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MAGE proteins regulate KRAB zinc finger transcription factors and KAP1 E3 ligase activity

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ABSTRACT

Expression of *M*elanoma *A*nti*G*en *E*ncoding (MAGE) genes, particularly MAGE-A3, has been correlated with aggressive clinical course, the acquisition of resistance to chemotherapy and poor clinical outcomes of melanoma and other malignancies. MAGE proteins bind to KAP1, a gene repressor and ubiquitin E3 ligase which also binds KRAB domain containing zinc finger transcription factors (KZNFs), and MAGE expression may affect KZNF mediated gene regulation. To investigate mechanisms for these effects, we tested the hypothesis that differences in KRAB domain composition affect KZNF poly-ubiquitination and determine whether MAGE expression increases, decreases, or has no effect on KZNFs mediated gene repression. Using an integrated reporter gene responsive to repression by KRAB domain fusion proteins, we found that MAGE-A3 relieved KZNF mediated repression and induced KZNF poly-ubiquitination and degradation in association with expression of the A+B box KRAB domain. In contrast, MAGE-A3 enhanced KAP1 mediated repression of KZNFs expressing A or A+b box KRAB domains but caused no increase in poly-ubiquitination or degradation. MAGE-A3 has no significant impact on KZNFs with KRAB domains containing the Scan box motif. These data support our hypothesis by showing that the effects of MAGE-A3 on gene repression depend on the type of KZNF KRAB domain involved.

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Introduction

The MAGE antigens are proteins that were first discovered because they elicited cytotoxic T cell and humoral responses in patients with malignant melanoma [21]. The genes were called MAGE genes for the acronym "*M*elanoma *A*nti*G*en *E*ncoding gene" [1,32]. The original MAGE antigens were found to be normally expressed only in male gametes and became the first proteins identified in what is now a much larger group of antigens that are expressed in malignant tumors, placenta and testes, and that are known as Cancer Testes (CT)[1] antigens [29]. The original MAGE genes included MAGE-A, B, and C, which are encoded on the X chromosome and have been called CT-X MAGE proteins. A number of autosomal gene families, named MAGE D through MAGE L, have more recently been found to be homologous with the CT-X MAGE genes but appear to be more widely expressed and include some genes which are expressed in all normal tissues [8,27]. The CT-X genes are now also known as Class I MAGE genes and the non-X encoded genes are called Class II MAGE genes. All MAGE genes are characterized by a MAGE homology domain (MHD) a region encoded by a single exon, showing as much as 98% homology within MAGE families. In this report we will concentrate on Class I MAGE proteins which, because of their tendency for expression in many cancers and hematopoietic malignancies and their very limited expression in normal adult tissues, have been used as tumor specific targets for immunotherapy of melanoma and other malignancies [5,10,16,22,25,26,29,30,35]. The functions of most MAGE proteins remain unknown but several studies have shown correlations between Class I MAGE expression and tumor development, aggressive clinical course, or resistance to chemotherapeutic agents [2,12,18,19,26,29]. However, it has not yet been conclusively determined whether Class I MAGE gene expression is a functionally irrelevant by-product of cellular transformation or could actually contribute to the development of malignancies [29].

The high degree of homology between Class I MAGE family proteins and the fact they are often co-expressed, suggests that

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[1] *Abbreviations used:* MAGE, *M*elanoma *A*nti*G*en *E*ncoding; KZNFs, KRAB domain containing zinc finger transcription factors; CT, cancer testes; MHD, MAGE homology domain; KRAB, Kruppel-associated box; HP1, heterochromatin protein 1; H3me3K9, histone-3-lysine-9-trimethylation; DBD, Gal4-DNA binding domain; MGC, mammalian gene collection; HRP, Horseradish peroxidase, HDACs, histone de-acetylases; RBCC, RING Finger-B Box-Coiled Coil.

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many perform common or complementary functions [8,23,29]. For instance, MAGE-A3 and MAGE-A6 differ mainly in un-translated regions and show 98% identity at the nucleotide level in their coding regions. Similarly, the murine mMage-b family, composed of mMage-b1, b2, and b3, are 98–99% homologous at the nucleotide levels and 97–100% identical at the amino acid level. Due to these factors and due to a lack of antibodies that can differentiate between nearly identical sub-family members, most studies of MAGE gene expression rely on detection of mRNA, usually by reverse transcription followed by the polymerase chain reaction (RT-PCR) [8,23,29]. Our experiments in this report involve MAGE-A3, which is the most common of the Class I MAGE genes expressed in malignancies and is in many ways characteristic of the Class I MAGE genes.

We and others have previously established that MAGE expression suppresses p53 and enhances the cellular response to DNA double strand breaks [3,24,34]. We also discovered and others confirmed that a common function of the MHD is binding to KAP1, a universally expressed nuclear scaffolding protein and ubiquitin E3 ligase, also known as TRIM28, Tif1b, and Krip125 [11,34]. KAP1 causes chromatin condensation in several contexts, including a generalized condensation in response to DNA double strand breaks and more localized chromatin compaction causing repression of specific genes mediated by Kruppel-associated box (KRAB) domain containing zinc finger transcription factors (KZNFs) [13] (please see Fig. 1). KZNFs are the largest group of transcription factors in vertebrates and they direct KAP1 to specific chromatin sites recognized by their zinc finger motifs. KAP1 recruits SetDb1, histone de-acetylases, and heterochromatin protein 1 (HP1), inducing localized chromatin compaction and gene repression characterized by histone de-acetylation and histone-3- lysine-9- trimethylation (H3me3K9) an epigenetic signature also seen in senescence associated heterochromatin foci (SAHF) [6,13,17]. Functionally, MAGE-KAP1 co-binding may: (i) increase repression of specific genes, (ii) have no effect on gene repression, or (iii) increase KAP1 mediated ubiquitination of KZNFs with decreased target gene binding and de-repression of specific genes [33]. These variable effects mean that MAGE proteins can act as master transcription factors affecting cascades of gene activity.

To determine the mechanism(s) for the variable effects of MAGE proteins on gene repression and to identify MAGE effects on KZNFs, we used a comprehensive series of fusion proteins containing different KRAB domains fused to the Gal4-DNA binding domain (DBD), with a cell line containing an integrated KRAB-DBD responsive reporter gene, to test the hypothesis that differences in the composition of KZNF KRAB domains determine whether expression of MAGE proteins increases, decreases, or has no effect on KZNF mediated gene repression. Our work shows that the structure of KRAB domains is the main determinant of the outcome of MAGE mediated biological effects on KZNFS.

Materials and methods

Tissue culture and transfections

HEK293T were purchased from American Type Culture Collection and were cultured in DMEM with 10% fetal bovine serum and 1% antibiotics (penicillin, streptomycin). CHO-5xGAL4-UAS-TK-Luc-2p cells, the kind gift of Dr. Raul Urrutia, were cultured in F12 with 10% fetal bovine serum and 100 μg/ml hygromycin antibiotic [4,31]. For transfections with MAGE-A3 plasmid, we used calcium phosphate (Invitrogen) and Lipofectamine 2000 (Invitrogen), the latter according to the manufacturer's recommendations.

Expression vectors and strategy

FLAG-tagged wild type MAGE-A3 was expressed using p3xFLAG-CMV-2 plasmid (Sigma). We used the pSG424 vector for the construction and expression of GAL4-DBD fusion proteins in mammalian cells. The plasmid contains the SV40 ori/early promoter region fused to the coding sequencing for GAL4-DBD (AA 1-147), followed immediately by a polylinker and translation stop codons. Different KZNF KRAB domains were amplified and linked with GAL4 (1-147) at the 3′ end, to generate fused GAL4-DBD-KRAB constructs. Detailed information is show in Tables 1 and 2.

Construction of plasmids for GAL4-DBD-KRAB fusion protein expression

Mammalian Gene Collection (MGC)-verified full length human KZNF cDNA clones were purchased from Thermo Fisher Scientific (Waltham, MA). PCR was performed to amplify ZNF cDNA using HotStar HiFidelity PCR kit (Qiagen, Valencia, CA). Primers were

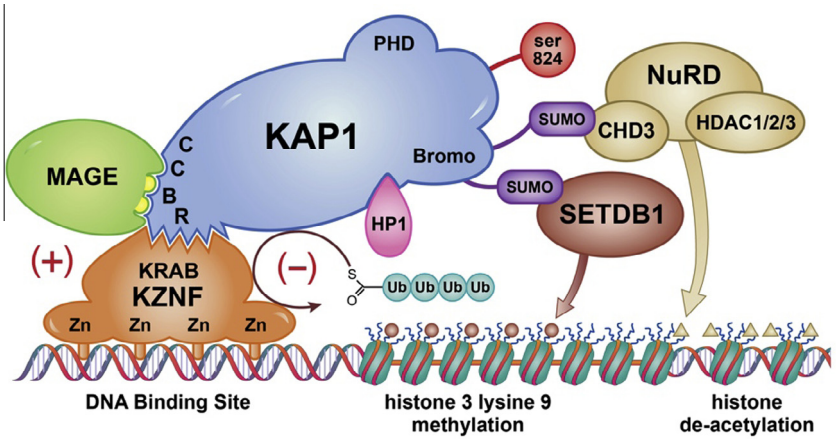

Fig. 1. KAP1 MAGE cartoon. The KAP1 RING Finger-B Box-Coiled Coil (RBCC) domain binds to the KRAB domains of KZNFs, which target KAP1 to specific DNA sequences through their zinc finger DNA binding motifs. KAP1 mediates localized compaction of chromatin necessary for repression of gene transcription, and is associated with chromatin modifications including histone de-acetylation, histone 3 trimethylation on K9, and HP1 binding to both DNA and histones. All Class I MAGE proteins contain two leucines (yellow semi-circles) which enable binding to the same KAP1 RBCC region as binds KZNFs. In some cases MAGE expression enhances KAP1 E3 ubiquitin ligase activity, resulting in KZNF ubiquitination and degradation, thereby de-repressing the genes targeted by that KZNF. In other cases MAGE expression increases KAP1 binding causing increased gene repression. Modified from [33]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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