The ARE-dependent mRNA-destabilizing activity of BRF1 is regulated by protein kinase B

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Butyrate response factor (BRF1) belongs to the Tis11 family of CCCH zinc-finger proteins, which bind to mRNAs containing an AU-rich element (ARE) in their 3' untranslated region and promote their deadenylation and rapid degradation. Independent signal transduction pathways have been reported to stabilize ARE-containing transcripts by a process thought to involve phosphorylation of ARE-binding proteins. Here we report that protein kinase B (PKB/Akt) stabilizes ARE transcripts by phosphorylating BRF1 at serine 92 (S92). Recombinant BRF1 promoted *in vitro* decay of ARE-containing mRNA (ARE-mRNA), yet phosphorylation by PKB impaired this activity. S92 phosphorylation of BRF1 did not impair ARE binding, but induced complex formation with the scaffold protein 14-3-3. *In vivo* and *in vitro* data support a model where PKB causes ARE-mRNA stabilization by inactivating BRF1 through binding to 14-3-3.

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Introduction

The stability of mRNA varies considerably among different species of transcripts and is in many cases regulated in a complex fashion in response to external stimuli. Short-lived transcripts carry *cis* elements that appear to regulate the access to the mRNA decay machinery. A major element is the so-called AU-rich element (ARE), which is located in the 3' untranslated region (3'UTR) of many short-lived transcripts from cytokines, proto-oncogenes, growth factors or cell cycle regulators (Shaw and Kamen, 1986; Chen and Shyu, 1995;

Bakheet *et al*, 2001). AREs promote deadenylation and decapping (Gao *et al*, 2001), followed by degradation of the mRNA body (Shyu *et al*, 1991; Xu *et al*, 1997). Mammalian ARE-containing mRNA (ARE-mRNA) is thought to be degraded mainly by the exosome, a multiprotein complex containing 3'–5' exonucleases and helicases (Chen *et al*, 2001; Mukherjee *et al*, 2002). Stabilization of short-lived ARE-containing transcripts by exogenous signals leads to a rapid accumulation of mRNA, with a consequent increase of protein levels. Physiological examples are found in T-cells activated by immune stimulation (Lindsten *et al*, 1989), mast cells responding to IgE-linked allergens (Wodnar-Filipowicz *et al*, 1989; Wodnar-Filipowicz and Moroni, 1990), or macrophages stimulated by IL-1/TNF α (Huang *et al*, 2000), where increased cytokine production occurs. Deregulated ARE-dependent mRNA turnover can contribute to oncogenic transformation (Schuler and Cole, 1988; Nair *et al*, 1994; Stoecklin *et al*, 2003), inflammation (Carballo *et al*, 1998) and immunopathology (Kontoyiannis *et al*, 1999), underlying the physiological relevance of this process. Also, stress stimuli, including UV exposure (Gorospe *et al*, 1998; Wang *et al*, 2000), heat shock (Laroia *et al*, 1999) and hypoxia (Paulding and Czyzyk-Krzeska, 2000), can lead to stabilization of ARE-mRNA. In addition to mRNA turnover, the ARE has been reported to regulate translation (Kontoyiannis *et al*, 1999; Piecyk *et al*, 2000).

Several signal transduction pathways have been implicated in mRNA decay control. Activation of stress-induced c-jun N-terminal kinase (JNK) (Chen *et al*, 1998; Ming *et al*, 1998), p38 mitogen-activated protein kinase (MAPK) (Dean *et al*, 1999; Winzen *et al*, 1999; Brook *et al*, 2000; Clark *et al*, 2003; Frevel *et al*, 2003), MAPKAP kinase 2 (MK2) (Neininger *et al*, 2002), phosphatidylinositol 3-kinase (PI3-K) (Ming *et al*, 2001) and wnt- β -catenin pathway (Briata *et al*, 2003) has been shown to trigger stabilization of various transcripts.

Several ARE-binding proteins (AUBPs) affecting mRNA turnover have been identified. HuR, a member of the embryonic-lethal abnormal vision (ELAV in *Drosophila melanogaster*) family of RNA-binding proteins (Ma *et al*, 1996), stabilizes ARE-containing transcripts (Fan and Steitz, 1998). AUF1 (hnRNPD) exerts a stabilizing as well as a destabilizing function depending on cell type (Zhang *et al*, 1993; Chen *et al*, 2002) and isoform (Raineri *et al*, 2004). The Tis11 protein family members tristetraprolin (TTP) and butyrate response factors (BRF1 and BRF2) share a highly conserved CCCH tandem zinc-finger (Zn-finger) motif that binds RNA (Lai *et al*, 1999). TTP was initially identified by the Blackshear group as a positive regulator of TNF α mRNA decay (Carballo *et al*, 1998) by studying the autoimmune-like phenotype of TTP knockout (k.o.) mice. Subsequently, it was found to act by targeting ARE-mRNA to the exosome (Chen *et al*, 2001). BRF1 was recently identified by a functional genetic screen aimed at finding genes responsible for ARE-dependent decay (Stoecklin *et al*, 2002). Interestingly, BRF1 has been found to be a circadian gene (Storch *et al*, 2002) and its k.o. is lethal

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at embryonic day 11 (Stumpo *et al*, 2004). Both TTP and BRF1 can stimulate deadenylation (Lai *et al*, 2003). TIA/TIAR are RNA recognition motif (RRM)-containing proteins that regulate translational silencing of TNF α mRNA and recruitment into stress granules (Gueydan *et al*, 1999; Piecyk *et al*, 2000). Lastly, KSRP, a K homology (KH)-motif RNA-binding protein originally described as a neuronal specific splicing enhancer of c-src (Min *et al*, 1997), has been recently demonstrated to be an ARE-mRNA-specific destabilizing factor both *in vitro* and *in vivo* (Gherzi *et al*, 2004). An ARE-binding helicase has been described by Tran *et al* (2004).

AUBPs are obvious candidate targets for signaling pathways regulating mRNA turnover. HuR has been shown to act in concert with p38 in stabilizing IL-3 mRNA (Ming *et al*, 2001), although direct phosphorylation of HuR was not detected. TTP was found to be phosphorylated by p38, which inhibited its ARE-binding activity (Carballo *et al*, 2001; Zhu *et al*, 2001). In addition, the extracellular signal-related kinase 2 (ERK2) (Taylor *et al*, 1995) and MK2 (Mahtani *et al*, 2001) are able to phosphorylate TTP *in vitro*. Phosphorylation of TTP by MK2 leads to 14-3-3 binding, inhibition of TTP activity and exclusion of TTP from stress granules (Johnson *et al*, 2002; Chrestensen *et al*, 2003; Stoecklin *et al*, 2004). 14-3-3 may also interact with BRF1, as suggested by results from a yeast two-hybrid screening (Bustin and McKay, 1999) and from GST-pulldown experiments (Johnson *et al*, 2002).

Considerable progress in the analysis of mRNA turnover has been made by the development of *in vitro* decay systems. As control can be exercised over the choice of reagents to be added/omitted, different aspects of post-transcriptional regulation, including deadenylation, decapping and degradation of ARE-containing RNA, can be studied (Ford *et al*, 1999; Chen *et al*, 2000; Gao *et al*, 2001). *In vitro* AREs destabilize RNA through binding of AUBPs such as KSRP and TTP, which in turn recruit both the exosome and the deadenylase, thus targeting the RNA for exosomal decay (Chen *et al*, 2001; Lai *et al*, 2003; Gherzi *et al*, 2004). *In vitro* systems therefore provide a powerful tool to study the regulation of ARE-dependent decay.

Here we show that BRF1 is able to promote ARE-dependent decay in an *in vitro* system, and that this activity is strongly reduced by phosphorylation of BRF1 by protein kinase B (PKB) at serine 92 (S92). Using a phospho-specific antipeptide antibody, we present evidence that BRF1 phosphorylation at S92 mediates insulin-induced mRNA stabilization *in vivo*. Furthermore, BRF1 phosphorylation was found to provide a docking site to 14-3-3, suggesting that this protein may sequester BRF1 from the cellular decay-promoting machinery.

Results

Activated PKB stabilizes ARE-containing reporter mRNA

Previously, we have shown that activated PI3-K is able to stabilize ARE-containing reporter mRNA upon transfection into NIH 3T3 cells (Ming *et al*, 2001). We tested whether PKB, a downstream effector of PI3-K, mediates this effect. An activated form of PKB (m/pPKB) (Andjelkovic *et al*, 1997) was transfected together with a tetracycline-sensitive β -globin reporter construct containing the ARE of IL-3 (Tet- β -globin-IL3UTR) (Stoecklin *et al*, 2002) into NIH 3T3 B₂A₂-

23 mouse fibroblasts (Xu *et al*, 1998). These cells stably express the Tet-responsive transcriptional activator tTA. When transcription of the reporter was inhibited by the addition of tetracycline, rapid degradation of the message with a half-life of less than 1 h was observed, while the reporter remained stable in the presence of activated PKB (Figure 1A and B). Activation of PKB was therefore sufficient to stabilize the ARE reporter transcript.

BRF1 is phosphorylated at S92 *in vitro*

Inspection of the coding sequence of BRF1 (SwissProt database; accession number Q07352) revealed the presence of two overlapping RXRXXS motifs, including S90 and S92, corresponding to PKB consensus phosphorylation sites (Alessi *et al*, 1996). Interestingly, this PKB recognition motif is highly conserved in human, mouse and *Xenopus leavis* BRF1 (Figure 2A), but it is not present in TTP, although these closely related proteins show considerable homology throughout other regions.

To address the question whether BRF1 is indeed a substrate of PKB, we first performed *in vitro* phosphorylation experiments. Either full-length recombinant BRF1 or three fragments consisting of the N-terminus (aa 3–110), the central Zn-finger domain (aa 111–179) or the C-terminus of BRF1 (aa 180–338) were tested as substrates for PKB β . Only the full-length rBRF1 and the N-terminal fragment containing S90 and S92 proved to be efficiently phosphorylated (Figure 2B, lanes 5–12).

We used the N-terminal fragment (aa 3–110) of BRF1 to identify the site phosphorylated by PKB via mass spectro-

Figure 1 ARE-mRNA stabilization by PKB. (A) The Tet- β -globin-IL3UTR reporter gene was transfected alone (lanes 1–3) or in combination with constitutively activated m/pPKB (lanes 4–6). After 24 h, transcription was stopped by addition of doxycycline. Cytoplasmic RNA was isolated at the indicated time points and processed for northern blotting. (B) The graph shows the quantifications of five independent decay assays normalized to the actin signal. Standard errors are shown unless too small to be represented.

Figure 2 PKB phosphorylates BRF1 on S92. (A) Alignment of BRF1 sequences from human, mouse, rat and *X. leavis*. S90 and S92 are indicated. (B) *In vitro* phosphorylation reactions were performed for the times indicated using 40 ng of activated recombinant PKB and 20 ng of either full length rBRF1-wt, a N-terminal fragment (aa 3–110), the Zn-finger domain (aa 111–179) or a C-terminal fragment (aa 180–338). The asterisk denotes the full-length BRF1 protein, lower bands are degradation products. (C) *In vitro* phosphorylation reactions were performed for the times indicated, using 40 ng of activated recombinant PKB and 20 ng of either rBRF1-wt, rBRF1-S90A or rBRF1-S92A.

metry (see Materials and methods). This revealed that BRF1 is preferentially phosphorylated at S92. Accordingly, a peptide containing the S92 residue (DSRFRDRSFSEG) was phosphorylated *in vitro* by purified activated PKB α and β at 60% of the rate of the GSK3 peptide (RPRTSSFAEG), which is the best-characterized substrate of this kinase (data not shown). To investigate whether S92 is indeed the major PKB phosphorylation site, we mutated S90 or S92 to alanine (S90A, S92A), and tested the recombinant proteins for substrate activity. In line with the mass spectrometry data, BRF1-wt and BRF1-S90A were equally phosphorylated by PKB (Figure 2C, lanes 5–12), whereas phosphorylation was considerably reduced by the S92A mutation (Figure 2C, lanes 13–16). It appears that in the absence of the S92 phosphorylation site PKB can phosphorylate S90, which represents an alternative consensus site.

BRF1 S92 phosphorylation regulates ARE-dependent RNA decay *in vitro*

We were curious to see whether BRF1 may show similar *in vitro* decay activity as reported for its close homolog TTP (Chen *et al*, 2001), as this would allow us to study the nature of the S92 phosphorylation under controlled *in vitro* conditions. We co-incubated S100 extracts from slowC cells, a line lacking BRF1 expression as the result of frameshift mutations

in both alleles (Stoecklin *et al*, 2002), with a radio-labeled RNA containing the 59 nt long ARE from IL-3 (ARE_{IL3}). While ARE_{IL3} was stable in the presence of slowC extracts (Figure 3A, lanes 1–4), it decayed rapidly when 5 ng or more of rBRF1 was added (Figure 3A, lanes 5–20). As a control for both ARE specificity of the observed decay and equal loading, a 250 nt RNA containing the IL-3 3'UTR lacking the ARE (Δ ARE) was added at the onset of the reaction. No degradation of the Δ ARE control could be observed under all conditions tested. We concluded that rBRF1 is able to promote rapid RNA decay in this ARE-dependent *in vitro* system.

We next investigated whether phosphorylation of rBRF1 by PKB would affect the *in vitro* decay rate. Unphosphorylated rBRF1-wt was compared to rBRF1-wt pretreated with activated PKB for 30 min (Figure 3B, lanes 5–12). A striking reduction of ARE_{IL3} decay was observed with phospho-rBRF1-wt (p-rBRF1-wt) as compared to unphosphorylated rBRF1-wt. To check whether this effect was due to phosphorylation of BRF1 at S92, we performed the same experiment using the rBRF1-S92A mutant. Again, rBRF1-S92A promoted degradation of ARE_{IL3} (Figure 3B, lanes 13–16), yet the mutant appeared refractory to inhibition by PKB (Figure 3B, lanes 17–20). Quantification (Figure 3B, lower panel) revealed an intermediate decay pattern, which may result from alternative phosphorylation at S90 (see also Figure 2C). From these data, we concluded that, *in vitro*, the decay-promoting activity of BRF1 is inhibited by phosphorylation at S92.

BRF1 is phosphorylated at S92 *in vivo*

To extend this study to endogenous BRF1, we first raised an antibody directed to a trisdekapeptide containing phosphorylated S92 (FRDRSFpSEGGGERL). Affinity-purified antibody recognized rBRF1 phosphorylated by PKB with a high specificity (Figure 4A). As an additional control, we preincubated either the antibody described above or an antibody recognizing the C-terminus of BRF1 (Raineri *et al*, 2004) with the S92-containing phosphopeptide (PP; see above). In the case of the phospho-antibody, the western signal could specifically be competed away, while detection of unphosphorylated BRF1 was not impaired (Figure 4B, compare lanes 3 and 6).

We then examined whether BRF1 becomes phosphorylated at S92 *in vivo*. Insulin, a known activator of PKB (Burgering and Coffey, 1995) was used to stimulate HIRc-B rat fibroblasts, which express the human insulin receptor and are widely used to investigate insulin signaling (McClain *et al*, 1987). To reduce PKB background activity, we starved the cells overnight in serum-free medium. After stimulation with insulin for 15 min, PKB was strongly activated (Figure 4C, lane 2, middle panel) and we monitored BRF1 phosphorylation by western blotting using the p-BRF1 antibody. Phosphorylation of BRF1 at S92 could be detected under conditions of PKB activation (Figure 4C, lane 2, upper panel). BRF1 levels did not change in response to insulin stimulation (data not shown). This phosphorylation was partially inhibited by pretreating the cells with the PI3-K inhibitor wortmannin (WM), which completely abolishes PKB activation (Figure 4C, lanes 2 and 3). As WM treatment was not able to block BRF1 phosphorylation completely, we hypothesize that, in addition to PKB, another unidentified kinase may also phosphorylate BRF1 at S92. Phosphorylation of a single serine by more than one

Figure 3 BRF1 S92 phosphorylation regulates ARE-dependent RNA decay *in vitro*. **(A)** Radioactively labeled ARE_{IL3} and ΔARE transcripts were incubated together in S100 extract from slowC cells. Decay reactions were performed at 37°C in the presence of increasing amounts of rBRF1-wt, stopped at the indicated time points and resolved on a 10% urea-polyacrylamide gel. ΔARE transcripts served as specificity and loading control. **(B)** Decay reactions were carried out in the absence (lanes 1–4) or in the presence of 20 ng of rBRF1-wt (lanes 5–12) or rBRF1-S92A (lanes 13–20) after pretreatment with buffer (lanes 5–8/13–16) or activated PKB (lanes 9–12/17–20). The graph below shows quantification of at least four independent experiments after normalizing to the ΔARE signal. Standard error bars were too small to be represented.

kinase is known to occur (Johannessen *et al*, 2004). To obtain *in vivo* evidence that indeed PKB is phosphorylating BRF1, we transfected BRF1-wt or the BRF1-S90A/S92A double mutant construct together with m/pPKB or a kinase-dead variant of PKB (PKBkd) into 3T3 cells. Strong phosphorylation of BRF1-wt, but not of the mutant, was observed in m/pPKB-transfected cells (Figure 4D). Together, these data indicate that PKB does phosphorylate BRF1 *in vivo* at S92.

ARE-mRNA is stabilized under conditions that phosphorylate BRF1

We then wanted to know whether phosphorylation of BRF1 impairs its decay-promoting activity *in vivo*. We co-transfected the Tet-β-globin-IL3UTR reporter together with the tTA transactivator (pTetOff, Clontech) into HIRc-B cells. When transcription of the reporter was turned off by adding doxycycline, we observed rapid decay of the reporter mRNA with a half-life of approximately 1 h (Figure 5A, lanes 1–3). After pretreatment of cells with insulin for 15 min, the mRNA was more stable (lanes 4–6), and this effect was sensitive to WM (lanes 7–9). We concluded that *in vivo* ARE-mRNAs become stabilized under conditions where phosphorylation of BRF1 at S92 occurs. Quantification from three parallel experiments indicated that the observed changes are statistically significant (Figure 5B).

To further evaluate the involvement of PKB in stabilizing ARE-mRNA, we made use of mouse embryonic fibroblasts with k.o. of PKBα (MEF-PKBα^{-/-}) (Yang *et al*, 2003). When MEF-PKBα^{-/-} and +/+ cells were compared by western blotting, we noticed strong PKB phosphorylation upon insulin stimulation even in -/- cells, apparently via the PKBβ and γ isoforms. In unstimulated MEF-PKBα +/+ cells, there was background phosphorylation of BRF1 (Figure 5C), presumably by a kinase other than PKB (see also Figure 4C). Insulin stimulation increased this signal and stabilized the reporter mRNA in a WM-sensitive fashion. In MEF-PKBα cells, the p-BRF1 signal was low with no response to insulin. When mRNA stability of the reporter was examined, decay was generally more rapid in -/- cells and insulin failed to induce stabilization (5D). These data support an *in vivo* role for PKBα in stabilization of ARE-mRNA.

BRF1 phosphorylation leads to 14-3-3 binding

To elucidate the mechanism how PKB inhibits BRF1, we first asked whether BRF1 phosphorylation affects ARE binding, as it was found for TTP (Carballo *et al*, 2001). By electrophoretic mobility shift assay, we compared increasing amounts of unphosphorylated rBRF1 to rBRF1 pretreated with activated PKB for its ability to bind a radio-labeled ARE_{IL3} probe. Phosphorylation did not impair ARE binding

Figure 4 BRF1 is phosphorylated *in vivo* at S92. (A) Indicated amounts of rBRF1-wt were incubated with buffer alone or activated PKB for 30' at 30°C and processed for western blotting. Phosphorylated (upper panel) or unphosphorylated BRF1 (lower panel) were detected by corresponding antibodies directed against a p-S92 containing or a C-terminal peptide (see Materials and methods). (B) rBRF1 phosphorylated as in (A) was processed for western blotting. Antibodies recognizing phosphorylated BRF1 (upper panel), BRF1 (middle panel) or PKB (lower panel) were pre-incubated on ice for 30' with or without 50 μ g of the PP used for immunization before adding to the blot. (C) HIRc-B cells were serum starved overnight, treated with WM (200 nM) for 30' where indicated before stimulation with insulin (20 μ g/ml) for 15' (lanes 1–3). Whole-cell extracts were analyzed by western blot, detecting p-BRF1, phospho-PKB and α -tubulin as a loading control. (D) NIH3T3 B₂A₂-23 cells were transfected with m/pPKB or kinase-dead PKB (PKBkd) and BRF1-wt or BRF1S90A/S92A (BRF1AA). Whole-cell extracts were analyzed by western blot, detecting p-BRF1, phospho-PKB and α -tubulin as a loading control.

(Figure 6A). The inhibitory effect of S92 phosphorylation on BRF1-destabilizing activity could therefore not be explained by a change in the RNA-binding capacity of BRF1. An alternative mechanism responsible could be binding of phosphorylated BRF1 to an inhibitory factor. A candidate binding partner was 14-3-3, as various isoforms of this protein bind to targets phosphorylated by PKB (Tzivion and Avruch, 2002) and, in addition, 14-3-3 has been found to interact with BRF1 (Bustin and McKay, 1999; Johnson *et al*, 2002). To test this possibility, His-tagged rBRF1, either untreated or phosphorylated by PKB, was added to slowC cell extracts, purified by Ni-NTA beads and processed for western blotting. Phosphorylated but not unphosphorylated BRF1 pulled down 14-3-3 (Figure 6B, lanes 1 and 2). Furthermore, addition of the PP containing phosphorylated S92 antagonized 14-3-3 binding in a dose-dependent fashion (lanes 3–5), whereas the analogous peptide containing the S92A substitution (AP) was unable to interfere with 14-3-3 binding (lanes 6–8).

If interaction with 14-3-3 was responsible for BRF1 inhibition, we would expect the PP to affect binding and hence BRF1 activity in the *in vitro* decay assay. Indeed, addition of PP could restore decay activity of phosphorylated BRF1 (Figure 6C, lanes 9–12), whereas the AP peptide was ineffective (Figure 6C, lanes 13–16). Taken together, these data indicate that PKB-induced inhibition of BRF1 occurs through binding of 14-3-3.

Finally, *in vivo* experiments were performed to verify that PKB regulates the interaction between BRF1 and 14-3-3. For this experiment we chose COS7 cells, as complex formation between phosphorylated TTP and 14-3-3 was recently demonstrated in this line (Stoecklin *et al*, 2004). Cells were transiently transfected with wt or mutant BRF1 constructs carrying a His-tag. BRF1 was purified from the cell extracts using Ni-NTA beads, and western blotting revealed co-precipitation of 14-3-3 with BRF1-wt (Figure 6D, lane 2). Mutation of BRF1 at S90/92 strongly prevented the interaction with 14-3-3 (lane 3). In order to see whether activation of the PKB pathway would stimulate 14-3-3 binding, cells were co-transfected with m/pPKB and serum-starved to reduce endogenous PKB activity. m/pPKB strongly induced binding of 14-3-3 to BRF1-wt (lane 6). Notably, induced binding of 14-3-3 was not observed with the S90A/S92A double mutant (BRF1AA, lane 8). Together, these data suggest that S92 phosphorylation by PKB leads to interaction of 14-3-3 with BRF1. Whereas this interaction does not impair RNA binding, it may inhibit recruitment of the BRF1-RNA complex to the exosomal decay machinery and thereby lead to stabilization of ARE-mRNA.

Discussion

In this report, we provide evidence that phosphorylation of BRF1 by PKB stabilizes ARE-mRNA. As initially reported for PI3-K (Ming *et al*, 2001), its downstream target PKB efficiently stabilized ARE-containing reporter transcripts in transfection experiments (Figure 1). BRF1 contains two overlapping consensus sequences for PKB phosphorylation at S90 and S92, both of which are conserved between mammals and *Xenopus* (Figure 2). Mass spectrometry analysis of a recombinant BRF1 N-terminal peptide phosphorylated by PKB revealed that phosphorylation occurs exclusively at S92. *In vitro* kinase assays with full-length wt protein and S90A/S92A mutants as substrates confirmed S92 as the major phosphorylation site.

To explore the role of S92 phosphorylation, we took advantage of the fact that rBRF1, as previously reported for TTP, is able to promote degradation of ARE-RNA when added to an S100 extract. Interestingly, the decay-promoting activity of BRF1 (Figure 3), but not its ARE-binding activity (Figure 6A), was abrogated by PKB phosphorylation. The rBRF1-S92A mutant was competent in promoting decay, but insensitive to PKB inhibition, establishing S92 as a regulatory site. To our knowledge, this is the first example where specific phosphorylation was shown to affect ARE-dependent mRNA decay *in vitro*.

To validate these findings *in vivo*, we analyzed endogenous BRF1 phosphorylation using a S92 phospho-specific antibody. In HIRc-B cells expressing the human insulin receptor, treatment with insulin triggered rapid phosphorylation of both PKB and BRF1 (Figure 4). The latter effect was only

Figure 5 Effect of insulin on reporter mRNA decay. (A) At 24 h after co-transfection of the Tet- β -globin-IL3UTR reporter gene and the Tet-responsive transcriptional activator tTA (pTET-on), HIRc-B cells were split into fresh medium for 16 h. Where indicated cells were pretreated with WM (200 nM) for 30' before addition of insulin (20 μ g/ml) for another 15'. Then doxycycline was added (2 μ g/ml) to stop transcription, cytoplasmic RNA was isolated at the indicated time points and processed for northern blotting. (B) Quantification of three independent experiments is shown normalized to the 18S rRNA signal. (C) MEF-PKB α -/- and +/+ cells were pretreated with WM for 30' prior to insulin (Ins) stimulation for 15' as indicated. p-BRF1, phospho-PKB, PKB and tubulin protein levels were analyzed by western blot. (D) MEF-PKB α -/- and +/+ cells were treated as described for HIRc-B in (A). Reporter RNA was analyzed by northern blot. One representative experiment is shown.

partially blocked by the PI3-K inhibitor WM, suggesting that a second kinase in addition to PKB may also phosphorylate S92. Support for this notion was found in MEF-PKB α +/+ cells, where in the absence of PKB activation background BRF1 phosphorylation was observed (Figure 5C). A single serine can indeed be phosphorylated by more than one kinase, as known from nuclear factor CREB, which becomes phosphorylated at position 133 not only by PKA, but also by PKB and other AGC kinases (Johannessen *et al*, 2004). That PKB can phosphorylate *in vivo* BRF1 at S92 was obvious from transfection experiments, where activated PKB phosphorylated BRF1-wt but not BRF1-S90A/S92A (Figure 4D).

When ARE reporter mRNA decay following insulin stimulation was examined, we observed WM-sensitive stabilization in HIRc-B (Figure 5A) and MEF-PKB α +/+ cells, but not in MEF-PKB α -/- cells. In the latter, the p-BRF1 signal was low under all conditions and correlated with rapid decay.

To explore the consequences of BRF1 phosphorylation, we turned our attention to the 14-3-3 protein. Members of the family of 14-3-3 proteins are known to bind a variety of target proteins phosphorylated by kinases belonging to the AGC kinase family, which includes PKB. The optimal binding site for 14-3-3 is RSXpSXP (Tzivion and Avruch, 2002), with preference for an aromatic or positively charged amino acid at position -1. The sequence surrounding S92 of BRF1

(RSFpSEG, see Figure 2) represents a 14-3-3 binding site only lacking a P at position +2 compared to the optimal consensus. However, BRF1 has been shown to interact with 14-3-3 in yeast two-hybrid assays and pulldown experiments (Bustin and McKay, 1999; Johnson *et al*, 2002). Our experiments demonstrate that PKB induces complex formation between BRF1 and 14-3-3 in the same extracts in which BRF1-dependent *in vitro* RNA decay is regulated by PKB. The S92-containing PP, which competitively dissociated the BRF1/14-3-3 complex, also specifically antagonized the inhibitory effect of PKB on BRF1 activity. As our BRF1 antibody was not able to pull down endogenous protein from HIRc-B cell (data not shown), we overexpressed His-tagged BRF1 in COS7 cells. 14-3-3 co-purified with BRF1-wt, but not with BRF1-S90A/S92A. In addition, co-transfection of activated PKB strongly stimulated binding of 14-3-3 to BRF1-wt, but not to mutant BRF1. Together, our results suggest that BRF1 phosphorylation at S92 leads to binding of 14-3-3, which inhibits the decay-promoting activity of BRF1. Interaction with 14-3-3 has been reported to have diverse functional consequences depending on the targets involved, including inhibition of apoptosis (BAD protein), increasing nuclear export rates (FKH transcription factor), enhancement of DNA binding (p53 protein), protection from proteolysis or by simply serving as a phosphorylation-dependent scaffold

Figure 6 S92 phosphorylation of BRF1 induces complex formation with 14-3-3. **(A)** Electrophoretic mobility shift assay. Indicated amounts of rBRF1 were pre-incubated with or without activated PKB, mixed with radio-labeled ARE_{IL3} RNA and resolved on 4% nondenaturing PAGE. **(B)** His-tagged rBRF1 pretreated with buffer (lane 1) or activated PKB (lanes 2–11) was mixed with S100 extract from slowC cells. Increasing concentrations of the phosphorylated peptide surrounding S92 (aa 86–98) of BRF1 (PP) or the corresponding S92A mutant peptide (AP) were added. rBRF1 was purified using Ni-NTA beads, and 14-3-3 and BRF1 were detected with the corresponding antibodies. The lower band represents 14-3-3, the upper His-tagged rBRF1. **(C)** *In vitro* decay reactions were carried out as described in Figure 3 in the presence of 0.4 mM of PP (lanes 9–12) or AP (lanes 13–16). The graph on the right shows quantification of four independent experiments after normalizing to the ΔARE signal. Standard error bars were too small to be represented. **(D)** COS7 cells were transfected with vector, bsdHisBRF1 wt, bsdHisBRF1 aa, either alone (lanes 1–3) or in combination with m/pPKB (lanes 5–8), as indicated. In the experiment shown on the right (lanes 4–8), FCS was withdrawn for 40 h. Expression of His-tagged BRF1, phospho-PKB and 14-3-3 in the cell extracts was determined by western blotting (load). After purification of BRF1 by Ni-NTA beads, co-precipitation of 14-3-3 was tested by western blotting (Co-P).

(for references, see Tzivion and Avruch, 2002). A reasonable assumption, therefore, is that 14-3-3 sequesters BRF1 *in vivo* and thereby restricts its access to the decay machinery. Interestingly, Stoecklin *et al* (2004) have recently reported that phosphorylation of TTP by MK2 at S178 and S52 leads to formation of a similar complex with 14-3-3, which reduces TTP activity and excludes TTP from the stress granules. While a homologous site to S178 in TTP is present in BRF1 (S201 or 203), the N-terminal S52 is not. On the other hand, TTP does not contain a homolog to S92 in BRF1. It will be interesting to see whether S201 or 203 in BRF1 is also phosphorylated by MK2 or another kinase, as this may allow cooperative regulation of BRF1 by two independent signal pathways. As 14-3-3 proteins generally bind as dimers to two phosphorylated residues on their target, dual phosphorylation of BRF1 may be required for full binding of 14-3-3 and robust inhibition of its activity.

An emerging model of regulated ARE-dependent mRNA decay has the following features: In resting cells, AUBPs such as BRF1, TTP, KSRP or AUF1 form part of a multiprotein complex that binds the ARE and facilitates access of the RNA to the decay machinery. More than one AUBP may act on a given species of mRNA, as shown for KSRP and BRF1 in HT1080 cells (Gherzi *et al*, 2004), and the relative contributions of AUBPs may depend on the cell type. The two Zn-finger domains of the Tis11 gene family proteins recognize the UUAUUUAUU nonamer, the hallmark of class-II AREs, by binding to two adjacent 5'-UAAU-3' subsites (Hudson *et al*, 2004). Thus, an ARE containing more than one nonamer is likely to bind several AUBPs. Cell activation or stress leads to a change in the phosphorylation status of TTP, BRF1 or AUF1 (Wilson *et al*, 2003a,b), changing their interacting partners and thereby altering the composition of the multiprotein complex targeting the ARE. In such a situation, the stabilizing

HuR protein may be able to bind preferentially to the ARE, while the poly(A)-ribonuclease (PARN), the exosome, the decapping machinery and the 5' to 3' exonucleases lose access to the ARE-mRNA. In addition, mRNA has not only the binary choice of being degraded or delivered to the translation machinery, but also may alternatively be stored in stress granules for later translation. Elucidation of the interplay between these complex and dynamic processes requires the identification of the major proteins involved, as well as their post-translational modification and their functional significance.

Materials and methods

Plasmids

The plasmids bsdHisBRF1_{S90A} and bsdHisBRF1_{S92A} were generated from bsdHisBRF1_{wt} (Stoecklin *et al*, 2002) by site-directed mutagenesis, using as upstream primers TV43 (5'-CCGCGCC TTCTCGGAAGGGGCGAG-3') and M2521 (5'-GGAGCGGTCTCGG AAGCGG-3'), respectively, and as downstream primers TV44 (5'-TCTCGGAAGCGGTCTCTCGGAGC-3') and M2518 (5'-TTCGCC GAAGGGGCGAGCG-3'), respectively. bsdHisBRF1_{S90A} served as template to construct bsdHisBRF1_{AA} using M2518 and M2522 (5'-GGCGCGGTCTCGGAAGCGG-3'). For plasmid SP6-ARE, a 59 nt fragment spanning the ARE of IL-3, including six AUUUA pentamers, was amplified from murine IL-3 cDNA by PCR using primers M1977 (5'-ATGGATCCTTCCATTAAGGC-3') and M1978 (5'-ATAGATCTTCACAGAAGGC-3'). The amplicon was digested with *Bam*HI and *Bgl*II, and ligated into the *Bgl*II site of pSP73 (Promega). Plasmids (m/p)-HA-PKB α and T7-ARE⁻ have been described previously (Andjelkovic *et al*, 1997; Stoecklin *et al*, 2002). For recombinant BRF1, the constructs used were pQE-30-BRF1_{S90A}, pQE-30-BRF1_{S92A} and pQE-30-BRF1_{AA}, which were constructed in the same way as pQE-30-BRF1_{wt} (Stoecklin *et al*, 2002), using bsdHisBRF1_{S90A}, bsdHisBRF1_{S92A} and bsdHisBRF1_{AA}, respectively. To generate pQE-30-N-term., pQE-30-BRF1_{wt} was digested with *Hind*III and *Hinc*II, blunt-ended and religated. For pQE-30-C-term., the *Sap*I (blunt)-*Xho*I fragment of bsdHisBRF1_{wt} was inserted into the *Sma*I-*Sal*I sites of pQE-30 (Qiagen). For pQE-30-Zn-finger, the *Hinc*II(blunt)-*Sap*I(blunt) fragment of pQE-30-BRF1_{wt} was inserted into the *Sal*I (blunt) site of pQE-30. Recombinant proteins were produced as described (Stoecklin *et al*, 2002).

Cell culture and transfection

SlowC (Stoecklin *et al*, 2002), NIH 3T3 B₂A₂-23 (Xu *et al*, 1998) and COS7 cells were grown in Iscove's modified Dulbecco medium (IMDM) supplemented with 10% fetal calf serum (FCS), 50 μ M 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. HIRc-B cells (McClain *et al*, 1987), MEF-PKB α -/- and +/- cells (Yang *et al*, 2003) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with FCS, 2-mercaptoethanol, glutamine, penicillin and streptomycin as described above. To reduce the background PKB activity, cells were starved overnight in serum-free medium before stimulation with insulin (20 μ g/ml). Transfection was performed using Lipofectamine 2000 reagent (Life Technologies) following the manufacturer's protocol.

In vitro phosphorylation

The reaction was performed in 30 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM DTT, 0.2 mM ATP γ S (unlabeled or ³²P-labeled) with 4 ng/ μ l rBRF1 and 10 ng/ μ l activated PKB for 30' at 30°C (Yang *et al*, 2002).

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay was performed as described (Stoecklin *et al*, 2002), with the exception that ARE_{IL3} RNA transcribed from SP6-ARE was used as probe.

ARE-mRNA decay analysis

In vivo measurement of RNA decay in NIH 3T3 B₂A₂-23 and HIRc-B cells using the tetracycline-sensitive Tet- β -globin-IL3UTR reporter gene was performed as described previously (Stoecklin *et al*, 2002).

For *in vitro* analysis of RNA decay, S100 fractions were prepared from slowC cells and RNA decay reactions were performed as described previously (Chen *et al*, 2001), with the addition of 100 nM of okadaic acid. ³²P-labeled transcripts from SP6-ARE and T7-ARE⁻ (Stoecklin *et al*, 2002) were synthesized *in vitro* using SP6 or T7 RNA polymerase (Promega), respectively. Both transcripts were co-incubated in all reactions.

Northern blotting analysis

RNA extraction and northern blotting analysis have been described previously (Stoecklin *et al*, 2000).

Western blot analysis

The following antibodies were used for Western blotting: sc-629 rabbit anti 14-3-3 β (Santa Cruz), sc-7270 mouse monoclonal anti-omni probe (Santa Cruz), phospho-Akt (Ser 473) 587F11 Monoclonal Antibody (Cell Signaling Technology), rabbit polyclonal anti-AKT (Cell Signaling) and mouse monoclonal anti α -tubulin (236-10501) (Molecular Probes).

Anti-p-BRF1 and anti-BRF1 antibodies were generated by immunizing rabbits with the KLH-linked peptides F-R-D-R-S-F-S(PO₃H₂)-E-G-G-E-R-L (aa 86–98) and S-D-Q-E-G-Y-L-S-S-S-S-H-S-G-S-D-S-P-T (aa 300–320) (Neosystem). Sera were affinity-purified using the above-mentioned peptides linked to activated CH Sepharose 4B (Pharmacia) according to the manufacturer's protocol. Proteins were visualized using ECL Advance (Amersham) or CDP-star (Roche).

Pulldown assay

In vivo: At 24–48 h after transfection of COS7 cells using lipofectamine 2000, cells were lysed in buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 10% glycerol, 20 mM 2-mercaptoethanol, 10 mM imidazole, 1 mM Na-vanadate, 50 mM NaF, 20 nM okadaic acid and EDTA-free 'complete' protease inhibitors (Roche). After incubation of the cytoplasmic fraction for 1 h at 4°C with 40 μ l of Ni-NTA magnetic agarose beads (Qiagen), the beads were washed four times in lysis buffer containing 20 mM imidazole, eluted in SDS-sample buffer and resolved on 4–20% gradient polyacrylamide tris-glycine gels (Invitrogen).

In vitro: In all, 40 ng of phosphorylated or unphosphorylated rBRF1 and different amounts of the peptides PP (F-R-D-R-S-F-S(PO₃H₂)-E-G-G-E-R-L corresponding to aa 86–98) or AP (F-R-D-R-S-F-A-E-G-G-E-R-L; Neosystem) were incubated with 40 μ g of S100 slowC extract in 100 mM Tris-HCl (pH 8.0), 1 mM Mg-acetate, 1.5 mM K-acetate, 150 mM NaCl, 10% glycerol, 10 mM imidazole and 100 nM okadaic acid in a total volume of 800 μ l. After incubation at 37°C for 30 min, Ni-NTA affinity purification was performed as described above.

Mass spectrometry

The N-terminal BRF1 band (aa 3–110) was excised from the gel, reduced with 10 mM DDT, alkylated with 55 mM iodoacetamide and cleaved with 0.5 μ g LysC (Achromobacter, Wako BioProducts) at 37°C overnight in 25 mM ammonium bicarbonate buffer (pH 8.0). After the LysC cleavage, the gel was dried and 0.5 μ g of Asp-N (Roche; sequencing grade) was added in 40 μ l of 50 mM sodium phosphate buffer (pH 8.0) at 37°C for 6 h. The extracted peptides were analyzed by capillary liquid chromatography tandem mass spectrometry (LC-MSMS) using a Magic C18 100 μ m $\times$ 10 cm HPLC column (Spectronex) connected on-line to an iontrap Finnigan DecaXP (ThermoFinnigan). PPs were identified with the neutral loss function, searching for fragment ions formed by the loss of phosphoric acid, 32.3, 48.5 or 97 Da from the (M+3H)³⁺, (M+2H)²⁺ and (M+H)⁺ ions, respectively. The PP was isolated by LC-MSMS, followed by cleavage with 0.1 μ g trypsin (Promega; sequencing grade) in 50 mM ammonium bicarbonate (pH 8.0) at 37°C for 1 h. The peptide mixture was then analyzed by LC-MSMS as described above and the PP was additionally sequenced by an MS3 experiment.

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