THE AU-RICH ELEMENT mRNA DECAY-PROMOTING ACTIVITY OF BRF1 IS REGULATED BY MITOGEN-ACTIVATED PROTEIN KINASE ACTIVATED PROTEIN KINASE-2

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ABSTRACT

Regulated mRNA decay is a highly important process for the tight control of gene expression. Inherently unstable mRNAs contain AU-rich elements (AREs) in the 3’ untranslated regions that direct rapid mRNA decay by interaction with decay-promoting ARE-binding proteins (ARE-BPs). The decay of ARE-containing mRNAs is regulated by signaling pathways, which are believed to directly target ARE-BPs. Here, we show that BRF1 involved in ARE-mediated mRNA decay (AMD) is phosphorylated by MAPK-activated protein kinase 2, MK2. In vitro kinase assays using different BRF1 fragments suggest that MK2 phosphorylates BRF1 at four distinct sites, S54, S92, S203, and an unidentified site at the C-terminus. Co-expression of an active form of MK2 inhibits ARE mRNA decay activity of BRF1. MK2-mediated inhibition of BRF1 requires phosphorylation at S54, S92, and S203. Phosphorylation of BRF1 by MK2 does not appear to alter its ability to interact with AREs or to associate with mRNA decay enzymes. Thus, MK2 inhibits BRF1-dependent AMD through direct phosphorylation. Although the mechanism underlying this inhibition is still unclear, it appears to target BRF1-dependent AMD at a level downstream from RNA binding and the recruitment of mRNA decay enzymes.
DEDICATION

For my parents
ACKNOWLEDGEMENTS

I would like to thank Dr. Ching-yi Chen for his continued support and mentorship for the five years that I have spent in his laboratory. Dr. Chen has played a key role in designing these experiments and provided experimental proof for the PKB independent inhibition of BRF1 by MK2. I would like to thank my thesis committee members Drs. David Bedwell, Louise Chow, Peter King and Tim Townes for all the encouragement and ideas that they have provided over the years. I acknowledge the contribution of Dr. Kyung Lee, who developed the in vitro phosphorylation system and identified MK2 substrates. I thank Dr. Chu-Fang Chou for carrying out RNA-BRF1 immunoprecipitations and enzyme interaction studies reported in this thesis. I also thank Christian Luber and Dr. Matthias Mann for their Mass Spectrometric analysis of phosphorylated BRF1, their contribution enabled us to unambiguously prove that MK2 phosphorylates BRF1 fragments at Ser92 and Ser203. I thank fellow graduate student Wei-jye Lin for ideas, encouragement and mentorship. I also acknowledge the contributions of former lab members Chen-chung Lin, Melissa Baker and May Chen. I would like to thank my family and friends who have stood by me during the darker moments. Special thanks to the CMB program (especially the Class of 2002) and the Department of Biochemistry and Molecular Genetics for making graduate studies a fun experience.
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Eukaryotic cells employ intricate mechanisms to regulate gene expression. These mechanisms operate at several levels during the flow of genetic information from DNA to RNA and proteins. Transcription is regulated by a complex interplay of DNA-binding proteins and enzymes that modify chromatin structure surrounding genes. These factors influence the rates at which RNA polymerase synthesize different mRNA molecules. At the post-transcriptional level, gene expression can be regulated during splicing, mRNA export and translation. mRNA decay is another important post-transcriptional mechanism for gene regulation as the abundance of cytoplasmic mRNA correlates with protein synthesis rates. Most eukaryotic mRNA are 5’-capped with a 7-methyl Guanosine (7mG) residue and 3’ poly-adenylated co-transcriptionally. The mRNA is exported to the cytoplasm where the 5’-cap and 3’-poly (A) tail are recognized by cap binding protein, eIF4E and poly(A)-binding proteins (PABP), respectively. Interactions between eIF4E and PABP results in the circularization of the mRNA required for its efficient translation. In eukaryotes the 5’-cap and the poly (A) tail are important stability determinant and the shortening of the poly (A) tail is generally associated with increased instability (Wilusz et al, 2001).
The first step in the degradation of a majority of mammalian mRNA is deadenylation. Several enzymes have been shown to participate in mRNA deadenylation these include - PAN2-PAN3, CCR4-NOT and Poly (A) specific ribonuclease (PARN). In mammalian cells PAN2-PAN3 has been shown to shorten poly-(A) tail of a β-globin reporter to ~80 nucleotides, the remaining poly-A tail is thought to be removed by the CCR4-NOT complex (Yamashita et al., 2005). PARN has also been implicated in deadenylation of mammalian mRNA (Dehlin et al., 2000). While deadenylation is generally regarded as an early step in degradation, some mRNA may undergo readenylation (Garneau et al., 2007).

After deadenylation the body of the mRNA molecule may be degraded by either 3’-5’ or 5’-3’ pathways. The removal of the 5’ 7-mG cap by the decapping enzyme DCP1/DCP2 initiates 5’-3’ decay. The decapped message is degraded by a 5’-3’ exoribonuclease XRN1. Several accessory factors stimulate these events; the Sm-Like (LSM) proteins assemble as a heptameric complex onto the deadenylated 3’ end of the mRNA to stimulate decapping. The recently identified HEDLS/GE-1 protein has also been shown to stimulate decapping (Fenger-Gron et al., 2005). Several components of the 5’-3’ decay pathway including DCP2 and XRN1 have been shown to accumulate in cytoplasmic foci called P-bodies, where mRNA degradation is thought to occur. 3’-5’ degradation is carried out by the exosome, a large ribonuclease complex comprised of 10-11 subunits with 3’-5’ exonuclease activity. The cap is removed from the 5’ oligonucleotide by the scavenger decapping enzyme, DCPS in this pathway (Wang and Kiledjian, 2001). Eukaryotic mRNA may also be degraded by deadenylation dependent...
and endonucleolytic pathways. However several of these pathways employ exonucleases involved in deadenylation dependent mRNA decay to degrade 5’ and 3’ cleavage products.

Although majority of mRNA are degraded by the pathways described above, some mRNA are degraded by regulated mechanism that specifically target a particular transcript. RNA interference (RNAi), Nonsense- mediated decay (NMD) and AU-Rich element mediated decay are examples in which a single mRNA species or a group of mRNAs containing certain sequences are rapidly degraded. RNA interference was first identified in plants and nematodes as a mechanism by which a short double stranded RNA molecules mediates the degradation of mRNA containing sequences identical to the short interfering RNA (siRNA). RNA interference has also been demonstrated in several other animal species. Double stranded RNA molecules are cleaved into short 21-22 nucleotide long siRNA by the Dicer ribonuclease, a single strand complementary to the target mRNA is assembled into an RNA induced silencing complex (RISC) which degrades the target mRNA(Hannon, 2002). The RISC complex is composed of Argonaute proteins, which are thought to possess endonuclease activity (Liu et al., 2004). Recent studies have suggested that the Argonaute proteins localize to the P-bodies and certain components of the 5’-3’ pathway may be involved in RNA interference (Liu et al., 2005; Rehwinkel et al., 2005). Another mechanism for the regulated destruction of specific mRNA molecules is Nonsense-mediated decay. NMD involves the degradation of mRNA containing a premature termination codon (PTC), serving as a surveillance mechanism that prevents the translation of truncated proteins. In higher eukaryote NMD
involves the recognition of a PTC upstream of an exon-exon junction by UPF proteins that interact with Release Factors and components of the exon junction complex (EJC). Detection of a PTC results in the recruitment of degradative enzymes that rapidly degrade the PTC containing mRNA. NMD has been shown to require enzymes involved in both 5'-3' and 3'-5' decay pathways (Isken and Maquat, 2007). Finally mRNA containing cis acting sequences such as AU-rich element are also targeted for rapid degradation. These elements are discussed in the following sections in greater detail.

The AU- Rich Elements

mRNA transcribed from genes encoding growth factor, proto-oncogenes and inflammatory cytokines are short-lived as compared to those that encode proteins required for cellular metabolism. The variability in mRNA half-lives is attributed to the presence of cis-acting sequences located within the mRNA molecule. Several cis-acting elements have been identified that either stabilize or destabilize the transcripts that contain them. The AU-Rich elements (AREs) are prominent among the destabilizing sequences and are found in the 3’ untranslated regions (UTR) of most cytokine, proto-oncogene and growth factor mRNA. These AREs promote the rapid deadenylation and subsequent decay of ARE-containing mRNA by a process termed as ARE-mediated decay or AMD(Wilusz et al., 2001). AREs clearly play a crucial role in mRNA turnover as computational analysis of the 3’UTRs reveal that 8% of human mRNAs contain AREs.(Bakheet et al., 2006)
Classification of AREs

AREs are categorized into 3 classes based on their structure. Class I AREs contain 1-3 copies of a pentameric AUUUA motif flanked by uridylate rich regions. mRNA encoding c-fos and c-myc contain Class I AREs. Class II AREs are found mostly in cytokine mRNA, these elements are typically composed of several overlapping iterations of the AUUUA pentanucleotide. AREs belonging to Class III lack the AUUUA motif and contain several uridylate rich regions. The c-jun mRNA contains a class III ARE (Table 1). In addition to difference in the nucleotide composition, the degradation of Class I and Class III AREs differ kinetically from that of Class II. Class II ARE containing mRNAs (e.g. GM-CSF) are degraded in an asynchronous, biphasic manner. These messages are first deadenylated completely by a processive deadenylase prior to the degradation of the mRNA body. In contrast Class I and III ARE mRNA are deadenylated and degraded in a synchronous process by a distributive ribonuclease activity (Chen and Shyu, 1995).

Table 1: Classification AREs

<table>
<thead>
<tr>
<th>Class</th>
<th>Representative member</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>c-fos, c-myc</td>
<td>1-3 copies of AUUUA</td>
</tr>
<tr>
<td>II</td>
<td>GM-CSF, TNF-α, IFN-α, COX-2, IL-2, VEGF</td>
<td>Overlapping AUUUA</td>
</tr>
<tr>
<td>III</td>
<td>c-jun</td>
<td>U-Rich sequence, No AUUA repeats</td>
</tr>
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ARE-Binding Proteins

Studies aimed at understanding the regulation of AMD have led to the identification of several ARE-BPs. Many of these proteins specifically bind to ARE-containing mRNA promoting the degradation of the bound RNA molecule. AU-Binding Factor (AUF-1) (Chen et al., 2001; Laroia et al., 1999; Sarkar et al., 2003; Zhang et al., 1993), KH-Type splice regulatory protein (KSRP) (Chen et al., 2001; Chou et al., 2006; Gherzi et al., 2004), Tristetraprolin, Butyrate reponse factor 1 and 2 have all been shown to promote the rapid degradation of ARE-containing mRNA (Carballo et al., 1998; Lai et al., 2000). In addition to these factors, the RNA binding protein HuR has been shown to stabilize ARE-containing mRNA (Fan and Steitz, 1998).

_AU-Binding Factor 1_

AUF1 was the first ARE-BP to be identified. It is expressed as four isoforms (p37, p40, p42 and p45) generated from the alternate splicing of a single transcript (Zhang et al., 1993). Overexpression of AUF1 isoforms correlate with increased instability of several ARE-containing mRNA, although there are cell type and isoform specific variations in the degree of destabilization (Sarkar et al., 2003; Xu et al., 2001). Laroia et al have demonstrated that AUF1 forms a complex heat-shock proteins hsc60-hsp70, translation initiation factor eIF4G and poly (A) binding protein. Their results suggest that AMD is
dependent on the disruption of AUF1-eIF4G by the ubiquitination and subsequent proteasome mediated degradation of AUF1 (Laroia et al., 1999). Several AUF1 target mRNA encode proinflammatory cytokines, several groups have provide evidence implicating AUF1 in the turnover of TNF-α and GMCSF mRNA (Laroia et al., 1999; Xu et al., 2001). AUF1 knockout mice exhibit an increased susceptibility to Lipopolysaccharide (LPS) mediated endotoxic shock. This increased susceptibility has been attributed to abnormal stabilization of TNF-α and IL-1β mRNA in AUF1 deficient macrophages (Lu et al., 2006).

KH-Type Splice Regulatory Protein

KSRP was originally identified as a factor involved in the splicing of c-src. It contains four hnRNP K homology domains and is a member of the family of far upstream sequence binding proteins (FUBP) (Min et al., 1997). Chen et al co-purified KSRP with the human exosome and demonstrated its ability to specifically recognize the c-fos ARE. Using an in vitro mRNA degradation assay, Chen et al showed that KSRP promoted the degradation of reporter mRNA containing the c-fos ARE. RNAi-mediated downregulation of KSRP in HeLa cell results in the stabilization of an otherwise unstable reporter mRNA containing the IL-2 3’ UTR (Gherzi et al., 2004). At physiological level KSRP is thought to influence myogenesis by regulating the stability of ARE-containing p21 and Myogenin mRNA in C2C12 myoblast cell line (Briata et al., 2005).
Tristetraprolin

The CCCH family is composed of three closely related mRNA binding proteins that contain two conserved non-canonical zinc finger domains. The three proteins share strong sequence similarity within the zinc finger domains, but differ significantly within the N and C terminal domains. The zinc finger is composed of three cysteines and one histidine, which are thought to coordinate a single atom of zinc. Each zinc finger is separated by 18 residues containing a R(K)YKTEL like sequence leading into the zinc finger (Lai et al., 2000). Tristetraprolin is the prototypical member of this group and was identified as gene induced by the phorbol ester, Tetradecanoyl Phorbol Acetate (TPA) and was therefore named TPA-inducible sequence-11 or Tis11 (Varnum et al., 1991). Mice deficient in TTP function are normal at birth but develop a severe autoimmune phenotype soon after. The administration of monoclonal antibodies raised against TNF-α was able to rescue the phenotype suggesting that TTP played a role in TNF-α biosynthesis (Taylor et al., 1996). Carballo et al later demonstrated that TTP interacted with the TNF-α mRNA and promoted its rapid degradation. Mutations within the zinc finger abolish RNA binding thereby impairing its ability to promote the degradation of TNF-α mRNA (Carballo et al., 1998; Lai et al., 1999).

Butyrate Response Factor 1

The gene encoding BRF1 was cloned by Bustin et al in 1994 (Bustin et al., 1994). Like TTP, BRF1 is a potent destabilizer of ARE-containing transcripts. In an early study Lai et
al demonstrated that co-transfection of rat BRF1 with a TNF-α 3’UTR containing reporter led to a dramatic reduction of reporter mRNA (Lai et al., 2000). More recently, using chemical mutagenesis Stoecklin et al generated an HT1080 cell line called SlowC in which AMD is severely compromised (Stoecklin et al., 2000). Retrovirus mediated transfection of BRF1 cDNA restored AMD in SlowC cells. Sequence analysis revealed that both alleles of BRF1 had been disrupted in these cells (Stoecklin et al., 2002).

Although mRNA targeted by BRF1 have not been identified, BRF1 knockout mice die by embryonic day 11 due to abnormal placentation, suggesting that BRF1 target genes may be involved in early development (Stumpo et al., 2004). Wegmuller and colleagues have established a mouse embryonic stem (ES) cell line that stably expresses a tetracycline inducible short hairpin (sh) RNA directed against BRF1. Tetracycline induced shRNA knockdown of BRF1 results in differentiation of the ES cell into cardiomyocytes, also implicating BRF1 as a developmental regulator (Wegmuller et al., 2007). BRF1 has also been shown to destabilize IL-3 and GMCSF mRNA (Raineri et al., 2004; Stoecklin et al., 2002).

**Hu Antigen R**

Mammalian HuR is a ubiquitously expressed RNA binding protein, closely related to the *Drosophila melanogaster* embryonic lethal abnormal visual (ELAV) protein family. While HuR is predominantly localized in the nucleus it continuously shuttles to the cytoplasm, where it is thought to exert a stabilizing effect on unstable mRNA. In support of this view, overexpression of HuR in mouse L929 cells cells has been correlated with
stabilization of reporters containing both Class I and Class II AREs (Fan and Steitz, 1998; Peng et al., 1998). Conversely, RNAi-mediated downregulation of HuR resulted in the enhanced turnover of IL-3 3’UTR containing mRNA in HT1080 cells (Raineri et al., 2004). Although the mechanism by which HuR stabilizes these mRNA is still unclear, it is thought to achieve mRNA stabilization by displacing destabilizing ARE-BPs from the 3’UTR of unstable mRNA. HuR also plays a role in the translation of its mRNA targets. At the translational level HuR has been shown to act as both an activator and repressor. The association of HuR with several cytokine mRNA results in the inhibition of translation, despite increased stability of the mRNA (Katsanou et al., 2005). In contrast HuR promotes the translation of VEGF, HIF-1α and CAT-1 mRNA (Bhattacharyya et al., 2006; Galban et al., 2008). Other Hu family proteins include HuB, HuC and HuD. These proteins are predominantly expressed in neuronal cell types and are thought to have similar functions (Gao and Keene, 1996).

Mechanism of AMD

Destabilizing ARE-BPs interact with several components of the mRNA degradation machinery. BRF1 and TTP interact with XRN1, DCP2, CCR4 and components of the exosome (Lykke-Andersen and Wagner, 2005). KSRP has also been shown to interact with the exosome, DCP2 and PARN (Chen et al., 2001; Chou et al., 2006; Gherzi et al., 2004). RNAi mediated knockdown of PARN, CCR4, XRN1, LSM proteins and exosome subunits result in a moderate inhibition of AMD, suggesting that these factors are required for AMD but may be functionally redundant (Lin et al., 2007; Stoecklin et al., 2006).
Table 2: ARE-Binding proteins.

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<thead>
<tr>
<th>ARE-BP</th>
<th>Target RNA</th>
<th>Effect</th>
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<tbody>
<tr>
<td>AUF1</td>
<td>Cyclin D1</td>
<td>Destabilizes</td>
</tr>
<tr>
<td></td>
<td>GMCSF</td>
<td>Destabilizes</td>
</tr>
<tr>
<td></td>
<td>IL-3</td>
<td>Destabilizes</td>
</tr>
<tr>
<td>HuR</td>
<td>c-fos</td>
<td>Stabilizes</td>
</tr>
<tr>
<td></td>
<td>p21</td>
<td>Stabilizes</td>
</tr>
<tr>
<td></td>
<td>GMCSF</td>
<td>Stabilizes</td>
</tr>
<tr>
<td></td>
<td>Myogenin</td>
<td>Stabilizes</td>
</tr>
<tr>
<td>TTP</td>
<td>GMCSF</td>
<td>Destabilizes</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>Destabilizes</td>
</tr>
<tr>
<td></td>
<td>Cox-2</td>
<td>Destabilizes</td>
</tr>
<tr>
<td>BRF1</td>
<td>TNF-α</td>
<td>Destabilizes</td>
</tr>
<tr>
<td>KSRP</td>
<td>c-fos</td>
<td>Destabilizes</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>Destabilizes</td>
</tr>
<tr>
<td></td>
<td>IL-2</td>
<td>Destabilizes</td>
</tr>
<tr>
<td></td>
<td>c-jun</td>
<td>Destabilizes</td>
</tr>
</tbody>
</table>

These observations suggest that ARE-BPs recruit degradative enzymes onto the mRNA molecule targeting it for degradation (Figure 1). Additional unidentified enzymes may also be involved. In a recent study, Jing et al have implicated Dicer and Argonaute2 (Ago2), both components of the micro RNA pathway, in the turnover of ARE-containing mRNA. RNAi-mediated downregulation of Dicer and Ago2 resulted in the stabilization of a TNF-α ARE containing reporter. A similar stabilization was observed when microRNA-16 was downregulated. The miRNA-16 sequence is complimentary to the AUUUA motif within the TNF-α 3’ UTR. The role of miRNA-16 in AMD is sequence-specific and requires TTP. Although TTP does not directly bind to miRNA-16, it associates with Ago/eIF2C family members in complex with miRNA-16 thereby targeting the ARE (Jing et al., 2005).
Figure 1: Model for ARE-mediated decay. mRNA undergoing active translation is circularized due to interaction between cap-binding proteins and poly(A)-binding proteins (PABP). Destabilizing ARE-BPs, such as TTP and BRF1, bind AREs and displace PABP from the poly (A) tail. These proteins promote the deadenylation and degradation of the bound mRNA by recruiting deadenylases (CCR4), decapping enzymes (DCP1/2) and ribonucleases (XRN1, Exosome).
Mammalian cells stabilize several ARE-containing mRNA in response to external stimuli including – Lipopolysaccharide, cytokines, growth factors, oxidative and osmotic stress. Many of these agents are potent activators of the p38 MAP kinase pathway (Han et al., 1994; Raingeaud et al., 1995). Ridley et al have shown that that blockade of p38 activity by a SB203580, an inhibitor of p38 MAP kinase, impairs IL-1 stimulated prostaglandin (PG) production in human fibroblasts (Ridley et al., 1997). The decrease in PG-E2 was attributed to a reduction in the levels of mRNA encoding Cycloxygenase-2 (COX-2), an enzyme involved PG-E2 biosynthesis. The same group later showed that the activation of p38 MAP kinase in response to IL-1 resulted in the stabilization of ARE-containing COX-2 mRNA (Ridley et al., 1998). The effect of IL-1 treatment on the stability of COX-2 mRNA can be mimicked by expression of constitutively active forms of MKK6, an upstream activator of p38 or MAPKAP-K2, a protein kinase activated by p38. While the stabilization induced by active MKK6 can be blocked by SB203580, the compound does not block MK2 mediated stabilization (Lasa et al., 2000). Winzen et al have reported similar observations with the ARE-containing IL-8 mRNA. IL-8 mRNA is stabilized by the activation of p38 by MKK6. However, coexpression of a dominant negative MK2 mutant with active MKK6 severely compromised the stabilization of an IL-8 3’ UTR containing mRNA suggesting that MK2 acts as the downstream effector of p38 (Winzen et al., 1999).
Although the mechanism of MK2 mediated inhibition of AMD is not very clear, the phosphorylation dependent inhibition of destabilizing ARE-BPs has been an attractive hypothesis.

TTP is phosphorylated by both p38 and MK2. Chrestensen et al used recombinant MK2 to identify Ser52 and Ser178 as major MK2 phosphorylation sites in vitro. In BHK cells, both MK2 expression and anisomycin treatment up-regulate phosphorylation of TTP at Ser52 and Ser178. Chrestensen et al also show that phosphorylation of recombinant TTP by MK2 does not affect its ability to bind RNA. However, phosphorylation at Ser52 and Ser178 does result in the recruitment of 14-3-3 proteins (Chrestensen et al., 2004). The 14-3-3 proteins are a group of highly conserved phospho-serine/threonine binding proteins. These proteins are key regulators in several signaling pathways influencing the structure, function and localization of their targets (Darling et al., 2005). Although the significance of phospho-TTP and 14-3-3 complex is not fully understood, Stoecklin et al have shown that 14-3-3 association results in the exclusion of TTP from cytoplasmic foci composed of stalled translational machinery called stress granules. 14-3-3 proteins preferentially bind phosphoserine residues located within the context of either RXXXXS/T or a RXXS/T consensus motif, favoring proline residues at the +2 position. Both Ser52 and Ser178 are located within such motifs and are required for proper 14-3-3 association, substitution of Val54 and Ser180 with proline greatly enhances stress granule exclusion. Complex formation with 14-3-3s does not prevent TTP from binding the TNF-α ARE, however substitution of Ser52 and Ser178 with Alanine renders TTP resistant to MK2 mediated inhibition (Stoecklin et al., 2004). In conflict with the above findings Gringhuis
et al have reported that the TTP-14-3-3 interaction is necessary for TTP dependent AMD (Gringhuis et al., 2005). The p38 pathway also targets KSRP; Briata et al have reported the phosphorylation KSRP at Thr692, located within the C-terminal domain, by p38. Phosphorylation at this site prevents KSRP from binding p21 and mygogenin mRNA (Briata et al., 2005).

PI3- Kinase and AMD

The activation of the Phosphotidyl Inositol 3-Kinase (PI3-K) and its downstream protein kinase B (PKB) also results in the stabilization of ARE-containing mRNA (Ming et al., 2001). BRF1 is phosphorylated by PKB on at least two serine residues. Using in vitro and in vivo methods Schmidlin et al have shown that BRF1 is phosphorylated by PKB on Ser92. Ser92 phosphorylated recombinant BRF1 is unable to promote the degradation of a radiolabeled ARE containing probe in SlowC cell extracts indicating that phosphorylation at Ser92 is sufficient to impair BRF1 function. However, phosphorylation at Ser92 does not affect the RNA binding properties of recombinant BRF1 (Schmidlin et al., 2004). Benjamin and colleagues recently reported that PKB also phosphorylates BRF1 at Ser203, their data suggest that phosphorylation at Ser92 and Ser203 impair BRF1 dependent degradation of a reporter containing IL-3 ARE in a fibroblast cell line. Phosphorylation of both Ser92 and Ser203 results in the recruitment to 14-3-3 complexes associated with insoluble cytoskeletal fraction. They also suggest that the phosphorylation at Ser203 plays a role in the half life of the protein (Benjamin et al., 2006). In response to PI3-K activation, PKB also phosphorylates KSRP at Ser193.
Phosphorylation of Ser193 located within the first KH domain results in the reduced recruitment of exosome components (Gherzi et al., 2006).

Several other signaling pathways also stabilize unstable mRNA. Activation of the c-jun N-terminal kinase during T-cell activation results in the stabilization of the IL-2 mRNA (Chen et al., 1998; Chen et al., 2000). The activation of the Wnt/beta-catenin pathway stabilizes mRNA encoding transcription factor, Pitx2, as well as other unstable mRNAs, including c-Jun, Cyclin D1, and Cyclin D2. Pitx2 mRNA stabilization has been attributed to a a reduced interaction of Pitx2 3'UTR with KSRP and TTP as well as to an increased interaction with HuR (Briata et al., 2003). Table 3 summarizes the role played by protein kinases in AMD (Shim and Karin, 2002).

This research project was aimed at determining whether BRF1 is targeted by MK2 and to determine the mechanism underlying the phosphorylation dependent inhibition of BRF1. BRF1 recruits the RNA-decay machinery onto the ARE-mRNA, we hypothesized that MK2 could phosphorylate BRF1 thereby impairing its ability to recognize AREs or recruit degradative enzymes. We devised in vitro and transient transfection of cultured cells based experiments to test this hypothesis, which are described in the following section.
Table 3: Cellular Signaling Pathways and ARE-mediated mRNA Decay

<table>
<thead>
<tr>
<th>Pathway/Stimulus</th>
<th>mRNA stabilized</th>
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<tbody>
<tr>
<td>JNK</td>
<td>IL-3</td>
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<tr>
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<td>VEGF</td>
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<td>VEGF</td>
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<td></td>
<td>p21</td>
</tr>
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<td></td>
<td>Myogenin</td>
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<tr>
<td></td>
<td>BCL-2</td>
</tr>
<tr>
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<td>i-NOS</td>
</tr>
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<td>IL-1a</td>
</tr>
<tr>
<td>PI3-K</td>
<td>IL-3</td>
</tr>
<tr>
<td>Wnt/β-catenin</td>
<td>Pitx2</td>
</tr>
</tbody>
</table>
EXPERIMENTAL DESIGN

The In Vitro Kinase Assay

We initiated this study with the question are ARE-BPs direct targets of MK2? At the time TTP was the only known substrate of MK2 involved in AMD (Mahtani et al., 2001). We hypothesized that the MK2 mediated inhibition of AMD was an outcome of the phosphorylation dependent inhibition of destabilizing ARE-BPs or the activation of a stabilizing protein like HuR. The first step towards identifying potential substrates was the establishment of a system that would enable us to rapidly screen for ARE-BPs that could be potentially phosphorylated by MK2. In vivo MK2 is activated by dual phosphorylation of Thr205 and Thr317 by p38. Engel et al reported that the substitution of these residues with glutamic acid (MK2EE) resulted in constitutive p38 independent activation of MK2 (Engel et al., 1995). Transient transfection of MK2EE has been a popular method to study the effect of MK2 on various cellular processes (Winzen et al., 1999). However recombinant MK2EE has weak kinase activity and is therefore not suitable for in vitro experiments (Chrestensen et al., 2004). We established a protocol to activate GST-MK2 in vitro by incubating it with His-tagged p38 and constitutively active MKK6. MKK6 is phosphorylated by its upstream kinase at Ser207 and Thr211 resulting in its activation.
Glutamic acid substitution (MKK6EE) of both these residues results in the constitutive activation of MKK6 (Raingeaud et al., 1996). In our experiment incubation of His MKK6EE with His-p38 and GST-MK2 in the presence of ATP recreates the kinase cascade in vitro. MKK6 phosphorylates p38 and p38 phosphorylates MK2. The phosphorylated MK2 can then be purified using GST-agarose beads (Figure 2). Similar protocols for the in vitro activation of MK2 have also been reported by others (Chrestensen et al., 2004; Rousseau et al., 2005). The activity of activated GST-MK2 is approximately 12 fold higher than that of GST-MK2EE (Chrestensen et al., 2004). We have used in vitro activated GST-MK2 in all the in vitro phosphorylation experiments in this study.

The Tet-off System

Several early experiments employed transcriptional inhibitors such as actinomycin D and Dichlororibofuranosylbenzimidazole (DRB) to measure mRNA decay kinetics. In these studies the turnover of reporter mRNA was measured after the inhibition of transcription by a generalized transcriptional inhibitor. While these studies have led to the identification of some factors involved in mRNA decay (Gherzi et al., 2004), there is some evidence to suggest that these reagents stabilize mRNA containing Class I and Class II AREs (Chen et al., 1995). Ann-Bin Shyu’s laboratory has adapted the tet-off system to develop a transcriptional pulsing strategy that is now widely used study mRNA decay in mammalian cells (Xu et al., 1998). This system was originally established by
Figure 2: Activation of GST-MK2: GST-MK2 purified from E.coli is incubated with His-p38, His-MKK6EE and ATP. Phosphorylated GST-MK2 is purified with glutathione beads.
Manfred Gossen and Herman Bujard as a method for rapid induction of gene expression (Gossen and Bujard, 1992). Tet-off cells stably express a plasmid encoding a chimeric tetracycline controlled transcriptional activator called tTA. tTA is composed of the DNA binding domain of the E.coli tetR fused to the transactivation domain of the herpes simplex virus VP16 protein. Reporter expression is driven from a promoter containing multiple copies of the tetO element. When bound to tetracycline, tTA no longer binds DNA turning off reporter transcription. The amount of tetracycline or doxycycline required to inhibit tTA is very small and at these levels the tetracycline treatment does not significantly alter cell physiology.

To study mRNA decay a chimeric reporter consisting of the coding region of rabbit β-globin fused to an ARE derived from an unstable mRNA is used. Reporter expression is driven by a promoter containing a Tet-repressible element (TRE). Transcription is turned off by the addition of doxycycline. mRNA is harvested at different time intervals, reporter stability is analyzed by northern blotting. For effective quantitation of reporter expression, a control plasmid generated from the fusion of the rabbit β-globin coding region to a segment of the stable GAPDH mRNA, is included. The expression of the GB-GAPDH mRNA is driven by the human cytomegalovirus promoter and is insensitive to tetracycline. The GB-GAPDH mRNA is longer than the GB-ARE mRNA allowing effective detection of both mRNA with a single probe directed against the globin coding region.
Figure 3: mRNA decay assay using the tet-off system. Plasmids encoding chimeric reporter mRNA, generated from fusing the coding region of rabbit β globin to an ARE derived from an unstable mRNA are transfected into tet-off cell line. Transcription is driven from a promoter containing a tet-repressible element (TRE). The degradation of reporter mRNA can be measured after transcription is blocked by the addition of tetracycline to the culture media.
RESULTS

Activation of p38 MAP Kinase Stabilizes a GMCSF 3’UTR Containing Reporter

Previous work from other laboratories have suggested that ARE-BPs: HuR, BRF1, TTP and AUF1 are involved in the regulation of reporters carrying the GMCSF 3’UTR. Transient transfection experiments were carried out with pTRE-GB-ARE\textsubscript{GMCSF} in HeLa-TO cells to determine the effect of the p38 pathway on its stability. As expected the GMCSF reporter was found to be very unstable in HeLa cells. Stimulation with anisomycin, a p38 activator led to the dramatic stabilization of the reporter (Figure 4). A similar effect on reporter stability was also observed with other p38 activators sorbitol and H\textsubscript{2}O\textsubscript{2} (not shown). In all cases pretreatment with p38 inhibitors: SB203580 or SB202190 compromised p38 mediated stabilization (Figure 4 and data not shown). These results suggest that the turnover of GB-ARE\textsubscript{GMCSF} mRNA is regulated by the p38 pathway.
Figure 4: Activation of the p38 pathway stabilizes GB-ARE\textsuperscript{GMCSF} mRNA – HeLa-TO cells were transfected with the pTRE GB-ARE\textsuperscript{GMCSF}. The cells were untreated or pre-treated with DMSO, SB203580 (10µM) for 30 min and further treated with anisomycin (200 ng/ml) for 30 min. Transcription was repressed by the addition of 2 µg/ml doxycycline. Total RNA was harvested at hourly intervals. Reporter mRNA stability was analyzed by northern blotting.
Activation of p38 MAP Kinase Does Not Affect the Turnover of a Reporter Containing a Non-ARE Instability Element

The regulation of GB-ARE\textsuperscript{GMCSF} may be a result of functional inhibition of destabilizing ARE-BPs known to bind the ARE within the GMCSF 3’ UTR. However, there exists a possibility that these kinases may affect the turnover of all unstable mRNA by targeting mRNA decay pathways. In order to test whether the activation of p38 MAP kinase pathway had a specific effect on the GB-ARE\textsuperscript{GMCSF} reporter we carried out a similar transfection experiment with a reporter containing a non-ARE stem loop destabilizing element (SLDE). This element has been shown previously to promote the destabilization of granulocyte colony stimulating factor (G-CSF) mRNA (Brown et al., 1996). We transfected HeLa-TO cells with pTRE-GB-SLDE and measured the stability of the reporter in the presence and absence of anisomycin. We did not observe a significant stabilization of the reporter in cells stimulated with anisomycin (Figure 5). This data indicates that the stabilization of the GMCSF reporter is due to p38 mediated inhibition of AMD and not generalized stabilization of mRNA.

BRF1 is Phosphorylated by MK2 In Vitro

ARE-BPs are critical regulators of AMD and are therefore likely targets of signaling pathways that stabilize ARE containing mRNA. Protein kinases such as p38 and MK2 may possibly impair AMD by the phosphorylation dependent inhibition of destabilizing ARE-BPs. Alternatively these kinases may activate stabilizing ARE-BPs such as HuR.
Figure 5: Activation of the p38 pathway does not stabilize GB-SLDE mRNA – HeLa-TO cells were transfected with the GB-SLDE. The cells were treated with anisomycin (200 ng/ml) for 30 min. Transcription was repressed by the addition of 2 µg/ml doxycycline. Total RNA was harvested at hourly intervals. Reporter mRNA stability was analyzed by northern blotting.
For the purpose of identifying ARE-BPs that are phosphorylated by MK2, an in vitro kinase assay was established. GST-tagged ARE-BPs: KSRP, HuR, AUF1, BRF1 and TTP were incubated with activated GST-MK2 and $\gamma^{32}P$ labeled ATP. HSP27, a known substrate of MK2 was used as a positive control (Stokoe et al., 1992). Results from these experiments indicated that GST-MK2 phosphorylated recombinant TTP and BRF1 at levels comparable to HSP27. KSRP, AUF1 and HuR were not phosphorylated by GST-MK2 in this assay (Figure 6). These results are consistent with previous reports demonstrating the MK2 mediated phosphorylation of TTP and identify BRF1 as a novel substrate for MK2. GST-BRF1 was also efficiently phosphorylated by endogenous MK2 immunoprecipitated from HEK 293 cells stimulated with anisomycin (Figure 7). These results suggest that BRF1 is an MK2 substrate and raise the question whether MK2 mediated phosphorylation of BRF1 impairs its ability to promote AMD.

**MK2 Phosphorylates BRF1 at Ser54, Ser92 and Ser203 In Vitro**

To determine MK2 phosphorylation sites, we expressed four BRF1 fragments as GST fusion proteins designated as F1 to F4 (Fig. 8A). All four fragments were phosphorylated by MK2 in vitro (Fig. 8B). MK2 has been shown to preferentially phosphorylate the serine residue in the context of an RXXS consensus motif (Manke et al., 2005; Stokoe et al., 1993). Inspection of amino acid sequences of the four fragments revealed that each one contains at least one MK2 consensus motif (Fig. 8A). We substituted the Ser residue within the RXXS motif of each fragment with Ala and performed kinase assays with mutant proteins. Substitution of Ser54 abrogated phosphorylation of F1 fragment (amino
Figure 6: GST-BRF1 is phosphorylated by MK2 in vitro. His-HSP27, GST-TTP, and GST-BRF1 were incubated with or without an active MK2 in the presence of $\gamma^{\text{32}}$P-ATP at 37°C for 40 min. Reactions were terminated by addition of SDS sample buffer, separated by SDS-PAGE, and subjected to (A) autoradiography. (B) Coomassie brilliant blue (CBB) staining of the gel, target proteins are indicated by asterisks.
Figure 7: GST-BRF1 is phosphorylated by endogenous MK2. 293 cells were treated with anisomycin (200 ng/ml) for 30 min. Cell extracts were immunoprecipitated with an anti-MK2 antibody. The precipitates were subjected to kinase assays using GST-HSP27 or GST-BRF1 as substrates as described earlier. 10% input used for immunoprecipitation was analyzed by anti-MK2 immunoblotting.
Figure 8: MK2 phosphorylates BRF1 at a minimum of four sites. (A) Schematic diagram of BRF1 fragments used for kinase assays. The RXXS motifs and Ser residues substituted are indicated. (B) BRF1 fragments, F1, F2, F3 and F4, were incubated with activated MK2 and subjected to analysis as described in Figure 6.
acids 1 - 64) by MK2 (Figure 9A). F2 fragment (amino acids 65-185) contains two overlapping RXXS motifs (Figure 9B) with either or both Ser90 and Ser92, serving as potential phosphorylation sites. While substitution of Ser90 with Ala had no effect, no phosphorylation was detected in both S92A and S90/92A mutant proteins, suggesting that Ser92 is the preferred MK2 phosphorylation site (Figure 9B). Substitutions of Ser192 and Ser283 with Ala in F3 fragment (amino acids 186-257) and F4 fragment (amino acids 257-338), respectively, did not significantly affect phosphorylation (Figure 10A and 10C), suggesting that these Ser residues are not MK2 phosphorylation sites. Previous reports demonstrated that MK2 phosphorylates TTP at Ser178 and have suggested that Ser203 of BRF1 is homologous to Ser178 in TTP (Benjamin et al., 2006). We generated Ala substitutions at Ser203 and the adjacent Ser201 in F3 fragment. While substitution of Ser201 resulted in a moderate reduction in phosphorylation, substitution of Ser203 completely abrogated MK2-mediated phosphorylation of F3 fragment (Figure 10B), suggesting that Ser203 is critical for MK2-dependent phosphorylation.

To confirm that Ser92 and Ser203 are indeed phosphorylated by MK2, F2 and F3 fragments were phosphorylated by MK2 and subjected to mass spectrometric analysis. We identified a peptide derived from F2 fragment containing a phosphate moiety at Ser92 (Figure 11) and a peptide derived from F3 fragment with a phosphate group at Ser203 (Figure 12). We recovered ~95% of the peptides, no other phosphorylation sites were identified (Figure 13) and no peptides containing phosphate groups at either Ser90 or Ser201 were detected. It is therefore possible that substitution of Ser201 with
Ala reduces the phosphorylation efficiency of the adjacent Ser203. We also observed that the F3 fragment was more efficiently phosphorylated by MK2 than other fragments (Figure 8B). However, it is likely that Ser203 is the only phosphorylation site within this region and the increased phosphorylation maybe due to a other factor that make F3 a better MK2 substrate. Altogether, these results demonstrate that Ser92 and Ser203 are indeed phosphorylated by MK2. Furthermore, MK2 phosphorylates BRF1 at Ser54 and another unidentified site located within F4 fragment.

Generation of HT1080-TO and SlowC-TO Cell Lines

The SlowC cell line developed by Christoph Moroni’s laboratory serves as an ideal model system to study the function of BRF1. In these cells AMD is compromised as a result of the loss of function of both BRF1 alleles (Stoecklin et al., 2002). To study mRNA decay in these cells, SlowC and its parental HT1080 cell line were stably transfected with the tTA plasmid. Stable clones were selected based on Neomycin resistance. Neomycin resistant clones were tested for Tetracycline mediated repression in transient transfection experiments using pTRE-Luciferase. Luciferase assays were carried out with cell lysates prepared from transfected clones that were treated with tetracycline or left untreated. Clones #4 and #5 from both SlowC and HT1080 showed greater than <20 fold repression of luciferase activity in the presence of tetracycline and were expanded for further analysis. These cells were then transiently transfected with the pTRE-ARE<sup>GMCSF</sup>
Figure 9: Identification of phosphorylation sites within GST-F1 and GST-F2: (A) F1 and a mutant F1 containing Ala substitution at Ser54 were subjected to kinase assays. (B) F2 and mutants of F2 containing Ala substitutions at Ser90, Ser92, or both Ser90 and Ser92 were subjected to kinase assays. The predicted MK2 phosphorylation site is shown in red and the consensus motif is underlined.
Figure 10: Identification of phosphorylation sites within GST-F3 and GST-F4. (A) F3 and a mutant of F3 containing Ala substitution at Ser192 were subjected to kinase assays. (B) F3 and mutants of F3 containing Ala substitutions at Ser201, Ser203, or both Ser201 and Ser203 were subjected to kinase assays. (C) F4 and a mutant of F4 containing Ala substitution at Ser283 were subjected to kinase assays. The predicted MK2 phosphorylation site is shown in red and the consensus motif is underlined.
Figure 11: Mass spectra of a peptide derived from F2, phosphorylated Ser is indicated (top right).
Figure 12: Mass spectra of a peptide derived from F3, phosphorylated Ser is indicated (top right)
GST-BRF1 F3 fragment

Shown is the sequence of GST-BRF1 F3 fragment
Underlined sequence is BRF1 (amino acids 186-257)
Identified peptides shown in **blue bold**
Sequence coverage by mass spectrometry: > 95%
Phosphorylation site only found at Serine 203 (**red underlined**)

Figure 13. Sequence coverage of F3. Mass spectrometry of BRF1 F3 fragment phosphorylated by MK2 and digested with trypsin/AspN shows >95% sequence coverage and that only Ser203 contains a phosphate group.
plasmid and the expression of the GB-ARE\textsuperscript{GMCSF} reporter was determined by northern blotting. GB-ARE\textsuperscript{GMCSF} expression was detected in all four clones. However the expression of GB-ARE\textsuperscript{GMCSF} was found to be higher in SlowC #5 than SlowC #4. Consistent with previous data the reporter was more stable in the SlowC cell line than the HT1080 (Figure 14). For all subsequent experiments Clone#5 of both cell lines were used.

MK2 Inhibits BRF1-dependent AMD in SlowC-TO Cells

To examine the effects of MK2-mediated phosphorylation on the function of BRF1 in AMD, we carried out transient transfection experiments in HT1080 and SlowC cells. GB-ARE\textsuperscript{GMCSF} mRNA was degraded in the parental HT1080 cell line with a half-life (t\textsubscript{1/2}) of 1.0 h, while it was significantly stabilized in the SlowC cell line with a t\textsubscript{1/2} of 3.1 h (Figure 15). Expression of BRF1 in SlowC cells restored AMD (Figure 16). Co-expression of BRF1 with a constitutively active form of MK2 (MK2EE), in which both of the threonine residues phosphorylated and required for its activation by p38 are substituted with glutamic acid (Engel et al., 1995), decreased the BRF1-dependent decay of GB-ARE\textsuperscript{GMCSF} mRNA (Figure 16). These results indicate that MK2 impairs the function of BRF1 in AMD.
Figure 14: Screening of SlowC-TO and HT1080-TO stable clones: SlowC and HT1080 were stably transfected with pTet-off. Stable clones #4 and #5 from both cell lines were transiently transfected with pTRE-GB-ARE\textsuperscript{GMCSF}. Transcription was repressed by the addition of 2\(\mu\)g/ml doxycycline. Total RNA was harvested at hourly intervals. Reporter mRNA stability was analyzed by northern blotting. Because of better expression of the pTRE reporter, SlowC-TO#5 and HT1080-TO #5 were used in all further experiments.
Figure 15: BRF1 is required for AMD: HT1080-TO and SlowC-TO cells were transfected with pTRE-GB-ARE\textsuperscript{GMCSF}. Transcription was repressed by the addition of 2\(\mu\)g/ml doxycycline. Total RNA was harvested at hourly intervals. Reporter mRNA stability was analyzed by northern blotting.
Figure 16: MK2 inhibits BRF1-dependent AMD. GB-ARE$^{\text{GMCSF}}$ mRNAs was expressed in SlowC-TO cells with constructs expressing FLAG-BRF1, myc-MK2EE, or both. Total RNA was isolated at different time points after addition of doxycycline (Dox). The levels of GB-ARE$^{\text{GMCSF}}$ mRNAs were analyzed as described earlier.
Ser54, Ser92 and Ser203 Are Required for the MK2 Mediated Inhibition of BRF1

We next examined whether MK2-mediated phosphorylation is necessary for the inhibition of BRF1 function in AMD. We performed mRNA decay assays in SlowC cells to investigate the effect of MK2 on the decay-promoting activity of BRF1 containing Ser to Ala substitutions. Expression of some of these BRF1 mutant proteins restored AMD in SlowC cells, indicating that they are not functionally impaired in mRNA decay (Figure 17 and data not shown). While MK2EE coexpression strongly compromised the decay-promoting activity of the wild type BRF1, BRF1 (S203A) mutant, but not BRF1 (S92A) mutant protein, significantly resisted MK2-mediated inhibition (Fig. 18A). A BRF1 mutant protein (S54/92/203A) containing additional Ala substitutions at both Ser54 and Ser92 in combination with Ser203 mutation completely escaped MK2-mediated inhibition (Figure 18). These results suggest that phosphorylation of Ser92 and Ser203, and likely Ser54, by MK2 is necessary for the inhibition of BRF1-dependent AMD.

MK2 Can Act on BRF1 in a PKB-independent Manner

Treatment with drugs such as anisomycin results in the activation of both p38 and PI3-K pathways. The activated PI3 Kinase results in the recruitment of both PKB and its activating PDK1 to membrane bound phosphatidylinositol trisphosphate (PIP3). PDK1 phosphorylates PKB at Ser308; a second kinase mTORC2 catalyzes a second phosphorylation of PKB at Ser473 resulting in its complete activation. While the
Figure 17: Mutations within the three MK2 phosphorylation sites does not affect BRF1 function. SlowC-TO cells were transfected with constructs expressing GB-ARE\textsuperscript{GMCSF}, GB-GAPDH mRNA and constructs expressing either Flag-BRF1 (WT) or FLAG-BRF1 (S54/92/203A) as indicated. The decay of GB-ARE\textsuperscript{GMCSF} mRNA was analyzed. The calculated $t_{1/2}$ (n=2-3) of GB-ARE\textsuperscript{GMCSF} mRNA are shown.

<table>
<thead>
<tr>
<th>Dox (h)</th>
<th>GB-GAPDH</th>
<th>GB-ARE\textsuperscript{GMCSF}</th>
<th>$t_{1/2}$ (h)</th>
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<tr>
<td>0</td>
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<td>3</td>
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- BRF1: 3.1 ± 0.1
- BRF1 (S54/92/203A): 0.9 ± 0.2
- BRF1 (S54/92/203A): 1.0 ± 0.2
Figure 18: Ser54, Ser92 and Ser203 are required for the MK2 mediated inhibition of BRF1. GB-ARE$^{GMCSF}$ and GB-GAPDH mRNAs were expressed in SlowC-TO cells in the presence of different FLAG-tagged BRF1 mutants and myc-MK2EE. The decay of GB-ARE$^{GMCSF}$ mRNA was analyzed. The calculated t$_{1/2}$ (n=2-3) of GB-ARE$^{GMCSF}$ mRNA are shown. (B) Expression of transfected FLAG-BRF1 mutants and myc-MK2EE. Extracts from B were either immunoprecipitated with anti-FLAG and analyzed by immunoblotting using anti-BRF1 (top panels) or directly analyzed by anti-myc immunoblotting (bottom panels). Differential mobility of BRF1 observed in C is likely due to phosphorylation of Ser203 by MK2.
phosphorylation of Ser308 is sufficient for the activation of PKB, dual phosphorylation at Ser308 and Ser473 greatly enhances its activity. It is speculated that Ser473 may play a role in substrate selection as some substrates do not seem to require the phosphorylation Ser473 (Manning and Cantley, 2007). Recent studies have suggest that MK2, p38, HSP27 and PKB form a complex in some cell types. The formation of this complex results in the MK2 mediated phosphorylation of PKB at Ser473 (Rane et al., 2001; Wu et al., 2007; Zheng et al., 2006). Since MK2 phosphorylates BRF1 at the sites (Ser92 and Ser203) shared by PKB, which also inhibits BRF1 function in AMD (Benjamin et al., 2006; Schmidlin et al., 2004), we examined whether PKB activity is necessary for MK2-mediated inhibition of BRF1. We first examined whether expression of MK2EE leads to the activation of PKB in SlowC-TO cells. Co-expression of MK2EE moderately increased (~2-fold) PKB phosphorylation (Figure 19). Transfection efficiency in SlowC cells was typically 30% analyzed by expression of a GFP marker (data not shown). We then examined whether downregulation of PKB expression by RNAi could interfere with the inhibition of BRF1 by MK2. While PKB expression was downregulated by 50-70% after two rounds of siRNA transfection (Figure 20B), the ARE-mRNA decay activity of BRF1 was still inhibited by co-expression of MK2EE (Fig. 20A).
Figure 19: The expression of MK2EE activates PKB: SlowC-TO cells were either left untransfected or transfected without DNA (H₂O) or with plasmids expressing FLAG or myc-MK2EE. Cell extracts were analyzed by anti-phospho-Ser⁴⁷³-PKB, anti-PKB, or anti-myc immunoblotting.
Figure 20: MK2 mediated inhibition of BRF1 is PKB independent. SlowC-TO cells were transfected with constructs expressing GB-ARE\textsuperscript{GMCSF} and GB-GAPDH mRNAs and constructs expressing FLAG-BRF1 and myc-MK2EE in the presence of a control siRNA (CAT) or a PKB siRNA. The decay of GB-ARE\textsuperscript{GMCSF} mRNA was analyzed (A). The calculated $t_{1/2}$ (n=2) of GB-ARE\textsuperscript{GMCSF} mRNA are shown. (B) Downregulation of PKB by siRNA was analyzed with extracts from (A) by anti-PKB or anti-KSRP immunoblotting.
Phosphorylation of BRF1 by MK2 Does Not Affect its RNA-Binding Property or its Ability to Associate with mRNA Decay Enzymes

To investigate the mechanism of inhibition of BRF1, we examined whether phosphorylation of BRF1 by MK2 decreases its ARE-binding activity. Preliminary UV cross-linking experiments were carried out using ARE^{GMCSF} RNA and GST-BRF1 phosphorylated in vitro by GST-MK2. We detected no change in the RNA binding ability of phosphorylated BRF1 suggesting that phosphorylation does not directly inhibit RNA-binding (Figure 21). Further immunoprecipitation experiments were carried out to exclude the possibility that recruitment of factors such as 14-3-3 proteins may indirectly affect RNA binding. FLAG-tagged BRF1 was expressed in 293 cells, since transfected BRF1 was poorly expressed in HT1080 or SlowC cells, in the absence or presence of MK2EE. UV-crosslinking experiments using ARE^{GMCSF} RNA or a non-ARE RNA as substrates were performed to induce formation of RNA-BRF1 complexes, which were immunoprecipitated with an anti-FLAG antibody. The amounts of ARE RNA crosslinked to FLAG-BRF1 were similar regardless of whether MK2EE was co-expressed or not (Figure 22). No RNA/BRF1 complexes were detected using a non-ARE RNA (Figure 22), suggesting that BRF1 specifically binds the ARE RNA.

We next examined the effect of MK2EE expression on the association of BRF1 with mRNA decay enzymes, including the deadenylase, CCR4, the decapping enzyme, DCP2, and the exosome component RRP4. An HA-tagged CCR4 or a myc-tagged DCP2 was co-
Figure 21: Phosphorylation does not affect the RNA-binding property of BRF1. Unphosphorylated or MK2-phosphorylated GST-BRF1 was incubated with $^{32}$P-labeled ARE$_{GM-CSF}$ RNA. RNA-BRF1 complexes were subjected to SDS-PAGE and detected by autoradiography.
Figure 22: Co-expression of MK2 with BRF1 does not alter its ability to interact with an ARE. FLAG-BRF1 was expressed in 293 cells in the absence or presence of myc-MK2EE. Cytoplasmic extracts were prepared, incubated with $^{32}$P-labeled ARE$^{GMCSF}$ RNA or non-ARE E4 RNA (Gherzi et al., 2004), and UV crosslinking assays performed. The UV crosslinking reactions were immunoprecipitated with anti-FLAG and immunoprecipitates were analyzed by SDS-PAGE. The RNA-BRF1 complexes were detected by autoradiography (top panel). The immunoprecipitates were also analyzed by anti-FLAG immunoblotting (middle panel). 10% input used for UV crosslinking assays were also analyzed by anti-myc immunoblotting (bottom panel).
expressed with FLAG-BRF1 in the absence or presence of MK2EE. While HA-CCR4, myc-DCP2, and endogenous RRP4 were co-immunoprecipitated with FLAG-BRF1, no significant differences in the amounts of co-immunoprecipitated enzymes were detected when BRF1 was coexpressed with MK2EE (Figure 23). Co-expression of MK2EE with BRF1 increased its association with 14-3-3 proteins (Figure 23A) consistent with previous observations (Benjamin et al., 2006; Schmidlin et al., 2004), suggesting that MK2EE is active in transfected 293 cells. The interaction of BRF1 with 14-3-3 in the absence of MK2EE (lane 2, Fig. 23A) was due to the basal activities of PI3-K/PKB and p38 signaling pathways as treatment of cells with wortmannin or SB203580 disrupted the interaction (data not shown). In addition, FLAG-BRF1 mutant protein (S54/92/203A) failed to interact with 14-3-3, but retained its ability to associate with mRNA decay enzymes (data not shown), suggesting that the interaction with 14-3-3, but not the decay enzymes, are dependent on BRF1 phosphorylation. These results suggest that MK2-mediated inhibition of BRF1 function in AMD may not result from decrease in RNA binding or impairment of its ability to recruit the tested mRNA decay enzymes.
Figure 23: Co-expression of MK2 with BRF1 does not alter its ability to associate with mRNA decay enzymes. (A) FLAG-BRF1 was expressed in 293 cells in the absence or presence of myc-MK2EE. RNase-treated cell extracts were subjected to anti-FLAG immunoprecipitation. The precipitates were analyzed by anti-FLAG, anti-RRP4, or anti-14-3-3 immunoblotting. 5% input used for immunoprecipitation reactions was also analyzed by anti-RRP4, anti-14-3-3, or anti-myc immunoblotting. (B) FLAG-BRF1 and HA-CCR4 were expressed in 293 cells in the absence or presence of myc-MK2EE. RNase-treated cell extracts were subjected to anti-FLAG immunoprecipitation. The precipitates were analyzed by anti-FLAG or anti-HA immunoblotting. 5% input used for immunoprecipitation reactions was also analyzed by anti-HA or anti-myc immunoblotting. (C) FLAG-BRF1 and myc-DCP2 were expressed in 293 cells in the absence or presence of myc-MK2EE. RNase-treated cell extracts were subjected to anti-FLAG immunoprecipitation. The precipitates were analyzed by anti-FLAG or anti-DCP2 immunoblotting. 5% input used for immunoprecipitation reactions was also analyzed by anti-DCP2 or anti-myc immunoblotting.
DISCUSSION

Previous studies aimed at understanding the mechanisms by which signaling pathways stabilize ARE-containing mRNAs have led to identification of TTP and BRF1 as targets of MK2 and PKB, respectively (Benjamin et al., 2006; Schmidlin et al., 2004). Phosphorylation of TTP by MK2 or BRF1 by PKB inhibits their ARE-mRNA decay functions. While MK2 was shown to phosphorylate BRF1 (Benjamin et al., 2006), its effect on the function of BRF1 in AMD has not been previously investigated. In this study, we have shown that MK2 inhibits the ARE-mRNA decay-promoting activity of BRF1. This inhibition is dependent on MK2-mediated phosphorylation of BRF1. Thus, our data and the previous studies (Benjamin et al., 2006; Briata et al., 2005; Gherzi et al., 2006#16; Schmidlin et al., 2004) strongly suggest that decay-promoting ARE-BPs are targeted by signaling pathways through direct phosphorylation.

BRF1 is a phosphoprotein, SDS PAGE analysis of endogenous BRF1 or ectopically expressed BRF1 reveal that the protein exhibits a much lower mobility than what would be theoretically predicted. The protein contains 70 residues that can be potentially phosphorylated by Ser/Thr kinases and another 6 tyrosine residues that may be targets of tyrosine kinases. Primary sequence analysis with NetPhos and NetPhosK algorithms predict that BRF1 is phosphorylated by at least 12 different kinases at a minimum of 32
different residues (Appendix). These predictions are not exhaustive as the list of kinases used by the NetPHosK is far from comprehensive. However, these predictions do suggest that many of these kinases may phosphorylate the same residues. Due to the possibility of redundant phosphorylation sites and poor expression of epitope-tagged BRF1 in tested cell lines, we chose to use the in vitro system to identify MK2 phosphorylation sites. Using recombinant proteins expressed in *E. coli*, we found that BRF1 is phosphorylated by MK2 at a minimum of four distinct sites in vitro. Substitution of Ser54, Ser92, or Ser203 with Ala abrogates MK2-mediated phosphorylation of fragments containing each of these Ser residues, suggesting that they are MK2 target sites. Indeed, we confirmed that MK2 phosphorylates Ser92 and Ser203 by mass spectrometry. In addition, our results indicate that there may be one or more MK2 phosphorylation sites within the C-terminus (amino acids 257-338). Although our attempts have ruled out Ser283 as a potential MK2 site, it may be possible that MK2 phosphorylates BRF1 at Ser334 as this site shares significant homology with Ser316 in TTP shown to be phosphorylated by MK2 in a previous study. Currently, we do not know whether the identified sites are indeed phosphorylated by MK2 in vivo and additional or fewer sites may be identified in vivo in the context of full-length BRF1. BRF1 was previously shown to be phosphorylated by MK2. In that study, the sites phosphorylated by MK2 were not identified, however it was suggested that Ser203 is not an MK2 target site (Benjamin et al., 2006). Although we do not know the reason for the discrepancy between our phosphorylation assays and previous experiments performed by Benjamin et al., we speculate that the disparity may result from differences in the BRF1 fragments analyzed: amino acids 143-233 (Benjamin et al.) vs. amino acids 185-257 (our studies). In our own
hands Ser92, which was identified as a phosphorylation site in F2, is not phosphorylated in the context of a shorter fragment composed of amino acids 65-127. Similar experiments were carried out with fragment composed of the Zinc Finger (111-185), N (1-111) and C-terminal (185-338) domains (data not shown). We detected a greater than 100 fold difference between the phosphorylation efficiency of C-terminal and N-terminal fragments. These variations in the phosphorylation efficiency are probably consequences of unphysiological folding of these fragments that alter accessibility of the phosphorylation sites. Although these results point to a flaw in our experimental strategy, it should be noted that mass spectrometric analysis of GST-CT only identified Ser203 as phosphorylation site within the sequence shared between F3 and CT (Appendix). We cannot rule out the presence of other sites present within regions that were not sequenced, but all three sites reported in this study either lie within an RXXS motif or share strong homology with an MK2 phosphorylation sites in other proteins. While in-vitro phosphorylation experiments have their limitations, in our case where multiple kinases may phosphorylate the same residues, it is the easiest way to establish a direct relationship between a kinase and its substrate.

Our results from the phosphorylation experiments identify three serine residues at which MK2 may potentially phosphorylate BRF1. Previous experiments suggest that PKB also phosphorylates BRF1 at two of these sites. In agreement with those experiments we found that substitution of Ser203 with Ala severely compromised MK2 mediated inhibition of AMD, while Ser92 substitution had little or no effect. The combined substitution of Ser92 and Ser203 moderately enhanced the effect of the Ser203; however,
the substitution of Ser92 and Ser203 with Ser54 completely abrogated MK2 mediated inhibition. These results suggest that Ser54 plays an additional role in the regulation of BRF1 dependent AMD by MK2. At this moment the role of Ser92 is unclear, since its substitution has a marginal effect. It is tempting to speculate that Ser54 and Ser203 function in a manner similar to Ser52 and Ser178 in TTP and Ser92 has an additional regulatory function.

The interpretation of the above data is complicated by recent results, which suggest that MK2 activates PKB by phosphorylating Ser473. Consistent with these observations we found a two-fold increase in the phosphorylation of Ser473 in PKB in MK2EE transfected cells. Since both MK2 and PKB phosphorylate Ser92 and Ser203, there exists a possibility that MK2 exerts its inhibitory effect through the activation of PKB. RNAi-mediated downregulation of PKB does not affect the MK2EE induced stabilization of the GB-ARE^{GMCSF} mRNA suggesting that MK2 may indeed act independently of PKB. The sequence similarity between PKB (RXRXXS) and MK2 (RXXS) consensus sites indicate that it is likely that both kinases phosphorylate BRF1 independently at the same sites to elicit the same downstream effect. Similar observations have been made for HDM2, a protein involved in the regulation of p53. Weber et al have reported that both MK2 and PKB independently phosphorylate HDM2 at Ser166 in response anisomycin treatment (Weber et al., 2005). Other proteins phosphorylated by these kinases at the same site include transcription regulator CREB and HSP27 (Phospho.elm database).

Since MK2 and PKB share target sites, it is likely that both kinases employ similar mechanisms to block BRF1-dependent AMD. While it is known that phosphorylation
results in the inhibition of mRNA decay-promoting properties of TTP and BRF1, the mechanisms involved remains unclear. TTP and BRF1 bind to ARE-containing mRNAs and are thought to function by recruiting mRNA decay enzymes onto the mRNAs, consequently targeting them for degradation. In vitro RNA binding experiments carried out with GST-BRF1 phosphorylated in vitro suggest that MK2 mediated phosphorylation does not directly impair the function of the Zinc Finger domains. However, those experiments did not rule out the possibility that the recruitment of factors such as 14-3-3s to 14-3-3s may result in the masking of the domain involved in RNA-binding. However the results from the UV-crosslinking experiments suggest that this may not happen. Changes in RNA-binding may also be a consequence of localization effects, i.e. phosphorylated BRF1 may not be present within the same cellular compartment as the ARE-containing mRNA. Such a scenario would not be detected by our experimental system and would require PCR based detection of endogenous mRNA associated with BRF1 in the presence or absence of MK2EE.

We also examined the effect of MK2EE on the recruitment of degradative enzymes. Immunoprecipitation experiments suggest that co-transfection of MK2EE does not significantly alter recruitment of DCP2, CCR4 and the exosome subunit RRP4. Consistent with previous results, we did detect an increase in the association 14-3-3s with BRF1. Although BRF1 has been show to associate with 5’-3’ exonucleases, XRN1, it is more likely that MK2 would block an early step such as decapping or deadenylation. Unlike deadenylation, decapping is thought to be an irreversible step in 5’-3’ decay. It is therefore unlikely that MK2EE would act on XRN1 and not DCP2. However, we can not
exclude the possibility that there may be cell type-specific effects, in which the RNA binding and mRNA decay enzyme recruitment by BRF1 is decreased, or that the recruitment of other untested mRNA decay enzymes is decreased upon MK2-mediated phosphorylation. Alternatively, MK2 may interfere with the formation of a ternary complex containing ARE-RNA, BRF1, and mRNA decay enzymes, which is believed to be an active complex for mRNA decay. In these experiments we tested for factors previously shown to associate with BRF1. However, additional factors may be involved. We hypothesize that additional critical factors may regulate the activity of the recruited mRNA decay machinery. These factors can either play an inhibitory role in mRNA decay, which are recruited upon MK2-mediated phosphorylation, or might serve as activators of mRNA decay, which are no longer recruited upon MK2-mediated phosphorylation. Recent studies have implicated the 14-3-3 protein family in the regulation of ARE-mRNA decay activities of BRF1 and TTP (Benjamin et al., 2006; Schmidlin et al., 2004). However, it is unclear whether 14-3-3 proteins play a direct role in the inhibition of TTP and BRF1 by protein kinases.

Lykke-Andersen and Wagner previously reported that both the N-terminal (NTD) and C-terminal (CTD) domains of TTP and BRF1 possess mRNA decay promoting activities (Lykke-Andersen and Wagner, 2005). The NTD of TTP, but not the CTD, is responsible for the association with mRNA decay enzymes (Lykke-Andersen and Wagner, 2005). Our results demonstrate that MK2 phosphorylates BRF1 at both NTD and CTD and this phosphorylation plays a critical role in the inhibition of BRF1-dependent AMD. The fact that MK2 does not significantly disrupt BRF1 association with mRNA decay
enzymes suggests that phosphorylation of NTD by MK2 does not regulate the recruitment of mRNA decay enzymes. The CTD of TTP, and perhaps of BRF1, does not associate with any tested mRNA decay enzymes, but contains a second mRNA decay activation domain (Lykke-Andersen and Wagner, 2005). Although the mechanism by which the CTDs of BRF1 and TTP activate mRNA decay pathways is still unknown, we hypothesize that unidentified factors critical for the control of the activity of recruited mRNA decay machinery are recruited or no longer recruited by the CTD of BRF1 upon phosphorylation of Ser203 by MK2. The exact mechanisms for MK2-dependent inhibition of BRF1 require further investigation and the identification of additional factors that regulate the functions of BRF1 and TTP upon phosphorylation should help to elucidate the mechanism and regulation of ARE-mediated mRNA decay.
FUTURE DIRECTIONS

In this study we have demonstrated that MK2 phosphorylates BRF1 at three serine residues thereby inhibiting its ability to promote mRNA decay. However, these results have failed to provide a mechanism for the phosphorylation dependent inhibition of BRF1. As described in previous sections, our data suggests that the MK2 mediated inhibition of BRF1 occurs downstream of RNA-binding and the recruitment of DCP2, CCR4 and the exosome. There exists a possibility that different molecules of BRF1 associate with RNA and degradative enzymes, mRNA decay is activated by the dimerization of RNA-bound and enzyme-associated BRF1. Phosphorylation may impair dimerization thereby inhibiting BRF1 dependent AMD. Preliminary experiments carried out in our laboratory suggest that this may not be the case as results from gel shift assays performed with phosphorylated and unphosphorylated do not indicate changes in the mobility of radiolabeled ARE\(^{\text{GMCSF}}\) RNA upon phosphorylation. Further pull down experiments carried out with biotinylated poly (U)-RNA also suggest that phosphorylated BRF1 interacts with both mRNA and degradative enzymes in the presence of MK2EE.

Phosphorylation of TTP results in the exclusion of TTP from cytoplasmic stress granules thought to be the sites of mRNA degradation. Such a scenario may also exist for BRF1, immuno-fluorescence experiments are being planned to address this possibility.
We have previously employed an MS2-GFP fusion protein to track changes in the localization of ARE-mRNA containing MS2 binding sites (Lin et al., 2007). Such a strategy may be used to monitor changes in the localization of ARE-mRNA upon stimulation with p38 activators. Similar localization studies carried out with epitope tagged BRF1 or a GFP-BRF1 fusion protein, may provide clues as to the mechanism of its inhibition by MK2 and PKB.

BRF1 may also recruit unidentified nucleases onto the mRNA, recent studies have suggested that Ago proteins are involved in AMD and directly or indirectly interact with TTP (Jing et al., 2005). It is possible that BRF1 may also recruit Ago proteins. If such an interaction exist, MK2 mediated phosphorylation may affect the association of BRF1 with Ago2. Previous report have also suggested that 14-3-3 like SMG proteins recruit PP2A, a protein phosphatase to dephosphorylate UPF1, thereby limiting NMD (Isken and Maquat, 2007). Similar proteins maybe recruited by phosphorylated BRF1 that dephosphorylate activating phosphophoserine residues within degradative nucleases. We are currently purifying protein complexes associated with TTP from anisomycin treated or untreated cells. Similar experiments are being planned for BRF1. We hope that these studies will enable us to identify proteins that are preferentially recruited by phosphorylated BRF1 or those that only interact with unphosphorylated BRF1.

It has been suggested that BRF1 and TTP facilitate the transport of ARE containing mRNA from stalled polysomes to the P-bodies (Franks and Lykke-Andersen, 2007). The role of BRF1 in the regulation of translation has not been addressed. MK2 mediated
phosphorylation of BRF1 may result in the enhanced translation of BRF1 bound mRNA. Experiments aimed at determining the association of BRF1 with polysome fraction in the presence and absence of MK2 must be carried to address this possibility.

Signaling pathways regulate gene expression at several levels. Regulation of gene expression at multiple levels may serve as mechanism to temporally control cellular responses to external stimuli. Activation of MK2 has been shown to induce the expression of several stress responsive genes. In addition recent research suggests that MK2 may regulate gene expression post-transcriptionally. RNAi mediated downregulation of MK2 has been shown to sensitize cells to UV induced cell death (Manke et al., 2005). It may be speculated that MK2 could enhance cell survival by blocking decay promoting activity of BRF1 thereby stabilizing mRNA encoding anti-apoptotic factors such as cIAP2 and possibly the Class II ARE-mRNA encoding BCL-2. Future studies can be directed at determining whether SlowC cells are less sensitive to UV induced cell death and if MK2 controls cell survival decisions post-transcriptionally.
SIGNIFICANCE TO HUMAN HEALTH

AU-Rich elements regulate the production proto-oncogenes, pro-inflammatory cytokines and growth factors. It is therefore not hard to imagine the medical problems that may result from defects in AMD. The severe immunological consequences of loss of function TTP and AUF1 observed in knockout mouse models for TTP and AUF1 underscore the importance of AMD in the regulation of the immune system. In addition, stabilization of ARE-containing mRNA may also result in malignancies. In an early study Raymond et al demonstrated that the deletion proto-oncogene fos 3’UTR enhanced its ability to promote malignancies. The stabilization cyclin D1 mRNA due to loss of its ARE has been associated with mantle cell lymphomas (Rimokh et al., 1994). Increased expression of stabilizing ARE-BP, HuR has been observed in brain tumors. Overexpressed HuR in these tumors has been shown to associate with mRNA encoding VEGF, IL-8 and several other factors that may contribute to pathogenesis (Nabors et al., 2001). Destabilizing ARE-BPs may serve as tumor suppressors by downregulating potentially tumorigenic mRNA. A recent study has shown that overexpression of BRF1 enhances the sensitivity of certain squamous cell carcinomas to cisplatin, an anti-cancer drug.
The ability of BRF1 to sensitize cancer cells to chemotherapeutic agents such as cisplatin has been attributed its ability to promote the degradation of anti-apoptotic cIAP2 mRNA (Lee et al., 2005).

Aberrant stabilization of ARE-containing mRNA by the p38 MAP kinase pathway may also facilitate inflammatory disease or tumor formation. McCormick and Ganem have recently demonstrated that the Kaposi’s sarcoma associate herpes virus (KSHV) encodes a protein Kaposin B that enhances the production of several cytokine that mediate the pathogenesis of Kaposi’s sarcoma. Kaposin B exerts this effect by binding to and activating MK2 thereby stabilizing cytokine mRNA (McCormick and Ganem, 2005). The p38 MAP kinase pathway has also been suggested to inhibit destabilizing ARE-BPs in malignant gliomas (Suswam et al., 2008). Since TTP and BRF1 are now known to be inactivated MK2, understanding the mechanism underlying their inhibition is and important step towards identifying possible therapeutic agents.
MATERIALS AND METHODS

Plasmids

mRNA reporter plasmids, pTRE-GB-ARE\textsubscript{GMCSF} and pcDNA-GB-GAPDH, have been described previously (Chou et al., 2006; Lin et al., 2007). FLAG-tagged BRF1 was constructed by subcloning the coding region of human BRF1, amplified by PCR, between EcoRI and XhoI sites of pcDNA-FlagB (Lin et al., 2007). A BamHI/XbaI fragment containing the coding region of DCP2 was subcloned into a pcDNA-myc vector to generate pcDNA-myc-DCP2. Constructs expressing myc-MK2EE and HA-CCR4 were described previously (Engel et al., 1995; Yamashita et al., 2005). BRF1 and its fragments were amplified by PCR and subcloned into pGEX vectors (Amersham) to produce GST-tagged BRF1 and BRF1 fragments. Site-directed mutagenesis was carried out with Stratagene quick-change kit as per manufacturer’s protocol and mutations were confirmed by DNA sequencing.
Purification of recombinant proteins and activation of MK2

GST- and His-tagged proteins were expressed in BL21 (DE3) cells and purified with glutathione-sepharose 4B (Amersham) or NiNTA agarose (Qiagen) as per manufacturer’s instructions. To activate MK2, GST-MK2 was immobilized on glutathione sepharose beads and incubated with a constitutively active His-tagged MKK6 and a His-tagged p38 in a kinase buffer [20 mM Hepes (pH 7.5), 10 mM MgCl$_2$, 1 mM DTT, 20 mM β-glycerol phosphate, 10 mM PNPP, 50 µM sodium vanadate, and 20 µM ATP] at 37°C for 40 min. After which, the MK2-containing beads were washed five times with PBS. Activated GST-MK2 was eluted off the beads with elution buffer [50 mM Tris-HCl (pH 8.0) and 5 mM glutathione].

In vitro kinase assays

0.5-1 µg of recombinant proteins were incubated with 50 ng of activated GST-MK2 and γ-$^{32}$P-ATP in kinase buffer at 37°C for 40 min. To minimize background phosphorylation by any contaminated His-p38 during activation of MK2, kinase assays were carried out in the presence of 5 µM SB203580. The reactions were denatured in SDS sample buffer and subjected to polyacrylamide gel electrophoresis. Gels were stained with coomassie brilliant blue and subjected to autoradiography.
Antibodies

Mouse monoclonal anti-FLAG (M2), anti-myc (9E10), anti-HA (12CA5), anti-MK2, and anti-14-3-3β (H8), which cross-reacts several isoforms, were purchased from Sigma, Roche, Upstate Biotechnology, and Santa Cruz Biotechnology, respectively. Anti-DCP2 (Wang et al., 2002), anti-RRP4 (Mitchell et al., 1997), and anti-BRF1 (Schmidlin et al., 2004) have been described previously.

mRNA decay assays

To establish the tetracycline (Tet)-Off (TO) system in HT1080 and SlowC fibrosarcoma cell lines, cells were transfected with the pTet-Off plasmid (Clontech) expressing tetracycline-controlled transactivator (tTA) that activates transcription in the absence of doxycycline (Dox). Individual stable clones were selected with G418 and analyzed for the repression of luciferase reporter gene expression under the control of a Tet-regulatory promoter upon Dox addition. A cloned cell line exhibiting about 20-fold repression upon the addition of Dox was chosen for subsequent mRNA decay analysis. HT1080-TO or SlowC-TO cells were transfected using lipofectamine. After transfection, cells were incubated with tetracycline-free medium for 16 h, followed by addition of doxycycline (2 µg/ml). For analysis of mRNA decay, constructs expressing reporter mRNAs under the control of the cytomegalovirus promoter were used. Constructs expressing reporter mRNAs under the control of a Tet-regulatory promoter were used for analysis of mRNA half-life ($t_{1/2}$). To examine mRNA decay, 6h after transfection, cells were collected and
replated onto 35-mm plates. After another 12 h, the cells were treated with medium containing doxycycline (2 µg/ml), and total RNA was isolated at different times (Chou et al., 2006).

Extract preparation and immunoprecipitation assays

Cytoplasmic extracts were prepared from transfected cells and subjected to RNase A treatment as described (Lykke-Andersen, 2002). The RNase-treated extracts were incubated with 10 µl (bed volume) of anti-FLAG agarose (Sigma) for 4 h at 4°C. The beads were washed eight times with buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% NP-40. The immunoprecipitated materials were eluted with SDS sample buffer and subjected to immunoblot analysis.

RNA-binding analysis and immunoprecipitation of RNA-protein complex

RNA binding UV-crosslinking assays and immunoprecipitation of RNA-protein complex were performed as described (Gherzi et al., 2004).

Mass spectrometry and LC-MS/MS analysis

GST-BRF1 F2 and F3 fragments were phosphorylated by MK2 in vitro and subjected to in solution digestion followed by LC-MS/MS analysis. Phosphorylated samples were adjusted to 2 M urea/thiourea, reduced, alkylated and digested with sequenced grade
modified trypsin. If required, tryptic peptides were further digested with AspN. Peptides were concentrated and desalted on reversed phase C$_{18}$ disks and resuspended in 7 µL of 1% TFA/2% acetonitrile in water for LC-MS analysis. Liquid chromatography was performed on a 1100 nano-HPLC (Agilent) coupled to a Finnigan LTQ-Orbitrap (Thermo Electron). Peptides were separated on a C$_{18}$-reversed phase column packed with Reprosil (ReproSil-Pur C$_{18}$-AQ 3-µm resin) and directly electrosprayed in the source of a LTQ-Orbitrap using 53 min or 100 min gradients from 2% to 60% acetonitrile in 0.5% acetic acid at a flow of 250 nl/min. The LTQ-Orbitrap instrument was operated in the data dependent mode switching automatically between MS survey scans (acquired in the LTQ-Orbitrap cell) and MS/MS spectra acquisition in the linear ion trap. An inclusion list, containing all possible phosphorylated and non-phosphorylated peptides of BRF1, was used for preferential sequencing. Multi-stage activation was enabled in all MS/MS events to improve fragmentation spectra of phosphopeptides and “lock mass” option was enabled in all full scans to improve mass accuracy of precursor ions (Olsen et al., 2005). Thee raw data files were converted to the Mascot generic format and searched with the Mascot search engine (http://www.matrixscience.com) against the IPI human protein database (http://www.ebi.ac.uk) containing BRF1 fragments of interest. Carbamidomethylation was selected as a fixed modification. Oxidation of methionine, N-acetylation of the protein, and phosphorylation of serine, threonine, and tyrosine were used as variable modifications; precursor ion mass tolerance was 10 ppm and fragment ion mass tolerance was 0.5 Da. Mass spectrometry data were visualized and validated with MSQuant (http://msquant.sourceforge.net) and exported to Excel (Microsoft) for
further analysis. The probability for phosphorylation at each potential site was calculated from the PTM scores as described previously (Olsen et al., 2006).
LIST OF REFERENCES


arthritus, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity*, 4, 445-454.


APPENDIX A

BRF1 Protein Sequence
Human BRF1 protein/cDNA

1  atgcaccaccaccccctcgtctgctcaccatctctgacttggagccgaa
M  T  T  T  L  V  S  A  T  I  F  D  L  S  E
46  gtatttgcaaggtaacaagatctcaactatagctgtttccaggt
V  L  C  K  G  N  K  M  L  N  Y  S  A  P  S
91  gcaagggggttacctgctggacagaaggaagctgtgcctggcc
A  G  G  C  L  L  D  R  K  A  V  G  T  P  A
136  ggtgaggggtatctgccgagcacagctgtctccgactggcctg
G  G  G  F  P  R  R  H  S  V  T  L  P  S  S
181  aagtctccacagacacagctctctcaagctgctagggtagcaca
K  F  H  Q  N  Q  L  L  S  S  L  K  G  E  P
226  gcccccgctctgagtctggacagccaccccaagcaagccc
A  P  A  L  S  S  R  D  S  R  F  R  D  R  S
271  ttccgcaaggggagggctgtctgccaccaccaagcagccc
F  S  E  G  G  E  R  L  L  P  T  Q  K  Q  P
316  ggggccagccagctcactccacagcctgagctaggggagcag
G  G  G  Q  V  N  S  S  R  Y  K  T  E  L  C
361  cggcccttttgagaaaccgggtctgtgtacatcgaggggaaagtgc
R  P  F  E  E  N  G  A  C  K  Y  G  D  K  C
406  cagttgcaacagcagctcactccagcctgacagcctgagccc
Q  F  A  H  G  I  H  E  L  R  S  L  T  R  H
451  ccaagtccacagagccagctcactccacagcctgacagcctgac
P  K  Y  K  T  E  L  C  R  T  F  H  T  I  G
496  tttgtcctcctcgggctctccgcctccttacatcgacagccc
F  C  P  Y  G  P  R  C  H  F  I  H  N  A  E
541  gagcggctgttcttgccgagggcccagccagctctctcgtgagcgt
erglaA  g  a  r  d  l  s  A  D  R
586  cccgctccacagcatctctttttgtctggttttccagtgcc
P  R  L  Q  H  S  F  S  F  A  G  F  P  S  A
631  gctgcaagggcgcgtgcagccacgctgtcagcagtgcc
A  A  T  A  A  T  G  L  L  D  S  P  T  S
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t  I  T  P  P  I  L  S  A  D  D  L  L  G  S
721  cctaccccgcgtttggaaccccttttttgcctcttcagc
P  T  L  P  D  G  T  N  N  P  F  A  F  S  S
766  caggaggctggcagctctcttttcacctctagctgtggctcgccggg
Q  E  L  A  S  L  F  A  P  S  M  G  L  P  G
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G  G  S  P  T  T  F  L  F  R  P  M  S  E  S
856  cctcacaatgttttgacctcccccacagctcaggattctctctcg
P  H  M  F  D  S  P  P  S  P  Q  D  S  L  S
901  gacaccagggctctctgtacatgacagcagccacacctctctctggc
D  Q  E  G  Y  L  S  S  S  S  S  S  S  H  S  G
946  tcaagactcccgcaggttgagcttaagctcagccacagtgggc
t  S  D  S  P  T  L  D  N  S  S  R  R  L  P  I  F
991  agcaagcttttctcaacagtacacctcagagttctagqnnnnntann
S  R  L  S  I  S  D  D  L  E  S  R  X  X  X

Zinc Finger Domains
APPENDIX B

NetPhos Predictions
NetPhos 2.0 Server - prediction results
Technical University of Denmark

338 Sequence
MTTTLVSATIFDLSEVLCKGNKLNYSAASPAGGCLLDRKAVGTPAGGFPRRHSTLPSSKFWQNQLLSLKGEPAPALS
80
SRDSRFDRFSDSEGGERLLPTQPKQGGGQVNSSRYKTELCPFEENGAKYGDCKCQFAHGIHELRSLTRHP
KYKTELCRT 160
FHTIGFCYGRCHFIHNAERRALAGARDLSADRPRLQHSFSFAGFPSAAAMTAATGGLDSPTSITPPPLSADDLLGS
240
PTLPDGTVNPFNFFSLFAFSMLPLPGGSPTTLFRPMSEPHMFDSPPSPQDSLSDQEYLSSSSHGSQDSPT 320
LDNRSRLPFSRSLSISDD
400
...T..................................T..........S....S..........S.
.......S 80
...S.....S.S........................S....................................S.
.Y....... 160
..........................S........S....................S..S......
.S......S 240
..........................................S.S........S...S.S....Y.S.SSS
S.S.S.... 320
.............S....
400

Phosphorylation sites predicted: Ser: 28 Thr: 2 Tyr: 2

Serine predictions

<table>
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Threonine predictions

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APPENDIX C

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Highest Score: 0.88 PKC at position 104

Explain the output. Go back.

Currently NetPhosK covers the following kinases: PKA, PKC, PKG, CKII, Cdc2, CaM-II, ATM, DNA PK, Cdk5, p38 MAPK, GSK3, CKI, PKB, RSK, INSR, EGFR and Src.
APPENDIX D

Sequence Coverage of GST CT
BRFl-CT: Trypsin and AspN digestion

MSPILGYWKI KGLVQPTRLL LEYLEEKYEE HLYERDEGDK WRNKKFELGL
EFPPNLFYID GDVFKTLQSMMA IIRYIADKHN MLGGCPKERA EISMLEGAVL
DIRGYVSRIA YSKDFETLKV DELSKLPMLKMFEDRLCHKTLYNNGDVTH
PDFMLYDALD VVLYMDPMCL DAPPKLVCFK KRLEAIPOIQD KLYKSSKYIA
WPIQGWMQATF GGGDHPKXS LVERGSRRAS VGSPGISGGG GGIEGARDLS
ADRFRQHSSF SFAGFPSAAA TAAATGLLDPS TSTIPFPILS SADDLLGSPT
LPDGTNNPFA FSSLQELASLF APSMGDPGGE SPPFLFRPM SESPHMFDFS
PSPQDSLSDQ EGYLSSSSSS HSGSDSPTLD NSRRLPFSR LSISSDDTRAQA

green sequences: identified!
black sequences: not identified!
red sequences: psites

psites found for:

_LQHSP_FpSFAGFPSSAAATAAATGLLL_
_LQHSP_FpSFAGFPSSAAATRAATGLLL_
_DSPTpSITPPPILSA_

pSite probabilities:

LQHSP_FpSFAGFPSSAAATAAATGLLL: 99%
LQHSP_FpSFAGFPSSAAATAAATGLLL: 1%

DSPTpSITPPPILSA: 75%  
DpSPTSITPPPILSA: 25%

That pSite is very weak!!!
1) Peptide Score low!
2) If the peptide is correctly identified, I intend to assign the pSite to the first Serine because of the „SP“-motif.

That supports my previous finding using chymotrypsin
BRF1-CT: only Trypsin digestion

MSPILGYWKL KGLVQPTRLL LEYLEEKYEE HLYERDEGDK WRNKKFELGL
EFPNLPPYYID GDVKLTQSMA IIRYIADKHN MLGGCPKERA EISMLEGAVL
DIRYGVSRIL YSKDFETLKV DFLSKLPPEML KMFEDRLCHK TYLNGDHVTH
PDFMLYDALD VVLVMDPMCL DAFPKLVCFK KRIEAIPQID KYLKSSDKYIA
WFLQGWQATF GGGHPPFKSD LVPRGSRRAS VGSPGISGDD GGIEGARDLS
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LPDGTNNPPA FSSQELASLF APSMGLPGGG SPTTFLFRPM SESPHMFDSP
PSPOQDSLSDQ EGYLSSSSSS HSGDSPTLD NSRRLPIFSR LSISDDTRAQ
A

green sequences: identified!
black sequences: not identified!

no pSites identified!