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Identification, Expression, and Nuclear Location of Murine Mage-b2 Protein, a Tumor-associated Antigen

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MAGE-1, which was originally identified by reacting with cytolytic T lymphocytes derived from the blood of melanoma patients, is a member of a gene family consisting of 17 structurally related genes. The MAGE genes are expressed only in the testis among normal tissues and in a number of human tumors of various histological types. Murine MAGE (also called SMAGE or Mage) genes were found in a study aimed at detecting mouse genes homologous to human MAGE genes. However, the biological functions of MAGE and Mage are currently unknown. To understand the biological functions of Mage, in the present study a recombinant SMAGE2 (Mage-b2) protein of 43 kDa was produced and monoclonal antibodies reactive with Mage-b2 protein were generated. One monoclonal antibody, smpG4A, specifically recognized a 43 kDa protein in lysates of Mage-b2 mRNA-positive sarcoma cells and of the testis. Immunohistochemistry showed that Mage-b2 is located in the nucleus of Mage-b2 mRNA-positive sarcoma cells. These results should contribute to understanding the biological functions of Mage.

Keywords: MAGE; Mage-b2; SMAGE; Tumor-associated Antigen.

Introduction

Tumor-associated antigens (TAA) that are expressed in tumors but not in normal adult tissues, except for a few normal cell types, have been characterized and their corresponding genes cloned Houghton (1994), and the number of TAA is still growing. Since the cloning of the MAGE-1 gene by a gene transfection approach to identify antigens recognized by cytolytic T lymphocytes on a human melanoma cell line, 17 related genes have been cloned and characterized (van der Bruggen *et al.*, 1991; De Plaen *et al.*, 1994; Dabovic *et al.*, 1995; Lurquin *et al.*, 1997; Muscatelli *et al.*, 1995; Lucas *et al.*, 1998; Pold *et al.*, 1999). The MAGE gene family of 18 genes can be subdivided into four subgroups: MAGE-A, MAGE-B, MAGE-C, and MAGE-D. MAGE-A, which consists of 12 members, including MAGE-1 (also called MAGE-A1), is located in the Xq28 region (van der Bruggen *et al.*, 1991; De Plaen *et al.*, 1994). Another group of 4 genes, MAGE-B, is located in the Xp21.3 region (Dabovic *et al.*, 1995; Lurquin *et al.*, 1997; Muscatelli *et al.*, 1995), and MAGE-C1 and MAGE-D1 were recently found on bands Xq26 and Xp11.23, respectively (Lucas *et al.*, 1998; Pold *et al.*, 1999). Several members of the MAGE gene family are expressed in a number of human tumors of various histological types but not in normal adult tissues except the testis (van der Bruggen *et al.*, 1991; De Plaen *et al.*, 1994; Lurquin *et al.*, 1997). Immunologically, both humoral and cell-mediated immune responses against MAGE antigens were detected in tumor patients (Gaugler *et al.*, 1994; Hoon *et al.*, 1995); therefore, MAGE has been used as a model for cancer immunotherapy (Hoon *et al.*, 1995; Maeurer *et al.*, 1996; Park *et al.*, 1999; Weber *et al.*, 1999). However, the function of the MAGE protein is currently unknown.

Twelve murine genes homologous to human MAGE genes were identified by hybridizing mouse genomic libraries with MAGE-A probes (De Backer *et al.*, 1995; De Plaen *et al.*, 1999; Osterlund *et al.*, 2000). Two members (SMAGE1 and 2; also called Mage-b1 and b2, respectively) of Mage-b (originally called SMAGE), a group of at least four genes, are located between the Dmd and the Ar loci on the X chromosome, which is syntenic to the Xp21–p22 region on the human X

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Abbreviation: TTA, tumor-associated antigen.

chromosome (De Backer *et al.*, 1995). The third member of Mage-b (SMAGE3 or Mage-b3) is autosomal, but the chromosomal location of Mage-b4 is currently unknown. Mage-a, a group of eight genes, can be divided into two subgroups depending on chromosomal locations (De Plaen *et al.*, 1999). Mage-a4 and Mage-a7 genes are located in a region syntenic to either Xp21 or Xq28 of the human X chromosome. The other genes (a1, a2, a3, a5, a6, and a8) are clustered in a region syntenic to Xp22. Like their human counterparts, members of murine Mage are expressed in several tumor cell lines of different tissue origin but not in normal adult tissues except the testis.

Despite there being no direct information on the function of MAGE, some members of the MAGE family are expressed in nomal tissues other than the testis, suggestive of the function of MAGE: MAGE-A3 and -A4 and MAGE-B2 in the placenta (De Plaen *et al.*, 1994; Lurquin *et al.*, 1997); Mage-a1, a2, a3, a5, a6 and a8 in blastocysts of mice (De Plaen *et al.*, 1999); Mage-a1, a2, a3, a5, a6, a8, and a4, and Mage-b1, b2, and b3 in embryonic stem cells of mice (De Backer *et al.*, 1995; De Plaen *et al.*, 1999); and Mage-b3 in mouse embryos from 11 d to 15 d (De Backer *et al.*, 1995). In addition, MAGE-A1 mRNA was detected in human skin after wounding (Becker *et al.*, 1994) and in normal ovarian tissue in premenopausal women (Gillespie *et al.*, 1998). These findings, that some members of the MAGE or Mage gene families are expressed in normal tissues where cell proliferation and differentiation is very active, suggest that MAGE or Mage gene products may be involved in cellular proliferation and differentiation.

As a preliminary step to understanding the functions of murine Mage protein, we have developed monoclonal antibodies against the Mage-b2 protein and identified the cellular location of the Mage-b2 protein.

Materials and Methods

Rat Sprague-Doly rats, 6–12 weeks of age, were purchased from the Korean Institute for Chemistry (Taejon, Korea).

Cell lines and tissues MCA-102 and WEHI 164, methylcholanthrene-induced fibrosarcomas, CT-26, a murine colon carcinoma cell line, and M-MSV-BALB/3T3, an M-MSV-transformed fibroblast-like cell line, acquired from ATCC (Rockville, MD, USA) and maintained in DMEM with 10% FBS supplemented with antibiotics and glutamine, were described previously (Park *et al.*, 1998). Sp2/0 murine myeloma and hybridoma cell lines were maintained in an H-Y medium with 10% FBS supplemented with antibiotics and glutamine.

RNA detection and RT-PCR analysis The total cellular RNA was extracted from tumor cells using the RNAzol method (TEL-TEST, INC., Friendswood, TX, USA), and the RNA was used for PCR analysis. RT-PCR analysis was performed basically according to the procedures previously described (Park and Shin, 1996). The primers used are as follows: for Mage-b2 amplification, 5′-atcaccatggccatgcctaggggtcaaaagagt-3′ (sense) and 5′-atcaggtgaccatgttagaggacttttgggatg-3′ (antisense), both at exon 3; and for β-actin amplification, 5′-gcaccacaccttctacaatgag-3′ (sense) and 5′-aaatagcacagcctggatagcaac-3′ (antisense). The expected size of the amplified products is 1,012 bp (Mage-b2) and 150 bp (β-actin), respectively. Thirty amplification cycles (94°C for 30 s, 68°C for 30 s, 72°C for 30 s) were performed. The PCR products were confirmed to correspond to their original sequence by DNA sequencing. The PCR products were comparatively quantitated using a GS-700 densitometer (BIO-RAD, Hercules, CA). The 150-bp band specific for the β-actin transcript served as an internal control for the quantitation of input mRNA. For removal of residual DNA contaminating mRNA, 20 μg of RNA was treated with 3 U of RNase-free DNase I for 3 h in 50 μl of the reaction buffer solution (10× reaction buffer: 40 mM Tris-HCl, pH 8.0/6 mM $MgCl_2$/10 mM NaCl/10 mM $CaCl_2$).

Expression cloning and purification of the Mage-b2 gene product For expression cloning, Mage-b2 cDNA was synthesized from murine hepatocyte genomic DNA by PCR using a pair of primers: sense, 5′-atcaccatggccatgcctaggggtcaaaagagt-3′ at exon 3 of the Mage-b2 gene and antisense, 5′-atcaggtgaccatgttagaggacttttgggatg-3′ at exon 3 of the Mage-b2 gene. Amplification was performed for 30 cycles (30 s at 94°C, 30 s at 55°C and 30 s at 72°C for each cycle) and the expected size of the product was 1,012 bp. The amplified product was digested completely with NcoI and then partially with BstEII, then and cloned into plasmid pFC+H (Kim *et al.*, 1994), which has a 6× histidine tag in the 3′ region. *E. coli* (BMH71-18) was transfected with the ligation mixtures. Clones were analyzed by restriction mapping and DNA sequencing and named pFcsmage. Recombinant protein expression was induced by 0.25 mM isopropyl-β-D-thiogalactoside for 5 h and purification of the fusion protein by Ni-NTA resin was performed under denatured conditions following procedures recommended by the manufacturer (Qiagen, Chatsworth, CA). All the buffer solutions were supplemented with 5 mM imidazole. The protein was finally eluted with buffer E (8 M urea/0.1 M NaH_2PO_4/0.01 M Tris-HCl, pH4.5/5 mM imidazole) and 0.5 ml fractions were collected. Protein synthesis was monitored by SDS/PAGE electrophoresis and Coomassie blue staining. The protein yield was determined by colorimetric protein quantitation assay (Bio-Rad, Richmond, CA, USA).

Immunization and rat hybridoma production Immunization and rat hybridoma production were performed according to the protocols (Harlow *et al.*, 1988). Rats were immunized intraperitoneally at 2-week intervals, first with 100 μg of gel-purified Mage-b2 protein emulsified with complete Freund's adjuvant, next with 100 μg of Ni-NTA column-purified Mage-b2 protein emulsified with incomplete Freund's adjuvant, and finally with 50 μg of Ni-NTA column-purified Mage-b2 protein in PBS. Seven to ten days after the second injection, serum was collected and tested for interaction with

Mage-b2 antigen. Three days after the final, third injection, the animals were sacrificed and splenocytes were recovered and fused with Sp2/0 cells. Hybridoma supernatants were screened by ELISA.

ELISA assay ELISA assay was performed as described previously (Park *et al.*, 1999), using recombinant Mage-b2 protein.

Western blot analysis Immunoblot analysis was described previously (Park and Levitt, 1993). Briefly, cells or tissues were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), 50 μM sodium vanadate, 20 mM p-nitrophenylphosphate, 50 mM sodium fluoride, leupeptin (0.5 μg/ml), aprotin (10 μg/ml), and soybean trypsin inhibitor (10 μg/ml). Proteins size-fractionated on SDS/PAGE were transferred to PVDF membranes, and the blots were sequentially treated with 5% skimmed milk, anti-Mage-b2 mAb, and HRP-conjugated anti-rat IgG, with intermittent washing with PBS. To reduce background staining, both primary and secondary antibodies were used after being preabsorbed with Mage-negative cellular extracts. Detection of protein bands was done by a colorimetric method using diaminobenzidine (DAB) and H_2O_2.

Immunohistochemistry Cells were grown on glass slides and fixed with acetone as described previously. The slides were rinsed with PBS three times and incubated with 0.3% H_2O_2 in PBS for 10 min to block endogenous peroxidase activity. The slides were sequentially treated with 5% skimmed milk, anti-Mage-b2 mAb, biotinylated-anti-rat IgG, and streptavidin-HRP, with intermittent washing with PBS. Color development was performed with DAB and H_2O_2 and counterstaining was done with Harris hematoxylin.

Results

Expression cloning and purification of the Mage-b2 gene product The Mage-b2 gene encompassing the entire reading frame flanked by restriction enzyme sites, NcoI at the 5′ end and BstEII at the 3′ end, was amplified by 30 cycles of PCR of hepatocyte genomic DNA from BALB/c mice. After being digested with NcoI and BstEII, the PCR product was cloned into plasmid pFC + H which has a 6× histidine tag in the 3′ region. Expression of the recombinant Mage-b2 protein was induced by treatment of the bacterial culture with 0.25 mM IPTG and identified by SDS/PAGE. The expected fusion protein has 330 amino acids encoding Mage-b2 plus 6× histidine amino acids, with an expected molecular mass of about 36.8 kDa (De Backer *et al.*, 1995). We observed an about 43 kDa protein induced by IPTG (Fig. 1A). Further purification of protein extracts from IPTG-treated bacterial cultures by Ni-NTA resin gave rise to one dominant band migrating at 43 kDa (Fig. 1B). The recombinant Mage-b2 protein was larger than the 36.5 kDa protein product predicted on the basis of the Mage-b2 gene sequence or the 38 kDa *in vitro* translation product of the Mage-b2 (De Backer *et al.*, 1995), but was smaller than the MAGE-1 protein of 46 kDa (Chen *et al.*, 1994; Kocher *et al.*, 1995). The deduced amino acid composition of murine Mage-b2 proteins reveals a high content of basic residues (pI 10.3–10.5), as observed with human MAGE-B proteins (De Plaen *et al.*, 1999). Whether the high content of basic amino acid residues in the Mage-b2 protein or unknown posttranslational modifications may retard migration of protein on SDS/

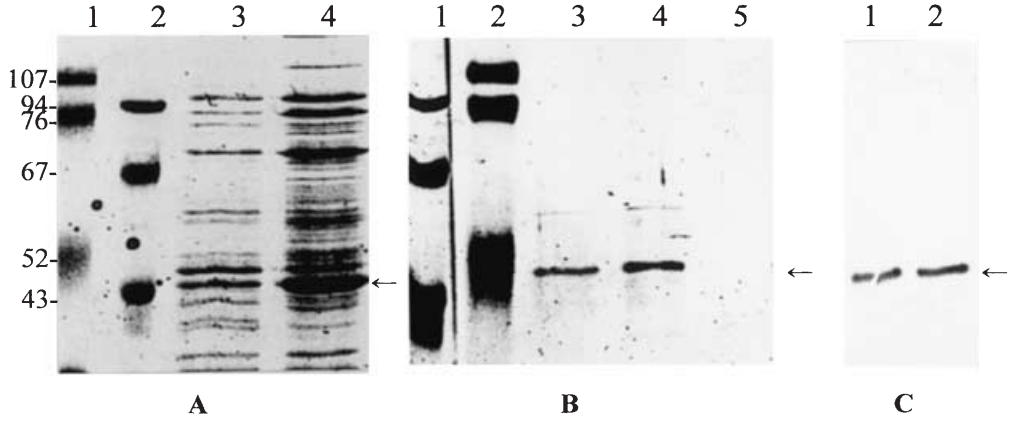

Fig. 1. Expression-cloning and immunodetection of recombinant Mage-b2 protein. (**A**) Coomassie blue-stained gel of crude extracts. Overnight cultures of *E. coli* transformed with pFcsmage were mock-treated (lane 3) or treated with 0.25 mM IPTG for 5 h (lane 4). Lanes 1 and 2 represent molecular mass markers in kilodaltons. One band migrating at 43 kDa was strongly induced by IPTG as indicated by an arrow. (**B**) Coomassie blue-stained gel of Ni-NTA affinity resin-purified Mage-b2 protein. Lanes 1 and 2 represent molecular mass markers in kilodaltons. Lanes 3–5 represent eluate fractions 1–3, respectively. One band migrating at 43 kDa was detected (lanes 3, 4). (**C**) Immunoblot analysis of the rMage-b2 protein. The rMage-b2 protein was run on lanes 1 (eluate fraction 1) and 2 (fraction 2), blotted, and assayed with monoclonal antibody smpG4A.

PAGE remains to be tested. The recombinant Mage-b2 protein was used for immunization and subsequent titration of anti-Mage-b2 sera by ELISA.

Generation of Mage-b2-specific monoclonal antibodies After purification of the recombinant Mage-b2 protein with a Ni-NTA column, the protein mixtures were SDS/PAGE run and further purified for the 43 kDa protein by gel elution. After repeated immunizations as described in **Materials and Methods**, fusions were made and hybridoma clones reactive with the Mage-b2 protein were screened by ELISA. Several clones of IgG isotypes including smpG4A and smpD1C specifically reacting with the Mage-b2 protein were isolated (Fig. 1C).

Mage-b2 expression in murine tumor cell lines There has been no report on expression of Mage-b2 mRNA in murine tumor cell lines; therefore, we screened several murine tumor cell lines of different tissue origin for Mage-b2 expression by RT-PCR. M-MSV-BALB/3T3 cells, a M-MSV transformed fibroblast-like cell line, expressed Mage-b2, whereas other cell lines, CT-26 (colon carcinoma) and MCA-102 and WEHI 164 (both methylcholanthrene induced-fibrosarcomas), did not (Fig. 2). Testis RNA was used as a positive control.

Detection of native Mage-b2 protein by monoclonal antibodies We then examined expression of the Mage-b2 protein in the tumor cell lines and normal murine tissues. Western blot analysis of M-MSV-BALB/3T3 cell lysates showed that a monoclonal antibody, smpG4A, recognized a protein exhibiting a molecular mass of 43 kDa (Fig. 3A); however, no corresponding band was detected from the lysates of Mage-b2-negative cell lines. Next, we examined the reactivity of smpG4A to the Mage-b2 protein expressed in adult tissues. The smpG4A mAb recognized in the lysates of the testis a 43 kDa protein which comigrated with the 43 kDa band detected in M-MSV-BALB/3T3 cell lysates (Fig. 3B). No corresponding band was detected from protein extracts of Mage-b2-negative tissues, including the liver, spleen, and kidney (Fig. 3B). Therefore, it is likely that the pattern of Mage-b2 protein expression in tumor cells and normal tissues determined by immunoblot analyses with smpG4A mAb is in agreement with the pattern of Mage-b2 mRNA expression in the same tumor cells as demonstrated in the present study and in normal tissues as described previously (De Backer *et al.*, 1995).

Intracellular location of the Mage-b2 gene product It was previously shown that the human MAGE protein is present in the cytoplasm or the nucleus (Kocher *et al.*, 1995; Takahashi *et al.*, 1995). To localize the Mage-b2 protein in the cell, immunohistochemical studies were performed using mAb smpG4A. The Mage-b2 protein was detected in the nucleus of Mage-b2-positive M-MSV-BALB/3T3 cells (Fig. 4A), but not in the nucleus of Mage-b2-negative cell line CT-26 (Fig. 4B). We found that, after streptavidin-HRP incubation, the nuclei of M-MSV-BALB/3T3 cells turned brown during chromogen treatment and became dark purple upon counterstaining with Harris hematoxylin. In contrast, the nuclei of CT-26 cells did not produce the brown color reaction, but appeared pale blue upon hematoxylin treatment. As a result, the nuclei of M-MSV-BALB/3T3 cells appeared black whereas those of CT-26 cells looked gray in black-and-white prints. No brown color reaction upon incubation with isotype-matched monoclonal antibodies (anti-CD4 mAb which reacts with mouse CD4 molecule on

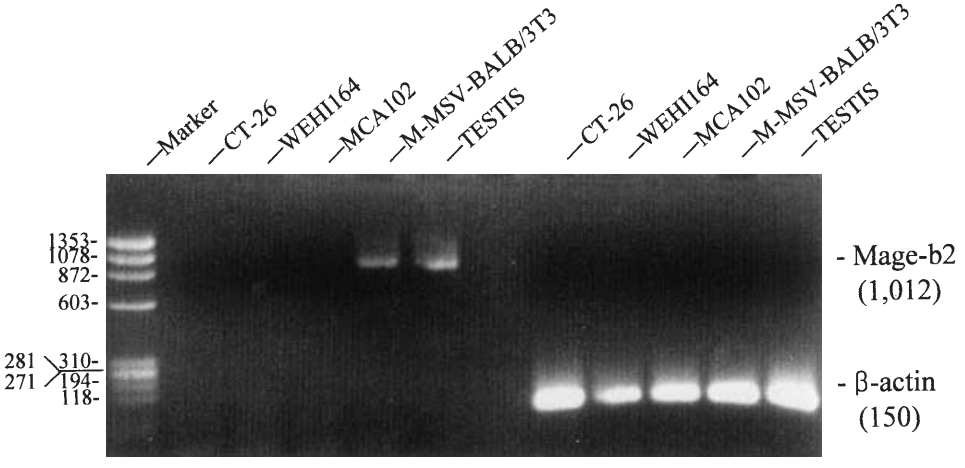

Fig. 2. RT-PCR analysis of Mage-b2 mRNA expression. mRNA, which was extracted from different types of tumor cells and tissues, was first reverse-transcribed, PCR-amplified using pairs of primers described in **Materials and Methods**, and resolved in 2.0% agarose gel. The expected size of the Mage-b2 product is 1,012 bp and that of β-actin 150 bp.

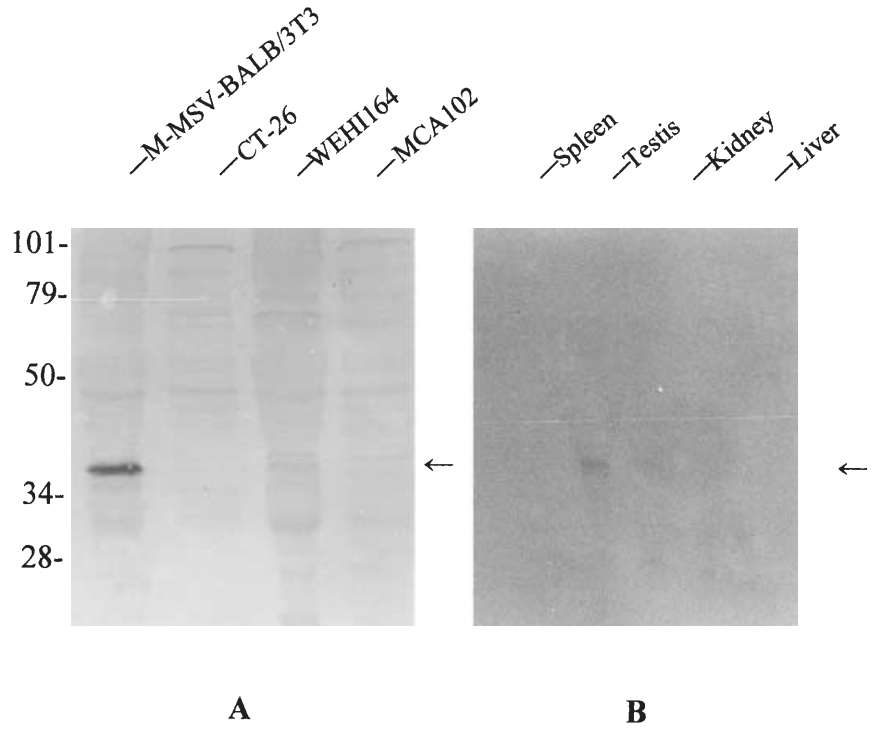

Fig. 3. Immunoblot analysis of anti-Mage-b2 mAb against lysates of various murine specimens. (**A**) anti-Mage-b2 mAb smpG4A against Mage-b2 mRNA-positive (lane 1, M-MSV-BALB/3T3) and Mage-b2 mRNA-negative (lanes 2–4, CT-26, WEHI164 and MCA102) cell lines. M-MSV-BALB/3T3 was positive for a species of 43 kDa which was not detected from the lysates from other tumor cell lines (CT-26, WEHI164 and MCA102). (**B**) anti-Mage-b2 mAb smpG4A against murine adult tissues. The lysate of testis which express Mage-b2 mRNA was positive for a species of 43 kDa, which was not detected from the lysates from other tissues (kidney, liver, and spleen).

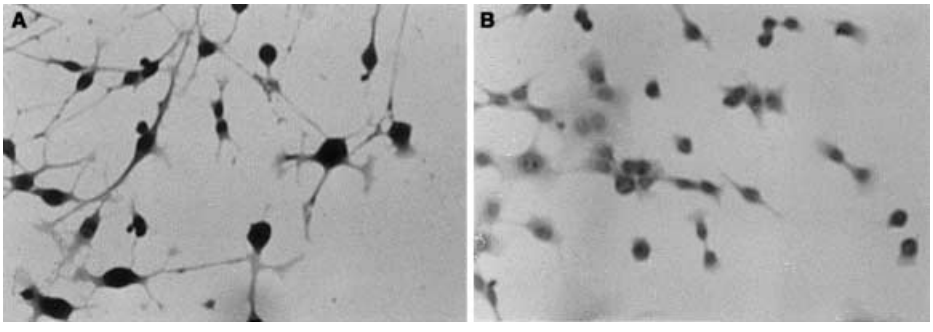

Fig. 4. Immunohistochemical analysis of the Mage-b2 protein. Acetone-fixed M-MSV-BALB/3T3 (**A**) and CT-26 (**B**) cells were stained with smpG4A (×400). The horseradish peroxidase method with diaminobenzidine as a chromogen was used and the slides were counterstained with Harris hematoxylin.

subsets of T lymphocytes) was observed in M-MSV-BALB/3T3 cells (data not shown).

Discussion

Human MAGE genes constitute a large family of genes, which have been found on three different loci on the X chromosome. Although a lot of studies on expression of MAGE genes in different tumors and normal adult tissues have been performed, no functional studies on the MAGE protein were reported. To understand the biological functions of MAGE molecules, we chose murine homologues of MAGE genes as a model. In the present study, we have demonstrated that monoclonal antibodies generated against recombinant Mage-b2 protein expressed in *E. coli* react specifically with native Mage-b2 protein in a Mage-b2-positive tumor cell line and the testis as well as with recombinant Mage-b2 protein and that the Mage-b2 protein is located in the

nucleus of the tumor cells. These mAbs will contribute to a better understanding of the biological functions of the Mage-b2 protein.

As described in the **Introduction**, some members of the human MAGE and murine Mage genes, in addition to the testis, are expressed in normal tissues: in the placenta, in blastocysts of mice, in embryonic stem cells of mice, in mouse embryos from 11 to 15 d, in human skin during wound healing, and in normal ovarian tissue in premenopausal women. It was recently reported that the tumor size of human hepatocellular carcinoma was significantly larger in MAGE-positive patients than in the MAGE-negative ones (Suzuki *et al.*, 1999). These findings suggest that MAGE or Mage gene products may be involved in cellular proliferation and differentiation. MAGE or Mage proteins lack signal sequences and contain a potential transmembrane domain that may function only in association with the transmembrane domain of other proteins (De Plaen *et al.*, 1994). A computer search of the protein data base showed that there is a moderate homology between MAGE or Mage proteins and mouse necdin protein (up to 31% homology). Necdin is a nuclear protein expressed in neurally differentiated embryonal carcinoma cells and in the brain of adult mice. Necdin suppresses the growth of postmitotic neuronal cells and was reported to interact with viral oncoproteins, SV40 T and adenovirus E1A, and with cellular transcription factor E2F1 (Maruyama *et al.*, 1991; Hayashi *et al.*, 1995; Taniura *et al.*, 1998). Hydrophobic cluster analyses of amino acid sequences of MAGE, Mage, and necdin proteins revealed a remarkable conservation of the hydrophobic regions, suggesting conservation of function (De Plaen *et al.*, 1994).

The cellular location of MAGE and Mage protein appears not to be restricted to one site. MAGE-1 and MAGE-4 proteins were identified in the cytoplasm and the nucleus of spermatogonia and spermatocytes, but in melanoma cells the MAGE-3 gene product was found to be expressed only in the cytoplasm. Transcripts of Mage-b1, -b2, and -b3 genes were previously shown to be expressed in postmeiotic spermatids by *in situ* hybridization using an antisense oligonucleotide corresponding to a sequence of exon 3 that is identical for Mage-b1, -b2, and -b3 genes (Chomez *et al.*, 1995); however, the cellular location of the Mage transcripts was not explored. In addition, it was very recently reported that the Mage-b4 protein, which has an expected molecular mass of 52 kDa, compared to 36 kDa of the Mage-b2 protein, but shows some homology to the Mage-b2 protein, is downregulated as germ cells enter meiosis in adult testis and is located in the cytoplasm of the germ cells (Osterlund *et al.*, 2000). In the present study, we have identified the Mage-b2 protein in the nucleus of M-MSV-BALB/3T3 tumor cells. Given the previous results and the results in the present study, it is not easy to draw a unified model for MAGE function. Using tumor cell lines, we observed protective functions of MAGE from harmful effects of cytotoxic drugs (unpublished observation). We are currently addressing this issue.

The recombinant Mage-b2 protein of 43 kDa was larger than the 36.5 kDa protein product predicted on the basis of the Mage-b2 gene sequence, and Western blot analyses revealed that the native Mage-b2 protein expressed in the testis and tumor cells has a molecular mass of 43 kDa. The molecular mass of MAGE-1 was previously estimated as 34.3 kDa on the basis of the primary MAGE-1 sequence (De Smet *et al.*, 1994); however, the apparent molecular mass of MAGE-1 prepared from several cell lines and testes analyzed by SDS/PAGE and Western blotting was 46 kDa or even higher. There are reports that proteins with stretches of acidic amino acids migrate in an anomalous fashion on SDS/PAGE, making the matter of determining the molecular mass of proteins complicated as evidenced with protein NO38 and a 165 kDa muscle-specific protein (Hofmann *et al.*, 1989; Schmidt-Zachmann *et al.*, 1987). The MAGE-1 amino acid composition deduced reveals a high content in acidic residues, which may reduce $NaDodSO_4$ binding and lower the polypeptide mobility during electrophoresis. The peculiar migration properties of the Mage-b2 protein may be attributed to stretches of basic residues present in the Mage-b2 protein (pI 10.3–10.5) as demonstrated with the Mage-b4 protein (pI 9–10.7), whose calculated molecular mass (52 kDa) is much smaller than 70–80 kDa on SDS/PAGE or to unknown properties peculiar to the Mage gene family members.

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