Biochemical and Biophysical Research Communications **288,** 8–15 (2001)
doi:10.1006/bbrc.2001.5719, available online at http://www.idealibrary.com on IDEAL®

Constitutive Activation of Wnt/β-Catenin Signaling Pathway in Migration-Active Melanoma Cells: Role of LEF-1 in Melanoma with Increased Metastatic Potential

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Received September 3, 2001

A constitutive complex of β-catenin and LEF-1 has been detected in melanoma cell lines expressing either mutant β-catenin or mutant APC (Rubinfeld *et al., Science,* 275, 1790–1792, 1997). However, it has been recently reported that β-catenin mutations are rare in primary malignant melanoma, but its nuclear and/or cytoplasmic localization, a potential indicator of Wnt/β-catenin pathway activation, is frequently observed in melanoma (Rimm *et al., Am. J. Pathol.,* 154, 325–329, 1999). In human malignant melanoma, the appearance of the tumorigenic phase represents a capacity for metastasis and is the significant phenotypic step in disease progression. Cell motility in invasive melanoma is thought to play a crucial role in metastatic behavior. In this work, we sought to determine which transcription factor of the LEF/TCF family was preferentially involved in human melanoma from different stages of tumor progression. We show that LEF-1 mRNA expression is predominant in highly migrating cells from metastatic melanomas. These actively migrating melanoma cells showed nuclear and cytoplasmic accumulation of β-catenin and active transcription from a reporter plasmid of the LEF/TCF binding site. These results may provide a new insight into the role of the Wnt/β-catenin signaling pathway in the tumor progression of malignant melanoma. © 2001 Academic Press

Key Words: **Wnt; β-catenin; LEF-1; melanoma; cell motility.**

Signal transduction mediated by Wnt/β-catenin plays diverse and important roles in the embryonic development of *Xenopus* and *Drosophila* (1, 2). In mammalian cells, several lines of evidence link deregulated Wnt signaling to tumor progression. The wnt-1 proto-oncogene, which stabilizes β-catenin (3, 4), promotes tumor formation when expressed in mouse mammary tissue (5). In colon carcinoma, enhanced β-catenin signaling may result from inactivation of the APC tumor suppressor gene, the product of which downregulates β-catenin in cultured cells (6). Moreover, missense mutations identified in the β-catenin gene in cancer cells promote the stabilization of the protein and prevent its suppression by the APC tumor suppressor protein (7, 8). Thus, these findings suggest that stabilized β-catenin contributes to the development of human tumors.

The LEF-1 transcription factor was originally identified as a T cell-specific high mobility group DNA binding protein that binds to a specific motif in the minimal T cell receptor α enhancer region (9, 10). The LEF-1 and a *Xenopus* homolog XTCF-3 were found to bind to β-catenin at a site localized to the amino-terminal region of LEF-1/XTCF-3 proteins (11, 12). Both proteins have been shown to functionally interact with β-catenin for axis duplication of *Xenopus* embryos. A *Drosophila* homolog of the LEF-1 protein has shown to act downstream of the β-catenin homolog *armadillo* in the *wingless* signaling pathway (13, 14). These observations suggest that deregulation of β-catenin may result in its active interaction with a LEF/TCF transcription factor and then activate specific target genes as an endpoint in carcinogenesis.

A constitutive complex of β-catenin and LEF-1 has been detected in melanoma cell lines expressing either mutant β-catenin or mutant APC (7). Recently, it has been reported that β-catenin mutations are rare in primary malignant melanoma, but its nuclear and/or cytoplasmic localization, a potential indicator of Wnt/β-catenin pathway activation, is frequently observed in melanoma (15, 16). In human malignant melanoma,

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0006-291X/01 $35.00

the appearance of the tumorigenic phase with the capacity for metastasis is a pathogenic or phenotypic step in disease progression (17–19). In invasive malignant melanoma, cell motility is thought to play a crucial role in metastatic behavior.

In this study, we sought to determine which LEF/TCF transcription factor of the family was preferentially involved in human melanoma from different stages of tumor progression. We show that LEF-1 mRNA expression is predominant in highly migrating cells from metastatic melanomas. These highly migrating cells activated transcription from a reporter plasmid containing LEF/TCF binding site. Furthermore, dominant negative LEF-1 inhibited the migration activity of the most actively migrating MM-RU cells. These results suggest that activation of β-catenin coupled with LEF-1 expression may contribute to the migration activity of malignant melanoma with metastatic potential.

MATERIALS AND METHODS

Cells, plasmids, and antibodies. Human melanoma cell lines, RPM-MC, MM-LH, MM-AN, MM-BP, RPM-EP, and MM-RU were isolated as described previously (20). Briefly, RPM-EP and RPM-MC were established from a dermal nodule of a recurrent primary cutaneous malignant melanoma, representing the vertical growth phase of primary cutaneous melanoma. MM-LH, MM-AN, MM-BP, and MM-RU were derived from lymph node metastases. These cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), penicillin G, and streptmycin sulfate. Normal human melanocytes were purchased from Clonetics and maintained in melanocyte growth media (Clonetics, San Diego, CA). The Jurkat T cell leukemia cell line was maintained in RPMI 1640 supplemented with 10% FCS. The cultures were kept in a 5% CO_2 and 95% air humidified atmosphere at 37°C.

The wild type LEF/TCF reporter plasmid (TOPFLASH) and mutant binding reporter plasmid (FOPFLASH) were obtained from Upstate biotechnology, NY. These contain three copies of the LEF/TCF binding sequence (5′-AGATCAAAGGG-3′) or the mutant sequence (5′-AGGCCAAAGGG-3′) upstream of the herpes simplex virus thymidine kinase (TK) minimal promoter and luciferase open reading frame. The pRL-TK vector contains the TK promoter upstream of the *Renilla* luciferase gene (Promega, Madison, WI), that serves for normalization to transfection efficiency.

Human LEF-1 cDNA was isolated by amplification of the entire coding sequence by polymerase chain reaction (PCR) from cDNA of Jurkat T cells. The cDNA was inserted into the Myc-taged pcDNA3.1 (Invitrogen, CA) expression vector carrying the cytomegalovirus promoter. A deletion mutant, ΔNLEF-1, was prepared similarly by using PCR, in which primers were designed by deleting the amino-terminal lesion (1–68 amino acid) of LEF-1 cDNA. The nucleotide sequences of the PCR products were determined using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, CA) followed by electrophoresis on an ABI PRISM 310 DNA autosequencer (Applied Biosystems, CA).

Anti-β-catenin and anti-APC monoclonal antibodies were obtained from Transduction Laboratories (KY) and Pharmingen (CA), respectively. Anti-Myc monoclonal antibodies were provided by Medical & Biological Laboratories (MBL) (Nagoya, Japan). Antibodies against α-tubuline and I-κB-α were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Reverse transcription (RT)-PCR. Total RNA was extracted from cells using Isogen (Nippon Gene, Toyama, Japan). The total RNA (1 μg) was used for a reverse transcriptional reaction. PCR was then carried out in a final volume of 50 μl containing 2.5 U of Ex Taq polymerase (Takara, Ohtsu, Japan) and 2 μl of the RT reaction in a buffer as described in the instruction manual. The following primers were used: LEF-1 sense, 5′-CCAGCTATTGTAACACCTCA-3′; LEF-1 antisense, 5′-TTCAGATGTAGGCAGCTGTC-3′; TCF-1 sense, 5′-TGACCTCTCTGGCTTCTACT-3′; TCF-1 antisense, 5′-TTGATGGTTGGCTTCTTGGC-3′; TCF-3 sense, 5′-AGGAAATCACCAGTCACCGT-3′; TCF-3 antisense, 5′-GTACTTGGCCTGTTCTTCTC-3′; TCF-4 sense, 5′-TTCAAAGACGACGGCGAACAG-3′; TCF-4 antisense, 5′-TTGCTGTACGTGATAAGAGGCG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5′-GTATCGTGGAAGGACTCATG-3′; and GAPDH antisense, 5′-AGTGGG-TGTCGCTGTTGAAG-3′. The PCR conditions for each set of primers were 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 56°C for 45 s, and 72°C for 1 min. The PCR products were analyzed on 2.0% agarose gel.

Northern blot analysis. The poly(A)$^+$ RNA was purified using Oligotex-dT30 Super (Takara, Ohtsu Japan). Then, 3 μg poly(A)$^+$ RNA was denatured with 2.2 M formaldehyde and electrophoresed in a formaldehyde-agarose gel. RNA was transferred to a nylon filter and hybridized overnight with ^{32}P-labeled cDNA at 42°C in a mixture containing 50% formamide, 4× SSC, 100 μg sonicated and denatured salmon sperm DNA, and 10 μg poly(A). The filter was washed in 0.1× SSC, 0.1% SDS at 68°C for 20 min.

Transfection, luciferase assay, and immunoblotting. Cells (1×10^5 cells per well) were seeded into 24-well plates. Reporter plasmids were transfected by means of lipofection using Lipofectamine 2000 (GibcoBRL, Gaithersburg, MD) after 36 h, and the luciferase activity was measured using the Dual-Luciferase Reporter assay system (Promega, Madison, WI). Each assay was independently repeated three times and its average value was indicated. The activity was dependent on the incubation time and protein concentration used for the assay.

For establishment of cells expressing ΔNLEF-1, plasmid DNA was transfected into 5×10^5 cells in a 60-mm petri dish, and cells were maintained with MEM supplemented with 10% FCS for 2 days and followed by the medium containing G418 geneticine (GibcoBRL, Gaithersburg, MD) (600 μg/ml) for 14 days. Fifteen geneticine-resistant clones were isolated and used for further cell tracking assays. Similarly, mock transfectants (vector plasmid alone) were also isolated.

For preparation of cytoplasmic and nuclear extracts, cells were lysed in hypotonic buffer (10 mM Hepes, pH 7.9, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 mM PMSF, 0.5% NP-40) and separated into cytoplasmic and nuclear fractions (21). The isolated nuclear extracts were prepared by suspending the pellet in a buffer consisting of 15 mM Hepes, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.4 M NaCl and collecting the supernatant after centrifugation. For preparation of total cell lysate, cells were disrupted by suspension in RIPA buffer (21) followed by sonication and centrifuged for 10 min at 4°C, and then the supernatant was used for immunoblotting. Each cell extract (10 μg of protein) was assayed for immunoblotting with appropriate monoclonal antibodies, biotinylated antibody pairs, and streptavidin-conjugated horseradish peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, UK). It should be noted that in the comparison of the cytoplasmic and nuclear fractions, the same amount of protein of each fraction was used; that is, the original cell number for the nuclear fraction was 6 times that of the cytoplasmic fraction.

Migration assays. Migration assays were performed as described previously with slight modifications (20, 22, 23). Briefly, subconfluent cultures of cells were seeded onto coverslips at a density between 0.4 and 1.0 cells per 10^4 μm^2. After a minimum of 4 h of incubation (37°C, 5% CO_2, 95% air humidified atmosphere), cell migration was studied over a 3-h period under a Nicon Diaphot inverted microscope in a hermetically sealed Plexiglass Nicon NP-2 incubator at 37°C in a 5% CO_2 and 95% air environment. Cell migration was recorded

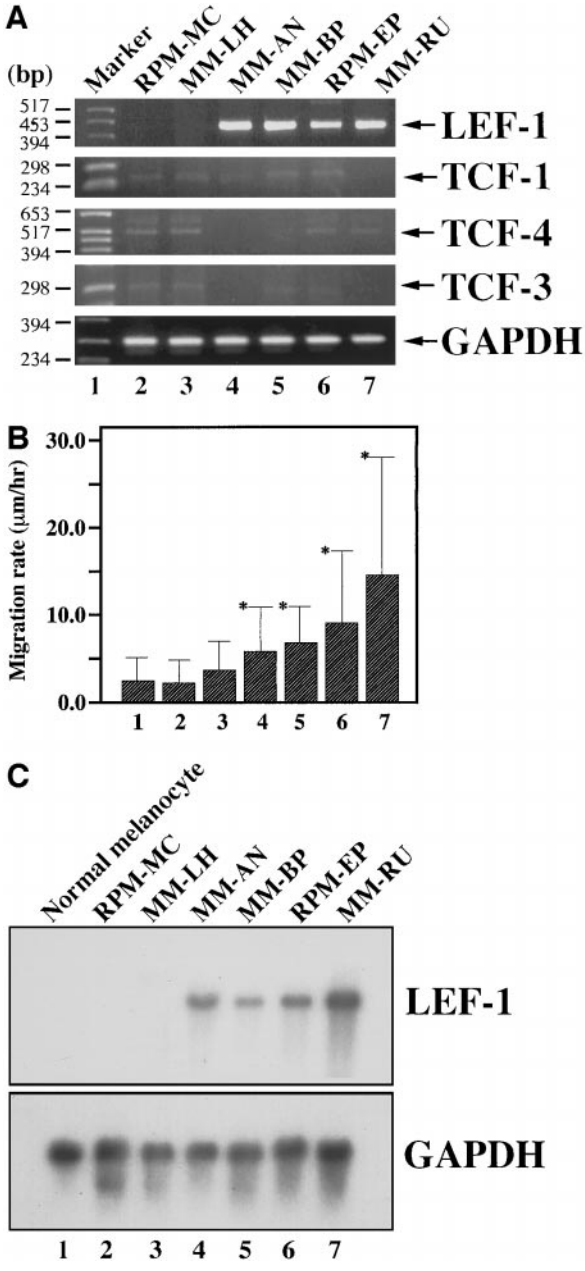

FIG. 1. Predominant expression of LEF-1 mRNA in highly migrating melanoma cells. (A) LEF/TCF mRNA expression was evaluated by RT-PCR analysis in melanoma cell lines. One microgram of total RNA that was extracted from each melanoma cell line was used for reverse transcription. PCR was then performed with the primers as described in Materials and Methods. The PCR condition was 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 56°C for 45 s, and 72°C for 1 min. Upper panel, LEF-1; upper middle panel, TCF-1; middle panel, TCF-4; lower middle panel, TCF-3; lower panel, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Lane 1, marker; lane 2, RPM-MC; lane 3, MM-LH; lane 4, MM-AN; lane 5, MM-BP; lane 6, RPM-EP; lane 7, MM-RU. (B) Random migration rate of the human melanoma cell lines and normal human melanocytes. Cell-migrating potentials have been analysed by time-lapse and image analysis as described under Materials and Methods. *Significant difference in migration rate (*t* test; $P < 0.001$) relative to normal melanocytes, RPM-MC, and MM-LH. Lane 1, normal human melanocyte; lane 2, RPM-MC; lane 3, MM-LH; lane 4, MM-AN; lane 5, MM-BP; lane 6, RPM-EP; lane 7, MM-RU. Error bar, SD ($n = 30$) (C) Analysis of LEF-1 mRNA level by Northern blotting. Poly(A)$^+$ RNA was extracted from melanoma cell lines and normal human melanocytes. Northern blot analysis was then performed on 3 μg poly(A)$^+$ RNA using ^{32}P-labeled cDNA probes described in the text. Upper panel, LEF-1; lower panel, GAPDH. Lane 1, normal human melanocyte; lane 2, RPM-MC; lane 3, MM-LH; lane 4, MM-AN; lane 5, MM-BP; lane 6, RPM-EP; lane 7, MM-RU.

using a video camera and a time-lapse videocassette recorder. Image analysis was performed by playing back video images, digital saving of images at hours 0, 1, 2, and 3, and tracing the migration paths of individual cells on the video monitor. The migration rate was defined by the algebraic sum of the hourly two-dimensional migration distances divided by 3 h, resulting in micrometers per hours. The migration of 30 cells was analyzed for each experimental condition.

For cell tracking assay, coverslips were coated with colloidal gold particles according to the procedure of Albrecht-Buehler (24) and thoroughly rinsed with MEM. Four thousand cells were seeded on these coverslips, which were then placed in 35-mm petri dishes and cultured with MEM supplemented with 10% FCS for 8 h. To assess cell migration, we employed computer analysis to monitor the microscopic image of the tracks made by cells and measured the distance of the tracks. Single cells in the field were chosen randomly for assessment, and 30 cells were monitored in each migration assay.

Statistical analysis. Data were presented as mean ± standard deviation (SD). Statistical differences between the two groups were evaluated using the unpaired Student's *t* test. The calculations were performed by means of the Statview software (Abacus Concepts, Berkley, CA). *P* values of less than 0.05 were considered significant.

RESULTS

Predominant Expression of LEF-1 mRNA in Migration-Active Melanoma Cell Lines

There are four known members of the LEF and TCF family in mammals: the lymphoid-specific factors LEF-1 and TCF-1, TCF-4 characterized in colon carcinoma, and the less well characterized TCF-3 (25). To determine which LEF/TCF transcription factor of the family is involved in human melanoma from different stages of tumor progression, expression of LEF-1, TCF-1, TCF-3, and TCF-4 was surveyed among melanoma cell lines using reverse transcription-polymerase chain reaction (RT-PCR). Although all of the melanoma cell lines expressed more than one of the LEF/TCF genes, only LEF-1 mRNA was expressed in essentially highly migrating melanoma cell lines (Fig. 1A). Their cell-migrating potentials have also been confirmed by time-lapse and image analysis (Fig. 1B), and well characterized by Byers *et al.* (20). Northern blot analysis showed that LEF-1 mRNA was predominantly expressed in highly migrating cell lines (Fig. 1C, lanes 4–7), but neither in normal melanocytes (lane 1) nor in low migrating melanoma cells (lane 2 and 3). The results demonstrate that the expression of LEF-1 transcription factor was augmented in actively migrating melanoma cells.

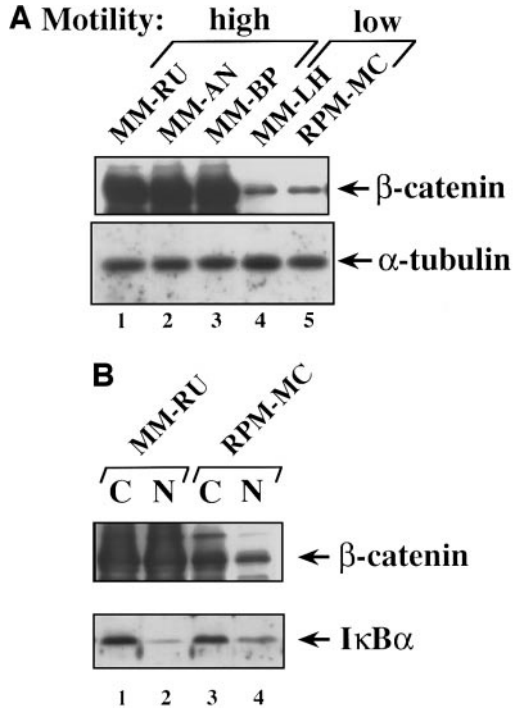

FIG. 2. Nuclear accumulation of β-catenin in actively migrating melanoma cells. (A) Abundant expression of β-catenin protein in actively migrating melanoma cells. Cells were disrupted by suspension in RIPA buffer, followed by sonication. The samples then were centrifuged for 10 min at 4°C, and the supernatant (10 μg of protein) was used for the Western blot analysis using anti-β-catenin (upper panel), and anti-α-tubulin (lower panel) antibodies. Lane 1, MM-RU; lane 2, MM-AN; lane 3, MM-BP; lane 4, MM-LH; lane 5, RPM-MC. Notably, level of β-catenin was increased in the actively migrating melanoma cells (lanes 1–3). (B) Nuclear accumulation of β-catenin in actively migrating melanoma cells. The cytoplasmic (C) and nuclear (N) fractions of the cells were analyzed by Western blotting using anti-β-catenin (upper panel), and anti-I-κBα (lower panel) antibodies. Lanes 1 and 2, MM-RU; lanes 3 and 4, RPM-MC. Notably, level of β-catenin in the nucleus was increased in the actively migrating melanoma MM-RU cells.

Nuclear Accumulation of β-Catenin in Highly Migrating Melanoma Cells

Previous studies have shown that activation of the Wnt/β-catenin pathway results in the free cytoplasmic pool of β-catenin and its nuclear translocation. Mutations in the APC tumor suppressor gene or the amino terminus of β-catenin itself have also been shown to result in increased levels of β-catenin, its nuclear translocation, and transcriptional activation of the LEF/TCF recognition site (7, 8, 26). We then tested the abundance and subcellular localization of β-catenin in the melanoma cell lines. Whole cell extracts from the melanoma cells were analyzed by Western blotting employing anti-β-catenin monoclonal antibodies. Figure 2A shows that the level of β-catenin was increased in the most actively migrating melanoma cells (lanes 1–3), compared to the less actively migrating cells (lanes 4 and 5). Furthermore, cytoplasmic and nuclear extracts were prepared from the cells, and then analyzed by immunoblotting using the anti-β-catenin monoclonal antibodies. As shown in Fig. 2B, β-catenin was accumulated in the nuclear fraction of the actively migrating melanoma MM-RU cells (lane 2), but not the less actively migrating RPM-MC cells (lane 4). Levels of cytoplasmic protein I-κBα (27) between MM-RU and RPM-MC cells were almost equal in each fraction. These results suggest that the Wnt/β-catenin signaling pathway be activated in actively migrating melanoma cells.

To assess the prevalence of mutations in β-catenin to the melanoma cell lines, we carried out sequencing studies on the amino terminus of β-catenin. However, we did not find any mutations in these cell lines (data not shown). Surveying the expression of APC tumor suppressor protein in the cell lines, neither large deletion nor truncation was observed (data not shown).

The LEF/TCF Reporter Plasmid Was Activated in Highly Migrating Melanoma Cells

In the present study, actively migrating melanoma cells showed nuclear accumulation of β-catenin. In order to investigate whether there was a correlation between LEF-1 expression and nuclear β-catenin accumulation in these more actively migrating melanoma cells, we employed a set of reporter plasmids in a β-catenin/LEF-1 reporter gene assay (28, 29). It contained three copies of the optimal LEF/TCF binding motif, or three copies of the mutant motif, upstream of a minimal herpes virus thymidine kinase promoter driving luciferase expression (TOPFLASH and FOPFLASH, respectively). Transient transfection was performed in those melanoma cell lines. The TOPFLASH reporter was significantly transcribed on actively migrating melanoma cells in which level of β-catenin was increased, but no enhancement of transcription was detected in cells transfected with the negative control plasmid FOPFLASH (Fig. 3A). These results suggest a functional correlation between LEF-1 expression and accumulation of β-catenin in the more actively migrating melanoma cells.

To evaluate whether this activation of LEF/TCF binding site is evoked via the transcription factor LEF-1, a dominant negative-type Lef-1 mutant (ΔNLEF-1) was expressed into the actively migrating MM-RU cells. The mutant ΔNLEF-1 deleted the 1–67 amino-terminus residues (amino acid 68–399) and was not able to bind to β-catenin (Fig. 3B) (30). The higher transcription of LEF/TCF binding site was reduced by enforced expression of ΔNLEF-1 in a dose-dependent fashion (Fig. 3C). This suggests that the enhanced transcription of the LEF/TCF site in migration-active MM-RU melanoma cells be via the transcription factor LEF-1 and β-catenin.

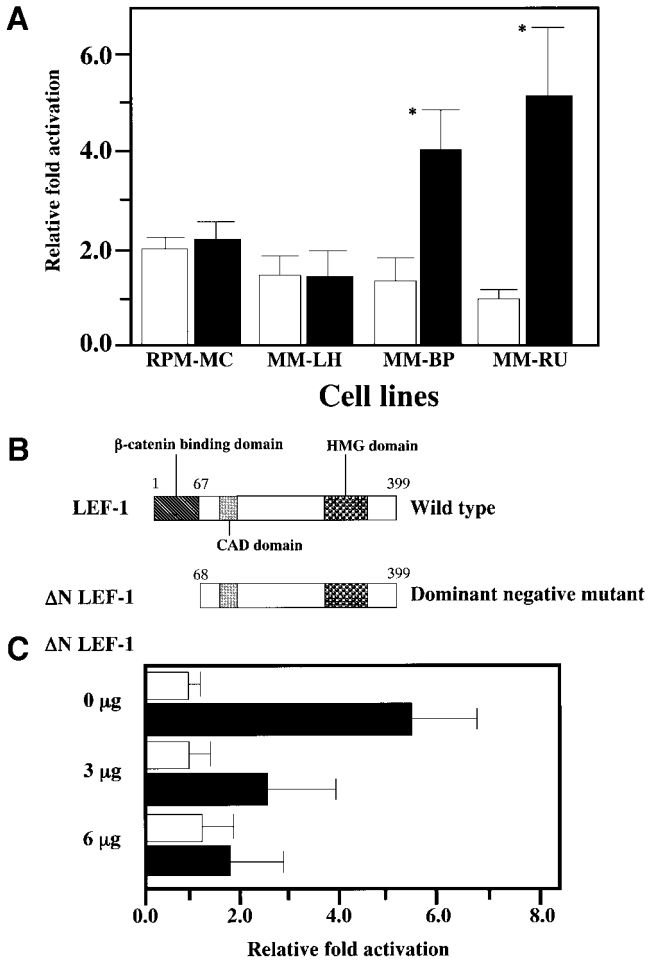

FIG. 3. The LEF/TCF reporter plasmid was activated in actively migrating melanoma cells. (A) Functional correlation between LEF-1 expression and accumulation of β-catenin. The luciferase reporter plasmid containing the three optimal LEF/TCF binding sites (TOPFLASF) was transiently transfected into the melanoma cell lines (RPM-MC and MM-LH, low migrating cells; MM-BP and MM-RU, highly migrating cells), and the luciferase activity was measured at 36 h posttransfection. Closed bar, TOPFLASF reporter plasmid; open bar, FOPFLASF mutant binding site. *Significant difference in the luciferase activity (t test; $P < 0.05$) relative to RPM-MC and MM-LH. Error bar, SD ($n = 3$). (B) Schematic representation of dominant negative LEF-1 mutant (ΔNLEF-1). ΔNLEF-1 (amino acid 68–399) is missing the β-catenin binding domain. (C) Dominant negative LEF-1 mutant inhibited the transcription of the LEF/TCF binding site in the actively migrating MM-RU cells. MM-RU cell were transfected with the reporter plasmid (TOPFLASH or FOPFLASF) and ΔNLEF-1 expression plasmid. The higher transcription of the TOPFLASH reporter plasmid was reduced by enforced expression of ΔNLEF-1 in a dose-dependent manner.

Dominant Negative LEF-1 Mutant Suppressed Migration Activity of MM-RU Cells

To examine whether ΔNLEF-1 can suppress the migration activity of MM-RU cells, we established the stable transformants expressing ΔNLEF-1, and then evaluated the random migration activity of the transformants by analyzing the cell-tracking image. Figure 4A shows representative results of cell tracking on gold particle-coated glass. The protein expression of ΔNLEF-1 was confirmed by immunoblotting (Fig. 4B). The TOPFLASH reporter was also inhibited in the cells expressing ΔNLEF-1 (data not shown). The distance showed by cell tracking was significantly reduced in ΔNLEF-1 transformants, compared to control transformants. The time-lapse and image analysis showed that the relative migration rate in the cells expressing ΔNLEF-1 was reduced to about a third of that of the control transfectants (Fig. 4C). These results indicate

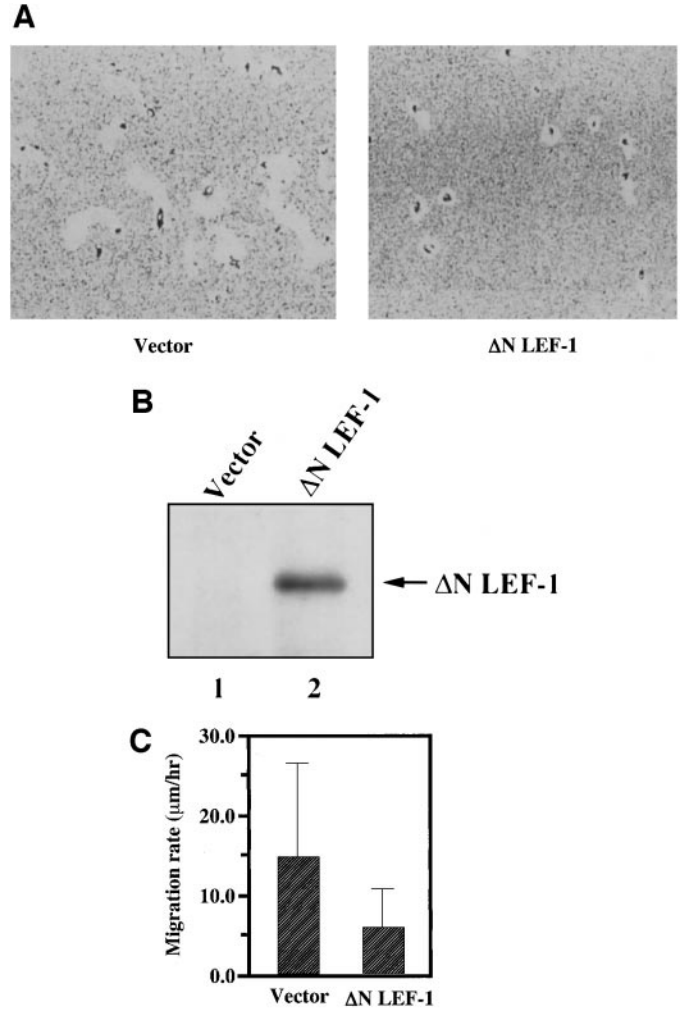

FIG. 4. Dominant negative LEF-1 mutant suppressed migration activity of MM-RU cells. ΔNLEF-1 that was fused by Myc-tag was constitutively expressed in MM-RU cells. The migration rate was assayed as described under Materials and Methods. (A) Representative results of cell tracking on gold particle-coated glass. Vector, mock transfectant of MM-RU cell; ΔNLEF-1, ΔNLEF-1-expressing MM-RU cell. (B) The stable expression of ΔNLEF-1 in MM-RU cells. The stable transformants were disrupted by RIPA buffer, and 10 μg of protein was analyzed by Western blotting anti-Myc antibodies. Lane 1, mock; lane 2, ΔNLEF-1 transformant. (C) Mean random migration rate was measured by time-lapse and image analysis. ΔNLEF-1 transformants suppressed mean random migration rate (t test; $P < 0.001$). Error bar, SD ($n = 30$).

that ΔNLEF-1 has the ability to inhibit the migration activity of MM-RU cells.

DISCUSSION

Invasive melanoma is proposed to involve at least three steps: (i) the capacity to bind the basement membrane; (ii) the ability to digest the basement membrane; and (iii) the capacity for cell migration (31–33). Especially, cell motility in the latter step is thought to play a crucial role in the metastatic behavior of melanoma. We have shown that LEF-1 in the TCF family was preferentially expressed in more actively migrating melanoma cells, compared to less actively migrating melanoma cells and normal melanocytes. The initial involvement of LEF-1 coupled with mutant β-catenin or mutant APC has been previously reported in melanoma cell lines (7), but its significance in the tumor progression had not been determined. On the other hand, activation of the target genes via LEF-1 has been shown to result in neoplastic transformation of chicken embryo fibroblasts (34), suggesting that LEF-1 has an oncogenic potential. Recently Hovanes *et al.* has reported that LEF-1, which is normally not expressed in intestinal epithelium, is a potent target gene ectopically activated in colon cancer (30), leading to inappropriate activation of Wnt/β-catenin signaling pathway. As LEF-1 is not expressed in normal melanocytes and in less actively migrating melanoma cells, it is likely that expression of LEF-1 during the development of melanoma may be involved in an increase of the migration activity as a step in tumor progression.

In the study of β-catenin mutations in melanoma cell lines by Rubinfeld *et al.* (7), 22% of the cell lines had mutations affecting the amino-terminal region of the protein, which is a region essential for the targeted degradation of β-catenin. However, based on immunohistochemical and DNA sequencing studies in 65 melanoma specimens, Rimm *et al.* (15) has reported that β-catenin mutations are rare in primary melanoma (2%: one of 50 melanomas). Nonetheless, nuclear and/or cytoplasmic localization of β-catenin, a potential indicator of the activation, was frequently observed in primary melanomas. Similar activation of β-catenin without mutations has been reported in prostate cancer (35). The discrimination between cell lines and clinical specimens might be explained by the possibility that β-catenin mutations may have arisen during *in vitro* culture, or that melanomas with β-catenin mutations may be more easily adapted to *in vitro* culture than melanomas lacking β-catenin mutations. In the present study, the more actively migrating melanoma cell lines showed that β-catenin without mutation was accumulated in the cytoplasm and nucleus: this was in sharp contrast to the low cell-motility cell lines. β-catenin activation has been suggested to be a focal and a subclonal alteration in the majority of tumor specimen (15). From this point of view, the cell lines used in this study appear to mimic the *in vivo* status of melanoma without β-catenin mutations. Taken together with these observations, a subclone with β-catenin activation may possess an active migrating activity, although its molecular mechanism remains be elucidated.

In the majority of colorectal cancers, mutations in the APC gene are responsible for β-catenin activation. Rubinfeld *et al.* (7) previously found evidence of β-catenin activation as a result of APC inactivation in two of the 27 melanomas. In the melanoma that we have studied, we did not observe large deletion in the APC protein (data not shown). Therefore, APC inactivation is unlikely to underlie β-catenin activation in the actively migrating melanoma cell lines. Rather than by APC inactivation, glycogen synthase kinase-3β (36–39), axin (37–42), or other elements in the Wnt signaling pathway may contribute to β-catenin activation in melanoma.

The neural crest is a multipotent population of ectoderm-derived cells that originate around the site and at the time of dorsal neural tube closure, migrate over defined pathways in the embryos, and differentiate into numerous cell types (43, 44). The process of cell lineage restriction and the path of cell migration are determined by a combination of environmental influences, intercellular interactions, and intrinsic factors (45). Melanocytes are specialized cells that produce melanin-based pigment and are responsible for coloration of the eye, skin, and hair. Vertebrate melanocytes are derived from the neural crest, and the Wnt signaling is important in many developmental processes including neural crest-derived melanocytes development (46). Dunn *et al.* (47) have reported that Wnt1 signaling is involved in both expansion and differentiation of migrating neural crest-derived melanocytes in the developing mouse embryo. Similarly, it is also likely that β-catenin activation coupled with LEF-1 expression is involved in cell-migration activity in transformed melanocytes.

In the present study, the dominant negative LEF-1 mutant could suppress the migrating activity of the actively migrating MM-RU cells, but its inhibitory effect on the cell line appears incomplete, compared to control normal melanocytes or less actively migrating cell lines. This suggests that the migrating activity of melanoma may not be simply regulated by activation of LEF-1 and β-catenin. Since multiple genetical alterations are thought to be accumulated during the development of melanoma, genes associated with cell adhesion and motility also may be disintegrated to the aberrant cell migration. Nonetheless, it appears that β-catenin and LEF-1 expression in melanoma cells contributes to their cell migration, and this would provide a new insight into the role of the Wnt signaling pathway in the tumor progression of malignant melanoma.

ACKNOWLEDGMENTS

We greatly appreciate the skillful technical assistance of Ms. Kyoko Okamaoto, Ms. Noriko Hayashi, and Ms. Izumi Nozawa. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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