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Nuclear Localization of Magphinins, Alternative Splicing Products of the Human Trophinin Gene

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Abstract Human magphinin proteins are translation products of differentially spliced transcripts from the 5′ region of the human trophinin gene (*TRO*), whose 3′ region encodes trophinin, a unique cell adhesion molecule involved in human embryo implantation. Magphinins belong to the MAGE (melanoma-associated antigen) family, and a previous study of mouse magphinins showed their expression in male and female germ cells, suggesting a role in germ cell development. Here, we characterized the structure and subcellular localization of human magphinins. Confocal microscopy analysis of ectopically expressed magphinins revealed that magphinin-α and -β localize in the cytoplasm, whereas magphinin-γ lacking the peptide encoded by exon-3 is nuclear. Following Triton X-100 extraction, DNA digestion, and high salt extraction magphinin-γ remained nuclear, suggesting strong association with the nuclear matrix. A series of magphinin-γ deletion mutants were generated and assayed for localization, which showed that the N-terminal region of the MAGE homology domain is necessary for nuclear localization. When magphinin-γ was expressed in NIH3T3 cells, cells underwent G1 arrest. These results suggest that human magphinin-γ inhibits cell cycle progression through nuclear activity. J. Cell. Biochem. 103: 765–777, 2008.

Key words: magphinin; trophinin; MAGE; alternative splicing; nuclear localization; nuclear matrix

Trophinin is a membrane protein potentially mediating the initial adhesion of the human embryo to the uterine epithelia through a unique apical cell adhesion between trophoblastic and endometrial cells [Fukuda et al., 1995; Suzuki et al., 1998; Fukuda and Nozawa, 1999; Nakayama et al., 2003; Sugihara et al., 2007]. Significant differences in embryo implantation are seen among different mammalian species [Nadano et al., 2002; Spencer et al., 2004]. These differences may be related to the fact that this type of cell adhesion may have been acquired by mammals relatively recently in evolution. Although mouse and human trophinin genes produce a transcript with an open reading frame encoding 196-kDa and 138-kDa proteins, respectively, previous studies did not detect this protein in vivo and in vitro [Nadano et al., 2002]. A previous study showed that the 5′ region of the mouse trophinin gene generates several alternatively spliced transcripts whose translated products were designated magphinins [Saburi et al., 2001]. Magphinins contain a polypeptide sequence homologous to the MAGE (melanoma-associated antigen) domain [Chomez et al., 2001].

Three major magphinin isoforms, α, β, and γ, have been identified in the mouse [Saburi et al., 2001]. Magphinin proteins are found in male and female germ cells, in which they localize to the cytoplasm and nucleus

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depending on the maturation stage of oocytes or spermatogenic cells. Magphinin-β is found mainly in the cytoplasm of male germ cells but it becomes nuclear around the time when meiosis I is completed. In female germ cells, magphinin-γ is nuclear when the oocyte divides into multi-layered follicles or when meiosis is complete. In vitro assays of mouse magphinins show that they inhibit cell proliferation. Overall, these results suggest that magphinin regulates cell proliferation during gametogenesis [Saburi et al., 2001].

Mouse magphinins belong to the MAGE family, a group of ~25 proteins distinguished by a 200 amino acid MAGE homology domain [Chomez et al., 2001; Barker and Salehi, 2002]. While the function of this class of proteins is not well understood, recent studies implicate several of them in cell cycle control or apoptosis. For example, ectopic expression of necdin, a member of the MAGE family expressed in the nervous system [Maruyama et al., 1991], strongly suppressed growth of proliferating cells [Hayashi et al., 1995; Taniura et al., 1998, 1999]. Necdin interacts with various proteins, including SV40 large T antigen, adenovirus E1A, E2F1, E2F4, p53, NEFA, heterogeneous nuclear ribonucleoprotein U and the p75 neurotrophin receptor (p75NTR) [Taniura et al., 1999; Taniguchi et al., 2000; Kobayashi et al., 2002; Taniura and Yoshikawa, 2002; Tcherpakov et al., 2002; Kuwako et al., 2004]. Another neuronal MAGE protein, NRAGE (Dlxin-1, MAGE-D1), interacts with p75NTR, blocks cell cycle progression, and promotes p75NTR-mediated apoptosis [Salehi et al., 2000; Barker and Salehi, 2002]. In addition, NRAGE associates with Msx2 and Dlx5 homeodomain proteins and regulates Dlx5 transcriptional activity [Masuda et al., 2001; Sasaki et al., 2002].

In humans, the trophinin gene—also called the MAGE-D3 gene [Chomez et al., 2001]—maps to Xp11.2 [Pack et al., 1997]. In all MAGE superfamily proteins except MAGE-Ds, each MAGE protein is encoded on a single exon [Lucas et al., 1999; Chomez et al., 2001; Saburi et al., 2001]. Since multiple alternatively spliced MAGE proteins generated from the human trophinin gene have not been defined, we identified and characterized three human MAGE-D3 products, magphinin-α, -β, -γ and determined both the relation between subcellular localization and domain structures and the activity of magphinin-γ in transfected cells.

MATERIALS AND METHODS

cDNA Clones of Human Magphinins

Human EST clones encoding sequences homologous to mouse magphinin-α (clone ID 2443944), -β (clone ID 2267673), and -γ (clone ID 74716) were obtained from Invitrogen and sequenced. Human magphinin-α, -β, and -γ cDNAs were obtained by EcoRI (5′ end) and XhoI (3′ end) digestion and subcloned into the pCMV/SPORT6 vector (magphinin-α and -β) or pBluescript II KS(+) (magphinin-γ). Full-length HA-tagged human magphinin-α and -β were constructed from magphinin-α/pCMV/SPORT6 and magphinin-β/pCMV/SPORT6, respectively, by digesting the vector with EcoRI and NotI. EcoRI/NotI fragments of each were ligated in frame into EcoRI/NotI-cut sites of the pHM6-derived vector (Roche). Full-length HA-tagged human magphinin-γ was constructed from magphinin-γ/pBluescript II KS(+) by digestion with EcoRI and XhoI and filling in the XhoI site. This fragment was subcloned into EcoRI and Eco72I sites of the pHM6-derived vector. To obtain the GFP-magphinin expression vector, EcoRI/XhoI fragments of magphinins were ligated into the EcoRI/SalI sites of pEGFP-C1 (Clontech). Deletion mutants of magphinin-α or -β (Δ1-406) were generated from HA-magphinin-α or -β using appropriate digestion, followed by fill-in reactions and ligation. Magphinin-γ deletion mutants (Δ1–139, Δ124–284, Δ173–284) were similarly constructed from GFP-magphinin-γ.

Antibodies

Monoclonal anti-HA antibodies (F-7 and Y-11) were obtained from Santa Cruz Biotechnology. Anti-GFP antibody was from Medical and Biological Laboratories. Horseradish peroxidase-conjugated $F(ab')_2$ of anti-rabbit Ig was from Amersham Biosciences. FITC-conjugated $F(ab')_2$ of anti-mouse IgG and TRITC-conjugated anti-mouse IgG were from BioSource International and Sigma–Aldrich, respectively.

Cells and Transfection

COS-1 or NIH3T3 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 5% fetal bovine serum (FBS) or 10% calf serum (CS) at 37°C in a humidified

incubator under 5% CO_2. Cells were transfected with a plasmid vector encoding each magphinin isoform or mutant forms using LipofectAMINE^{TM}2000 Reagent (Life Technologies) or TransIT Transfection Reagent (Mirus), according to the manufacturer's instructions, as reported [Yamaguchi et al., 2001; Kasahara et al., 2004; Aoyama et al., 2005; Nakayama and Yamaguchi, 2005].

Immunofluorescence Microscopy

Cells grown on a glass coverslip were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 20 min at room temperature and permeabilized in PBS containing 0.1% saponin and 3% bovine serum albumin for 30 min, as described [Yamaguchi and Fukuda, 1995; Kasahara et al., 2007a]. Cells were stained with anti-HA antibody for 1 h. After washing with PBS containing 0.1% saponin, cells were stained with FITC-conjugated anti-mouse IgG antibody for 1 h and treated with 200 μg/ml RNase A and 100 μg/ml propidium iodide for 30 min to stain DNA. Confocal and Nomarski differential-interference-contrast images were obtained using an FV500 laser-scanning microscope (Olympus). Emission signals were detected between 505 and 530 nm for fluorescein and at more than 650 nm for propidium iodide. Care was taken to ensure that there was no bleed-through from fluorescein into the red channel [Mera et al., 1999; Tada et al., 1999].

Western Blot Analysis

Western blotting was performed as described [Hirao et al., 1997; Mera et al., 1999; Yamaguchi et al., 2001; Matsuda et al., 2006]. Transfected COS-1 cells were washed three times with PBS and scraped and lysed in buffer containing Triton X-100 (50 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100, 4 mM EDTA, 100 mM NaF, and 1 mM Na_3VO_4) or RIPA buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 4 mM EDTA, 0.1% SDS, and 1% sodium deoxycholate) containing 50 μg/ml aprotinin, 100 μg/ml leupeptin, 25 μM pepstatin A, and 2 mM PMSF at 4°C. Extracted proteins were resolved by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with anti-HA (Y-11) or anti-GFP antibody, followed by horseradish peroxidase-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG antibody [Yamaguchi et al., 2001; Aoyama et al., 2005; Kasahara et al., 2007b]. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham Biosciences) using an image analyzer LAS-1000-plus (Fujifilm).

Nuclear Matrix Fractionation

Nuclear matrix fractionation was performed essentially as described [He et al., 1990; Aoyama et al., 2005; Nakayama et al., 2006]. Cells were washed and extracted for 3 min at 4°C with cytoskeleton (CSK) buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, and 1 mM EGTA) supplemented with 0.5% Triton X-100 and protease inhibitors. Chromatin was digested with DNase I (>2.5 U/100 μl) in CSK buffer at 37°C for 30 min and eluted serially with CSK buffer containing 0.25 M ammonium sulfate at 4°C for 5 min, with 2 M NaCl in CSK buffer for 5 min.

Flow Cytometry

NIH3T3 cells were detached by trypsinization 48 h post-transfection, fixed with 1.5% paraformaldehyde at 4°C for 1 h, and then permeabilized with 70% ethanol at −30°C for more than 1 h. Fixed cells were washed three times with PBS containing 3% FBS. After washing, cells were treated with 200 μg/ml RNase A and 50 μg/ml propidium iodide at 37°C for 30 min to stain DNA and subjected to flow cytometry using a MoFlo cell sorter (Dako Cytomation/TakaraBio) equipped with a 488-nm argon laser. Sort gates were defined by expression of GFP or GFP-magphinins. Debris was excluded by gating on forward scatter and pulse-width profiles, as described [Nakayama and Yamaguchi, 2005].

Apoptosis Assay

NIH3T3 cells were grown on a glass cover slip and were transfected by the expression vector either for magphinin-α, -β, or -γ. Three days after transfection, cells were subjected to TUNEL assay using ApopTag peroxidase in situ apoptosis detection kit (Chemicon). Apoptotic cells were visualized by color reaction with peroxidase substrate AEC (Zymed) and counter stained by hematoxylin.

RESULTS

Structural Characterization of Human Magphinin Isoforms

Sequencing of cDNAs encoding human magphinin revealed alternatively spliced

transcripts of the trophinin gene (Fig. 1). Although the full-length transcript contains 14 exons, transcripts of magphinin-α, -β, and -γ isoforms lack exon 13a, which encodes the almost entire trophinin protein.

The MAGE homology domain of human magphinins is encoded by genomic sequence from exons 4 to 12 and is highly homologous (93% amino acid identity) to the MAGE domain of mouse magphinins. The MAGE domain of human magphinins is also highly homologous to other human MAGE-D orthologues, whereas the N- and C-terminal regions flanking the MAGE domain are not homologous to other MAGE family proteins. Furthermore, the NH_2-terminal region encoded by exons 2 and 3 is 44% identical at the amino acid level between human and mouse sequences. By contrast, the C-terminal region flanking the MAGE domain of human magphinins shows no significant homology to mouse magphinins. This difference may contribute to differences in localization and function.

Ectopic Expression and Localization of Human Magphinin Proteins

To determine localization of human magphinins, expression vectors were constructed for each isoform tagged with the HA epitope at the N-terminus (Fig. 2A). COS cells were transfected with HA-magphinin-α, -β, or -γ,

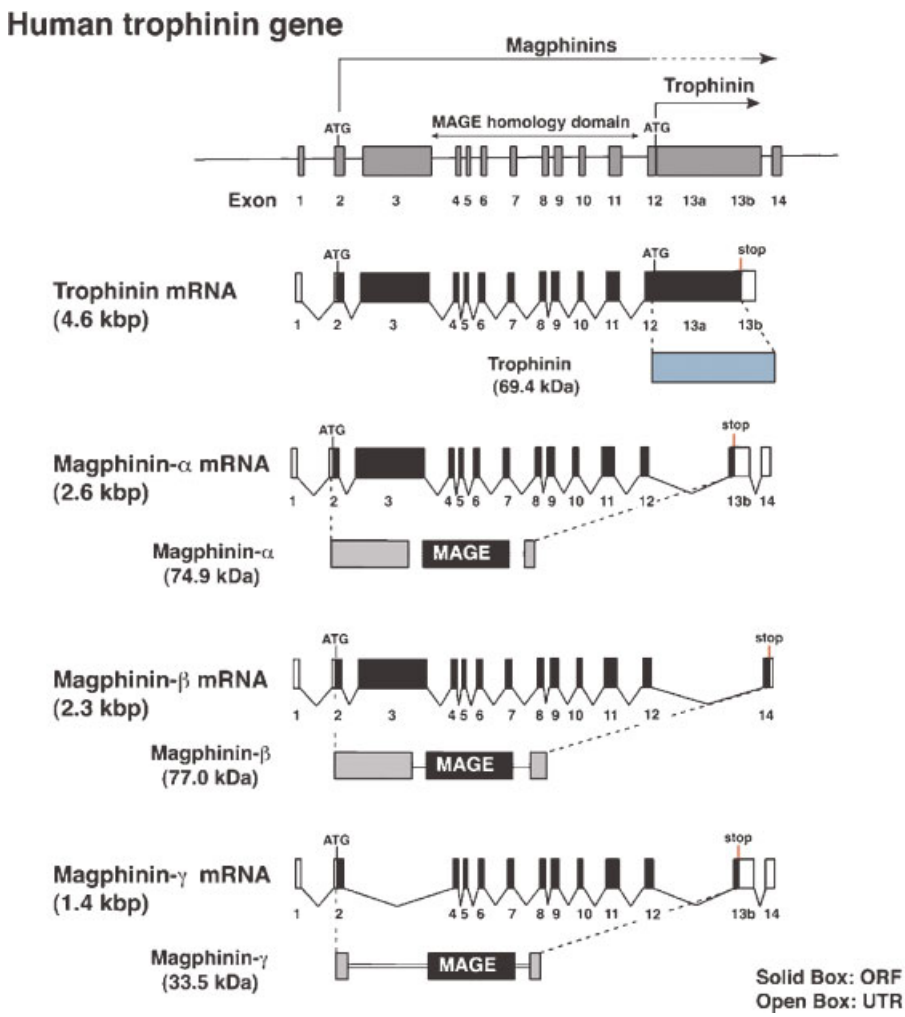

Fig. 1. Structure of the trophinin gene and predicted trophinin/magphinin transcripts and proteins. Predicted exons and introns of the human trophinin gene (*TRO*) are shown. Predicted alternatively spliced mRNAs and polypeptides of human trophinin and magphinins are based on the EST database and our previous study [Saburi et al., 2001]. Exons are shown by shaded boxes, open reading frames (ORFs) are shown by solid boxes, and untranslated regions (UTRs) are represented by open boxes. Human magphinin proteins (green) do not share peptide sequence with trophinin protein (blue). The predicted molecular sizes of human trophinin, magphinin-α, -β, and -γ are 69.4, 74.9, 77.0, and 33.5 kDa, respectively.

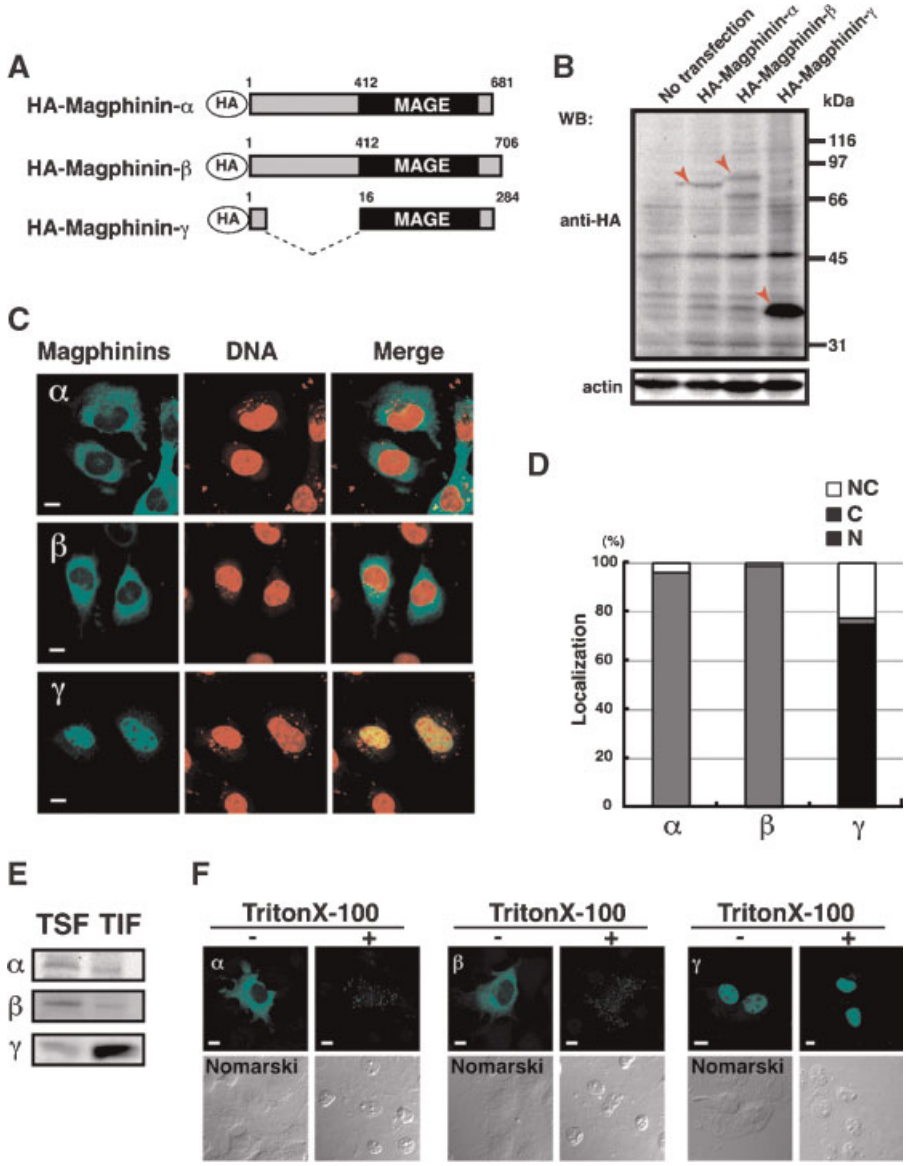

Fig. 2. Localization of human magphinin isoforms in COS cells. **A**: Schematic representations of HA-magphinins-α, -β, and -γ. Numbers refer to amino acid residues. **B**: Western blot analysis of HA-magphinin-α, -β, and -γ expressed in COS cells. Cells transfected with HA-magphinin expression vectors were solubilized and equal amounts of lysates were subjected to Western blot analysis with anti-HA antibody. Expected molecular sizes for HA-magphinin-α, -β, and -γ (red arrowheads) are 80.4, 82.5, and 38.0 kDa, respectively. **C**: Fluorescence micrographs of HA-magphinins expressed in COS cells. COS cells were transfected with the indicated expression vectors, and expressed proteins were detected with anti-HA antibody by confocal fluorescent microscopy. Note that magphinin-α and -β are cytoplasmic, whereas magphinin-γ is nuclear. Green, magphinins; red, DNA. Bars, 10 μm. **D**: Distribution of HA-magphinin-α, -β, and -γ in COS cells. The graph depicts quantitative data obtained from three independent experiments. In each experiment, more than 200 cells were counted. N, exclusively nuclear staining; NC, cytoplasmic and nuclear staining; C, exclusively cytoplasmic staining. **E**: Distribution of HA-magphinin proteins in detergent-soluble and -insoluble fractions. COS cells transfected with each HA-magphinin isoform were solubilized in Triton X-100-containing buffer for 30 min at 4°C, and soluble and insoluble fractions were analyzed by Western blotting using anti-HA antibody. TSF, Triton X-100-soluble fraction; TIF, Triton X-100-insoluble fraction. **F**: COS cells transfected with HA-magphinin isoforms were treated with Triton X-100-containing buffer for 30 min at 4°C, followed by fixation and immunocytochemistry. Most magphinin-α and -β was extracted with Triton X-100, whereas magphinin-γ remained nuclear after Triton X-100 treatment. Green, magphinins. Bars, 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and HA-magphinin expression was examined by Western blotting using an anti-HA antibody. As shown in Figure 2A, HA-magphinin-α, -β, and -γ were 80.4, 82.5, and 38.0 kDa, respectively, consistent with their predicted sizes (Fig. 2B). A 66–68 kDa band in HA-magphinin-β may be a degradation product, but we cannot exclude a possibility that this is produced a downstream translation initiation site. Indeed, magphinin-α and -β levels expressed in COS cells were lower than that of magphinin-γ, likely due to differences in translation efficiencies, as described previously [Saburi et al., 2001].

Localization of HA-magphinins in COS cells was examined by confocal fluorescence microscopy. Interestingly, approximately 70% of magphinin-γ was nuclear, whereas almost all magphinin-α or -β was cytoplasmic (Fig. 2C,D). In these experiments, levels of each isoform were comparable (Fig. 2C). To further examine the localization of magphinins, cells transfected with each HA-magphinin isoform were treated with Triton X-100-containing buffer before fixation and immunostaining. Magphinin-α and -β were found in the detergent-soluble fraction, whereas most magphinin-γ remained insoluble (Fig. 2E,F). Similar results were obtained with COS, HeLa, and NIH3T3 cells transfected with HA- or GFP-magphinins (data not shown). These results indicate that localization of human magphinin-γ differs from that of human magphinin-α and -β.

Requirement of the Peptide Encoded by the Exon-3 for Cytoplasmic Localization

Magphinin-γ structure differs distinctly from magphinin-α and -β in the region encoded by exon 3: magphinin-α and -β contain this region, whereas magphinin-γ lacks it. To determine whether the region contributes to protein localization, we deleted N-terminal regions of interest from magphinin-α or -β (Fig. 3A). COS cells transfected with deletion mutants α(Δ1–406) and β(Δ1–406) expressed high levels of the proteins (Fig. 3B). Confocal microscopy of transfected COS cells revealed that both deletion mutants localized to the nucleus (Fig. 3C,D). Furthermore, most of these proteins were not solubilized with Triton X-100, like magphinin-γ. These results suggest that the region encoded by exon 3 is critical for cytoplasmic localization of magphinin-α and -β.

Nuclear Localization of Magphinin-γ Requires N-Terminal Sequences

To identify the region determining the nuclear localization of magphinin-γ, we produced a series of deletion mutants of GFP-magphinin-γ. COS cells were transfected with GFP alone, GFP-magphinin-γ, or GFP-magphinin-γ mutants (Δ173–284, Δ124–284, and Δ1–139), and expression of proteins were confirmed by Western blotting using anti-GFP antibody (Fig. 4A,B). Immunocytochemistry showed that Δ173–284, Δ124–284 mutants were nuclear, whereas the Δ1–139 mutant was both cytoplasmic and nuclear (Fig. 4C). These results show that the N-terminus (aa1–123) of magphinin-γ is necessary for nuclear localization.

Association of Magphinin-γ With the Nuclear Matrix

To determine whether magphinin-γ associates with the nuclear matrix, we undertook sequential extraction [He et al., 1990; Aoyama et al., 2005; Nakayama et al., 2006] (Fig. 5). The fractionation is designed to (1) remove cytoplasmic, soluble proteins with cytoskeleton (CSK) buffer containing 0.5% Triton X-100, (2) release chromatin proteins by DNase I digestion followed by extraction with 0.25 M ammonium sulfate, and (3) remove residual proteins by washing with 2 M NaCl, leaving only structural nuclear matrix proteins and nuclear matrix-associated proteins. Intriguingly, magphinin-γ remained nuclear after CSK buffer extraction (Fig. 5A upper panel) and after treatment with DNase I digestion and high salt extraction (Fig. 5A lower panel). Like magphinin-γ, magphinin-α (Δ1–406) and magphinin-β (Δ1–406) mutants remained in the nuclear matrix after treatment with CSK buffer, DNase I and high salt (Fig. 5B,C; see Fig. 3). These results indicate that nuclear magphinin-γ and magphinin-α and -β mutants are associated with the nuclear matrix.

Induction of Morphology Associated With Cell Death by Magphinin-γ

A previous study of mouse magphinin showed that ectopically expressed mouse magphinin was nuclear and suppressed cell proliferation [Saburi et al., 2001]. When we transfected COS or HeLa cells with human magphinins,

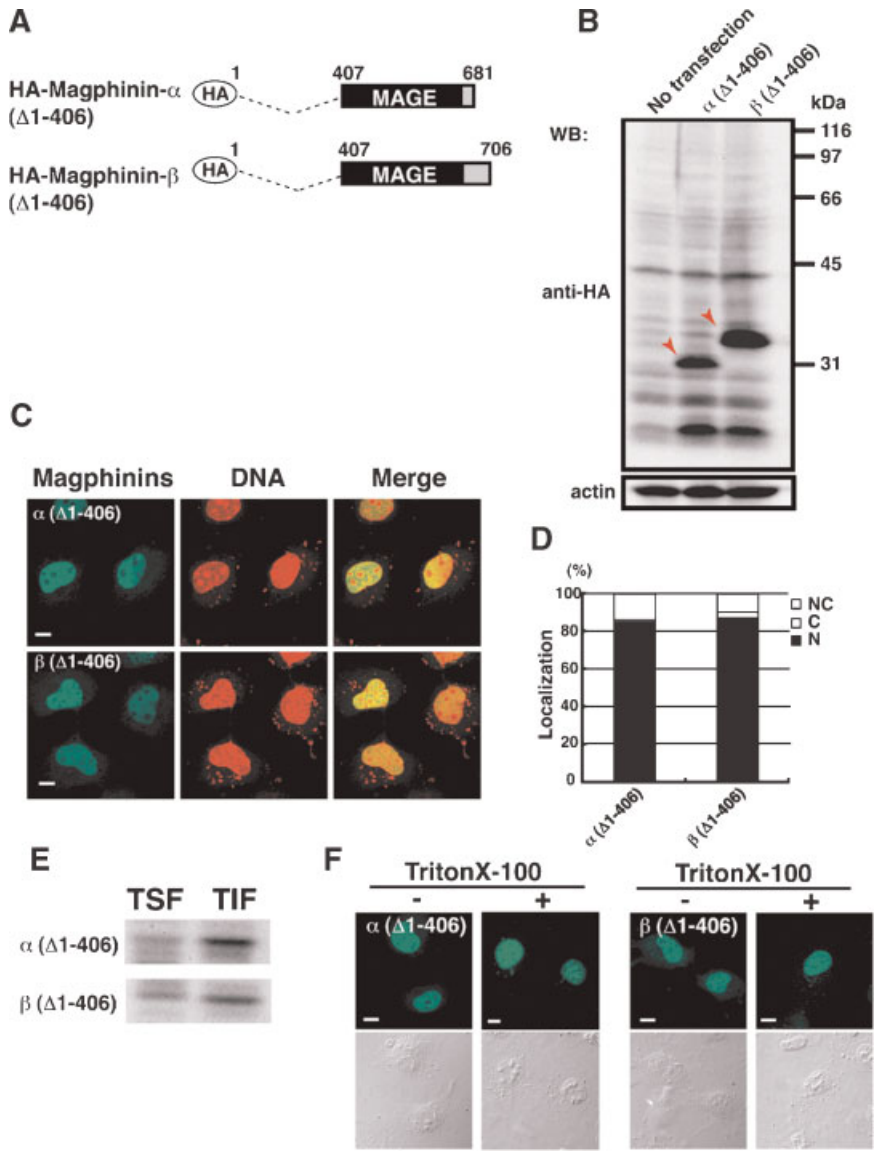

Fig. 3. Localization of magphinin-α and -β deletion mutants. **A**: Deletion mutants of HA-magphinin-α and -β. **B**: Western blot analysis of HA-magphinin-α and -β mutants expressed in COS cells. Cell lysates prepared from COS cells transfected with expression vectors for each HA-magphinin mutant were analyzed by Western blotting with an anti-HA antibody. Arrowheads indicate magphinin-α or -β deletion mutants. **C**: Immunofluorescence micrographs of COS cells transfected with magphinin-α and -β mutants. HA-magphinin proteins were visualized using an anti-HA antibody. Note that both deletion mutants (Δ1–406) of magphinin-α and -β changed their localization from the cytoplasm to the nucleus. Green, magphinins; red, DNA. Bars, 10 μm. **D**: Distribution of HA-magphinin-α and -β mutants in COS cells. The graph depicts quantitative data obtained from three independent experiments. In each experiment, more than 200 cells were counted in each group. N, exclusively nuclear staining; NC, cytoplasmic and nuclear staining; C, cytoplasmic staining. **E**: Western blot analysis of HA-magphinin deletion mutant proteins in detergent-soluble and -insoluble fractions. COS cells transfected with each magphinin mutant were solubilized with Triton X-100-containing buffer, and soluble and insoluble fractions analyzed by Western blotting using anti-HA antibody. TSF, Triton X-100-soluble fraction; TIF, Triton X-100-insoluble fraction. **F**: Immunofluorescence micrographs of COS cells transfected with HA-magphinin-α or -β mutants treated with Triton X-100-containing buffer before fixation. Magphinin-α and -β mutants remained nuclear after Triton X-100 extraction. Green, magphinins. Bars, 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

neither wild-type nor mutant proteins promoted growth arrest or cell death (data not shown). By contrast, when mouse fibroblast NIH3T3 cells were transfected with human magphinin-γ, we observed morphological signs of cell death in that cells became round (Fig. 6A). Similar changes were detected in NIH3T3 cells transfected with HA-magphinin-α (Δ1–

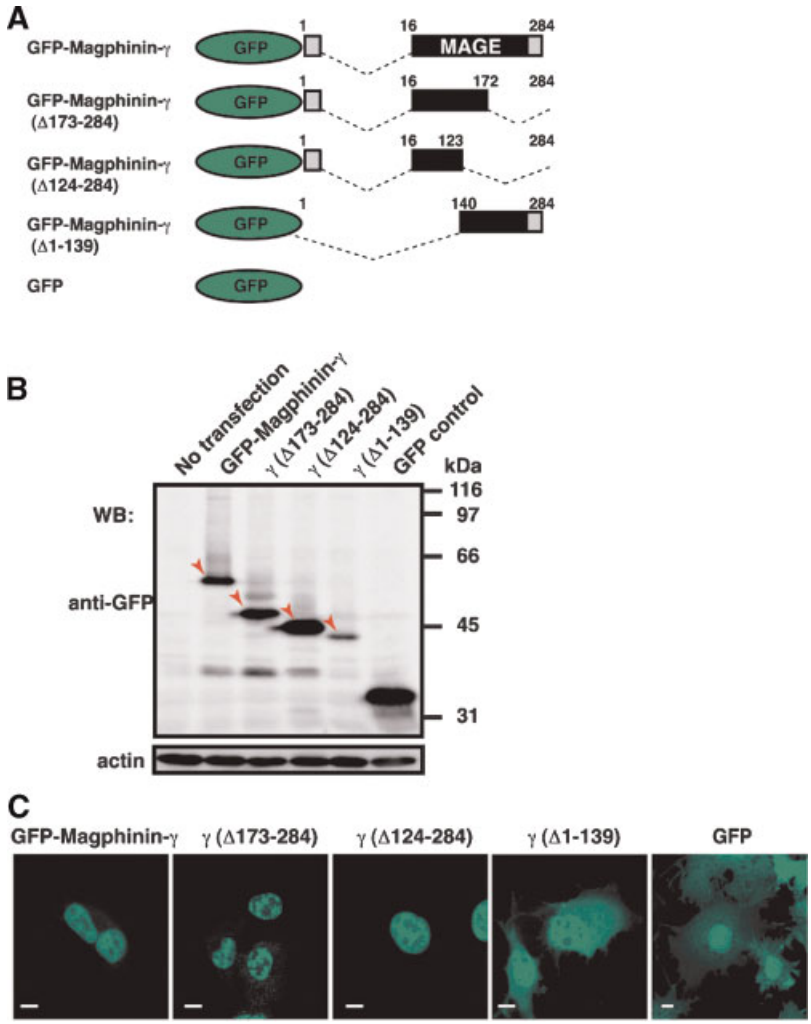

Fig. 4. Localization of magphinin-γ deletion mutants. **A**: Schematic representation of GFP-magphinin-γ and deletion mutants. **B**: Western blot analysis of GFP-magphinin-γ and deletion mutants expressed in COS cells. Equal amounts of RIPA-soluble lysates from transfected COS cells were subjected to Western blot analysis using anti-GFP antibody. Arrowheads indicate magphinin-γ or mutants. **C**: Fluorescence microscopy of COS cells transfected with GFP-magphinin-γ or mutant proteins. The Δ1–139 mutant does not accumulate in nuclei, suggesting that a nuclear localization signal is present in amino acid residues 1–123. Green, GFP-magphinin; red, DNA. Bars, 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

406) and HA-magphinin-β (Δ1–406) mutants (Fig. 6B,C).

To determine the specific stage of magphinin-γ-mediated effects on the cell cycle, cells expressing GFP-magphinins were analyzed by flow cytometry. While magphinin-α and -β showed no significant effect, GFP-magphinin-γ transfected cells showed increased numbers of cells in subG1 phase (Fig. 6D). The relative number of cells transfected with GFP-magphinin-γ in subG1 was 3.5 times higher than control ($P < 0.01$), whereas these numbers were 1–1.5 ($P > 0.1$) in GFP-magphinin-α or -β expressing cells (Fig. 6E). These results strongly suggest that magphinin-γ inhibits cell cycle progression and arrests cells at G1. Apoptosis caused by magphinin-γ was detected by TUNEL assay (Fig. 6F). No apoptosis was detected in NIH3T3 cells transfected by magphinin-α and magphinin-β (data not shown).

DISCUSSION

In this study, we characterized three isoforms, human magphinin-α, -β, and -γ, which are alternatively spliced products of the human trophinin gene. We show that magphinin-α and -β, are exclusively cytoplasmic, whereas

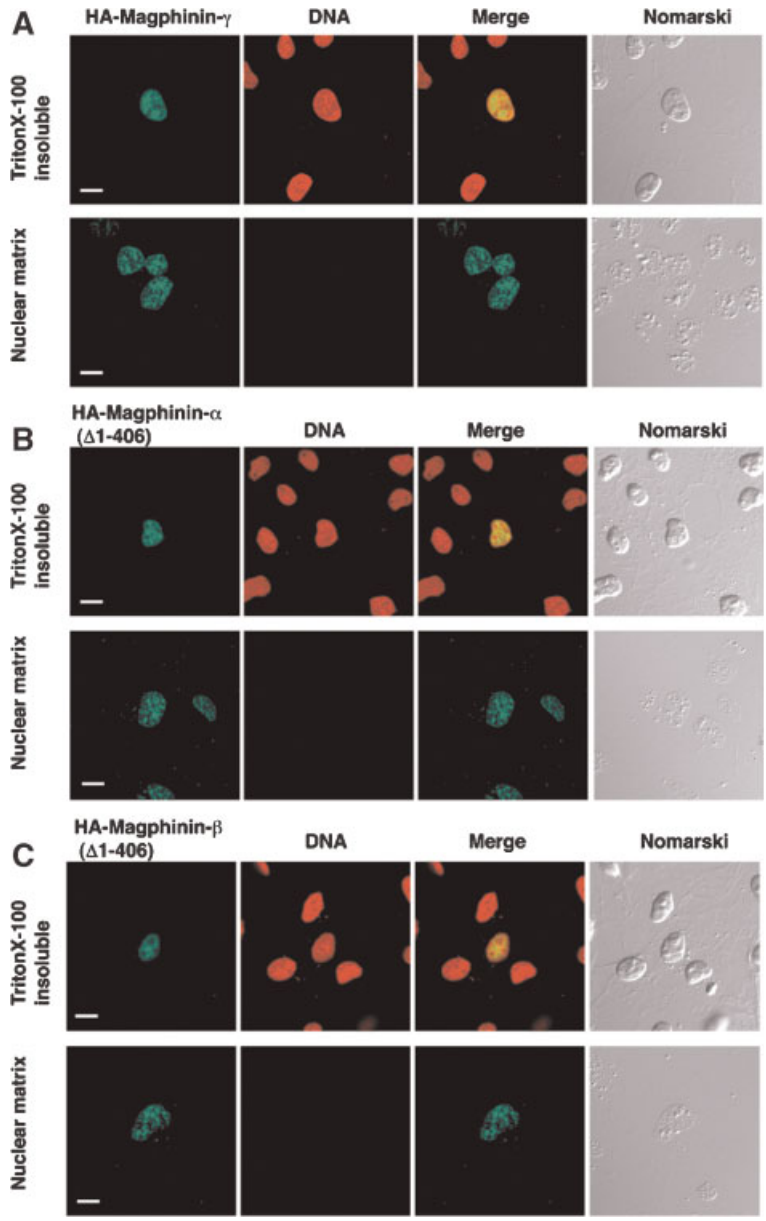

Fig. 5. Association of magphinin-γ, magphinin-α (Δ1–406) and magphinin-β (Δ1–406) with the nuclear matrix. COS cells transfected with HA-magphinin-γ (**A**), HA-magphinin-α (Δ1–406) (**B**) and HA-magphinin-β (Δ1–406) (**C**) were extracted with cytoskeleton (CSK) buffer to remove Triton-soluble fractions (**upper panels**) and then treated sequentially with DNase digestion and high salt extraction to isolate the nuclear matrix (**lower panels**). Bars, 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

magphinin-γ localizes in the nucleus. Deletion mutation of magphinin-α and -β showed that the 5′ region adjacent to the MAGE domain is necessary for cytoplasmic localization. While the N-terminus of magphinin-γ is necessary for nuclear localization, this signal is masked in magphinin-α and -β as they localize to the cytoplasm. Nuclear localization of magphinin-γ resulted in morphological changes in cells, which likely lead to cell death.

The MAGE family is a large group of proteins containing a well-conserved ~200 amino acid MAGE homology domain [Itoh et al., 1996; Kirkin et al., 1998]. Necdin, originally identified in a screen for proteins functioning in neural differentiation of P19 cells, is the

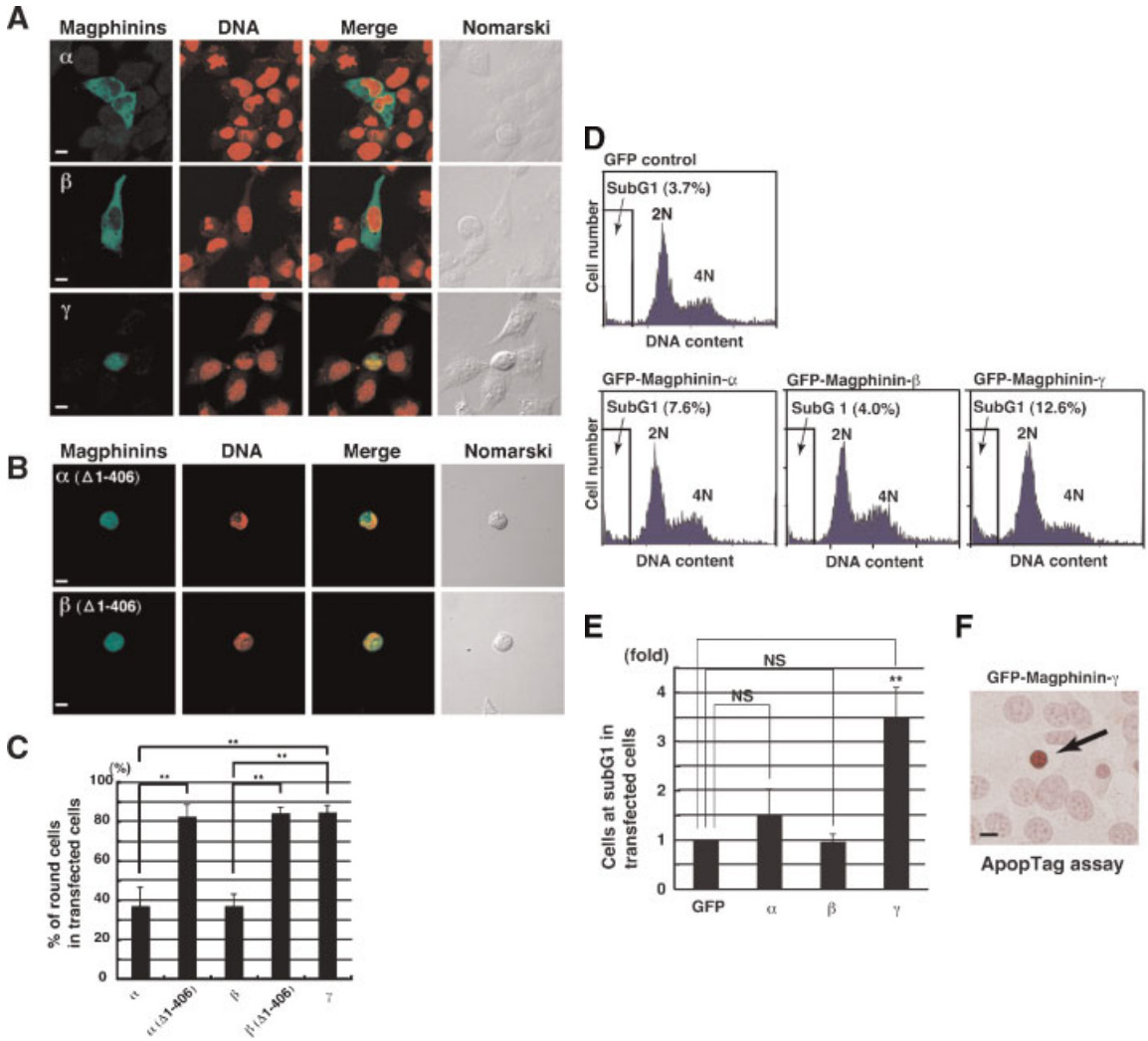

Fig. 6. Nuclear localized magphinins and cell cycle profiles of transfected cells. **A**: NIH3T3 cells were transfected with HA-magphinin-α, -β, or -γ. Twenty-four hours later, morphologies of transfected cells were compared to those in untransfected cells. Bars, 10 μm. **B**: NIH3T3 cells were transfected with HA-magphinin-α (Δ1–406) or HA-magphinin-β (Δ1–406) mutants, and morphologies inspected after 24 h. Bars, 10 μm. **C**: Quantitative analysis of rounded cells. Data represent means ± SD from three independent experiments. In each experiment, more than 200 transfected cells were counted. Asterisks indicate significant differences ($^{**}P < 0.01$) calculated by Student's *t*-test. **D**: Histograms of GFP or GFP-magphinin-expressing cells. NIH3T3 cells were transfected with GFP or GFP-magphinin isoforms and collected 48 h post-transfection when their cell cycle profiles were analyzed by flow cytometry. **E**: Quantity of DNA in subG1 phase expressing GFP-magphinins relative to that of cells expressing GFP alone. Data represent means ± SD from three independent experiments. Asterisks indicate significant differences ($^{**}P < 0.01$) calculated by the Student's *t*-test. NS, not significant. **F**: Apoptosis of NIH3T3 cells transfected for GFP-magphinin-g. Apoptotic cells were stained red, which were detected in 3T3 cells transfected for magphinin-g, but not detected in 3T3 cells transfected for magphinin-α or magphinin-β (data not shown). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

best-characterized MAGE family member [Maruyama et al., 1991]. Necdin overexpression causes cell cycle arrest in NIH3T3, HEK293, and SAOS2 cells [Hayashi et al., 1995; Taniura et al., 1998], which is potentially dependent on physical interactions of necdin with E2F-1 or p53 [Taniura et al., 1998, 1999]. It has also been reported that necdin interacts with a heterogenous nuclear ribonucleoprotein U (hnRNP U), which is a nuclear matrix-associated protein, and suppresses cell proliferation through this interaction [Dreyfuss et al., 1988; Kiledjian and Dreyfuss, 1992; Romig et al., 1992; Taniura and Yoshikawa, 2002]. Furthermore, Taniura et al. [2005] showed that amino acids 144–184 and 191–222 within the necdin MAGE domain are required for nuclear matrix targeting. We

showed here that when human magphinins localize to the nucleus, they associate with the nuclear matrix (Fig. 5). However, we did not observe a region in magphinins homologous to necdin sequences required for association with the nuclear matrix, suggesting that magphinin-γ and necdin interact with the nuclear matrix through different mechanisms.

Human magphinin-α and -β contain three potential nuclear localization signals (NLSs) at respective amino acids 65, 201, and 296 encoded in exon 3 (identified by a computer-based search using PSORT II as a predictor [Nakai and Horton, 1999]; http://psort.ims.u-tokyo.ac.jp/). However, all forms of magphinin-γ tested, and magphinin-α (Δ1–406) and magphinin-β (Δ1–406) localize to the nucleus (Figs. 2 and 3) but lack these specific NLS sequences. It is therefore likely that sequences not encoded in exon 3 are critical for magphinin nuclear localization. Indeed, our data indicate that motifs in the N-terminus of magphinin-γ (amino acids 16–123 of the MAGE domain) are required for nuclear localization (Fig. 4). Since the N-terminus does not contain a canonical NLS, these sequences remain unknown.

The cytoplasmic magphinins-α and -β may be exported to the cytoplasm through an intrinsic nuclear export signal (NES), since peptide sequences between amino acid residues 166 and 175 conform to a potential NES consensus motif [Bogerd et al., 1996; Henderson and Eleftheriou, 2000]. Magphinin-γ lacks this sequence and thus may remain nuclear. Alternatively, sequences encoded by exon 3 of magphinin-α and -β may interact directly or indirectly with a cytoplasmic protein, retaining those proteins in the cytoplasm. In magphinin-α and -β, the N-terminal region of the MAGE domain may be masked by the peptide encoded by exon 3.

To identify downstream targets of magphinin-γ, gene microarray analysis was performed. We obtained data that several nuclear proteins are down-regulated in magphinin-γ expressing cells (Aoyama, Yamaguchi and Fukuda, unpublished data). These results suggest that nuclear magphinin-γ suppresses expression of some nuclear proteins, which might be the first step for growth arrest and cell death.

Previously we showed that endogenous magphinin-α in human trophoblastic teratocarcinoma HT-H cells is nuclear [Aoyama et al., 2005]. However, here magphinin-α expressed in mouse NIH3T3 cells was cytoplasmic (Fig. 6A), suggesting that magphinin-α localization is cell type-dependent. Furthermore, COS and HeLa cells expressing nuclear HA-magphinin-γ did not show growth arrest or cell death, whereas NIH3T3 cells expressing magphinin-γ showed indications of cell death (Fig. 6). These results suggest that subcellular localization and nuclear activity of magphinins may be affected by diverse cellular milieus. Further studies are required to define activities of human and mouse magphinins expressed in different cell types.

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REFERENCES

Aoyama J, Nakayama Y, Sugiyama D, Saburi S, Nadano D, Fukuda MN, Yamagichi N. 2005. Apical cell adhesion molecule, trophinin, localizes to the nuclear envelope. FEBS Lett 579:6326–6332.

Barker PA, Salehi A. 2002. The MAGE proteins: Emerging roles in cell cycle progression, apoptosis, and neurogenetic disease. J Neurosci Res 67:705–712.

Bogerd HP, Fridell RA, Benson RE, Hua J, Cullen BR. 1996. Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel in vivo randomization-selection assay. Mol Cell Biol 16:4207–4214.

Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T, Lucas S. 2001. An overview of the MAGE gene family with the identification of all human members of the family. Cancer Res 61:5544–5551.

Dreyfuss G, Philipson L, Mattaj IW. 1988. Ribonucleoprotein particles in cellular processes. J Cell Biol 106:1419–1425.

Fukuda MN, Nozawa S. 1999. Trophinin, tastin, and bystin: A complex mediating unique attachment between trophoblastic and endometrial epithelial cells at their respective apical cell membranes. Semin Reprod Endocrinol 17:229–234.

Fukuda MN, Sato T, Nakayama J, Klier G, Mikami M, Aoki D, Nozawa S. 1995. Trophinin and tastin, a novel cell adhesion molecule complex with potential involvement in embryo implantation. Genes Dev 9:1199–1210.

Hayashi Y, Matsuyama K, Takagi K, Sugiura H, Yoshikawa K. 1995. Arrest of cell growth by necdin, a nuclear protein expressed in postmitotic neurons. Biochem Biophys Res Commun 213:317–324.

He D, Nickerson JA, Penman S. 1990. Core filaments of the nuclear matrix. J Cell Biol 110:569–580.

Henderson BR, Eleftheriou A. 2000. A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. Exp Cell Res 256:213–224.

Hirao A, Hamaguchi I, Suda T, Yamaguchi N. 1997. Translocation of the Csk homologous kinase (Chk/Hyl) controls activity of CD36-anchored Lyn tyrosine kinase in thrombin-stimulated platelets. EMBO J 16:2342–2351.

Itoh K, Hayashi A, Nakao M, Hoshino T, Seki N, Shichijo S. 1996. Human tumor rejection antigens MAGE. J Biochem 119:385–390.

Kasahara K, Nakayama Y, Ikeda K, Fukushima Y, Matsuda D, Horimoto S, Yamaguchi N. 2004. Trafficking of Lyn through the Golgi caveolin involves the charged residues on alphaE and alphaI helices in the kinase domain. J Cell Biol 165:641–652.

Kasahara K, Nakayama Y, Sato I, Ikeda K, Hoshino M, Endo T, Yamaguchi N. 2007a. Role of Src-family kinases in formation and trafficking of macropinosomes. J Cell Physiol 211:220–232.

Kasahara K, Nakayama Y, Nakazato Y, Ikeda K, Kuga T, Yamaguchi N. 2007b. Src signaling regulates completion of abscission in cytokinesis through Erk/MAPK activation at the midbody. J Biol Chem 282:5327–5339.

Kiledjian N, Dreyfuss G. 1992. Primary structure and binding activity of the hnRNP U protein: Binding RNA through RGG box. EMBO J 11:2655–2664.

Kirkin AF, Dzhandzhugazyan K, Zeuthen J. 1998. The immunogenic properties of melanoma-associated antigens recognized by cytotoxic T lymphocytes. Exp Clin Immunogenet 15:19–32.

Kobayashi M, Taniura H, Yoshikawa K. 2002. Ectopic expression of necdin induces differentiation of mouse neuroblastoma cells. J Biol Chem 277:42128–42135.

Kuwako K, Taniura H, Yoshikawa K. 2004. Necdin-related MAGE proteins differentially interact with the E2F1 transcription factor and the p75 neurotrophin receptor. J Biol Chem 279:1703–1712.

Lucas S, Brasseur F, Boon T. 1999. A new MAGE gene with ubiquitous expression does not code for known MAGE antigens recognized by T cells. Cancer Res 59: 4100–4103.

Maruyama K, Usami M, Aizawa T, Yoshikawa K. 1991. A novel brain-specific mRNA encoding nuclear protein (necdin) expressed in neurally differentiated embryonal carcinoma cells. Biochem Biophys Res Commun 178: 291–296.

Masuda Y, Sasaki A, Shibuya H, Ueno N, Ikeda K, Watanabe K. 2001. Dlxin-1, a novel protein that binds Dlx5 and regulates its transcriptional function. J Biol Chem 276:5331–5338.

Matsuda D, Nakayama Y, Horimoto S, Kuga T, Ikeda K, Kasahara K, Yamaguchi N. 2006. Involvement of Golgi-associated Lyn tyrosine kinase in the translocation of annexin II to the endoplasmic reticulum under oxidative stress. Exp Cell Res 312:1205–1217.

Mera A, Suga M, Ando M, Suda T, Yamaguchi N. 1999. Induction of cell shape changes through activation of the interleukin-3 common beta chain receptor by the RON receptor-type tyrosine kinase. J Biol Chem 274:15766–15774.

Nadano D, Sugihara K, Paria BC, Saburi S, Copeland NG, Gilbert DJ, Jenkins NA, Nakayama J, Fukuda MN. 2002. Significant differences between mouse and human trophinins are revealed by their expression patterns and targeted disruption of mouse trophinin gene. Biol Reprod 66:313–321.

Nakai K, Horton P. 1999. PSORT: A program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci 24:34–36.

Nakayama Y, Yamaguchi N. 2005. Multi-lobulation of the nucleus in prolonged S phase by nuclear expression of Chk tyrosine kinase. Exp Cell Res 304:570–581.

Nakayama J, Aoki D, Suga T, Akama TO, Ishizone S, Yamaguchi H, Imakawa K, Nadano D, Fazleabas AT, Katsuyama T, Nozawa S, Fukuda MN. 2003. Implantation-dependent expression of trophinin by maternal fallopian tube epithelia during tubal pregnancies: Possible role of human chorionic gonadotrophin on ectopic pregnancy. Am J Pathol 163:2211–2219.

Nakayama Y, Kawana A, Igarashi A, Yamaguchi N. 2006. Involvement of the N-terminal unique domain of Chk tyrosine kinase in Chk-induced tyrosine phosphorylation in the nucleus. Exp Cell Res 312:2252–2263.

Pack SD, Tanigami A, Ledbetter DH, Sato T, Fukuda MN. 1997. Assignment of trophoblast/endometrial epithelium cell adhesion molecule trophinin gene TRO to human chromosome bands Xp11.22 → p11.21 by in situ hybridization. Cytogenet Cell Genet 79:123–124.

Romig H, Fackelmayer FO, Renz A, Ramsperger U, Richter A. 1992. Characterization of SAF-A, a novel nuclear DNA binding protein from HeLa cells with high affinity for nuclear matrix/scaffold attachment DNA elements. EMBO J 11:3431–3440.

Saburi S, Nadano D, Akama TO, Hirama K, Yamanouchi K, Naito K, Tojo H, Tachi C, Fukuda MN. 2001. The trophinin gene encodes a novel group of MAGE proteins, magphinins, and regulates cell proliferation during gametogenesis in the mouse. J Biol Chem 276:49378–49389.

Salehi AH, Roux PP, Kubu CJ, Zeindler C, Bhakar A, Tannis LL, Verdi JM, Barker PA. 2000. NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. Neuron 27:279–288.

Sasaki A, Masuda Y, Iwai K, Ikeda K, Watanabe K. 2002. A RING finger protein Praja1 regulates Dlx5-dependent transcription through its ubiquitin ligase activity for the Dlx/Msx-interacting MAGE/Necdin family protein, Dlxin-1. J Biol Chem 277:22541–22546.

Spencer TE, Johnson GA, Bazer FW, Burghardt RC. 2004. Implantation mechanisms: Insight from the sheep. Reproduction 128:657–668.

Sugihara K, Sugiyama D, Byrne J, Wolf DP, Lowitz KP, Kobayashi Y, Kabir-Salmani M, Nadano D, Aoki D, Nozawa S, Nakayama J, Mustelin T, Ruoslahti E,

Yamaguchi N, Fukuda MN. 2007. Trophoblast cell activation by trophinin ligation: Possible role in human embryo implantation. Proc Natl Acad Sci USA 104:3799–3804.

Suzuki N, Zara J, Sato T, Ong E, Bakhiet N, Oshima RG, Watson KL, Fukuda MN. 1998. A cytoplasmic protein, bystin, interacts with trophinin, tastin, and cytokeratin and may be involved in trophinin mediated cell adhesion between trophoblast and endometrial epithelial cells. Proc Natl Acad Sci USA 95:5027–5032.

Tada J, Omine M, Suda T, Yamaguchi N. 1999. A common signaling pathway via Syk and Lyn tyrosine kinases generated from capping of the sialomucin CD34 and CD43 in immature hematopoietic cells. Blood 93:3723–3735.

Taniguchi N, Taniura H, Niinobe M, Takayama C, Tominaga-Yoshino K, Ogura A, Yoshikawa K. 2000. The postmitotic growth suppressor necdin interacts with a calcium-binding protein (NEFA) in neuronal cytoplasm. J Biol Chem 275:31674–31681.

Taniura H, Yoshikawa K. 2002. Necdin interacts with the ribonucleoprotein hnRNP U in the nuclear matrix. J Cell Biochem 84:545–555.

Taniura H, Taniguchi N, Hara M, Yoshikawa K. 1998. Necdin, a postmitotic neuron-specific growth suppressor, interacts with viral transforming proteins and cellular transcription factor E2 F1. J Biol Chem 273:720–728.

Taniura H, Matsumoto K, Yoshikawa K. 1999. Physical and functional interactions of neuronal growth suppressor necdin with p53. J Biol Chem 274:16242–16248.

Taniura H, Kobayashi M, Yoshikawa K. 2005. Functional domains of necdin for protein-protein interaction, nuclear matrix targeting, and cell growth suppression. J Cell Biochem 94:804–815.

Tcherpakov M, Bronfman FC, Conticello SG, Vaskovsky A, Levy Z, Niinobe M, Yoshikawa K, Arenas E, Fainzilber M. 2002. The p75 neurotrophin receptor interacts with multiple MAGE proteins. J Biol Chem 277:49101–49104.

Yamaguchi N, Fukuda MN. 1995. Golgi retention mechanism of β-1,4-galactosyltransferase: Membrane-spanning domain-dependent homodimerization and association with α- and β-tubulins. J Biol Chem 270:12170–12176.

Yamaguchi N, Nakayama Y, Urakami T, Suzuki S, Nakamura T, Suda T, Oku N. 2001. Overexpression of the Csk homologous kinase (Chk tyrosine kinase) induces multinucleation: A possible role for chromosome-associated Chk in chromosome dynamics. J Cell Sci 114:1631–1641.