectra were searched against a single AUF1 protein sequence database using SEQUEST (Bioworks software, v3.3.1;

Thermo Electron). The search parameters permitted a 0.05 Da peptide MS tolerance and a 1.4 Da MS/MS tolerance. Oxidation of methionine (M) and carboxymethylation of cysteines (C) were allowed as variable modifications. The other variable modification of AUF1 was set up as +14.0 and +28.0 on K,N,R to find possible methylated and dimethylated sites. Up to two missed tryptic peptide cleavages were considered. The cutoffs for SEQUEST assignment were cross-correlation (Xcorr) scores greater than 1.9, 2.5, and 2.9 for peptide charge states of 1, 2, and 3, respectively. All identified peptides were inspected manually.

2.9. Statistical analysis

Results are shown as mean $\pm$ standard deviation (SD). Significant differences were determined by two-tailed T-test using GraphPad Prism, and differences with $p < 0.05$ was considered significant.

3. Results

3.1. Effects of AUF1-RGG peptides on endogenous VEGF protein levels in stable cell lines

The amino acid sequences of AUF1-RGG peptides are shown in Figure 1. Plasmids expressing the “AUF1-Ex6+8” and “AUF1-QRGG” peptides were used to create G418-resistant RAW-264.7 monocyte-macrophage stable cell lines. Control G418-resistant cell lines (“PC”) were made using the pcDNA-3.1 plasmid containing no AUF1 sequence. In Figure 2, control PC clones or clones created using the AUF1-Ex6+8 peptide were immunoblotted for VEGF protein. Endogenous VEGF protein was reduced by approximately 50% in strongly positive AUF1-Ex6+8 clones (“6+8-2,” “6+8-3” and “6+8-5”). These results support previous experiments using transient transfection where the VEGF protein level was decreased by 35% for the AUF1-Ex6+8 peptide and 40% for the AUF1-QRGG peptide [18].

3.2. Effects of AUF1-RGG peptides on VEGF 3'UTR-dependent gene expression in stable cell lines

Expression of VEGF in macrophages increases with LPS cell activation, and the 3'UTR of VEGF mRNA mediates VEGF expression [4,18]. To confirm that activation of macrophages with LPS increased VEGF-3'UTR luciferase reporter, the “PC-2” control stable cell line was transfected with the reporter and treated with LPS. Reporter activity increased by 3-fold (Figure 3A). When AUF1-RGG stable cells were not treated with LPS, the VEGF-3'UTR reporter showed reduced reporter activity in AUF1-Ex6+8 cells (40%) and AUF1-QRGG cells (70%), as compared with the PC-2 control cell line (Figure 3B). If stable cell lines were treated with LPS, reporter activity was also reduced in AUF1-Ex6+8 cells (65%) and AUF1-QRGG cells (80%) (Figure 3C). In separate studies, luciferase reporters containing the AURE from TNF or glucose transporter-1 (GLUT1) were reduced by more than 50% in AUF1-Ex6+8 and AUF1-QRGG stable cells (data not shown). The results with TNF and GLUT1 reporters may demonstrate that AUF1-RGG peptides reduce gene expression by acting on AURE elements in the 3'UTR of mRNA.

3.3. The poly-glutamine and nuclear localization sequences in AUF1-RGG peptides are not required for AUF1-RGG peptide activity

Stable cells expressing the AUF1-QRGG or AUF1-Ex6+8 peptides both inhibited AURE-dependent reporters similarly (Figure 3), demonstrating that the eleven amino acids in the AUF1-Ex6+8 peptide N-terminal to the poly-glutamine motif (see Figure 1) are not essential for activity. The AUF1-Ex6+8 and AUF1-QRGG peptides retain (a) a poly-glutamine motif N-terminal to the RGG domain, and (b) a NLS-PY motif C-terminal to the RGG domain (see Figure 1).

QQQQQ motif—The role of poly-glutamine motifs has been considered with respect to the function of AUF1 [21]. Triplet repeat expansion in DNA results in poly-glutamine stretches, and poly-glutamine stretches suppress VEGF expression [30]. To evaluate the role of the poly-glutamine and NLS-PY motifs, a shorter “AUF1-3RGG” peptide was designed (Figure 1). The “AUF1-3RGG” retains all three RGG motifs but lacks the poly-glutamine and NLS-PY motifs. The functionality of the “AUF1-3RGG” peptide was examined by transient transfection assay with the VEGF-3′UTR reporter. The “AUF1-3RGG” peptide retained activity (inhibited 40%) (Figure 4).

NLS-PY motif—The NLS-PY motif in AUF1-RGG peptides may affect the activity of endogenous NLS-PY signals on AUF1 or other shuttling proteins (hnRNP A1, hnRNP M) [31]. To determine if the NLS-PY had an effect on gene expression, an “AUF1-1RGG-PY” peptide was designed that contains the C-terminal “PY” motif and the third RGG motif (Figure 1). The “AUF1-1RGG-PY” activity lacked activity (Figure 4).

Together, these results show that an AUF1-RGG peptide that only contains the three RGG motifs comprises an active RGG regulatory motif.

3.4. Methylation of arginines in the RGG domain of AUF1

To further understand the role of the RGG domain we next evaluated methylation of arginine residues in RGG motifs. Arginine methylation is a post-translational protein modification that alters protein function [22–24]. We have reported that blockade of arginine methylation affects cellular localization of the RNA binding protein hnRNP A2 [24] and decreases AUF1 protein levels [18]. Mass spectroscopy analysis showed that only the arginine residues in the RGG motifs of hnRNP A2 were methylated [24]. To determine how AUF1 is methylated in macrophages, and if cell activation affects methylation, RAW-264.7 cells were transfected with V5-tagged AUF1-p37 and were not treated or treated with LPS. The AUF1 protein was purified and the protein modifications identified by mass spectroscopy. Results show that AUF1 from untreated cells is monomethylated on arginines in the first and second RGG motifs (Figure 5A1 and A2). In contrast, LPS activation results in dimethylation of the arginine residues in the first and second RGG motifs (Figure 5B1 and B2). The “RAR” motif is a less frequent site of methylation, and the first arginine in the RAR motif between the first and second RGG motifs was monomethylated in LPS-activated cells (Figure 5B1). No other methylated arginines in the AUF1 protein were identified in untreated or LPS treated cells. In addition, one lysine residue (K164) was dimethylated in untreated cells (Figure 5A3). This lysine residue was not methylated in LPS-treated cells (data not shown). These results show that methylated arginines are found in the RGG domain of AUF1 and that methylation changes with cell activation. To evaluate if the arginine residues in the AUF1-3RGG sequence were essential for activity, we created arginine→lysine and arginine→alanine point mutants. All four methylatable arginines were mutated. In transient transfection studies, the wild-type AUF1-3RGG construct reduced VEGF-3′UTR-luciferase activity by 45%. In contrast, the AUF1-3RGG-R→K and AUF1-3RGG-R→A constructs were inactive (Figure 6). These mutation studies demonstrate that methylatable arginines in the RGG motif are required for AUF1-RGG activity.

3.5. PI3K regulation of VEGF and phosphorylation status of AUF1

A recent study demonstrated that inhibition of PI3K decreases VEGF protein in macrophages [32], but the mechanism was not explored. Several groups have reported that some isoforms of AUF1 are phosphorylated [33,34]. To determine if PI3K/AKT kinase might act directly on AUF1 to change 3′UTR-regulated gene regulation, we first confirmed the effect of the PI3K pathway on VEGF mRNA levels in RAW-264.7 cells using the PI3K

inhibitor LY294002. Four hr treatments with LY294002 (5 μ M) decreased VEGF mRNA by 60% (Figure 7A). To determine if inhibition of PI3K affects post-transcriptional gene regulation, we transfected cells with the VEGF-3'UTR reporter and treated with LY294002 for 4 hr. PI3K inhibition decreased reporter expression decreased by 25% (Figure 7B). The different magnitude of effects shown in Figures 7A and 7B likely reflects the molecular differences in half-life kinetics for mRNA and protein, as has been reported [50].

To determine if PI3K signaling kinases may act directly on AUF1, preliminary studies explored the phosphorylation status of AUF1-p37 in macrophages. Cultured RAW-264.7 cells were transfected with AUF1-p37-V5 and then not treated or treated with LY294002, or LPS, or both. Purified AUF1-p37-V5 was immunostained with antibodies to phosphorylated amino acids. Both phospho-threonine and phospho-serine signals increased with LPS treatment and the effect was reversed with LY294002 treatment (data not shown). To determine if AUF1-p37 was phosphorylated, we analyzed mass spectroscopy results for the phosphorylation status of AUF1-p37. However, no residues in p37 were phosphorylated with or without LPS treatment. This result suggests that the changes in phosphorylation shown by immunostaining may result from another protein that associates with AUF1-p37 and co-migrates with AUF1-p37 on PAGE gels. A candidate phosphoprotein known to associate with AUF1-p37 is AUF1-p40 [34]. However, AUF1-p40 was not identified by mass spectroscopy analysis. Three other phosphoproteins were identified in pulldowns of (a) untreated but not LPS-treated cells (60S acidic ribosomal protein P0, *Rplp0*), or (b) LPS-treated but not untreated cells (hnRNP-A/B2, *Hnrnpab*, and hnRNP-A3, *Hnrnpa3*) (phosphorylation status determined using PhosphoSitePlus, <http://www.phosphosite.org>). Further study is planned to determine if phosphorylation of these proteins is controlled by PI3K signaling, and if these proteins affect the activity of AUF1.

4. Discussion

Gene expression is regulated at many stages. A small subset of genes is regulated by AU-rich element sequence elements in the 3'-untranslated regions of mRNA [28,35,36,38]. The AURE sequences are recognized by mRNA binding proteins that control movement of mRNA (a) from the nucleus to the cytoplasm, (b) to ribosomes for translation, (c) to storage sites, or (d) to degradation complexes. As recently reviewed [37], AUF1 is a multi-functional gatekeeper that works in concert with other mRNA regulatory proteins to control translation of mRNA. The N- and C-termini of AUF1 contain regulatory motifs that control AUF1 activity. The C-terminal RGG domain of AUF1 is similar to RGG domains in other mRNA regulatory proteins, and protein-protein binding by RGG domains may be affected by methylation of arginines in RGG motifs. This report explores the role of the RGG motif of AUF1 and post-translational protein modifications that affect AUF1.

The AUF1-RGG peptides were designed after we showed that deletion of the RGG domain inactivated AUF1 [18]. Two known functions of the RGG domain are protein-RNA [21] and protein-protein binding [25]. With respect to protein-RNA binding, the p37 AUF1 isoform has the strongest affinity for RNA among the four AUF1 isoforms, but deletion of most of the RGG region from the full-length protein did not affect affinity for RNA [21]. Protein-protein interactions affecting nucleo-cytoplasmic shuttling of AUF1 have been characterized [25,31]. The RGG domain binds the 14-3-3 σ protein that blocks nuclear import of AUF1 [25]. In addition, a "PY" NLS on the C-terminus of AUF1 (Figure 1) is homologous with the "PY" NLS of other proteins and mediates interaction with transportin-1 [39]. The poly-glutamine motif N-terminal to the RGG domain may affect AUF1 activity [21]. To address the possible role of motifs proximal to the RGG domain, a smaller AUF1-RGG peptide, AUF1-3RGG (Figure 1), was designed that lacks both the poly-glutamine motif and the "PY" NLS motif. The AUF1-3RGG peptide inhibited VEGF-3'UTR activity as effectively

as full-length AUF1 (Figure 4). Hence, it appears unlikely that AUF1-RGG peptides act on transportin-1 binding to increase AUF1 in the cell cytoplasm. Whether the AUF1-RGG peptides affect binding of AUF1 to the 14-3-3 σ protein remains to be determined. These results establish that a minimal AUF1-RGG peptide that contains three RGG motifs is a functional reagent that decreases VEGF gene expression. Next we examined post-translational modifications of AUF1, methylation and phosphorylation, that result from cell activation.

Many mRNA binding proteins contain repeating RGG motifs. Arginine residues in some mRNA binding proteins (hnRNP-A1, hnRNP-A2/B1, AUF1/hnRNP-D, hnRNP-K and Hrp1) are methylated, and methylation is largely restricted to arginines in RGG motifs [for Review, see 40]. Arginine residues are enzymatically methylated by protein arginine N-methyltransferase-1 (PRMT1) or other methyltransferases [22,24], and methylation may affect protein-RNA or protein-protein binding [40]. One well-documented functional effect of arginine methylation is cellular localization [24,40]. Arginine residues in RGG motifs of hnRNP A2 are methylated, and blockade of methylation with adenosine dialdehyde (AdOx) eliminated preferential nuclear localization of hnRNP A2 [24]. However, treatment of several cell types with AdOx did not affect cytoplasmic/nuclear localization of AUF1 [26]. To determine if methylation is altered by cellular activation of RAW-264.7 cells, AUF1 was immunopurified from untreated and LPS-treated cells and assayed for methylation by mass spectroscopy (Figure 5). Unlike results reported for the largest AUF1 isoform (AUF1-p45) in HeLa cells [27], the third arginine of AUF1-p37 was not methylated in RAW-264.7 cells. Instead, arginines in the first and second RGG motifs were monomethylated in unstimulated RAW-264.7 cells and dimethylated after LPS stimulation. In previous studies we evaluated the significance of arginine methylation on AUF1 activity [18]. Blockade of arginine methylation with adenosine dialdehyde dramatically reduced AUF1 protein levels, suggesting a role for arginine methylation in protein stability [18]. The role of methylation and protein stability in cellular activation is not clear, but these findings support the findings of our group [18,24] and others [41,42] that the RGG domain is a regulatory region, and arginine methylation affects the activity of RGG proteins. In point mutation studies reported here, replacing all methylatable arginines with lysine or alanine in the AUF1-3RGG peptide inactivated the AUF1-RGG peptide. This loss of activity shows that methylation may be essential for AUF1 and the AUF1-RGG peptide in controlling 3'UTR-dependent gene expression.

The most important binding partner for the RGG domain may be another AUF1 protein. The C-terminus of AUF1 is required for assembly of homodimers and higher order homo-oligomers [21]. Dimerization of AUF1 monomers is an initiating step in the formation of the RNA degradation machinery [37]. If arginine methylation affects AUF1 self-association, it is possible that dimethylation of AUF1 following LPS stimulation may favor dissociation of AUF1 dimers and inactivation of the mRNA degradation complex. By this mechanism, dimethylation of AUF1 and dissociation of the degradation complex would increase the half-life of AURE-regulated mRNA. An alternative mechanism would involve increased binding of dimethylated AUF1 to an inhibitory protein. In the case of the mRNA splicing factors Smd1 and Smd3, which contain RG (not RGG) repeats, methylated RG arginines favor binding to SMN [43,44]. With AUF1, dimethylation of the RGG domain may favor binding of a repressor protein that blocks AUF1 dimerization.

A surprising result of the mass spectroscopy studies was evidence that in resting cells lysine-164 was dimethylated (Figure 5A3). Lysine methylation is important in transcriptional regulation of histones, where lysine monomethylation is associated with gene activation. In contrast, lysine dimethylation is associated with gene silencing [45]. Lysine methylation of non-histone proteins is rare, but the important p53 onco-protein, which is

expressed upon DNA damage, is methylated on lysine residues. Methylation of lysine-382 in p53 may block acetylation of that residue and prevent activation of p53 [46]. In AUF1, lysine-164 is the residue that defines the N-terminal border of the second RNA recognition motif [47]. Methylation of lysine-164 in unstimulated cells may reduce RNA binding. In the nucleus, AUF1 has been reported to bind DNA [48], and lysine methylation may also affect DNA binding in this cellular compartment.

Activation of signaling pathways results in phosphorylation of regulated proteins. Isoforms of AUF1 may be phosphorylated on tyrosine, serine or threonine residues [33,34]. A recent report shows that PI3K/AKT kinase controls gene expression through another mRNA regulatory protein, KSRP [16], and inhibition of PI3K decreases VEGF levels [32,49]. We hypothesized that PI3K, or one of its dependent kinases, might affect VEGF gene expression by phosphorylating AUF1. To test this hypothesis, we confirmed that PI3K inhibitors suppress VEGF mRNA levels in RAW-264.7 cells and suppress VEGF-3'UTR reporter activity (Figure 7). In addition, our preliminary immunoblotting studies suggested that phosphorylation of AUF1-p37 is increased by LPS and decreased by PI3K inhibitors. However, mass spectroscopy analysis of the immunoprecipitated AUF1-p37 isoform did not identify phosphorylated residues. We assume from these results that LPS and PI3K inhibitors affect another protein that associates with AUF1-p37. Although the AUF1-p40 isoform is phosphorylated on multiple residues in the Exon 2 coding region [34], we did not identify p40 in our pulldowns. Other phosphoproteins were found in the pulldowns and further studies are needed to determine if these phosphoproteins associate with AUF1-p37 and are affected by cell activation.

In sepsis, severe inflammation results from cytokine activation initiated by a bolus of invading pathogens. When severe inflammation is discovered, a rapid response is critical. Just as macrophages are first responders to infection, mRNA regulatory proteins are first responders for gene expression in the cytoplasm. Although treatments that act directly on VEGF, such as anti-VEGF antibodies, act faster than agents affecting transcription or translation, large molecule drugs like anti-VEGF antibodies are very expensive to administer, require injection, and have the risk of infection and possible organ damage. Drugs like the AUF1-RGG peptides may be a novel and rapid means to activate AUF1 and reduce cytokines that promote severe inflammation. Hopefully, efficacious small molecule drugs can be designed to activate AUF1 and reduce expression of AURE-regulated disease genes.

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ABBREVIATIONS

AUF1	AUF1/hnRNP-D mRNA binding protein
AURE	AU-rich element
GLUT1	glucose transporter-1
LPS	lipopolysaccharide
MW	molecular weight
NLS	nuclear localization signal

NLS-PY	proline-tyrosine NLS
nt	nucleotide
RGG	arginine-glycine-glycine
TNF	tumor necrosis factor- α
UTR	untranslated region of mRNA
VEGF	vascular endothelial growth factor

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Highlights

- AUF1-RGG peptides are modeled on the RGG regulatory domain of AUF1
- AUF1-RGG peptides decrease expression of VEGF
- Arginines in RGG motifs are methylated and are essential for AUF1-RGG peptide activity in macrophages
- The PI3K/Akt signaling pathway affects 3'UTR-dependent VEGF gene expression

Figure 1. Amino acid sequence of AUF1-RGG peptides and NLS-PY motifs

Top. Shown are the sequences of four peptides used to study AURE-regulated gene expression: AUF1-Ex6+8, AUF1-QRGG, AUF1-3RGG, and AUF1-1RGG-PY. Amino acid numbering on left and right is for the AUF1-p37 isoform. Each peptide contains an initiating methionine residue (not shown). The "RGG domain" contains three "RGG" motifs and is marked. *Bottom.* The "NLS-PY" consensus sequence [39] for nuclear localization is shown in italic font. The corresponding NLS-PY sequences from AUF1, hnRNP-M and hnRNP-A1 are shown with the NLS-PY motif amino acids underlined. Spacing is added to achieve alignment. The RGG, PY, and poly-glutamine (QQQQQ) motifs are shown in bold font.

Figure 2. Expression levels of endogenous VEGF protein in AUF1-Ex6+8 stable cell lines

Lysates from G418-resistant stable RAW-264.7 cell lines (transfected with the AUF1-Ex6+8 plasmid) were immunoblotted for VEGF protein. Clones 2, 3, and 5 have reduced VEGF protein when compared to “pcDNA” control clones. Clone “6+8–1” was negative, and Clone “6+8–4” shows an intermediate effect on VEGF protein. Results are representative of two experiments.

Figure 3A

Figure 3B

Figure 3C

Figure 3. Effects of AUF1-RGG stable cell lines on VEGF-3'UTR-regulated gene expression

The VEGF-3'UTR luciferase reporter construct was transfected into stable cell lines that express the AUF1-Ex6+8 peptide, the AUF1-QRGG peptide, or the control cell stable line, "PC-2." Some wells were treated with LPS two hr after transfection (50 ng/mL). Cells were lysed after 24 hr. Shown are mean and SD, and are representative of two or more experiments. (A) Luciferase in control PC-2 stable cells treated without or with LPS. LPS treatment increased luciferase activity ($p < 0.002$). (B) Luciferase activity in cell lines not treated with LPS. Compared to PC-2, cell lines show significant decreases (6+8, $p < 0.002$; QRGG, $p < 0.002$). (C) Luciferase activity in cell lines treated with LPS. Compared to PC-2, cell lines show significant decreases (6+8, $p < 0.0001$; QRGG, $p < 0.0001$).

Figure 4. Effects of “AUF1-3RGG” and “AUF1-1RGG-PY” peptides on VEGF-3’UTR regulated gene expression

Two AUF1-RGG peptides were created in plasmids that (a) retain only the RGG domain (“AUF1-3RGG”), or (b) retain only the third RGG and the NLS-PY motif (“AUF1-1RGG-PY”) (see Figure 1). A negative control utilized the empty pcDNA-3.1 plasmid (“pcDNA”). A positive control utilized the plasmid expressing the full length AUF1. Plasmids were co-transfected with VEGF-3’UTR-luciferase and lysed after 24 hr. All results are normalized to the pcDNA-3.1 control. Results are mean ± SD of six wells, and are representative of two experiments. AUF1 and AUF1-3RGG inhibited luciferase compared to pcDNA-3.1 (AUF1, $p < 0.02$; AUF1-3RGG $p < 0.04$). Reporter levels of transfections with AUF1-3RGG were not significantly different from transfections with AUF1. The luciferase activity of cells transfected with AUF1-1RGG-PY were not significantly different from the pcDNA control.

Figure 5A1

K.EQYQQQQQWGSR⁺.G

Figure 5A2

R.GR⁺GGDQQSGYGK.V

Figure 5A3

K.K@IFVGGLSPDTPEEK.I

Figure 5B1

K.EQYQQQQQWGSR@GGFAGR^A

Figure 5B2

R.GR@GGDQQSGYGK.V

Figure 5. Effects of LPS activation on methylation of arginines in AUF1

Cells were not treated or treated with LPS (50 ng/mL) for 24 hr. Immunopurified AUF1-p37 proteins were separated on SDS-PAGE and bands excised for analysis. Tryptic peptide fragments were analyzed for methylation by LC-MS/MS. Figures show neighboring residues at cleavage sites. **(A)** AUF1 peptides from cells not treated with LPS: peptide EQYQQQQWGSRA is monomethylated on one arginine residue (**A1**); peptide of GR^GGDQQSGYGK is monomethylated on one arginine residue (**A2**); peptide K@IFVGGLSPDTPEEK is dimethylated on one lysine residue (**A3**). **(B)** AUF1 peptides from cells treated with LPS: peptide EQYQQQQWGSRA@GGFAGR^ is dimethylated on one arginine residue and monomethylated on one arginine residue (**B1**); peptide GR@GGDQQSGYGK is dimethylated on one arginine residue (**B2**). Monomethylated residues are marked with “^” and dimethylated residues are marked with “@”.

Figure 6. Effects of the AUF1-3RGG peptide and AUF1-3RGG arginine-to-lysine/alanine mutant peptides on VEGF-3'UTR reporter activity

Two AUF1-RGG peptides were created in plasmids that replace methylatable arginines with lysine ("3RGG-R→K") or alanine ("3RGG-R→A"). The sequence of the 3RGG peptide is shown in Figure 1, and mutated arginine residues are described in Materials and Methods. A negative control utilized the empty pcDNA-3.1 plasmid ("pcDNA"). Plasmids were co-transfected with VEGF-3'UTR-luciferase and lysed after 24 hr. All results are normalized to the pcDNA-3.1 control. Results are mean ± SD of four wells, and are representative of two experiments. The AUF1-3RGG inhibited luciferase compared to pcDNA-3.1 ($p < 0.003$). Reporter levels of transfections with AUF1-3RGG-R→K and AUF1-3RGG-R→A were significantly greater than reporter levels of transfections with AUF1-3RGG ($p < 0.0001$ and $p < 0.02$, respectively). Transfections with mutants were not significantly different from transfections with the pcDNA-3.1 control.

Figure 7A

Figure 7B

Figure 7. Effects of PI3K on VEGF gene expression

(A) Effects of PI3K inhibition on VEGF mRNA levels. Cultured RAW-264.7 cells were treated with the PI3K inhibitor, LY294002, for 4 hr. Real-time PCR was performed on cDNA prepared from total RNA. Results are the average of four separate experiments with VEGF Ct normalized to actin Ct measured in the same sample. In each experiment, control samples (DMSO treated) were set to unity. (B) Effects of PI3K inhibition on VEGF-3'UTR-dependent gene expression. Cultured RAW-264.7 cells were transfected with the VEGF-3'UTR luciferase reporter. After 24 hr cells were treated with LY294002 for 4 hr and reporter activity measured in cell lysates. Shown are mean and SD. Treatments with 5 and 25 μ M LY294002 are significantly less than control ($p<0.05$ and $p<0.003$, respectively).