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mRNA Degradation Plays a Significant Role in the Program of Gene Expression Regulated by Phosphatidylinositol 3-Kinase Signaling▽†

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Control of gene expression by the phosphatidylinositol (PI) 3-kinase/Akt pathway plays an important role in mammalian cell proliferation and survival, and numerous transcription factors and genes regulated by PI 3-kinase signaling have been identified. Because steady-state levels of mRNA are regulated by degradation as well as transcription, we have investigated the importance of mRNA degradation in controlling gene expression downstream of PI 3-kinase. We previously performed global expression analyses that identified a set of approximately 50 genes that were downregulated following inhibition of PI 3-kinase in proliferating T98G cells. By blocking transcription with actinomycin D, we found that almost 40% of these genes were regulated via effects of PI 3-kinase on mRNA stability. Analyses of β-*globin*–3′ untranslated region (UTR) fusion transcripts indicated that sequences within 3′ UTRs were the primary determinants of rapid mRNA decay. Small interfering RNA (siRNA) experiments further showed that knockdown of BRF1 or KSRP, both ARE binding proteins (ARE-BPs) regulated by Akt, stabilized the mRNAs of a majority of the downregulated genes but that knockdown of ARE-BPs that are not regulated by PI 3-kinase did not affect degradation of these mRNAs. These results show that PI 3-kinase regulation of mRNA stability, predominantly mediated by BRF1, plays a major role in regulating gene expression.

The phosphatidylinositol (PI) 3-kinase pathway is a major regulator of mammalian cell survival and proliferation. PI 3-kinase activates its main downstream effector, the serine/threonine kinase Akt, which phosphorylates a variety of substrates that function to suppress apoptosis and promote progression through the cell cycle (6, 7, 18). Targets of Akt include the Bcl-2 family member Bad (19, 20) and the protein kinase glycogen synthase kinase 3 (GSK-3) (17, 44), both of which are inhibited by Akt phosphorylation. The transcriptional program controlled by PI 3-kinase has been well studied, and a variety of transcription factors that are regulated by Akt and GSK-3 have been identified (6, 7, 22, 33). In addition, global studies of gene expression have identified sets of target genes and transcription factors that are regulated by PI 3-kinase signaling in different cell types and in response to different stimuli (11, 21, 30, 31, 55–57).

In addition to transcription, however, the steady-state levels of mRNA can be regulated by degradation, raising the possibility that control of mRNA stability may also contribute to the regulation of gene expression by PI 3-kinase. The stability of many mRNAs is regulated by AU-rich elements (AREs) located within the 3′ untranslated region (UTR), which generally lead to rapid turnover of the mRNA (14). Although AREs are loosely defined and differ in both length and sequence, they usually contain several copies of the AUUUA pentamer and are rich in uridine (14). These sequence elements serve as binding sites for proteins that regulate mRNA stability, which can lead to either mRNA stabilization or mRNA degradation. Interaction of the ARE with destabilizing proteins, such as butyrate response factor 1 (BRF1), tristetraprolin (TTP), or KH-type splicing regulatory protein (KSRP), results in decay, which is thought to occur by the recruitment of mRNA decay enzymes onto the mRNA, leading to deadenylation, 5′ decapping, and subsequent degradation by the exosome (13, 16, 27, 28, 39). In contrast, binding of HuR to the ARE stabilizes the mRNA (23, 40), whereas binding of AUF1 can result in either stabilization or degradation (48, 50).

A number of signal transduction pathways have been implicated in regulating mRNA decay, including the PI 3-kinase (29, 43, 49, 52), p38 mitogen-activated protein (MAP) kinase (9, 25), MAP kinase-activated protein kinase 2 (59), c-Jun N-terminal kinase (12), and Wnt/β-catenin (10) pathways. Activation of PI 3-kinase has been shown to induce mRNA stabilization by Akt phosphorylation of the ARE binding proteins (ARE-BPs) BRF1 and KSRP (4, 28, 52). Although phosphorylated BRF1 and KSRP are still able to bind to the mRNA, they do so in a complex that includes 14-3-3 proteins, which prevents the mRNA from being degraded (4, 28, 52).

Continuous PI 3-kinase signaling is necessary for the survival and proliferation of cells maintained in the presence of growth factors, and we have previously characterized the global alterations in gene expression that result from inhibition of PI 3-kinase (55). These studies identified a set of approximately 50 genes, many of which function to control cell proliferation and survival, that were downregulated within 4 h of inhibition of PI 3-kinase (55). Approximately one-third of these genes were regulated at the transcriptional level by NF-κB (55). In the present study, we have investigated whether PI 3-kinase-

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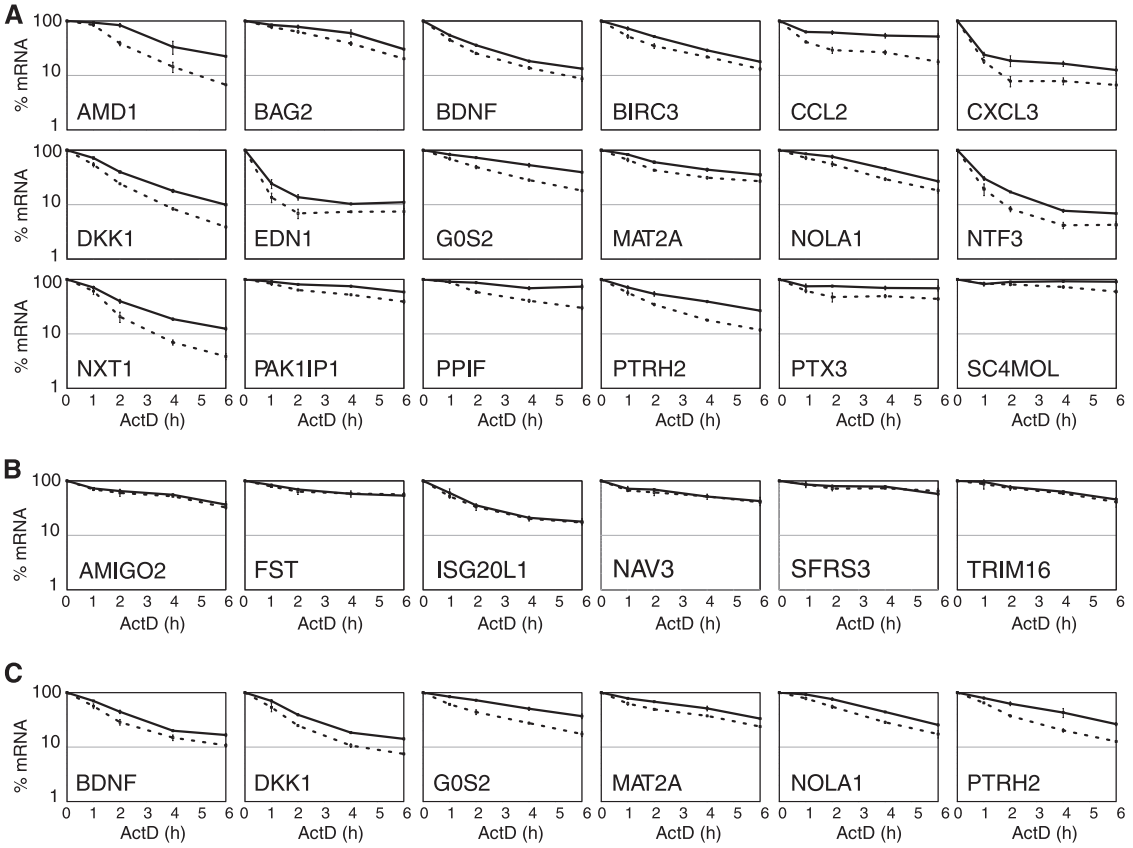

FIG. 1. Inhibition of PI 3-kinase increases mRNA degradation. Proliferating T98G cells were treated with actinomycin D (ActD) in either the presence (dashed lines) or the absence (solid lines) of the PI 3-kinase inhibitor LY294002 (A and B) or PI-103 (C). Total RNA was isolated after 1, 2, 4, or 6 h, and the mRNA levels of 48 genes were determined by real-time RT-PCR. Inhibition of PI 3-kinase with LY294002 increased the mRNA degradation of 18 genes (A). The results for six representative genes for which degradation was not affected by inhibition of PI 3-kinase with LY294002 are shown in panel B. PI 3-kinase inhibition using PI-103 increased mRNA degradation, with the results for six representative genes shown in panel C. Data are presented as the percentages of mRNA relative to the level for a DMSO vehicle control at each time point and are the means of results from two (B and C) or three (A) independent experiments ± standard errors (SE).

regulated mRNA degradation also plays a role in the downregulation of these genes. We found that inhibiting PI 3-kinase resulted in an increase in the mRNA decay of almost 40% of the downregulated genes whereas knockdown of the ARE-BPs BRF1 and KSRP resulted in the stabilization of over 60% of the downregulated mRNAs. In contrast, knockdown of either AUF1 or HuR, neither of which has been shown to be regulated by PI 3-kinase, did not affect the degradation of the mRNAs. In addition, sequences within the 3′ UTRs appeared to be the primary determinants of mRNA decay. Importantly, the genes that showed the greatest response to PI 3-kinase inhibition were the most affected by BRF1 knockdown, indicating that BRF1 plays a major role in regulating mRNA stability downstream of PI 3-kinase signaling. These results provide evidence that regulation of mRNA degradation mediated by BRF1 and KSRP is a significant component of the overall program of gene expression controlled by the PI 3-kinase pathway.

MATERIALS AND METHODS

Cell culture. T98G human glioblastoma cells were cultured in minimal essential medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (HyClone). HeLa Tet-off cells (Clontech) were cultured in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies) with 10% fetal bovine serum and 100 μg/ml of G418 (Calbiochem).

Analysis of mRNA degradation. T98G cells were treated with 2.5 μg/ml of actinomycin D (Enzo Life Sciences) in the presence or absence of 50 μM LY294002 (Enzo Life Sciences) or in the presence or absence of 5 μM PI-103 (EMD Chemicals) for 1 to 6 h. RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Real-time reverse transcription-PCR (RT-PCR) was then performed as previously described, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a normalization control (30). The primer sequences for the real-time PCR are listed in Table S1 in the supplemental material. The statistical significance of the change in mRNA half-life resulting from treatment with LY294002 was determined using a *t* test.

Analysis of 3′ UTR role in mRNA degradation. The 3′ UTR from a gene of interest was inserted downstream of a β-*globin* reporter gene under the control of a Tet-off promoter in the plasmid pTet-BBB, which was a gift from A.-B. Shyu (62). The complete 3′ UTR for each gene was amplified from T98G genomic DNA using Phusion DNA polymerase (New England BioLabs) and the PCR products ligated into the BglII site of the pTet-BBB plasmid. The primer sequences used in the PCR are listed in Table S1 in the supplemental material. All constructs were verified by sequencing, using either a universal primer for the cytomegalovirus (CMV) promoter or insert-specific primers (see Table S1 in the supplemental material). HeLa Tet-off cells were then transfected with the constructs using TransIT-HeLa MONSTER (Mirus) according to the manufacturer's protocol. Briefly, cells were seeded on 6-well plates at 9×10^4 cells/ml in 2 ml of medium 24 h prior to transfection and then transfected with 100 μg of the ptet-BBB-3′UTR reporter plasmid and 900 μg of pcDNA-3. Following 24 h of

incubation at 37°C, 100 μg/ml of doxycycline was added to stop the transcription of the pTet constructs for the times indicated in the text, after which RNA was extracted using TRIzol reagent. Levels of β-*globin* mRNA were measured by real-time RT-PCR as described above, using an intron-spanning β-*globin* primer (see Table S1 in the supplemental material for the primer sequence).

RNA interference. Transfections of T98G cells were performed using predesigned small interfering RNAs (siRNAs) against BRF1 (s2090; Ambion), KSRP (s16322; Ambion), AUF1 (s6724; Ambion), HuR (s4610; Ambion), or a nonspecific negative control (4390843; Ambion). Shortly before transfection, 10^5 cells/ml were seeded on 60-mm plates in 4 ml of medium containing 10% fetal bovine serum. Transfection reaction mixtures containing either 10 nM siRNA (for BRF1) or 20 nM siRNA (for KSRP, AUF1, and HuR), 20 μl of HiPerfect (Qiagen), and 100 μl of serum-free medium were incubated for 10 min at room temperature and added dropwise onto the cells. Cells were incubated at 37°C for 72 h and then treated with 50 μM LY294002 for the times indicated in the text, after which RNA was extracted and analyzed by real-time RT-PCR.

Immunoblot analysis. Whole-cell extracts were prepared by lysing cells with radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, with protease inhibitor cocktail [Roche]). Proteins were separated by electrophoresis in 12% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, and incubated with an appropriate primary antibody: anti-BRF1 (ab42473; Abcam), anti-KSRP (sc-33031; Santa Cruz), anti-AUF1 (07-260; Millipore), anti-HuR (sc-5261; Santa Cruz), or anti-β-actin (Sigma). Immunoblots were visualized using species-specific horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and chemiluminescence (PerkinElmer). Densitometry was performed using ImageJ software (NIH).

RESULTS

PI 3-kinase signaling regulates mRNA degradation. We previously identified a set of approximately 50 genes whose mRNAs were downregulated at least 1.8-fold after 2 to 4 h of inhibition of PI 3-kinase in proliferating T98G cells (55). Since the decrease in the steady-state levels of these mRNAs could result from regulation of either transcription or mRNA degradation by PI 3-kinase signaling, we sought to directly determine whether inhibition of PI 3-kinase affected the stability of these mRNAs. We therefore used actinomycin D to inhibit transcription and investigated the effect of PI 3-kinase on mRNA degradation.

Proliferating T98G cells were treated with actinomycin D in the presence or absence of the PI 3-kinase inhibitor LY294002 and mRNA levels measured after 1 to 6 h of incubation. For 18 of the mRNAs tested, inhibition of PI 3-kinase increased the amount of degradation approximately 1.3- to 2.7-fold (Fig. 1A). This corresponded to a significant decrease ($P < 0.05$) in the half-lives of these transcripts, calculated from the data in Fig. 1A. In contrast, degradation of the mRNAs of the remaining 30 genes tested was not affected by inhibition of PI 3-kinase (the results for six representative genes are shown in Fig. 1B). To confirm that the increase in mRNA decay was a result of PI 3-kinase inhibition and not due to off-target effects of LY294002, we investigated the effects of PI-103, another widely used and specific inhibitor of PI 3-kinase (1). When cells were treated with actinomycin D in the presence of PI-103, a similar increase in mRNA degradation occurred (the results for six representative genes are shown in Fig. 1C).

We next assessed the relationship between the half-lives of the mRNAs and the increase in degradation resulting from 4 h of PI 3-kinase inhibition (Fig. 2). Because actinomycin D treatment stabilized some of the mRNAs, as had been observed by others (15), we first excluded the nine genes that had half-lives greater than 10 h from any analyses (Fig. 2A). Interestingly, we found that the mRNAs with short half-lives were much more likely to be regulated by PI 3-kinase ($r^2 = 0.3$; $P = 0.0001$). To further rule out the possibility that the longer half-lives were an artifact of actinomycin D treatment, we also performed the correlation using only those mRNA half-lives that were less than 5 h (Fig. 2B). In support of the conclusion that PI 3-kinase is more likely to regulate short-lived mRNAs, this analysis also showed that there was a strong correlation between half-life and PI 3-kinase regulation ($r^2 = 0.4$; $P = 0.0001$). These results, together with the fact that PI 3-kinase inhibition increased the degradation of 18 out of 48 downregulated mRNAs, indicate that regulation of mRNA stability plays a significant role in control of gene expression by the PI 3-kinase pathway.

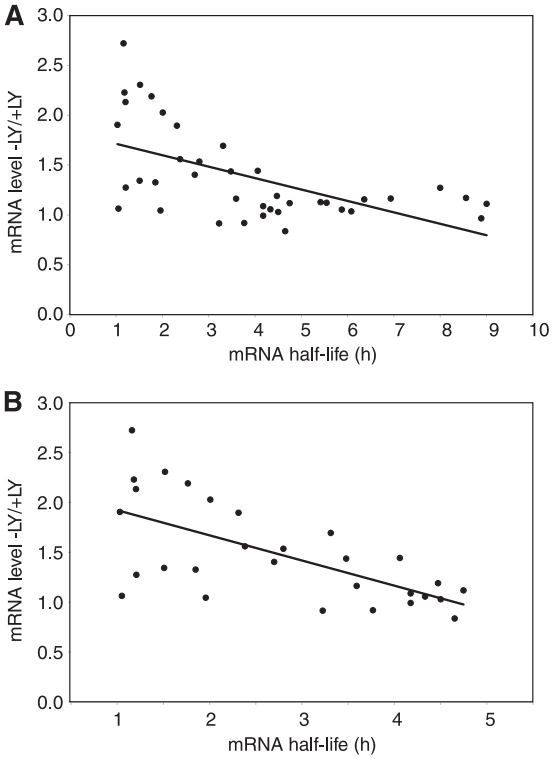

FIG. 2. Correlation between mRNA half-lives and effects of PI 3-kinase on mRNA degradation. The half-lives of mRNAs that were <10 h (A) or <5 h (B) following treatment with actinomycin D and LY294002 (LY) were compared to the ratio of mRNA levels in cells treated for 4 h with actinomycin D with and without LY294002, as calculated from the data in Fig. 1. For panel A, correlation coefficient $r^2 = 0.3$, $P = 0.0001$, and $n = 39$. For panel B, correlation coefficient $r^2 = 0.4$, $P = 0.0001$, and $n = 29$.

3′ UTRs mediate mRNA degradation. AREs within the 3′ UTRs of mRNAs are the most common elements involved in regulating mRNA stability (14). These sequences serve as binding sites for proteins that regulate mRNA degradation, and the activity of some ARE binding proteins has been shown to be modulated by Akt (29, 49, 52). Therefore, we sought to determine the roles of the 3′ UTR in the degradation of the mRNAs of genes that were downregulated in response to PI 3-kinase inhibition.

First, we determined if ARE sequences were enriched in the downregulated genes. Using the UCSC Table Browser Database (34), we obtained the 3′ UTR sequences for 50 genes that

TABLE 1. AU-rich element motifs located within the 3′ UTRs of the downregulated genes[a]

GenBank accession no.	Gene product	No. of instances of indicated motif				
		AUUUA	UUAUUUAUU	UUAUUUAWW	UAUUUAU	UA[U]$_{2-5}$AU
NM_001634	AMD1	6	0	0	1	2
NM_181847	AMIGO2	7	0	0	0	1
NM_004282	BAG2	4	0	0	0	0
NM_001709	BDNF	9	0	0	3	5
NM_001165	BIRC3	4	1	1	2	3
NM_001048249	C5orf43	9	0	0	0	2
NM_002982	CCL2	1	0	0	0	3
NM_053056	CCND1	7	0	0	1	6
NM_002090	CXCL3	7	1	3	3	3
NM_000104	CYP1B1	10	0	2	2	6
NM_000788	DCK	4	0	0	0	2
NM_013989	DIO2	11	1	1	4	8
NM_012242	DKK1	3	0	0	0	0
NM_004414	DSCR1	2	0	0	0	1
NM_001955	EDN1	4	0	0	1	1
NM_001993	F3	3	0	1	1	3
NM_002006	FGF2	18	0	0	1	11
NM_006350	FST	1	0	0	0	0
NM_015714	G0S2	0	0	0	0	1
NM_002041	GABPB2	1	0	0	1	1
NM_002053	GBP1	4	0	0	1	2
NM_018284	GBP3	2	0	0	0	0
NM_006851	GLIPR1	10	0	0	1	4
NM_006143	GPR19	1	0	0	0	0
NM_000830	GRIK1	0	0	0	0	0
NM_000599	IGFBP5	7	0	1	2	2
NM_002185	IL7R	2	0	0	0	0
NM_022767	ISG20L1	3	1	1	2	2
NM_032860	LTV1	0	0	0	0	3
NM_005911	MAT2A	3	1	1	1	2
NM_002422	MMP3	1	0	1	0	1
NM_014903	NAV3	5	1	1	1	4
NM_181782	NCOA7	12	1	1	3	8
NM_018983	NOLA1	0	0	0	0	0
NM_002527	NTF3	1	1	1	1	2
NM_013248	NXT1	1	0	0	0	0
NM_017906	PAK1IP1	0	0	0	0	0
NM_002658	PLAU	2	0	1	2	4
NM_006622	PLK2	2	1	1	2	3
NM_005729	PPIF	2	0	0	0	0
NM_016077	PTRH2	0	0	0	0	0
NM_002852	PTX3	2	0	0	1	2
NM_003702	RGS20	3	0	0	1	3
NM_006745	SC4MOL	3	0	0	0	0
NM_003020	SCG5	0	0	0	0	0
NM_003017	SFRS3	3	0	0	0	1
NM_003714	STC2	12	0	1	3	4
NM_002546	TNFRSF11B	3	0	1	1	4
NM_006470	TRIM16	0	0	0	0	0
NM_033035	TSLP	7	0	0	0	3

[a] An R script was used to identify the number of ARE motifs located within the 3′ UTRs of 50 genes that were downregulated following inhibition of PI 3-kinase.

were downregulated after inhibition of PI 3-kinase, in addition to a background set of 691 genes that were not differentially regulated by PI 3-kinase inhibition. The 3′ UTRs were examined for the presence of motifs traditionally defined to be AREs, including the AUUUA pentamer, the conserved UUA UUUAUU nonamer, and consensus nonamers UUAUUUA (A/U)(A/U) (14) (Table 1). Because it has also been shown that TTP, a BRF1 family member, has a high affinity for a UAUUUAU heptamer as well as UA[U]$_{2,4,5}$AU variations (8), we also included these sequences in our analyses. The frequencies of the ARE motifs in the downregulated genes were then compared to the frequencies in the background set using a Wilcoxon test. All of the variations of the motifs were highly enriched in the downregulated genes ($P < 0.01$), with the pentamer present in 84% of the genes and at least one hexamer present in 70% of the genes (Table 2). These sequence elements were also found to be significantly overrepresented when the 18 genes with PI 3-kinase-regulated mRNA degradation rates were compared to the background gene set ($P < 0.01$; data not shown). GU-rich elements with the sequence UGUUUGUUUGU have also been shown to mediate mRNA degradation by serving as binding sites for CUG repeat binding

TABLE 2. Numbers of genes with at least one ARE motif located within the 3′ UTR[a]

Gene set	AUUUA		UUAUUUAUU		UUAUUUAWW		UAUUUAU		UA[U]$_{2-5}$AU	
	No. (%) of genes	*P*	No. (%) of genes	*P*	No. (%) of genes	*P*	No. (%) of genes	*P*	No. (%) of genes	*P*
Downregulated	42 (84)	6.8×10^{-6}	9 (18)	3.3×10^{-4}	16 (32)	3.6×10^{-4}	25 (50)	4.5×10^{-7}	35 (70)	5.5×10^{-7}
Background	393 (57)		39 (6)		95 (14)		136 (20)		264 (38)	

[a] Indicated in parentheses is the percentage of genes with the motif, out of 50 genes downregulated upon PI 3-kinase inhibition or 691 background genes that did not show differential expression upon PI 3-kinase inhibition. The *P* values were determined using a Wilcoxon test to compare the frequencies of ARE motifs in the downregulated genes to the frequencies in the background set of genes.

protein 1 (58); therefore, we also examined the 3′ UTRs for the presence of this element and its variations. This motif, however, was not overrepresented ($P = 0.73$).

To test whether the 3′ UTRs of downregulated genes mediated the degradation of their mRNAs, the 3′ UTRs of representative transcripts were fused to a β-*globin* reporter gene under the control of a Tet-off promoter in the plasmid pTet-BBB (62). We cloned the 3′ UTRs for 14 downregulated genes, including 11 genes that exhibited an increased rate of mRNA degradation when PI 3-kinase was inhibited and 3 genes whose degradation was not affected by inhibition of PI 3-kinase (Fig. 1). In addition, we chose genes with various rates of mRNA degradation and with 3′ UTRs containing a variety of ARE sequences, ranging from one to nine pentamers, zero or one nonamer, or zero to five heptamers. The constructs were then used to transfect HeLa Tet-off cells expressing the tetracycline-regulated transactivator. Doxycycline was added to stop transcription, and β-*globin* mRNA levels were measured after 1 to 6 h of treatment. The mRNA decay rates of the constructs containing the 3′ UTRs of downregulated genes were compared to that of the highly stable β-*globin* transcript, allowing for a direct measure of the effect of the 3′ UTRs on mRNA stability. We found that the mRNA of the β-*globin* reporter gene had a half-life of approximately 6 h and that the addition of the 3′ UTRs significantly decreased the half-life to less than 2.5 h for seven of the downregulated genes that were tested (Fig. 3). Overall, the genes fell into three groups, consisting of three genes whose 3′ UTRs induced rapid decay corresponding to half-lives of approximately 1 h (Fig. 3A), four genes whose 3′ UTRs induced moderate decay (half-lives of 2 to 2.5 h) (Fig. 3B), and seven genes for which the 3′ UTRs did not increase the rate of decay of the β-*globin* reporter gene (Fig. 3C).

We assessed the role of 3′ UTR sequences in mediating the degradation of these genes by comparing the rates of degradation of the intact transcripts in actinomycin D-treated cells (Fig. 1) with the rates of degradation of the β-*globin*–3′ UTR fusion transcripts (Fig. 3). As illustrated in Fig. 3D, there was a strong correlation between the half-lives of mRNAs following actinomycin D treatment and the half-lives of the corresponding β-*globin*–3′ UTR fusion transcripts ($r^2 = 0.4$; $P = 0.009$), indicating that the 3′ UTRs are the primary determinants of the rates of degradation of these mRNAs. Interestingly, the number of ARE sequences within the 3′ UTRs did not significantly correlate with either the rates of mRNA degradation following actinomycin D treatment ($r^2 < 0.03$; $P > 0.1$) or the rates of degradation of β-*globin*–3′ UTR fusion transcripts ($r^2 < 0.2$; $P > 0.1$) (data not shown). This was true for all variations of ARE motifs, including the pentamer, nonamer, hexamer, and total number of elements; thus, the number of motifs alone was not a good indicator of mRNA degradation.

BRF1 and KSRP regulate mRNA degradation downstream of PI 3-kinase signaling. Having established the role of 3′ UTRs in the degradation of mRNAs that are downregulated in response to PI 3-kinase inhibition, we next assessed whether ARE-BPs regulate these mRNAs. Because Akt phosphorylation has been shown to inactivate BRF1 and KSRP (29, 52), two of the ARE-BPs that function to degrade mRNA, we tested whether knocking down these proteins stabilized mRNA levels and blocked the downregulation that occurs when PI 3-kinase is inhibited. Proliferating T98G cells were transfected for 72 h with either BRF1 or KSRP siRNA, which reduced the respective protein levels by 80 to 90% (Fig. 4A). The cells were then treated with either dimethyl sulfoxide (DMSO) vehicle control or LY294002 to inhibit PI 3-kinase and mRNA levels measured after 1 to 8 h of incubation. Results for three representative genes, all of whose mRNA degradation rates were increased by PI 3-kinase inhibition (Fig. 1A), are shown in Fig. 4B, demonstrating that knockdown of either BRF1 or KSRP substantially reduced the rate of mRNA degradation following inhibition of PI 3-kinase.

Analyses of the effects of BRF1 and KSRP knockdown on the degradation of mRNAs for the full set of downregulated genes following 4 h of inhibition of PI 3-kinase are summarized in Fig. 5. Knockdown of BRF1 significantly reduced the degradation of 31 out of the 48 mRNAs tested ($P < 0.05$), resulting in 1.3- to 4.6-fold increases in mRNA levels (Fig. 5A). KSRP knockdown significantly reduced the downregulation of 11 mRNAs in response to inhibition of PI 3-kinase, 9 of which were also stabilized by BRF1 siRNA, with an average increase in mRNA levels of 1.3- to 2.3-fold ($P < 0.05$) (Fig. 5B). We also measured the effects of knocking down AUF1 and HuR, two ARE-BPs that have not been shown to be regulated by PI 3-kinase signaling. Proliferating T98G cells were treated with siRNA for the respective proteins for 72 h, which reduced AUF1 and HuR protein levels by 90% (Fig. 6A). Cells were then treated with either DMSO or LY294002 for 4 h, after which mRNA levels were measured. In contrast to the results for BRF1 and KSRP, knockdown of either AUF1 or HuR had no effect on the mRNA degradation resulting from PI 3-kinase inhibition (Fig. 6B).

To further assess the role of BRF1 in PI 3-kinase-regulated mRNA decay, we investigated the relationship between the rates of mRNA degradation and the extent of mRNA stabilization resulting from BRF1 knockdown. The half-lives of

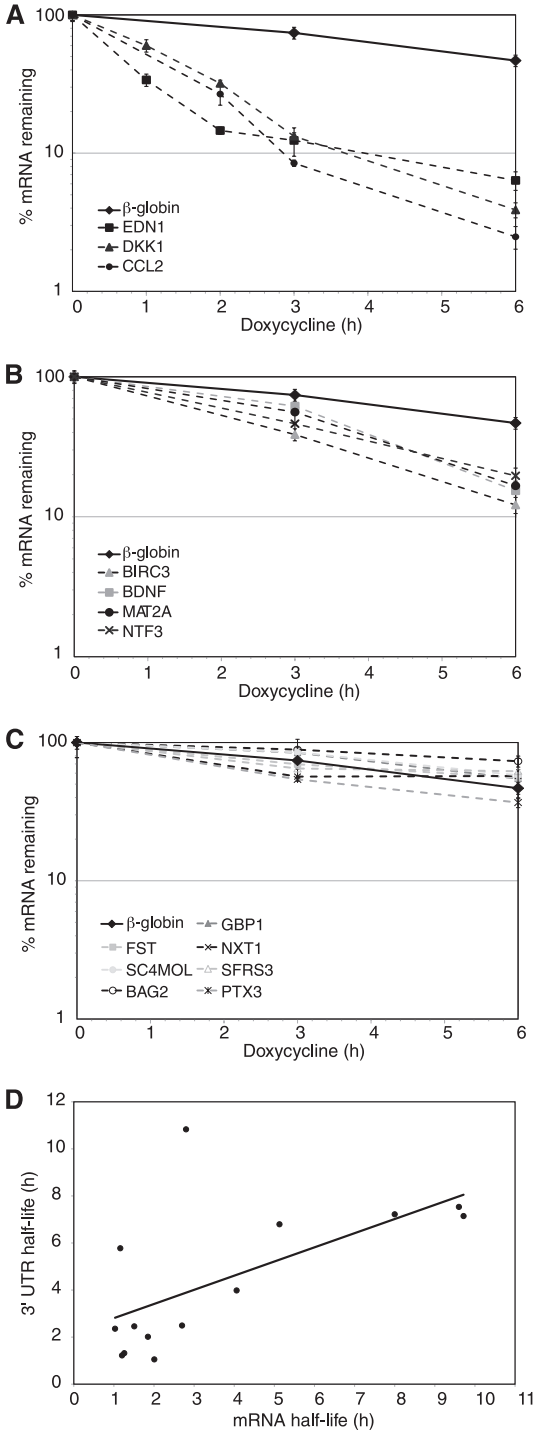

FIG. 3. mRNA degradation is induced by the 3′ UTRs of genes downregulated upon PI 3-kinase inhibition. HeLa Tet-off cells were transfected for 24 h with either a β-*globin* reporter gene (solid line) or a β-*globin*–3′ UTR fusion transcript (dashed lines). Transcription was stopped by the addition of doxycycline. Total RNA was harvested after the indicated times and the levels of β-*globin* transcript determined by real-time RT-PCR. The 3′ UTRs either induced rapid decay of the β-*globin* reporter gene, with half-lives of <1 h (A), induced moderate decay, with half-lives of 2 to 2.5 h (B), or had no effect (C). Data represent the percentages of mRNA relative to the control level and are representative of 2 to 6 experiments (means ± SE). Shown in panel D is the correlation between mRNA half-lives following treatment with actinomycin D plus LY294002 (Fig. 1) and half-lives of 3′ UTR fusion transcripts. Correlation coefficient $r^2 = 0.4$, $P = 0.009$, and $n = 14$.

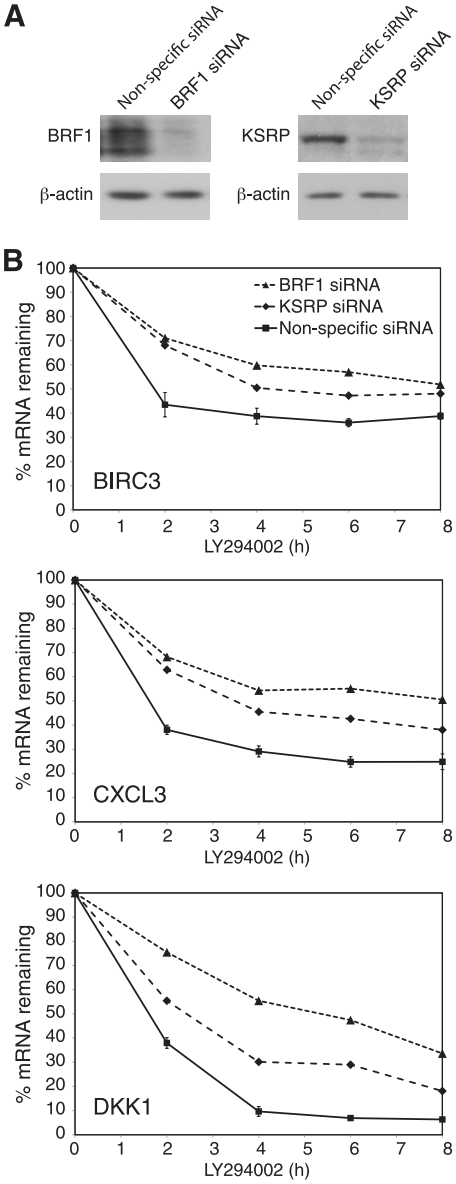

FIG. 4. Stabilization of mRNA degradation by knockdown of BRF1 or KSRP. Proliferating T98G cells were transfected for 72 h with a nonspecific control siRNA, BRF1 siRNA, or KSRP siRNA. Immunoblots were used to measure knockdown of BRF1 and KSRP (A). mRNA degradation was measured by real-time RT-PCR following treatment with either DMSO or LY294002 for the indicated times (B). Data are presented as the percentages of mRNA compared to the level for the vehicle control at each time point.

mRNAs following treatment with actinomycin D strongly correlated with the extent of mRNA stabilization that resulted from BRF1 knockdown ($r^2 = 0.13$; $P = 0.01$) (Fig. 7A), as did the half-lives conferred by 3′ UTRs in β-*globin* fusion transcripts ($r^2 = 0.3$; $P = 0.02$) (Fig. 7B). Thus, the most rapidly degraded 3′ UTRs were also those most stabilized by knockdown of BRF1, indicating that BRF1 is a major factor in mediating mRNA degradation via 3′ UTR sequences. Notably, there was also a striking correlation between the increase in mRNA degradation that resulted from inhibiting PI 3-kinase

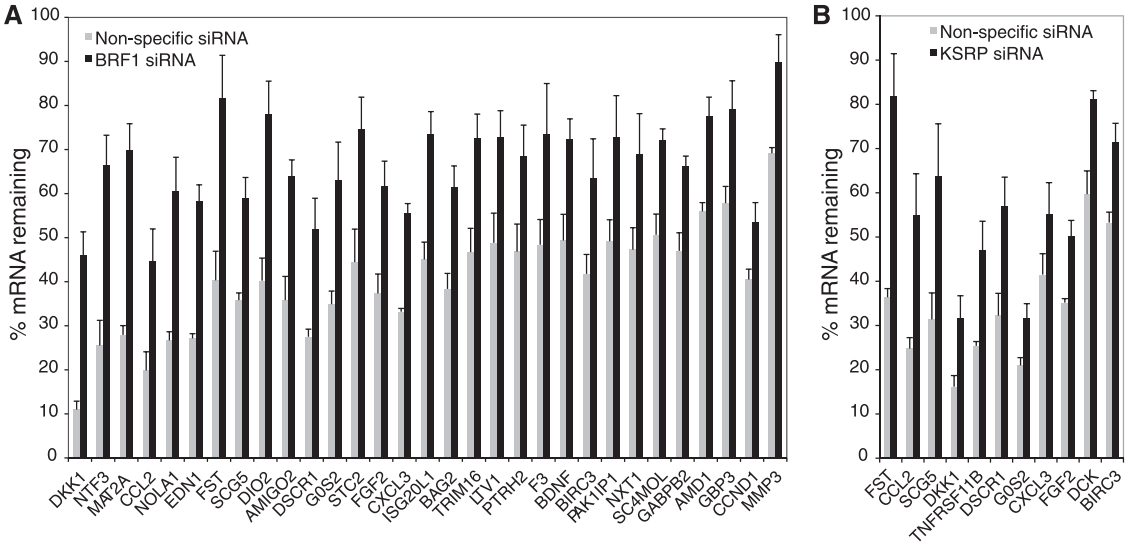

FIG. 5. Global effects of BRF1 and KSRP knockdown on mRNA degradation following PI 3-kinase inhibition. Proliferating T98G cells were transfected for 72 h with BRF1 siRNA (A), KSRP siRNA (B), or a nonspecific control siRNA. Cells were treated with DMSO or LY294002 for 4 h, after which levels of 48 downregulated mRNAs were measured by real-time RT-PCR. Data are presented only for those mRNAs that were significantly affected by either BRF1 or KSRP knockdown ($P < 0.05$) and represent the percentages of mRNA compared to the level for the DMSO vehicle control (means of results from four [A] or three [B] independent experiments ± SE).

and the extent of stabilization that resulted from knocking down BRF1 ($r^2 = 0.2$; $P = 0.005$) (Fig. 7C). Therefore, those mRNAs whose degradation was most affected by inhibition of PI 3-kinase were also most affected by knockdown of BRF1.

Overall, 33 of the 48 mRNAs tested were stabilized by knockdown of either BRF1 or KSRP, indicating that these ARE-BPs, particularly BRF1, play significant roles in mediating mRNA degradation following inhibition of PI 3-kinase. This relationship is further supported by the fact that 16 out of the 18 genes with PI 3-kinase-regulated mRNA degradation rates were also found to be regulated by BRF1 (Fig. 8). Taken together, these results show that PI 3-kinase signaling can regulate mRNA degradation by ARE-mediated decay, primarily mediated by BRF1.

DISCUSSION

We previously identified over 50 genes that were downregulated at least 1.8-fold in response to inhibition of PI 3-kinase in proliferating T98G cells (55), and the present study has demonstrated that ARE-mediated mRNA degradation plays a key role in regulating their expression. By blocking transcription, we determined that the degradation of almost 40% of these downregulated mRNAs increased when PI 3-kinase was inhibited. Notably, the half-lives of mRNAs were highly correlated with the effect of PI 3-kinase inhibition on the rate of degradation, such that the degradation of mRNAs with short half-lives was most likely to be regulated by PI 3-kinase. In fact, of the 18 genes that exhibited PI 3-kinase-regulated mRNA degradation, only 3 had half-lives that were greater than 3 h. Therefore, our results suggest that PI 3-kinase regulates the mRNA stability of many of its target genes, the majority of which have mRNAs that are rapidly degraded.

Rapid and selective degradation of many mRNAs is mediated by AREs within their 3′ UTRs (14), and statistical analysis of the genes that were downregulated following inhibition of PI 3-kinase indicated that their 3′ UTRs were enriched in sequences defined as common ARE motifs. Using a β-*globin* reporter gene fused to the 3′ UTRs of PI 3-kinase-regulated mRNAs, we found that there was a strong correlation between the half-lives of the β-*globin*–3′ UTR transcripts and the half-lives of intact mRNAs in actinomycin D-treated cells, indicating that degradation of these mRNAs was primarily mediated by sequences within their 3′ UTRs. PI 3-kinase signaling has previously been shown to stabilize ARE-containing transcripts, including *interleukin 3* and β-*catenin*, as a result of phosphorylation of the ARE-BPs KSRP and BRF1 by Akt (29, 43, 49, 52). We therefore performed siRNA experiments against BRF1 and KSRP and found that the mRNAs of almost 70% of the downregulated genes were stabilized upon knockdown of either BRF1 or KSRP. BRF1 was the dominant factor in controlling mRNA stability downstream of PI 3-kinase signaling, with 31 out of 48 mRNAs stabilized by knockdown of BRF1. In contrast, knockdown of KSRP stabilized 11 mRNAs, with 9 of these also stabilized by knockdown of BRF1. We also showed that knockdown of ARE-BPs that have not been shown to be regulated by PI 3-kinase did not affect mRNA levels following PI 3-kinase inhibition, further illustrating the significance of BRF1 and KSRP in mediating mRNA degradation downstream of PI 3-kinase. Importantly, 16 out of the 18 genes that exhibited PI 3-kinase-regulated mRNA degradation were stabilized by BRF1 knockdown, indicating that BRF1 was the major factor involved in controlling mRNA stability downstream of PI 3-kinase signaling.

Other global studies have previously identified mRNAs that are targeted for degradation by KSRP. Ruggiero et al. compared mRNAs that were bound by KSRP and those that were upregulated by overexpression of Akt and identified a set of eight mRNAs that both were targets of KSRP and were in-

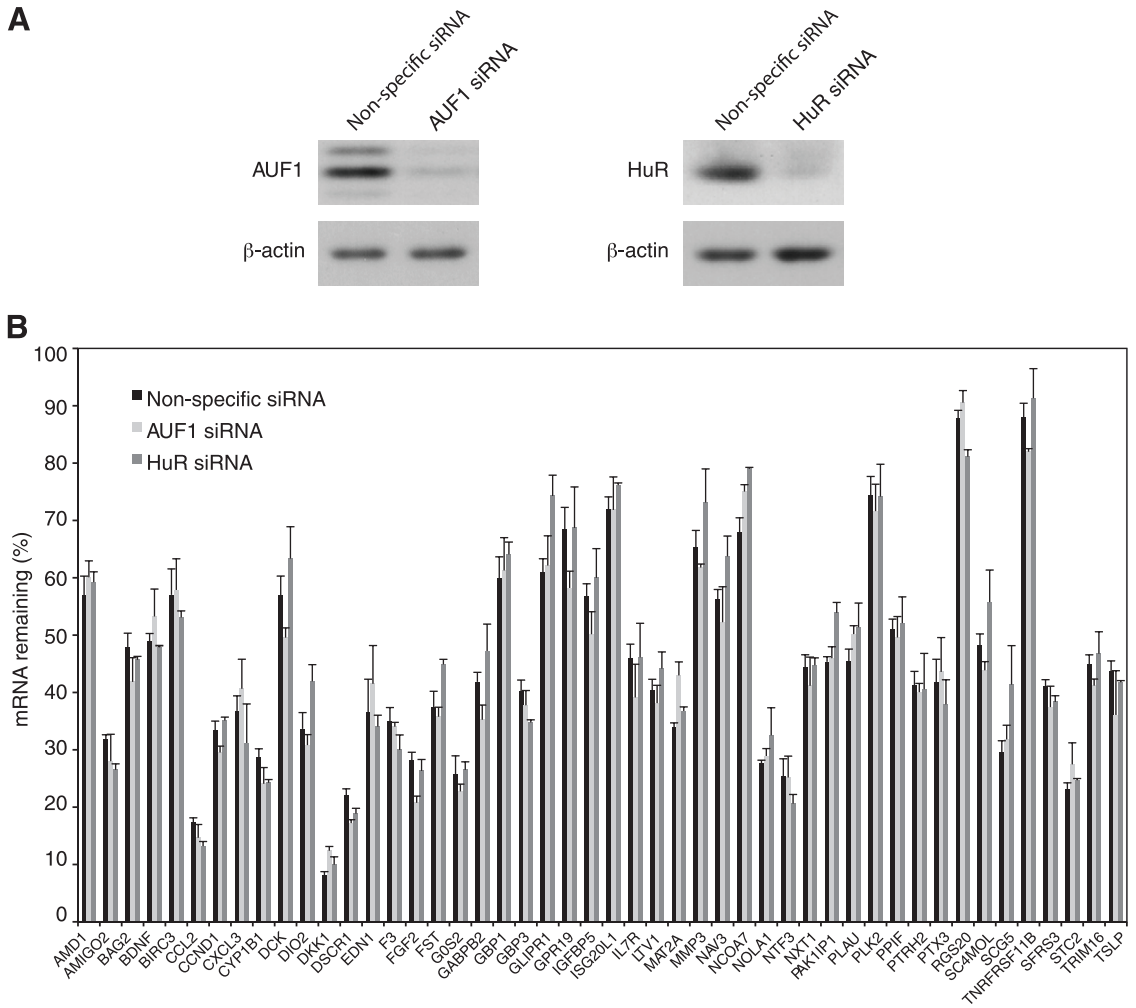

FIG. 6. Knockdown of either AUF1 or HuR does not affect PI 3-kinase-regulated mRNA degradation. Proliferating T98G cells were transfected for 72 h with AUF1 siRNA, HuR siRNA, or a nonspecific control siRNA. Immunoblot analysis was used to measure knockdown of AUF1 and HuR (A). Cells were treated with DMSO or LY294002 for 4 h, after which levels of 48 downregulated mRNAs were measured by real-time RT-PCR (B). Data represent the percentages of mRNA compared to the level for the DMSO vehicle control and are the means of results from five (nonspecific control), three (AUF1), or two (HuR) independent experiments ± SE.

duced by Akt overexpression (49). In a similar manner, Winzen et al. identified a set of 100 mRNAs that were induced in response to stimulation with the inflammatory cytokine interleukin 1 and were both bound by KSRP and stabilized by KSRP knockdown (60). Of the 11 mRNAs that we found to be stabilized by knockdown of KRSP following inhibition of PI 3-kinase, 9 had previously been identified by Winzen et al. as bound and/or stabilized by KSRP knockdown (60), while *CCL2* and *G0S2* are newly identified targets of KSRP.

Although Akt has also been shown to inhibit the activity of BRF1, which led to the stabilization of a reporter gene containing the 3′ UTR of interleukin 3 (4, 52), other genes whose mRNA degradation is regulated by the PI 3-kinase pathway via BRF1 have not been identified prior to this study. Furthermore, only a few BRF1 target genes have been determined, although its family member TTP has been much more extensively studied (2). Of the 31 mRNAs that we found to be stabilized by BRF1 knockdown, only *BIRC3*/*c-IAP2* has previously been reported to be regulated by BRF1 (38), although TTP has been shown to regulate *CCL2* (51) and *CCND1* (41).

Although there was a significant enrichment of sequences defined as ARE motifs within the 3′ UTRs of genes downregulated by PI 3-kinase inhibition, the presence of these motifs alone is not sufficient to predict ARE-mediated decay and may reflect the need for additional sequence features to make a functional ARE. Using multiple variations of the motif, including the pentamer, nonamers, conserved heptamer, variations on the heptamer, and total number of all AREs, we did not find any significant correlation between the number of ARE motifs and the half-lives of the downregulated mRNAs. Although the nonamer was present in 30% of the rapidly degraded mRNAs (half-lives of <3 h), it was also present in 10% of the stable mRNAs (half-lives of >3 h). Furthermore, both the pentamers and the heptamers were found in 85% and 70% of the mRNAs in both groups. Thus, although many short-lived mRNAs contain an ARE

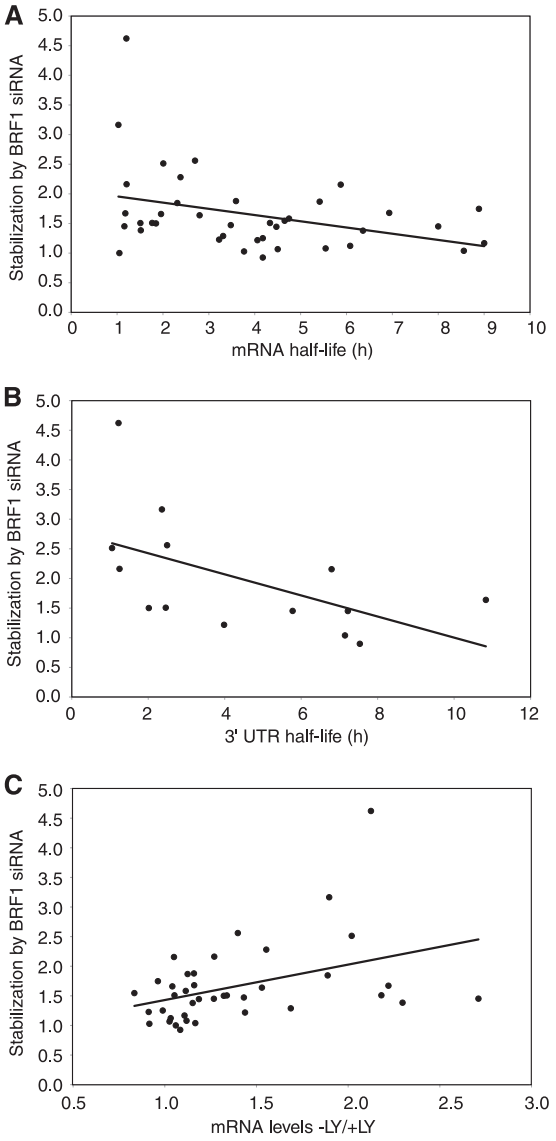

FIG. 7. Correlations between mRNA stabilization by BRF1 siRNA, mRNA half-lives, and PI 3-kinase-regulated mRNA degradation. The mRNA stabilization resulting from BRF1 knockdown (the ratio of mRNA levels in cells transfected with BRF1 siRNA compared to the level for control siRNA) (Fig. 5) was compared to the mRNA half-lives following treatment with actinomycin D plus LY294002 (LY) (Fig. 1) ($r^2 = 0.13$; $P = 0.01$; $n = 39$) (A), the half-lives of the β-*globin*–3′ UTR fusion transcripts (Fig. 3) ($r^2 = 0.3$; $P = 0.02$; $n = 14$) (B), or the ratio of mRNA levels in cells treated for 4 h with actinomycin D with or without LY294002 (Fig. 1) ($r^2 = 0.2$; $P = 0.005$; $n = 39$) (C).

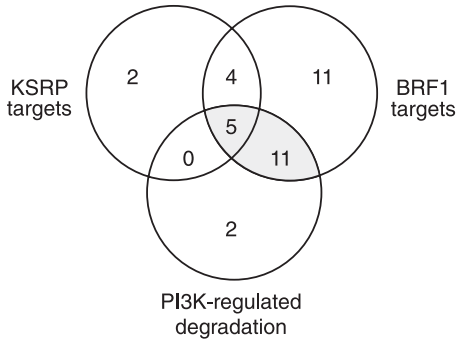

FIG. 8. Relationship between genes regulated by PI 3-kinase and ARE-BPs. Shown is the distribution of the 48 mRNAs downregulated upon PI 3-kinase inhibition according to whether their mRNA degradation increased upon PI 3-kinase inhibition (PI3K-regulated) or was stabilized by KSRP and/or BRF1 knockdown.

motif, these elements are also present in more stable mRNAs. Conversely, many rapidly degraded mRNAs do not have an ARE sequence. Several global studies have investigated the relationship between mRNA decay rates and the presence of typical ARE motifs, which have also determined that the presence of an ARE motif does not predict a short mRNA half-life (25, 37, 47, 63). Our results and those obtained by others demonstrate that these sequence elements are not an accurate indicator of rapidly degraded mRNAs, suggesting that other signals are also responsible for degradation. Of note, a functional ARE may also include a U-rich region, an overall U-rich content, or multiple motifs (3, 14). Therefore, simply identifying the presence of an ARE motif or motifs does not reflect the complexity of regulation of these elements and does not recognize other sequence features that may be necessary to mediate mRNA degradation.

Additionally, we investigated the relationship between the presence of an ARE-BP binding motif and mRNA degradation regulated by an ARE-BP. Because it has previously been shown that the multiple domains of KSRP allow it to bind to a broad range of motifs as a result of each domain recognizing different sequences (26), we focused on mRNAs regulated by BRF1. The optimal binding sequence for TTP family members, which includes BRF1, has been identified as the UUAU UUAUU nonamer (5, 8, 32, 35, 61). TTP can also bind to variations of this sequence, namely, a UAUUUAU heptamer (36), where the internal U sequence can consist of either two or four Us with minimal loss of affinity (8). Of the mRNAs stabilized by BRF1 knockdown, 19% contained a nonamer and 58% contained some variation of the heptamer; however, we did not find that any of these motifs were sufficient to predict BRF1 regulation. There was no strong correlation between AREs and amount of stabilization, and in fact, several mRNAs that were stabilized by BRF1 knockdown did not contain any of these AREs. Conversely, some mRNAs that did contain a motif were not affected by knockdown of BRF1. These results are similar to what has been observed in studies of TTP, in which only 33 out of 250 mRNAs stabilized in $TTP^{-/-}$ cells were found to have at least two of the heptamer sequences (36), and in a separate study, where fewer than half of TTP-bound mRNAs contained a consensus nonamer (54). Overall, this suggests that the sequence to which these ARE-BPs bind may be more flexible than previously thought or that these proteins bind indirectly to the mRNA via interactions with other proteins. Indeed, it has previously been shown that not only can many mRNAs bind to multiple ARE-BPs but there can be functional cooperation between the ARE-BPs, with either synergistic or antagonistic effects (3). Moreover, mRNA stability can be influenced by sequences flanking the ARE (42, 53), by mRNA structure (24, 45, 46), or by other elements,

such as miRNAs, further illustrating the complexity involved in regulating mRNA decay.

Previous studies of the control of gene expression by PI 3-kinase signaling have focused primarily on transcriptional regulation. Our present results indicate that regulation of mRNA stability, predominantly mediated by BRF1, also constitutes a significant component of the overall program of gene expression controlled by PI 3-kinase in proliferating cells. Since it is well known that PI 3-kinase signaling is a critical regulator of cell survival and proliferation, these results imply a significant role for regulation of mRNA decay in these vital processes.

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