ZAS3 represses NFκB-dependent transcription by direct competition for DNA binding

Joung-Woo Hong1 & Lai-Chu Wu2,3,*

1The Graduate School of East-West Medical Science, Kyung Hee University, Yongin 446-701, Korea, Departments 2Molecular and Cellular Biochemistry, 3Internal Medicine, College of Medicine and Public Health, The Ohio State University, Columbus, OH, 43210, USA

NFκB and ZAS3 are transcription factors that control important cellular processes including immunity, cell survival and apoptosis. Although both proteins bind the κB-motif, they produce opposite physiological consequences; NFκB activates transcription, promotes cell growth and is often found to be constitutively expressed in cancer cells, while ZAS3 generally represses transcription, inhibits cell proliferation and is down-regulated in some cancers. Here, we show that ZAS3 inhibits NFκB-dependent transcription by competing with NFκB for the κB-motif. Transient transfection studies show that N-terminal 645 amino acids is sufficient to repress transcription activated by NFκB, and that the identical region also possesses intrinsic repression activity to inhibit basal transcription from a promoter. Finally, in vitro DNA-protein interaction analysis shows that ZAS3 is able to displace NFκB by competing with NFκB for the κB-motif. It is conceivable that ZAS3 has therapeutic potential for controlling aberrant activation of NFκB in various diseases. [BMB reports 2010; 43(12): 807-812]

INTRODUCTION

NFκB controls key cellular processes, including immunity, cell survival and apoptosis by activating transcription of target genes through the κB motif (GGGRNNYCC) (1, 2). NFκB is ubiquitously expressed in the cell, and its cellular activity is subjected to tight post-translational control. In most cell types, NFκB exists as an inactive form in the cytoplasm by associating with an inhibitor, IκB. Many stimuli, such as TNFα and lipopolysaccharide (LPS), activate cellular signaling pathways that trigger the assembly of the IκB kinase (IKK) complex. IKK phosphorylates IκB and marks it for ubiquitination and degradation (3). NFκB then translocates into the nucleus (at which time it is referred to as activated) and induces NFκB-dependent transcription of target genes including anti-apoptotic genes. Thus, mutations in components of the NFκB signaling pathway that make NFκB constitutively expressed are often implicated in certain types of cancers (4).

The Zα family of large, separated-paired-C2H2 zinc finger proteins also regulates transcription through binding κB-motif (5, 6). Unlike NFκB that promotes tumorigenesis, the Zα proteins are most likely tumor suppressors. Down-regulation of ZAS2 (HIVEP2) is often found in breast cancer (7), and functional loss of ZAS1 and ZAS2 is associated with poor prognosis for chronic lymphocytic leukemia (8). Furthermore, ZAS3 deficiency has resulted in tumor formation, cell immortalization and growth acceleration (9). Hence, NFκB and Zα, the two major families of cellular κB-binding proteins, appear to have opposing effects on the regulation of transcription and growth. A recent study shows that ZAS3 inhibits NFκB by directly associating with TNF receptor-associated factor 2 (TRAF2), thereby blocking nuclear translocation of NFκB (10). Consistent with these results, NFκB is constitutively expressed in ZAS3 knock-out cells (11).

Although NFκB and ZAS3 proteins are able to bind similar DNA binding sites, how they cooperatively regulate transcription of downstream target genes and thereby how they control the switch between cell survival and death remains a mystery.

In the present study, we show that the N-terminal 645 amino acids of ZAS3 compete efficiently with NFκB for κB-motif and abolish NFκB-dependent transcription. In addition, the N-terminal region possesses intrinsic repression activity. Furthermore, ZAS3 does not prevent overexpressed NFκB from translocating into the nucleus. Together, these results suggest that DNA competition by ZAS3 alone is sufficient to repress transcription activated by NFκB and that ZAS3 is an endogenous κB-motif competitor with intrinsic repression activity.

RESULTS

ZAS3 represses NFκB-activated transcription by binding the κB-motif

Previously, ZAS3 was shown to bind the κB-motif and mediate transcriptional repression (11). To extend our understanding of ZAS3 function, we asked if ZAS3 could repress transcription of target genes including anti-apoptotic genes. Thus, mutations in components of the NFκB signaling pathway that make NFκB constitutively expressed are often implicated in certain types of cancers (4).

The Zα family of large, separated-paired-C2H2 zinc finger proteins also regulates transcription through binding κB-motif (5, 6). Unlike NFκB that promotes tumorigenesis, the Zα proteins are most likely tumor suppressors. Down-regulation of ZAS2 (HIVEP2) is often found in breast cancer (7), and functional loss of ZAS1 and ZAS2 is associated with poor prognosis for chronic lymphocytic leukemia (8). Furthermore, ZAS3 deficiency has resulted in tumor formation, cell immortalization and growth acceleration (9). Hence, NFκB and Zα, the two major families of cellular κB-binding proteins, appear to have opposing effects on the regulation of transcription and growth. A recent study shows that ZAS3 inhibits NFκB by directly associating with TNF receptor-associated factor 2 (TRAF2), thereby blocking nuclear translocation of NFκB (10). Consistent with these results, NFκB is constitutively expressed in ZAS3 knock-out cells (11).

Although NFκB and ZAS3 proteins are able to bind similar DNA binding sites, how they cooperatively regulate transcription of downstream target genes and thereby how they control the switch between cell survival and death remains a mystery.

In the present study, we show that the N-terminal 645 amino acids of ZAS3 compete efficiently with NFκB for κB-motif and abolish NFκB-dependent transcription. In addition, the N-terminal region possesses intrinsic repression activity. Furthermore, ZAS3 does not prevent overexpressed NFκB from translocating into the nucleus. Together, these results suggest that DNA competition by ZAS3 alone is sufficient to repress transcription activated by NFκB and that ZAS3 is an endogenous κB-motif competitor with intrinsic repression activity.
activated by NFκB by binding to the κB-motif. Increasing amounts of expression vectors encoding ZAS protein (amino acid 106 to 2013) were cotransfected into HEK293 cells together with a constant amount of expression vector encoding p65 and p50, and a reporter plasmid (p3xκB-Fos-Luc, see Fig. 1A top) containing three consecutive copies of κB-motifs and a c-fos minimal promoter (−65 to +109, relative to transcription start site as +1) immediately upstream of the firefly luciferase gene. As expected, ectopic expression of p65 and p50 increased luciferase activity 10-fold more than basal level. Co-expression of ZAS3 repressed the activity of p65 and p50 up to almost the basal level in a dose-dependent manner (Fig 1A). An essentially identical result was obtained even when luciferase expression was activated up to 100-fold by increased expression of p65 and p50 (data not shown). The fact that ZAS3 expression could significantly inhibit luciferase activity induced by p65 and p50 expression suggests that ZAS represses NFκB-activated transcription. To determine whether the repression activity of ZAS is exerted through binding the κB-motif, a similar co-transfection experiment was performed with a c-fos minimal promoter containing a reporter plasmid (p3xκB-Luc; see Fig. 1B top), but no κB-motif. In the absence of the κB-motif, neither p65/p50 nor ZAS3 had a negative effect on luciferase activity. Taken together, these data indicate that ZAS3 represses transcription that is activated by NFκB and this repression requires binding of ZAS3 to the κB-motif.

The N-terminal region of ZAS3 is required for repression of NFκB-activated transcription

To determine which part(s) of the ZAS3 protein are necessary for transcriptional repression, a number of systemic truncations were made by deleting different structural components, one at a time. To assay repression, p65 and p50 were used as an activator and p3xκB-Fos-Luc as the reporter. First, either the N- or C-terminal half was deleted from full-length ZAS3, and its repression activity was examined (Fig. 2A). Deleting the C-terminal half containing the ZASC domain had little effect, if any,
on the repression activity of ZAS3 (106-1186), but deleting the N-terminal half containing the ZASN domain resulted in a significant loss of its repression activity (1186-2153). Next, we deleted the zinc fingers in the ZASN domain, the nuclear localization signal (NLS) region or the entire ZASN domain from ZAS106-1186 (261-1092, 106-750 and 750-1186, respectively). None of the two ZAS3 vectors, 261-1092 or 750-1185, gave rise to strong repression. However, a ZAS3 vector containing the ZASN domain and zinc finger 3, 107-750, did display the strongest repression activity among all ZAS3 constructs tested. Although repression activity of more the truncated form of the C-terminal half, 1497-2012, was also measured, it was inactive as a repressor and resulted in weak activation. These findings indicate that a 1.9 kb region around the ZASN domain is sufficient for the full repression activity of ZAS3.

Recently, it was reported that ZAS3 inhibits NFκB activity induced by TNF signaling pathway through direct interaction with TRAF2 in the cytoplasm (10). This finding made it hard to exclude the possibility that the observed repression activity of ZAS3 is due to direct interaction with other proteins such as TRAF2 involved in transcription and/or signaling pathway. To measure the expression and localization of the ZAS3 proteins, all ZAS3 proteins were tagged with a FLAG octapeptide (D-Y-K-D-D-D-D-K) following a methionine (M), and nuclear and cytoplasmic extracts were prepared after transfection and western blot analysis with a monoclonal anti-FLAG antibody (M2, Sigma) were performed (Fig. 2B). These results make several points. First, while there were reproducible differences in the levels of ZAS3 protein accumulation in nuclear extracts, there was no correlation between the amount of protein detected and its repression activity. A second point is that repression activity was also not correlated with protein size. Finally, regardless of NLS, all proteins were localized in the nucleus and none were detectable in the cytoplasm. This result strongly supports the previous finding that ZAS3 acts as transcription factor mainly in the nucleus, but not in the cytoplasm (11), although this notion is not consistent with the previous observation that ZAS3 blocks translocation of NFκB into the nucleus by interacting with TRAF2 in the cytoplasm (10). The separated nuclear and cytoplasmic extracts were verified by measuring nuclear and cytoplasmic marker proteins, histone H1 and hsp90 (Fig. 2B, bottom two panels). These findings indicate that ZAS3 is a nuclear protein and the N-terminal region containing the ZASN domain is required for the repression activity of ZAS3, suggesting that ZAS3 binding to DNA is essential for its repression activity.

**ZAS3 represses basal transcription**

In our co-transfection experiments (Fig. 1A, last lane), ZAS3 strongly blocked luciferase activity in the absence of p65 and p50 proteins, providing evidence that ZAS3 represses basal transcription in the absence of activators, as in a direct repression model (12). To determine if ZAS3 could function as a direct repressor, GAL4-ZAS3 fusion proteins containing GAL4 DNA binding domain (DBD) and a reporter plasmid containing GAL4-binding sites (p4xG-SV-Luc) (13, 14) were used (Fig. 3A). GAL4-ZAS3 expression vectors were co-transfected into HEK293 cells with a reporter plasmid containing four consecutive GAL4-binding sites. Transcription from this SV40 basal promoter of p4xG-SV-Luc was weak, but readily detectable, and could be quantified accurately (Fig. 3A). All GAL4 DBD-ZAS3 fusion proteins containing a ZASN domain (GLA41186-2153) repressed basal transcription from the SV40 minimal promoter (up to ~20-fold) (Fig. 3A), whereas a GAL4-ZAS3 protein containing a ZASC domain (GLA41186-2153) failed to repress the transcription in the absence of activators. Since there are no endogenous transcriptional regulators that bind to the GAL4-binding sites (15), our data support the hypothesis that ZAS3 can also...
ZAS3 inhibits NFκB by DNA competition
Young-Woo Hong and Lai-Chu Wu

Fig. 4. The competing effect of ZAS3 on binding of p65/p50 to 32P-NFκB-DNA. (A) Electrophoretic mobility shift assay (EMSA) of 32P-NFκB-DNA. GST-tagged recombinant p65 and p50 (GST-p65/p50) were mixed with 32P-NFκB-DNA and ZAS3 fusion proteins, and then the mixture was incubated for 10 minutes before the addition of excess unlabeled NFκB-DNA. The final mixture including the excess amount of unlabeled NFκB-DNA was incubated for another 10 minutes before gel loading. (C) Protein competition assays showing that ZAS3 competes with NFκB for NFκB-DNA. EMSA of GST-p65/p50 (10 ng), varying amounts of ZAS3 fusion proteins, GST106-750 or GST1497-2012, and 32P-NFκB-DNA. Control GST (10 ng) did not form DNA-protein complexes.

ZAS3 displaces NFκB from xB-motif.

Given the fact that ZAS3 and NFκB have the ability to bind the xB-motif and ZAS3 represses transcription activated by NFκB, it is possible that ZAS3 represses NFκB-activated transcription through a competition mechanism. To determine whether ZAS competes with NFκB for binding the xB-motif, an electrophoretic mobility shift assay (EMSA) was performed with bacterially expressed ZAS3 and p65/p50. The N- and C-terminal 645 and 516 amino acids of ZAS3 (106-750 and 1497-2012), which presented the strongest and the weakest repression activities, were fused with glutathione-S-transferase (GST). The GST106-750 contains the ZASN domain, zinc finger 3 and the first proline-rich region, and the GST1497-2012 contains the ZASC domain and the linker region that is conserved among ZAS proteins (6).

In EMSA, both GST-p65/p50 and GST-ZAS3, but not GST alone, formed a complex with 32P-labeled xB-motif with instantly distinguishable gel mobility and the specificity of binding was verified with unlabeled competitors (Fig. 4A). The relative affinity of GST106-750 was higher than that of GST1497-2012, indicating that there is a correlation between relatively strong DNA-binding affinity and repression activity. Namely, the repression activity of ZAS3 is proportional to the strength of the DNA-binding affinity. This result supports a notion that a competition model is the most likely mechanism underlying the observed repression activity of ZAS3 against NFκB-activated transcription.

We also assessed whether the GST-ZAS3 fusion proteins could compete with NFκB for binding the xB-motif. To address this, we pre-incubated GST-p65/p50 with one of the GST-ZAS3s and 32P-labeled xB-motif probe, and then we added the indicated amount of unlabeled xB-motif probe (cold xB-motif-DNA) to the mixture. GST-p65/p50 and GST106-750 remained to bind xB-motif, even with an excess amount of the unlabeled xB-motif, whereas the smallest amount of the unlabeled xB-motif was sufficient to displace GST1497-2012 from the preformed complex. Furthermore, when increasing amounts of one of the GST-ZAS3s were added to the mixture, where the constant amount of GST-p65/p50 had been pre-incubated with the labeled xB-motif, only GST106-750, but not GST1497-2012, efficiently abrogated the interaction between xB-motif and GST-p65/p50. Together, the above results indicate that the N-terminal 645 amino acids of ZAS3 can displace NFκB from the xB-motif, suggesting that competition between ZAS3 and NFκB for a common DNA site is mainly responsible for the repression activity of ZAS3.

DISCUSSION

Here, we provide the first evidence that the large zinc finger protein, ZAS3, represses NFκB-activated transcription in a xB-motif-dependent manner. The repression activity of ZAS3 ap-
pears to result from the combination of competition with NFκB for the κB-motif and its intrinsic repression activity.

Based on the concept of a genetic switch proposed by Ptashne (16), we propose a "dual-switch" model, whereby ZAS3 and NFκB can independently bind one or multiple copies of κB-motifs to regulate transcription of NFκB-target genes. The transcription activity of the target gene depends on the occupancy of ZAS3 and NFκB on the κB-motifs. At least one mechanism can be envisioned for the model. In the resting state, ZAS3 not only masks the κB-motif, but it also prevents NFκB target genes from unnecessary expression by using its direct repression activity. A recent study demonstrated that NFκB does not constantly reside in the cytoplasm, but rather, shuttles continuously in and out of the nucleus in the unstimulated state (17), indicating the possible existence of a small amount of NFκB in the nucleus. In this condition, a relatively large amount of ZAS3 competes with the residual NFκB for κB-motifs and occupies the κB-motifs, resulting in repression of NFκB target genes. A quenching repression model (12) is the least likely scenario to explain the repression ability of ZAS3 on the NFκB-dependent transcription, because of the observation that when ZAS3 and NFκB are competing for the κB-motif, each forms only one DNA-protein complex in vitro (Fig. 4B, C). This result suggests that the two proteins do not directly interact with each other. However, the possible involvement of a cofactor protein(s) between ZAS3 and NFκB as well as the physical interaction of ZAS3 with other Rel family members, aside from p65 and p50, still remains to be tested.

A functional domain(s) that possesses the intrinsic repression ability of ZAS3 is located in the N-terminal region of ZAS3. The 645 amino acids surrounding the ZASN domain did display repression activity that is stronger than or at least equivalent to that of full-length ZAS3, whereas the C-terminal half containing the ZASC domain had little, if any, effect on the repression activity and actually produced weak activation. Functional repression domains have been shown to require general hydrophobicity (i.e. proline and alanine) (18, 19). Additionally, the abundance of serine, threonine and acidic residues is a common feature of transcriptional activation domains (20, 21). The existence of serine, threonine and an acidic stretch, as well as the lack of hydrophobic abundance in the N-terminal ZAS3, were unexpected. However, the N-terminal 645 amino acids strongly repressed both basal and activated transcription and the result was reproducible, although there was a minor variation in the strength. It is not clear how this seemingly paradoxical observation could explain the repression ability of the N-terminal region. The simplest explanation is that other N-terminal regions, aside from the ZASN domain, may have the novel class of a repression domain, or that an unknown combination of known features, such as serine/threonine stretch and acidic residues, may produce novel repression domain in the N-terminal 645 amino acids. The possibility is speculative and is only one of several that might be consistent with our currently available results. Nonetheless, defining the minimal repression domain of ZAS3 provides a future framework for developing a specific inhibitor to block aberrant expression of NFκB target genes.

An accumulating number of studies have shown that NFκB activates transcription of numerous genes involved in cell survival and death in addition to immunity, development and diseases. Although, to date, several proteins that synergistically enhance or attenuate transcriptional activation competency of NFκB have been identified (22-24), a transcription factor that represses NFκB-activated transcription via binding κB-motif has not been reported. As presented here, the ability of ZAS3 to repress transcription of NFκB-target genes by binding a κB-motif indicates that the transcription of the NFκB-target genes does not rely solely on NFκB, but also on the cooperation with at least one more factor, ZAS3. Based on the existence of several κB-motif-binding proteins in addition to NFκB [reviewed in (11)], however, it is not likely that NFκB and ZAS3 are the only proteins that are able to bind to κB-motif. At least other ZAS family members, ZAS1 and ZAS2, have been demonstrated to specifically interact with the κB-motif [reviewed in (6)]. Therefore, a combinational effect of ZAS1, ZAS2, ZAS3 and NFκB on the κB-motif should be examined to fully understand the mechanism of NFκB-dependent transcription.

MATERIALS AND METHODS

Detailed information is described in Supplementary Material.

Acknowledgements

We would like to give the deepest thank to Dr. Denis C. Guttridge for pFos-Luc, p3xB-Fos-Luc, p65 and p50. This work was supported by a grant from the National Cancer Institute (p3CA15058).

REFERENCES


