

MAGE-A1 interacts with adaptor SKIP and the deacetylase HDAC1 to repress transcription

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ABSTRACT

MAGE-A1 belongs to a family of 12 genes that are active in various types of tumors and silent in normal tissues except in male germ-line cells. The MAGE-encoded antigens recognized by T cells are highly tumor-specific targets for T cell-oriented cancer immunotherapy. The function of MAGE-A1 is currently unknown. To analyze it, we attempted to identify protein partners of MAGE-A1. Using yeast two-hybrid screening, we detected an interaction between MAGE-A1 and Ski Interacting Protein (SKIP). SKIP is a transcriptional regulator that connects DNA-binding proteins to proteins that either activate or repress transcription. We show that MAGE-A1 inhibits the activity of a SKIP-interacting transactivator, namely the intracellular part of Notch1. Deletion analysis indicated that this inhibition requires the binding of MAGE-A1 to SKIP. Moreover, MAGE-A1 was found to actively repress transcription by binding and recruiting histone deacetylase 1 (HDAC1). Our results indicate that by binding to SKIP and by recruiting HDACs, MAGE-A1 can act as a potent transcriptional repressor. MAGE-A1 could therefore participate in the setting of specific gene expression patterns for tumor cell growth or spermatogenesis.

INTRODUCTION

The gene *MAGE-A1* was identified because it encodes a human melanoma antigen recognized by cytolytic T lymphocytes (1). It belongs to a family of 12 genes, the *MAGE-A* cluster located on the X chromosome in region q28 (2). The *MAGE-A* genes are expressed in tumors of various histological types (1,3,4), but they are silent in normal adult tissues except in male germ-line cells (5–7).

A search for the gene responsible for the sex-reversal phenotype led to the identification of a second cluster of *MAGE* genes, *MAGE-B*, located in Xp21.3 (8–11). A third cluster,

MAGE-C, was identified and located in Xq26–27 (11,12). The expression pattern of the *MAGE-B* and *C* genes is similar to that of the *MAGE-A* genes.

In contrast to the *MAGE-A*, *B* and *C* genes, two genes constituting a distantly related fourth cluster, *MAGE-D*, were found to be expressed in many normal tissues (13,14). Additional *MAGE* genes families with such a ubiquitous expression pattern, *MAGE-E* to *L*, were identified through database screening (15).

By comparing the sequences of all the *MAGE* proteins, we identified a stretch of ~200 amino acids which was named the ‘MAGE conserved domain’ (15). The rest of the protein sequences vary widely from one family to another.

The function of protein MAGE-D1 has been partially elucidated. The rat protein NRAGE (neurotrophin receptor-interacting MAGE homolog), the ortholog of human MAGE-D1 and mouse Dlxin-1, was found to bind to the cytosolic domain of the p75 neurotrophin receptor (p75NTR) (16). Upon binding, NRAGE prevents the formation of a complex between p75NTR and tyrosine kinase receptor TrkA. This retards cell cycle progression and initiates an apoptotic cascade leading to neuronal cell death (16,17). In agreement with these data, NRAGE and p75NTR are co-expressed in a region of the developing nervous system where p75NTR mediates developmental apoptosis. In addition, NRAGE was found recently to induce apoptosis through interaction with the axon guidance receptor, UNC5H1 (18). On the other hand, Dlxin-1, the murine ortholog of NRAGE, exerts a function in the nucleus: it acts as a transcriptional activator of Dlx5, a homeodomain protein of the Dlx family that plays a critical role in skeletal development (19). This transcriptional function may be regulated by the tyrosine kinase receptor, Ror2 since this receptor appeared capable of sequestering Dlxin-1 at the plasma membrane and endoplasmic reticulum (20).

Computer search of the Protein Sequence Database revealed that *MAGE* proteins display a weak similarity with necdin because it also contains a ‘MAGE conserved domain’. The murine necdin gene (*Ndn*) is highly expressed in post-mitotic neurons in the brain stem and hypothalamus (21,22). Mice deficient in necdin show early postnatal lethality due to respiratory distress and hypotonia, as observed in Prader–Willi syndrome (23–25). Necdin seems to act as a neuron-specific

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post-mitotic growth suppressor, functionally similar to the retinoblastoma suppressor protein, Rb. Ectopic expression of *Ndn* suppresses proliferation of NIH/3T3 fibroblasts and colony formation of SAOS-2 osteosarcoma cells (26,27). Necdin interacts with the E2F1 and p53 transcription factors, repressing their transcriptional activity (27,28). Furthermore, necdin associates with the p75 neurotrophin receptor (29). These observations suggest that necdin induces cell cycle arrest and controls neuronal apoptosis through interactions with E2F1 and p75NTR (30).

The functions of the MAGE-A group of proteins, which are present only in tumor and germ-line cells, are largely unknown. MAGE-A4 has been identified as binding to gankyrin, an abundant protein in hepatocellular carcinomas (31). This protein has been reported to associate with Rb and to compete with p16 for binding to cyclin-dependent kinase CDK4, increasing both the phosphorylation and degradation of Rb. MAGE-A4 was shown to suppress both anchorage-independent growth *in vitro* and tumor formation of gankyrin-expressing cells in nude mice. Interaction with gankyrin was not observed for proteins MAGE-A1, MAGE-A2 and MAGE-A12 (31).

The subcellular localization of MAGE-A proteins seems to vary from one member of the family to another. MAGE-A1 and MAGE-A3 were reported to be located in the cytosol of melanoma cells (32–34). MAGE-A1 and MAGE-A4 have been detected in both the cytoplasm and the nucleus of spermatogonia (7). MAGE-A10 and MAGE-A11 have been shown to be located predominantly in the nucleus of tumor cells (35,36). MAGE-A proteins may exert different functions according to their subcellular localizations. To gain insight into MAGE-A functions, we used the yeast two-hybrid system to identify protein partners of MAGE-A1.

MATERIALS AND METHODS

Plasmids

Yeast expression plasmids. Plasmids pGBT9 and pAS2-1 encoding the Gal4(1–147) DNA-binding domain, pACT2 and pGAD424 encoding the Gal4(768–881) activation domain, pTD1 encoding SV40 large T antigen (84–708) and pVA3 encoding mouse p53 (72–390) were purchased from Clontech. The MAGE-A1 open reading frame (ORF) and truncated versions of the MAGE-A1 ORF were obtained by PCR amplification with native *Pfu* DNA polymerase (Stratagene). The PCR products were cloned in frame with the Gal4 DNA-binding domain sequence of pGBT9 or pAS2-1. The MAGE-A1 ORF was excised from pGBT9 and subcloned in frame with the Gal4 activation domain of pGAD424. A BamHI/BglII fragment carrying the Ski Interacting Protein (SKIP)-coding region was isolated from the pACT2 plasmid and ligated into the BamHI site of pGBT9 in frame with the Gal4 DNA-binding domain. The human testis cDNA library in pACT2 was purchased from Clontech.

Eukaryotic expression plasmids. Expression vectors encoding the Gal4(1–147) DNA-binding domain (GH250), Gal4(1–147)–SKIP (JH274) and the full-length cytoplasmic domain (amino acids 1747–2531) of rat Notch1-IC (pBOS-FCDN1) were provided by Diane Hayward (Johns Hopkins University School of Medicine, Baltimore, MA) (37). The

reporter plasmid 5xGal4TK-CAT was also obtained from Diane Hayward. Expression vectors for Luciferase (pTKluc) and Gal4(1–147) fused to the activation domain of HNF-6 (Gal4BD Nterm) were provided by F.Lemaigre (Hormone and Metabolic Research Unit, Université Catholique de Louvain and Institute of Cellular Pathology, Brussels). The pcDNAI/Amp expression vector carrying a complete MAGE-A1 cDNA (pcDNAI/Amp-M1) was provided by C.Lurquin (Ludwig Institute, Brussels).

The sequence encoding a 9 amino acid hemagglutinin (HA) epitope tag (YPYDVPDYA) was obtained by PCR on vector pACT2 with primers LAD 31: 5'-CGGGATCCGACTATGGCTTACCCATAC-3' and LAD32: 5'-CGGAATTCGAGCGTAATCTGGAAC-3'. The PCR product was digested with restriction enzymes BamHI and EcoRI. An EcoRI/XhoI fragment carrying the SKIP coding region was excised from the pACT2 plasmid and ligated with HA into the BamHI/XhoI sites of pcDNAI/Amp, placing the SKIP-coding region in frame with (and downstream of) the HA epitope. MAGE-A1 ORF was excised from plasmid pGBT9 and cloned in frame with the Gal4 DNA-binding domain of GH250.

Vectors expressing deleted versions of MAGE-A1 were obtained by cloning PCR products obtained with native *Pfu* DNA polymerase in vector pcDNAI/Amp digested with HindIII and XhoI. The deleted MAGE-1 ORFs were inserted between their native 5'- and 3'-untranslated regions obtained by PCR. The deleted ORF encoding MAGE-A1_{1–279} and the 5'-untranslated region were obtained by PCR on vector pcDNAI/Amp-M1 with primers LAD 108 (forward): 5'-CCCAAGCTTCCATTCTGAGGGACG-3' and LAD14 (reverse): 5'-GCGGATCCTCAGACTTTCACATAGCTGGTTTC-3'. The 1040 bp product was digested with HindIII and BamHI and ligated to a 540 bp fragment corresponding to the 3'-untranslated region. This fragment was obtained by PCR with primers LAD 110 (forward): 5'-CGGGATCCGATCGCATGAGTTGCAGCCA-3' and LAD 111 (reverse): 5'-CCGCTCGAGACAGGAAGAATTCTTTA-3' and was digested with BamHI and XhoI. The ORF encoding MAGE-A1_{99–309} and the 3'-untranslated region were obtained with primers LAD 112 (forward): 5'-CGGGATCCGTCATCATGCGAGCAGTAATCA-3' and LAD 111 (reverse). The 1170 bp product was digested with BamHI and XhoI and ligated to a 200 bp fragment corresponding to the 5'-untranslated region. This fragment was obtained by PCR with LAD 108 (forward): 5'-CCCAAGCTTCCATTCTGAGGGACG-3' and LAD 109 (reverse): 5'-CGGGATCCTCTCGTCAGGCAGCA-3' and digested with HindIII and BamHI. The ORF encoding MAGE-A1_{99–279} was obtained by PCR with primers LAD 112 (forward) and LAD14 (reverse). The 540 bp product was digested with BamHI and ligated to both the 200 bp fragment corresponding to the 5'-untranslated region and the 540 bp fragment corresponding to the 3'-untranslated region.

pcDNA3-HDAC1-F, pING14A-HDAC1 and the reporter construct (Gal4)₄-tkLuc have been described previously (38,39).

Yeast two-hybrid assays

Plasmid DNA was transformed into yeast cells using the lithium acetate method and the Clontech's protocol.

DNA of plasmid pAS2-1/MAGE-A1 was cotransformed with DNA of the testis cDNA library into yeast strain CG-1945 (Clontech). Transformed His⁺ CG-1945 cells were selected onto medium lacking histidine (–His). In our conditions, 5 mM 3-aminotriazole (3-AT), a chemical inhibitor of imidazoleglycerolphosphate dehydratase (*HIS3*) which restores histidine auxotrophy, was always required in the –His medium. It completely abrogated the growth of yeast cells containing only plasmid pAS2-1/MAGE-A1. His⁺ colonies were grown in 5 ml –His liquid medium to eliminate some false positives. Plasmids isolated from His⁺ colonies were then transferred in Y190 yeast strain (Clontech). This strain has a higher *LacZ* reporter expression. Transformed His⁺ Y190 cells were assayed for β -galactosidase with *O*-nitrophenyl β -D-galactopyranoside (ONPG) as substrate according to a Clontech's protocol. β -Galactosidase units were calculated as in the Clontech's protocol. To obtain more reproducible values in the β -galactosidase assays, plasmid DNA of His⁺*LacZ*⁺ yeast colonies was isolated and transformed with the pGBT9/MAGE-1 construct into yeast strain PJ69-4A, a kind gift of Philip James (40). This strain contains three reporter genes (*HIS3*, *ADE2* and *LacZ*), each driven by a different promoter (*GAL1*, *GAL2* or *GAL7* promoter) inducible by the Gal4 transcription factor. According to a protocol sent out by Philip James with the strain, transformed PJ69-4A cells were first selected on medium lacking histidine in the presence of 1 mM 3-AT. His⁺ colonies were then plated on a medium lacking adenine to screen for the *ADE2* marker. Finally, liquid cultures of His⁺Ade⁺ colonies were assayed for β -galactosidase.

Sequence analysis

Sequencing was performed using the dideoxy-mediated chain termination method with the BigDye Terminator Cycle sequencing kit and an ABI Prism 310 genetic analyser (Applied Biosystems).

Co-immunoprecipitations and western blot analysis

COS-7 cells seeded at 10⁶ cells per 10 cm diameter culture dish were transiently transfected with 8 μ g of expression plasmid and 48 μ l of LIPOFECTAMIN reagent (Invitrogen). Approximately 48 h after transfection, cells were washed with phosphate-buffered saline (PBS) and lysed for 30 min on ice in 1 ml of ice-cold NP-40 lysis buffer [150 mM NaCl, 1% NP-40, 50 mM Tris–HCl, pH 8.0, and 1/50 per milliliter of a protease inhibitor cocktail tablet (Complete, Roche) in PBS]. For the co-immunoprecipitation of HA–SKIP with the truncated forms of MAGE-A1, cells were lysed in DH buffer (0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 0.5% NP-40 and 1/50 per milliliter of a protease inhibitor cocktail tablet in PBS). The cell extract was clarified by centrifugation for 10 min at 12 000 g at 4°C in a Sigma 1K15 microcentrifuge. The supernatant was preincubated for 3 h at 4°C with 50 μ l of a protein G-agarose suspension (Roche) on a rocking platform. Beads were pelleted by centrifugation for 20 s at 12 000 g. Precleared lysate was incubated at 4°C for 1–2 h on a rocking platform with 5 μ l of polyclonal anti-MAGE-A1 serum LBK7-2 [a goat K7 serum directed against the recombinant MAGE-A1 (1–309) protein made by Chiron]. A mixture of 5 μ l of polyclonal anti-MAGE-A1 serum

LBK7-2 and 100 μ l of monoclonal anti-MAGE-A1 antibody supernatant 6C1 (a gift of Donata Rimoldi, LICR Lausanne) (41), was used in the co-immunoprecipitation of HA–SKIP with the truncated forms of MAGE-A1. Fifty microliters of protein G-agarose were added to the cell extract and the mixture was incubated for 3–12 h at 4°C on a rocking platform. The beads were then washed 4 to 5 times with 1 ml of DH buffer and mixed with 50 μ l of gel-loading buffer. Samples (10 μ l) were subjected to electrophoresis using a 10% denaturing polyacrylamide gel. For the co-immunoprecipitation of HA–SKIP with MAGE-A1, western blot analysis was performed using anti-HA high-affinity rat monoclonal antibody (1/1000) (Roche), sheep anti-rat IgG-biotin F(ab')₂ fragments (1/400) (Roche), streptavidin–horseradish peroxidase (HRP) conjugate (1/2500) (Amersham Pharmacia Biotech) and the ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech). Alternatively, western blot analysis was performed using the anti-MAGE-A1 6C1 hybridoma supernatant (one-half to one-fiftieth) and the goat anti-mouse IgG–HRP (1/2000) (BD Transduction Laboratories).

For the co-immunoprecipitation of histone deacetylase 1 (HDAC1) with MAGE-A1, COS cell lysates in 100 mM Tris–HCl, pH 7.4, 10% glycerol, 0.5% NP-40 and 1/50 per milliliter of a protease inhibitor cocktail tablet were incubated with 5 μ l of pre-immune or 5 μ l of polyclonal anti-MAGE-A1 goat serum, as described above. Immunoprecipitates were washed 4 times with 500 μ l of IPH buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40 and 1/50 per milliliter of a protease inhibitor cocktail tablet). Western blots of the immunoprecipitates were analyzed either with the anti-MAGE-A1 6C1 antibody as above or with the anti-HDAC1 mouse monoclonal antibody (clone 2E10, Upstate) (1/2000), the goat anti-mouse IgG–HRP (1/2000) (Santa Cruz Biotechnology) and the Supersignal West Pico chemiluminescent substrate (Pierce).

Cell culture, transfection, CAT and luciferase assays

HeLa cells were maintained in DMEM plus 10% fetal calf serum and plated at 1.2 $\times$ 10⁵ cells per well in 12-well plates (Nunc) 1 day prior to transfection. Cells were transfected using LIPOFECTIN and PLUS Reagent (Invitrogen). Cells received 0.25 μ g of the expression plasmid encoding Gal4(1–147)–SKIP, Notch1-IC, Gal4(1–147), MAGE-A1 (intact or deleted), 0.4 μ g of the 5xGal4TK–CAT reporter plasmid and 0.5 μ g of pTKluc plasmid as an internal control for transfection efficiency. Plasmid DNA was purified with the Endofree Plasmid Maxi kit (Qiagen). At 48 h after transfection, cells were washed twice with PBS, lysed in 100 μ l of Reporter Gene Assay Lysis Buffer (Roche) for 15 min at 4°C. Cell debris were removed by centrifugation for 2 min at maximum speed at 4°C. For luciferase assays, 40 μ l of cell lysate were loaded into a luminometer TD20/20 (Promega) and reaction was started by addition of 100 μ l of Luciferase Assay Reagent (Roche) and chemiluminescence was counted for 12 s. For chloramphenicol acetyltransferase (CAT) assays, 40 μ l of cell lysate were incubated for 5 min at 65°C to destroy deacetylases. The test was then performed in 1.5 ml translucent, colorless, microcentrifuge tubes with the *Quant*-T-CAT assay system, as recommended by Amersham Pharmacia Biotech. Each experiment was repeated at least twice.

Transfected HeLa cells were separated into nuclear and cytosolic fractions using the Nuclear/Cytosol fractionation kit (BioVision). They were analyzed by western blotting with the following antibodies: anti-MAGE-A1 clone 6C1, anti-HDAC1 clone 2E10 (Upstate) and anti- α -Tubulin clone B-7 (Santa Cruz Biotechnology, 1/2000).

Monolayer cultures of U2OS human osteosarcoma cells were maintained in DMEM supplemented with 10% fetal calf serum at 37°C in 5% CO₂ atmosphere. Twenty-four hours after plating, the exponentially growing U2OS cells were transfected with polyethylene imine (PEI) (Euromedex). All transfections were carried out with the same total amount of DNA (1 μ g). Four hours after transfection, the cells were washed once in PBS and recovered with fresh medium. After 24 h of incubation, luciferase assays were performed with the Promega luciferase Assay System according to the manufacturer's instructions. Transfection efficiencies were normalized using a cotransfected plasmid encoding β -galactosidase. For trichostatin A (TSA) treatment, U2OS cells were transfected with the (Gal4)₄-tkLuc reporter and a vector encoding Gal4bd/MAGE-A1, and incubated for an additional 24 h, either in the presence or absence of TSA (100 nM; Waco BioProducts) before harvesting. Luciferase assays were then performed as described above.

MBP fusions proteins, *in vitro* translations and pull-down assays

The vector expressing a fusion protein of maltose-binding protein (MBP) with MAGE-A1 was constructed by ligating a BamHI/EcoRI fragment carrying the MAGE-A1 coding region isolated from the pGBT9 plasmid into vector pMAL-C2 (New England Biolabs). For *in vitro* transcription and translation, we used the following constructs: pcDNA3.1-Myc HDAC1-F and pcDNA3.1MycHis HDAC4. We expressed MBP and MBP fusion proteins in *Escherichia coli* TOP 10F⁺ (Invitrogen) and proteins from crude bacterial lysates were purified using Amylose resin (New England Biolabs) according to the manufacturer's instructions. *In vitro* transcription/translation was performed using the TNT system (Promega). MBP pull-down experiments were performed essentially as described previously (42).

RESULTS

MAGE-A1 interacts with SKIP in yeast cells

To identify proteins interacting with MAGE-A1, we used a yeast two-hybrid system with reporter genes controlled by the Gal4 transcription factor. The bait was protein MAGE-A1 fused to the Gal4 DNA-binding domain (Gal4bd). It was obtained by cloning the full-length ORF of *MAGE-A1* into vector pAS2-1, which produces a high level of fusion protein. The preys, fused with the Gal4 transcriptional activation domain (Gal4ad), were produced from a human testis cDNA library prepared in vector pACT2. The first round of screening was conducted in yeast strain CG-1945 which contains reporter gene *HIS3* controlled by Gal4, using medium deficient in histidine. Since Gal4bd/MAGE-A1 exerted a certain degree of transactivation on its own, selection occurred in the presence of 5 mM 3-aminotriazole to increase histidine

Table 1. *In vivo* interaction of MAGE proteins with SKIP

Gal4bd fusions	Gal4ad fusions	β -Gal activity
MAGE-A1	—	8.8
—	SKIP	0.8
MAGE-A1	SKIP	52.1
p53	SV40 T	123.6

Yeast PJ69-4A cells were transformed with vectors expressing Gal4 DNA-binding domain (Gal4bd) and Gal4 activation domain (Gal4ad) fusion proteins. β -Galactosidase activity was quantified using liquid cultures. Interaction between murine p53 and SV40 large T antigen was taken as a positive control.

dependence. Under these conditions, no colonies were observed with pAS2/MAGE-A1 alone. From approximately 7×10^5 yeast transformants, nine His⁺ colonies were recovered.

For the second round of screening, pACT2 plasmids were isolated from the His⁺ colonies and tested for their ability to encode proteins interacting with Gal4bd/MAGE-A1 produced by vector pGBT9 at a much lower level than by vector pAS2-1. This screening used a yeast strain in which gene *LacZ* is controlled by Gal4. One plasmid was repeatedly positive. Interaction was conserved when the testis/Gal4ad and Gal4bd/MAGE-A1 associations were switched. The positive clone contained a 2.1 kb cDNA including the complete ORF of gene *SKIP*, also designated *NcoA-62* (Nuclear receptor coactivator; 62 kDa) (43,44). This gene codes for an adaptor protein involved in transcription regulation.

The β -galactosidase activity was reproducibly 2 to 6 times higher in cells co-expressing Gal4bd/MAGE-A1 and Gal4ad/SKIP than in cells expressing Gal4bd/MAGE-A1 or Gal4ad/SKIP alone (Table 1 and Figure 1). To identify in the 309 amino acids MAGE-A1 protein the region that interacted with SKIP, constructs were prepared that coded for truncated versions of MAGE-A1 fused to Gal4bd (Figure 1). A comparison of MAGE-A1₁₋₂₉₅ (amino acids 1–295) and the complete MAGE-A1₁₋₃₀₉ indicated that the negatively charged region composed of the 14 C-terminal residues of MAGE-A1 is required for SKIP interaction. The ability of MAGE-A1 to autonomously stimulate the reporter gene was lost when amino acids 279–295 were deleted.

MAGE-A1 interacts with SKIP in mammalian cells

To verify the interaction observed in yeast, we used a co-immunoprecipitation approach in mammalian cells. To this end, the simian COS-7 cells were cotransfected with vectors expressing MAGE-A1 and SKIP. The latter was tagged with an influenza HA epitope. Immunoprecipitations were carried out on cell extracts with an anti-MAGE-A1 antiserum, followed by western blotting using an anti-HA antibody. As shown in Figure 2, MAGE-A1 interacted specifically with SKIP.

Using constructs producing truncated forms of MAGE-A1, we found that MAGE-A1₉₉₋₃₀₉ also coprecipitated with HA-SKIP (Figure 3A, lane 7), whereas MAGE-A1₁₋₂₇₉ and MAGE-A1₉₉₋₂₇₉ did not (Figure 3A, lanes 5 and 9). These results indicated that the C-terminal region of MAGE-A1 was required for the interaction with SKIP in mammalian cells as well as in yeast.

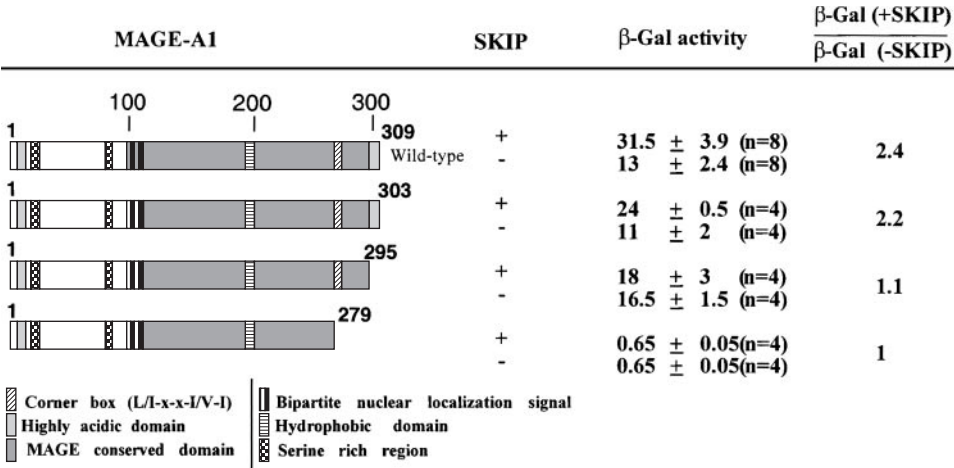


Figure 1. Interaction between deleted forms of MAGE-A1 and SKIP in the yeast two-hybrid system. Truncated *MAGE-A1* sequences were inserted in vector pGBT9 and *SKIP* in vector pACT2. These constructs were transformed in yeast PJ69-4A cells. β -Galactosidase activity was quantified using liquid cultures. Results are presented as mean $\pm$ S.D. of *n* tests. CoRNR box (L/I-x-x-I/V-I) involved in interactions with nuclear hormone receptors is found in nuclear corepressors like SMRT and N-CoR (61).

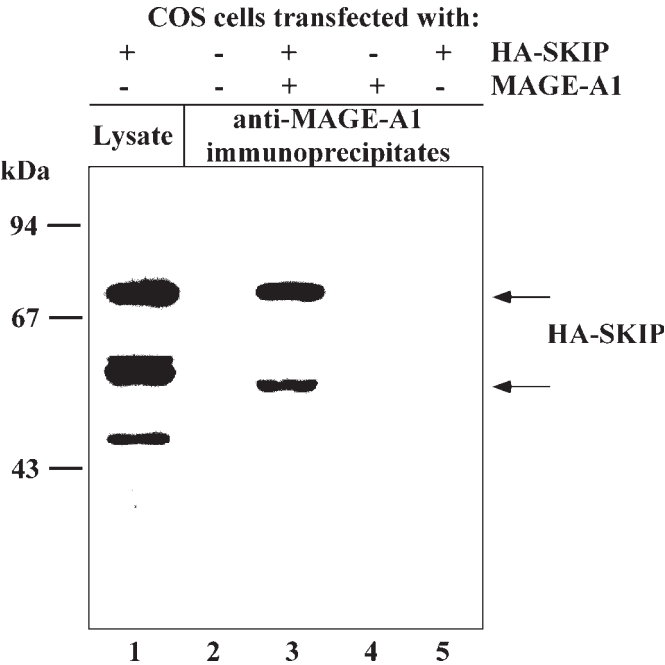


Figure 2. Immunoprecipitation of MAGE-A1 and HA-SKIP. Approximately 2×10^6 COS-7 cells were transiently transfected with HA-SKIP (lanes 1, 3 and 5) and MAGE-A1 expression plasmids (lanes 3 and 4). Cell extracts were incubated with an anti-MAGE-A1 polyclonal antibody. One-hundredth of whole cell lysate of SKIP-positive cells (lane 1) and immune complexes were separated by SDS-polyacrylamide gel electrophoresis, blotted and probed with an anti-HA antibody. HA-SKIP has already been observed as two bands of ~66 and 55 kDa in COS cells (44).

MAGE-A1 counteracts Notch1-IC-mediated transactivation

Having shown the association of MAGE-A1 with SKIP, we wished to assess its functional consequences. SKIP has been described to be involved in the Notch1 signaling pathway. Notch1 is a transmembrane receptor that mediates intercellular communications and directs cell fate decisions during

development. Ligand binding to Notch1 induces an intramembrane proteolytic cleavage that releases the intracellular domain of the receptor, Notch1-IC, which then enters the nucleus and regulates the expression of a number of genes. One of the DNA-binding proteins that interact with Notch1-IC is the C-promoter binding factor 1 (CBF1) (45). In the absence of Notch1-IC, CBF1 inhibits transcription by binding SKIP and the SMRT-corepressor complex which contains HDACs (Figure 4) (37). Repression is relieved by the presence of Notch1-IC, which binds to SKIP and CBF1, displaces the repression complex and activates transcription by recruiting histone acetyltransferases (HATs) and also coactivators such as Mastermind (Mam), which stabilizes the complex of Notch1-IC and CBF1 (37,46,47).

We examined whether MAGE-A1 could interfere with the SKIP-dependent transcriptional activity of Notch1-IC. Transient transfection into HeLa cells of a plasmid encoding a Gal4bd/SKIP fusion protein together with a *CAT* reporter gene containing Gal4 DNA-binding sites near its promoter, led to transcriptional repression of the low level of expression observed with the reporter gene alone (Figure 5A). As shown previously (37), when Gal4bd/SKIP was cotransfected with Notch1-IC, a considerable increase in transcription was observed, whereas in the absence of Gal4bd/SKIP, the effect of Notch1-IC was negligible. Finally, when we cotransfected MAGE-A1 together with Gal4bd/SKIP and Notch1-IC, this led to significant transcriptional repression. Increasing the amount of Notch1-IC with a constant amount of MAGE-A1 gradually increased the expression of the *CAT* reporter, but even at the highest dose of transfected Notch1-IC plasmid, repression by MAGE-A1 remained efficient (Figure 5A).

Conversely, MAGE-A1 repressed transcription of the reporter activated by Gal4bd/SKIP and Notch1-IC in a dose-dependent manner (Figure 5B). This was not due to an autonomous non-specific repressor activity of MAGE-A1, as we did not observe a dose-dependent repression when the same promoter was activated by Gal4bd fused to the activation domain of HNF-6 in the presence of increasing amounts of MAGE-A1 (Figure 5C).

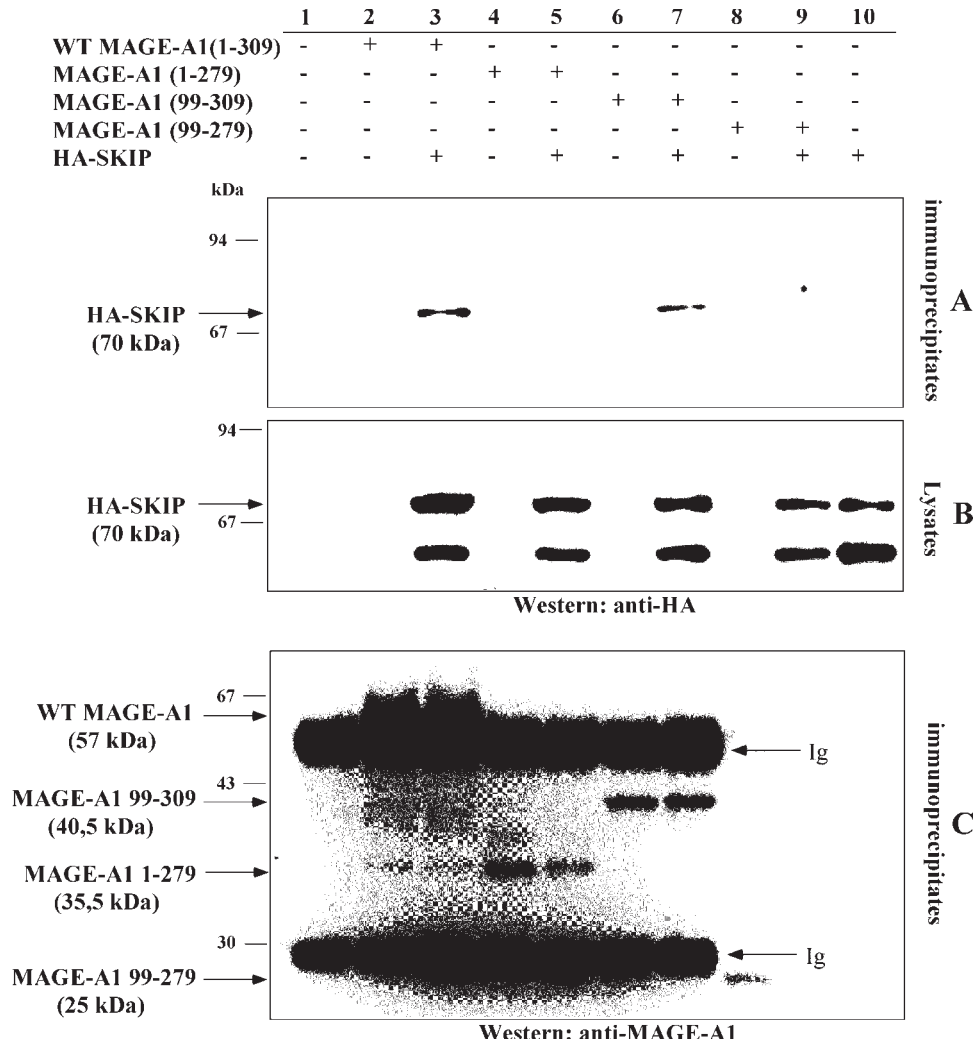


Figure 3. Binding of HA-SKIP to truncated forms of MAGE-A1 in mammalian cells. Approximately 2×10^6 COS-7 cells were transiently transfected with a HA-SKIP expression plasmid (lanes 3, 5, 7, 9 and 10) and with plasmids expressing intact or deleted forms of MAGE-A1: wild-type MAGE-A1 (WT MAGE-A1) (lanes 2 and 3), MAGE-A1₁₋₂₇₉ (lanes 4 and 5), MAGE-A1₉₉₋₃₀₉ (lanes 6 and 7), MAGE-A1₉₉₋₂₇₉ (lanes 8 and 9). Half of each cell lysate was immunoprecipitated with a mixture of a polyclonal anti-MAGE-A1 antibody and a monoclonal anti-MAGE-A1 antibody [(A,C) lanes 1–7] or only the polyclonal anti-MAGE-A1 antibody [(A,C) lanes 8–10]. Immune complexes (A,C) or one-hundredth of whole cell lysates (B) used for immunoprecipitation were separated by SDS-PAGE, blotted and probed with a monoclonal anti-HA antibody (A,B) or with a monoclonal anti-MAGE-A1 antibody (C). Bands marked as Ig display the light and the heavy chains of the immunoglobulins used for the immunoprecipitation.

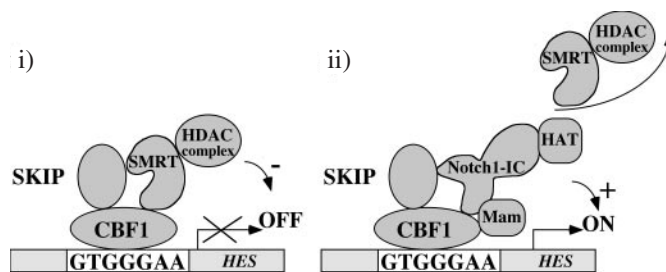


Figure 4. Transactivation by the intracellular part of Notch. Model for Notch1-IC activation on the *HES* (Hairy/Enhancer of Split) promoter regulated by CBF1 according to Zhou *et al.* (37). (i) The SMRT-corepressor complex containing HDAC binds to SKIP; (ii) Notch1-IC interacts with SKIP and CBF1, displaces the SMRT-corepressor complex and activates the transcription of the *HES* genes through the recruitment of Mastermind (Mam) and HATs.

To determine the cellular localization of the exogenous MAGE-A1, cell fractionation experiments were performed on transfected HeLa cells. These experiments revealed that cytosolic expression of MAGE-A1 was predominant, but that a significant fraction of MAGE-A1 was present in the nucleus (Figure 5D). We could not detect Notch1-IC on these immunoblots. This was not unexpected since Notch1-IC is known to be unstable and to act at very low nuclear concentrations (48). However, in COS cells transfected in exactly the same conditions as the HeLa cells of Figure 5B, we found that transfected *Notch1-IC* was expressed and that the levels of Notch1-IC did not decrease when the amounts of MAGE-A1 in the cell increased (data not shown).

To determine the region of MAGE-A1 that is required for the inhibition of Notch1-IC-mediated transactivation, we tested deleted forms of MAGE-A1 in the reporter assay.

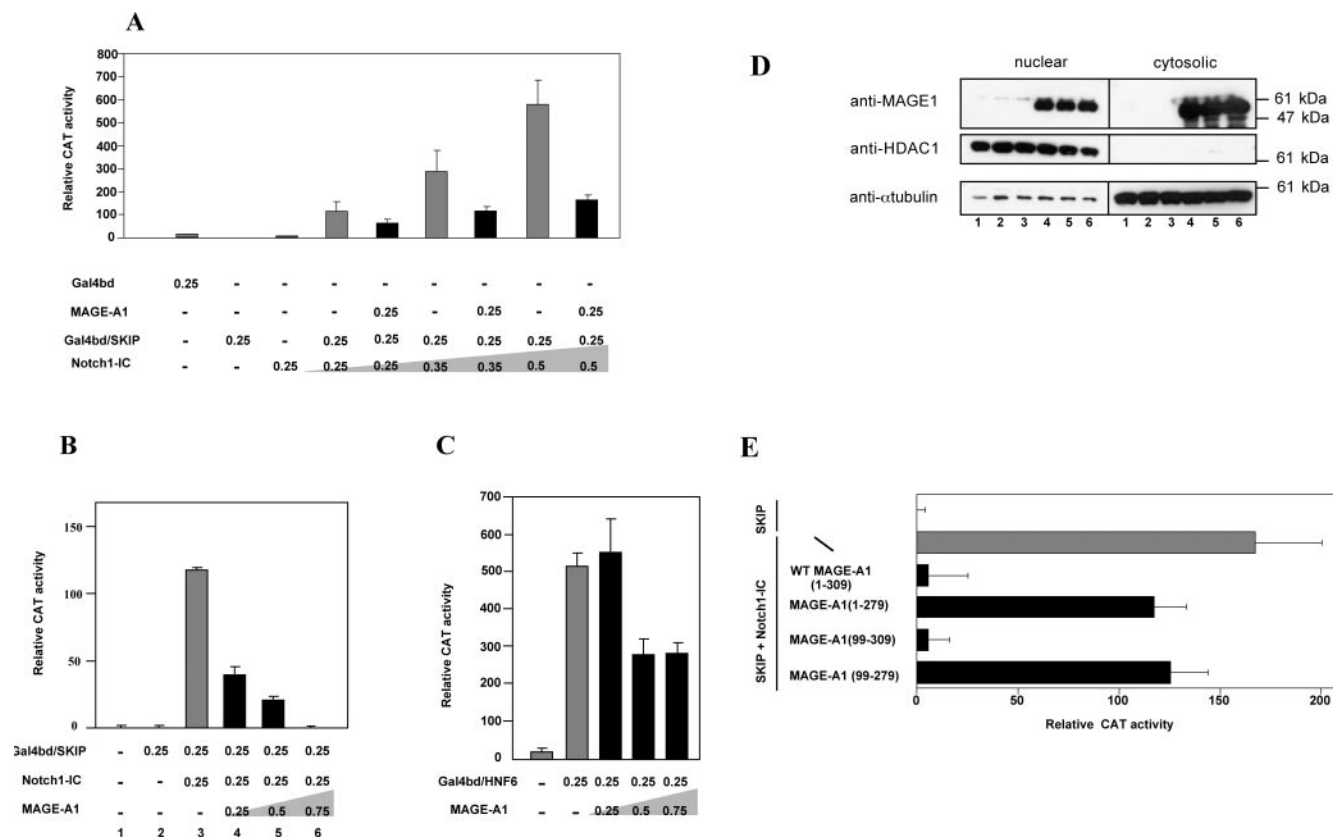


Figure 5. MAGE-A1 prevents Notch1-IC transactivation. (A) An aliquot of 0.25 μ g of an expression vector encoding Gal4bd, Gal4bd/SKIP, Notch1-IC or MAGE-A1 was transfected into HeLa cells together with 0.4 μ g of the 5xGal4TK-CAT reporter plasmid. CAT activities measured on cell lysates were normalized to the luciferase activity obtained by transfecting 0.5 μ g of pTKluc. The amount of DNA was kept constant at 1.65 μ g by adding an irrelevant pcDNA1/Amp recombinant plasmid. Data are means of duplicates. (B) Relative CAT activity measured in HeLa cells transfected with the 5xGal4TK-CAT reporter (0.4 μ g), increasing amounts (0.25–0.75 μ g) of the expression vector coding for MAGE-A1 and the vectors producing Gal4bd/SKIP (0.25 μ g) and Notch1-IC (0.25 μ g). Data are means of triplicates. Comparable total amounts of DNA were transfected. (C) Relative CAT activity measured in HeLa cells transfected with the 5xGal4TK-CAT reporter (0.4 μ g), increasing amounts (0.25–0.75 μ g) of the expression vector coding for MAGE-A1 and a vector encoding Gal4bd fused to the activation domain of HNF-6 (0.25 μ g). Data are means of quadruplicates. Comparable total amounts of DNA were transfected. (D) Cellular localization of MAGE-A1 in transfected HeLa cells. Transfections were performed as described in Figure 5B. Nuclear and cytosolic extracts were analyzed by western blotting for the presence of MAGE-A1 with the 6C1 monoclonal antibody. Purity of the fractions was evaluated by incubating the western blot with antibodies against HDAC1 (a nuclear localized protein) and α -tubulin (a cytoplasmic localized protein). Molecular weights in kiloDaltons are indicated on the right. (E) Repression of Notch1-IC transactivation requires that MAGE-A1 binds to SKIP. An aliquot of 0.25 μ g of a vector producing Gal4bd/SKIP, Notch1-IC, wild-type MAGE-A1 (WT MAGE-A1) or deleted versions of MAGE-A1 (two clones per construct) together with 0.4 μ g of the 5xGal4TK-CAT reporter plasmid were transfected into HeLa cells. Data are means of quadruplicates for each MAGE-A1 construct.

MAGE-A1_{99–309} reduced Notch1-IC transactivation by a factor of about 30, whereas MAGE-A1_{1–279} or MAGE-A1_{99–279} had no significant effect (Figure 5E). As the MAGE-A1 C-terminus mediates the binding to SKIP (Figures 1 and 3), this suggests that the repression of Notch1-IC transactivation requires an interaction between SKIP and MAGE-A1.

As reported previously, Gal4bd/SKIP repression is likely to be due to recruitment of the SMRT–HDAC repressor complex whereas Notch1-IC upregulation is caused by Notch1-IC competition with the SMRT–corepressor complex and recruitment of coactivators (Figure 6) (37,49). Our experiments indicate that, by binding to SKIP MAGE-A1 inhibits Notch1-IC transactivation. MAGE-A1 could achieve this inhibitory effect either by displacing Notch1-IC from SKIP or by masking Notch1 sites which bind transcriptional coactivators while leaving Notch1-IC attached to SKIP. A third possibility

would be that, after binding to SKIP MAGE-A1 functions as an active repressor.

MAGE-A1 recruits deacetylase HDAC1

We investigated whether MAGE-A1 had the ability to repress transcription upon binding autonomously to the promoter. To this end, we transiently transfected cells with a Gal4bd/MAGE-A1 construct together with a luciferase reporter gene driven by a tk promoter preceded by four Gal4 DNA-binding sites. MAGE-A1 inhibited transcription in a dose-dependent manner when linked to the Gal4 DNA-binding domain (Figure 7A). This suggests that MAGE-A1 can autonomously repress transcription.

Active repression of transcription can be mediated through recruitment of HDAC, resulting in nucleosome

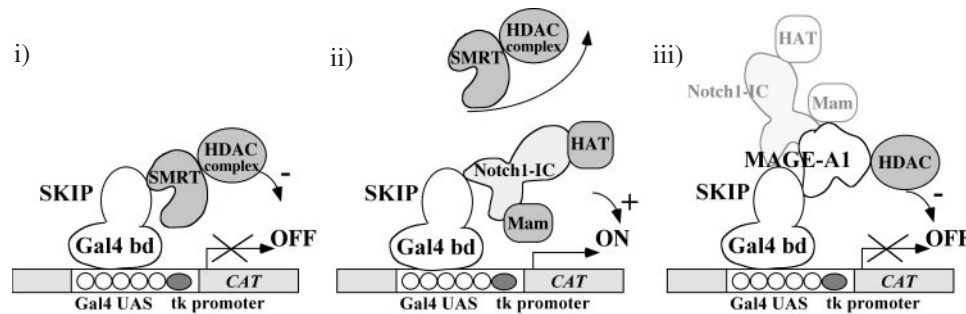


Figure 6. MAGE-A1 counteracts Notch1-IC transactivation and recruits HDAC. (i and ii) Summary of experimental data using Gal4bd/SKIP, Notch1-IC and the CAT reporter according to the model of Zhou *et al.* (37). (iii) By binding to SKIP and recruiting HDAC, MAGE-A1 counteracts Notch1-IC transactivation and thereby represses transcription. Endogenous proteins are indicated by shaded forms; proteins expressed upon transfection are indicated by unshaded forms.

remodeling (50). To test whether MAGE-A1 may repress transcription through HDAC, we incubated cells expressing Gal4bd/MAGE-A1 with the HDAC inhibitor, TSA. This resulted in a significant relieve of MAGE-A1-mediated transcriptional repression (Figure 7B).

We, therefore, examined whether MAGE-A1 could associate with HDAC1 and/or HDAC4 that belong to Class I and Class II HDAC, respectively. To this end, we performed *in vitro* pull-down experiments using a MBP-MAGE-A1 fusion protein produced in *E. coli* and *in vitro* translated ³⁵S-labelled HDAC1 and HDAC4. As shown in Figure 7C, MBP-MAGE-A1 bound specifically to full-length HDAC1 (lane 3) whereas MBP alone did not (lane 2). No binding was observed with HDAC4 (Figure 7C).

We then examined whether the association between MAGE-A1 and HDAC1 could occur *in vivo*, in COS cells that were transiently transfected with HDAC1 and MAGE-A1 expression plasmids. MAGE-A1 was immunoprecipitated with the anti-MAGE-A1 goat serum. Co-immunoprecipitated HDAC1 was detected by western blotting using an anti-HDAC1 antibody (Figure 7D, lane 3). No HDAC1 was detected when a goat preimmune serum was used to immunoprecipitate the lysate (Figure 7D, lane 2).

Finally, we tested the functional relevance of the interaction between MAGE-A1 and HDAC1 by using transient transfection of a reporter gene containing Gal4 DNA-binding sites and a construct expressing a Gal4bd/MAGE-A1 fusion protein. In this assay, we used a limiting amount of Gal4bd-MAGE-A1 that moderately repressed luciferase activity. Cotransfection of Gal4bd/MAGE-A1 with HDAC1 synergistically enhanced transcriptional repression (Figure 7E), indicating a co-operative effect of MAGE-A1 and HDAC1. Taken together, these experiments strongly suggest that MAGE-A1 can actively repress transcription through the recruitment of HDAC1.

DISCUSSION

Our results indicate that MAGE-A1 interacts with transcriptional regulator SKIP, through its C-terminal part. In line with these results, we observed that MAGE-A4, the only other MAGE in which this region is conserved, also binds to SKIP, whereas MAGE-A10 and MAGE-D2, which display different C-terminal parts, do not bind to SKIP in the yeast two-hybrid system (data not shown).

SKIP intervenes in signaling pathways involving vitamin D, retinoic acid, estrogens, glucocorticoids, Notch1-IC and

TGF- β (37,43,51,52). It can have either an activator or repressor role in transcription because it is an adaptor protein that connects DNA-binding proteins such as Smad3, the vitamin D receptor, CBF1 or MyoD, to proteins that activate or repress transcription. For example, the steroid receptor coactivator (SRC), which has a HAT activity, forms a ternary complex with SKIP and the liganded vitamin D receptor (51,53). Ski, the protein that was used as bait to fish SKIP, similarly binds to the DNA-binding protein Smad and recruits a repression complex including HDAC (54).

Another repression complex was shown to bind to SKIP and CBF1 (37). In the presence of Notch1-IC, the repression complex is detached from SKIP and recruitment of an activation complex including HATs is facilitated by SKIP (37,47). In our experimental system, MAGE-A1 counteracted the transcriptional activation mediated by Notch1-IC (Figure 6). A possible explanation of this effect is that MAGE-A1 functions as a passive repressor dissociating Notch1-IC from SKIP, thereby preventing the recruitment of the transcriptional activation complex. However, the observation that the repressive effect of MAGE-A1 was not relieved by increasing the amount of transfected Notch1-IC plasmid (Figure 5A) suggests that MAGE-A1 and Notch1-IC did not compete to bind to SKIP. Another possible mechanism of passive repression could be that, upon binding to SKIP, MAGE-A1 masked the activation domain of Notch1-IC. Such a mechanism of passive repression was reported for the MAGE-related protein, necdin, which binds to the transactivation domain of E2F1 and p53 (27,28). MAGE-A1 is not involved in this pathway, as we found that it does not interact with E2F1 or p53 in the yeast two-hybrid system (data not shown).

We investigated whether MAGE-A1 could actively repress transcription. Active repression could be exerted by interfering with the assembly of the basal transcription complex near the transcription-initiation site, by disrupting the interaction between DNA-bound transcription activators and the transcription machinery or by recruiting corepressors, such as HDAC. We observed that MAGE-A1 binds and recruits HDAC1, which modifies chromatin, but may also target non-histone proteins involved in transcriptional regulation (38). Recruitment of HDAC by MAGE-A1 could be sufficient to inhibit Notch1-IC transactivation, but we cannot exclude that, in our experimental setting, MAGE-A1 also inhibited transcription by destabilizing the binding of transcriptional coactivators to Notch1-IC.

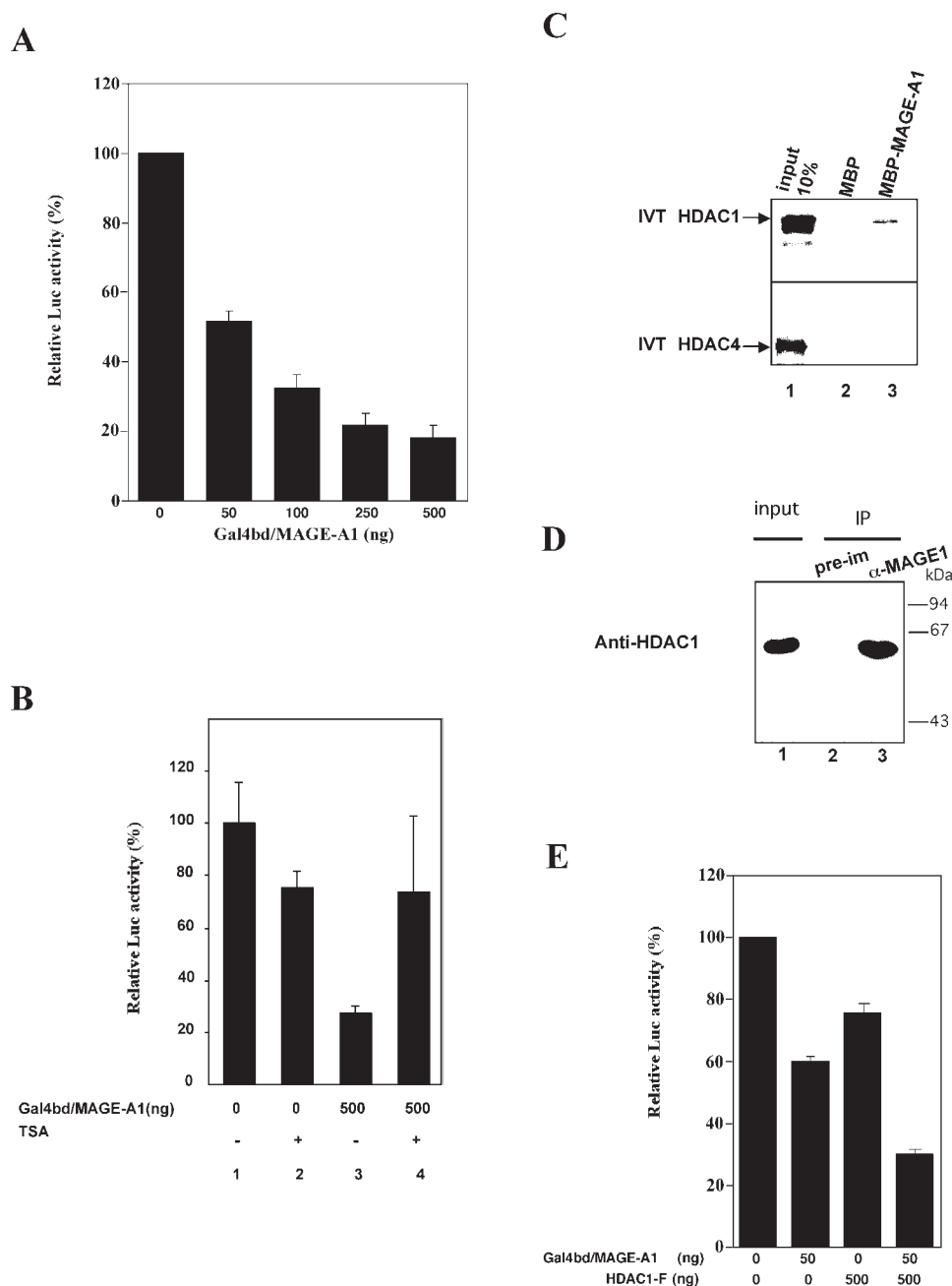


Figure 7. MAGE-A1 actively represses transcription through the recruitment of the histone deacetylase, HDAC1. (A) MAGE-A1 actively inhibits transcription when fused to Gal4bd. U2OS cells were transiently transfected with 250 ng of the (Gal4)₄-tkLuc reporter with increasing amounts (50–500 ng) of a vector coding for Gal4bd/MAGE-A1. The amount of transfected DNA was kept constant to 500 ng by adding DNA of a vector producing Gal4bd. Whole cell extracts were used in luciferase assays. The basal activity of the reporter in the presence of Gal4bd is normalized to a value of 100%. Transfection efficiencies were normalized using β-galactosidase activity. The results are the average of seven independent transfections with error bars displaying standard deviations. (B) MAGE-A1-mediated repression is sensitive to the HDAC inhibitor TSA. U2OS cells were transiently transfected with 250 ng of the (Gal4)₄-tkLuc reporter and 500 ng of the vector encoding Gal4bd/MAGE-A1. Four hours after transfection, cells were treated (lanes 2 and 4) or not (lanes 1 and 3) with 200 nM TSA. The relative luciferase activities were determined as described in Figure 7A. The results are the average of at least two independent transfections with error bars displaying standard deviations. (C) MAGE-A1 interacts with HDAC1. Full-length ³⁵S-radiolabelled HDAC1 and HDAC4 were obtained by *in vitro* translation (IVT) and incubated with equivalent amounts of maltose binding protein (MBP) (lane 2) or MBP-MAGE-A1 fusion protein (lane 3). IVT HDAC1 and HDAC4 are indicated by an arrow on the left. Lane 1, ³⁵S-radiolabelled HDAC1 (top) or HDAC4 (bottom), input (10%). (D) HDAC1 co-immunoprecipitates with MAGE-A1. COS cells were cotransfected with expression vectors encoding MAGE-A1 and HDAC1. A lysate was analyzed by immunoblotting with an anti-HDAC1 monoclonal antibody, directly (lane 1, input 4%) or after immunoprecipitation (IP) with preimmune goat serum (lane 2) or anti-MAGE-A1 goat serum (lane 3). (E) MAGE-A1 requires the deacetylase HDAC1 for transcriptional repression. U2OS cells were transiently transfected with 250 ng of the (Gal4)₄-tkLuc reporter and, as indicated, with 50 ng of a vector encoding Gal4bd/MAGE-A1 and/or 500 ng of a vector producing HDAC1-F (HDAC1 tagged with a Flag epitope). Cells were then harvested and assayed for luciferase activity. The basal activity of the reporter is normalized to a value of 100%. Transfection efficiencies were normalized using β-galactosidase activity. The results of a representative experiment done in duplicate are shown. In the absence of synergy, the expected value for the relative luciferase activity is 46%, which corresponds to the product of the activity measured in the presence of MAGE-A1 alone (60%) × the activity measured in the presence of HDAC1 alone (76%).

Such a combination of passive and active repression mechanisms was previously demonstrated for retinoblastoma protein, Rb. It binds to the activation domain of transcription factor E2F1, preventing it to interact with the CBP coactivator (55,56). In addition, Rb recruits HDAC and actively represses the E2F-regulated promoters of genes required for the S phase of the cell cycle (38,57).

Since MAGE-A1 acts as a transcriptional regulator, a fraction of the MAGE-A1 protein produced in mammalian cells must reach the nucleus. In transfected HeLa cells, we observed that MAGE-A1 was not exclusively localized in the cytosol, but that a significant fraction of it was present in the nucleus (Figure 5D). Some reports described that MAGE-A1 was located only in the cytosol of melanoma cells and spermatogonia (32,33,58), but another report described that MAGE-A1 and MAGE-A4 proteins are located in both the cytoplasm and the nucleus of spermatogonia (7). MAGE-A1 may be located predominantly in the cytoplasm and may translocate to the nucleus upon appropriate stimulation of the cell.

Experiments performed in our experimental system clearly indicated that, upon binding to SKIP, MAGE-A1 exerted a potent transcriptional repression. Since SKIP is involved in several signaling pathways, repression by MAGE-A1 bound to SKIP may influence gene expression from many different promoters. As the testis is the only healthy adult organ where human *MAGE-A* and murine *Mage-a* genes are expressed, proteins encoded by these genes may play a role in spermatogenesis (7,59). It is therefore possible that, by binding to SKIP and by inhibiting Notch1-IC transactivation, MAGE-A1 participates in cell fate choices during spermatogenesis. However, we were not able to demonstrate that MAGE-A1 could repress the expression of an endogenous Notch1-IC target gene (data not shown). This may be because MAGE-A1, in contrast to Notch1-IC or SMRT, does not bind to the activator CBF1 but binds only to SKIP, a protein that could not be absolutely required for Notch1-IC signal transduction. The possibility that MAGE-A1 regulates the expression of genes involved in spermatogenesis through other signaling pathways remains to be investigated. On the other hand, MAGE-A1 is expressed in tumor cells of different histological types. Altered HAT and/or HDAC activities have been observed in many cancer cells (60). By recruiting HDAC1 to genes that remain to be identified, MAGE-A1 may contribute to these alterations and favor tumor cell growth.

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