Morphological Transformation Induced by Activation of the Mitogen-activated Protein Kinase Pathway Requires Suppression of the T-type Ca\(^{2+}\) Channel*

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Transformation of fibroblasts by various oncogenes, including ras, mos, and src accompanies with characteristic morphological changes from flat to round (or spindle) shapes. Such morphological change is believed to play an important role in establishing malignant characteristics of cancer cells. Activation of the mitogen-activated protein kinase (MAPK) pathway is a converging downstream event of transforming activities of many oncogene products commonly found in human cancers. Intracellular calcium is known to regulate cellular morphology. In fibroblasts, Ca\(^{2+}\) influx is primarily controlled by two types of Ca\(^{2+}\) channels (T- and L-types). Here, we report that the T-type current was specifically inhibited in cells expressing oncogenically activated Ras as well as gain-of-function mutant MEK (MAPK/extracellular signal-regulated kinase (ERK) kinase, a direct activator of MAPK), whereas treatment of ras-transformed cells with a MEK-specific inhibitor restored T-type Ca\(^{2+}\) channel activity. Using a T-type Ca\(^{2+}\) channel antagonist, we further found that suppression of the T-type Ca\(^{2+}\) channel by the activated MAPK pathway is a prerequisite event for the induction and/or maintenance of transformation-associated morphological changes.

Oncogenically transformed cells display many properties that are not observed in normal cells. One such transformed phenotype is the change in cell morphology. For example, the introduction of oncogenically activated Ras into fibroblasts induces a round-up or spindle-shaped morphology, which in turn disrupts the regulatory mechanisms controlling cell-cell contact and cell-substratum adhesivity and, thus, is believed to contribute to malignant (metastatic) phenotypes of cancer cells (reviewed in Ref. 1). Intracellular calcium is known to play an important role in establishment of cellular morphology. Early studies showed the calcium sensitivity of actin and actin-binding proteins (reviewed in Ref. 2), major cytoskeletal components (reviewed in Refs. 3 and 4). Moreover, calcium ions regulate the formation of actin bundles and networks (5, 6). It has also been shown that ectopic expression of calmodulin, a major intracellular Ca\(^{2+}\)-binding protein (reviewed in Ref. 7) or administration of calmodulin-antagonists disrupts Ca\(^{2+}\) homeostasis, leading to changes in cell morphology and cytoskeleton organization (8). Microinjection of villin, a Ca\(^{2+}\)-regulated F-actin bundling and nucleating protein (reviewed in Refs. 9 and 10), into NIH 3T3 cells results in disruption of the stress fiber networks, leading to morphological changes (11). These findings indicate that calcium signaling is important for maintaining proper cell morphology.

In fibroblasts, two major voltage-dependent Ca\(^{2+}\) channels are present: L (long-lasting, large conductance)-type and T (transient)-type channels (12, 13). L- and T-type channels are distinguished by differences in their electrophysiological and pharmacological properties (reviewed in Refs. 14–19). The L-type channel requires strong depolarization for activation, shows high sensitivity to dihydropyridines, and inactivates slowly. In contrast, the T-type is a rapidly inactivating channel activated by weak depolarizations and is resistant to organic blockers of L-type channels but relatively sensitive to mibebradil. Because of the low fluctuation of membrane potential in fibroblasts, T-type channels are thought to impart a greater influence on fibroblast physiology than L-type (13, 20, 21). In Swiss 3T3 fibroblasts transformed by various oncogenes (i.e., v-ras, v-fms, or polyoma virus middle T oncogenes), T-type channel currents have been shown to be specifically suppressed (12).

Ras is a plasma membrane-localized GDP/GTP-binding protein that is active in the GTP-bound state and functions as an extracellular mitogenic signal transducer. Activating mutations of Ras results in constitutive signaling to downstream elements, leading to cellular transformation (reviewed in Refs. 22 and 23). To date, three major effector pathways of Ras have been characterized in detail: the mitogen-activated protein kinase (MAPK)1 pathway, the phosphatidylinositol 3-kinase pathway, and the Ras/Raf/MEK/GSK3 pathway (40–42). In the MAPK pathway, activated Ras promotes the movement of Raf to the plasma membrane where it becomes a functional kinase (26–31). Raf then phosphorylates MAPK kinases (MEK) (32–36), which in turn activates MAPK through phosphorylation (37). In the phosphatidylinositol 3-kinase pathway, Ras binds and activates phosphatidylinositol 3-kinase (38, 39), which results in activation of a variety of effector molecules including Akt/PKB, Vav, SOS, and GRP1 (40–42). In the Ras/Raf/GSK3 pathway, Ras binds and activates Raf/GSK3 (40–45), which in turn activates effectors such as...
Using various Ha-Ras effector loop mutations (39, 48–50), we first attempted to identify which of the downstream effector pathway(s) of Ras are responsible for inhibiting the T-type Ca\(^{2+}\) channel current. We found that Ras mutants lacking the ability to activate the MAPK pathway failed to inhibit T-type Ca\(^{2+}\) channel current, whereas Ras mutants that activate only the MAPK pathway efficiently inhibit T-type channel activity. Moreover, the introduction of the constitutively active MEK results in inhibition of the T-type Ca\(^{2+}\) channel current. By use of the T-type channel blocker, mibebradil, we further found that the suppression of the T-type Ca\(^{2+}\) channel is essential for the induction and/or maintenance of spindle-shape transformation morphology in fibroblasts.

**Experimental Procedures**

**Cells, Plasmids, and Transfection—**Swiss 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml) (this medium will be referred to as complete medium hereafter) at 37 °C in 10% CO\(_2\). Plasmids were transfected by the calcium phosphate method previously described (51). Approximately 1 × 10\(^6\) cells were plated in 100-mm dishes 24 h before transfection. The Swiss 3T3 cells were transfected with the following Ras mutant plasmids: 12V, 12V3S5, 12V37G, 12V40C, and MEK were recorded using the Axopatch 200A patch-clamp amplifier and analyzed using the pClamp (version 6.02, Axon instruments, Inc.) software. Whole cell capacitive transients were compensated on-line, and linear leakage currents were cancelled using a P/4 subpulse protocol. The data were filtered at 2 kHz and digitized at 10 kHz via a Lambaster interface (Axon instruments Inc., Burlingame, CA). L-type currents were elicited by ten 10-mV depolarizing test pulses between −20 to +70 mV from a holding potential of −30 mV, where T-type was completely inactivated. T-type currents were evoked by using ten 10-mV step pulses from −40 to +50 mV, with a holding potential of −80 mV in the presence of 1 µM nifedipine. Identity of L- and T-type currents were confirmed by their sensitivity to nifedipine and mibebradil, respectively.

**Immunoblot Analysis—**To prepare cell lysates, cells were washed twice with PBS and lysed in SDS-Nonidet P-40 lysis solution (10% normal goat serum in PBS) for 1 h on ice. The lysates were sonicated, boiled for 5 min, and then centrifuged at 20,000 g for 30 min at 4 °C for 10 min at 20,000 × g. The lysates were further denatured for 5 min at 95 °C in sample buffer (2% SDS, 10% glycerol, 60 mM Tris (pH 6.8), 5% β-mercaptoethanol, 0.01% bromphenol blue). The proteins were fractionated by SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membranes (Millipore). The membranes were blocked with TBST (20 mM Tris HCl (pH 7.6), 137 mM NaCl, and 0.2% Tween 20) with 5% (w/v) dry milk at room temperature (20–22 °C). After multiple washes with TBST, the membranes were incubated with primary polyclonal antibodies: anti-HA (clone Y-11, Santa Cruz), anti-MEK-1 (clone 12-B, Santa Cruz), or anti-phosphorylated MAPK (New England Biolab). The blots were then washed thoroughly with TBST and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. The antibody-antigen complex was detected using the enhanced chemiluminescence procedure (ECL, Amersham Pharmacia Biotech).

**Morphological Transformation Assay—**Because of the p53-dependent toxicity associated with overexpression of Ras, Swiss 3T3 cells stably transfected with human p53 mutant (Val at amino acid residue 143 was changed to Ala) were transiently transfected with ras mutant plasmids (25 µg/100-mm plate). Cells were then incubated in media supplemented with 1% FBS and appropriate Ca\(^{2+}\) channel antagonists. Five to 7 days after transfection, cells were fixed, and either Giemsa-stained or processed for immunostaining as described below.

**Indirect Immunofluorescence—**Transiently transfected cells grown on slides with 1% FBS and respective Ca\(^{2+}\) channel antagonist were washed with PBS and then fixed with 10% formalin. The cells were then washed with PBS and permeabilized with 1% Nonidet P-40 in PBS for 5 min at room temperature. The cells were incubated with blocking solution (10% normal goat serum in PBS) for 1 h and probed with an anti-HA monoclonal antibody (Roche Molecular Biochemicals) for 1 h. The antibody-antigen complexes were detected with rhodamine-conjugated goat anti-mouse IgG antibody. After 30 min of incubation at room temperature, the samples were washed three times with PBS. Cells were also stained with 4’,6-diamidino-2-phenylindole (DAPI) DNA dye for visualization of nuclei.
mutant-expressing cell lines are shown in Fig. 2 (columns A and B), and the corresponding current-voltage relationships of peak $I_{Ba}$ density are shown in column C. L-type currents were detected in all the cell lines tested. The average L-type current density for control Swiss 3T3 cells was $3.21 \pm 0.58$ pA/pF ($n = 9$) and $2.82 \pm 0.37$ pA/pF ($n = 12$) for 12V, $2.33 \pm 0.51$ pA/pF ($n = 8$) for 12V40C, $1.68 \pm 0.34$ pA/pF ($n = 12$) for 12V35S, and $2.20 \pm 0.37$ pA/pF ($n = 9$) for 12V37G (Fig. 3). The L-type channel current peaked at $+30$ mV, and there was no significant difference in the voltage dependence among the controls and mutant Ras expressors.

To isolate T-type current, Ba$^{2+}$ currents were elicited from a holding potential of $-80$ mV in the presence of the L-type channel blocker, nifedipine (1 $\mu$M). The control cells elicited a T-type peak $I_{Ba}$ with a current density of $0.62 \pm 0.10$ pA/pF ($n = 5$). The 12V37G mutant line had a current density of similar amplitude ($0.55 \pm 0.14$ pA/pF ($n = 5$)), whereas the 12V40C line displayed a slightly higher T-type current density ($0.87 \pm 0.12$ pA/pF ($n = 8$)). The 12V mutant had a T-type current density of $0.008 \pm 0.003$ pA/pF ($n = 7$) that was significantly less than that of the control ($p < 0.001$). Similar to 12V, diminished T-type current was observed in the 12V35S expressor, which had a T-type current density of peak $I_{Ba}$ of $0.005 \pm 0.003$ pA/pF ($n = 12$), which is also significantly less than that of the control ($p < 0.001$). The threshold current for the L-type channel activation in both the mutants and control was approximately $-10$ mV (Fig. 2, column C). The T-type currents in the control, 12V37G, and 12V40C cells were activated approximately at a threshold of $-30$ mV and peaked at $+10$ mV with no significant difference in their voltage dependence (Fig. 2, column C). The absence of T-type currents in both 12V- and 12V35S-expressing cells suggests that the activation of the MAPK pathway may be responsible for suppression of T-type Ca$^{2+}$ channel activity.

Mibebradil is a T-type-selective Ca$^{2+}$ channel antagonist (54, 55). As such, we sought to determine the sensitivity of endogenous T-type Ca$^{2+}$ channels to mibebradil in Swiss 3T3 cells. The treatment of Swiss 3T3 cells with 1 $\mu$M mibebradil caused approximately 50% reduction in T-type Ca$^{2+}$ channel currents (Fig. 4). In the presence of 3 $\mu$M mibebradil, T-type Ca$^{2+}$ channel currents were completely abolished (Fig. 4), whereas there was no change in L-type channel currents (data not shown). Similar results have been previously reported for the recently cloned a1H T-type Ca$^{2+}$ channel from heart (56).

**Activation of the MAPK Pathway Is Responsible for Inhibition of T-type Ca$^{2+}$ Channels**—To test whether the activation of the MAPK pathway is responsible for inhibition of the T-type Ca$^{2+}$ channel, Swiss 3T3 cells were stably transfected with a gain-of-function MEK mutant (ΔN3-S218E-S222D (52)). The expression of this MEK mutant results in constitutive activation of MAPK (52). The G418-resistant colonies were subcloned (3T3/MEK1 and -2), and subjected to immunoblot analysis for expression of MEK (Fig. 5A). Both 3T3/MEK1 and -2 expressed higher levels of MEK than the control Swiss 3T3 cells that were transfected with a vector plasmid. The 3T3/MEK1 and -2 cell lines were further tested for activation of MAPK by immunoblot analysis using anti-phosphorylated (activated) MAPK antibody (Fig. 5B). As expected, activated MAPK was present in both MEK-transfected cell lines.

**Fig. 2.** Whole cell currents recorded from the control Swiss 3T3 cells and cells expressing H-ras effector domain mutants. A, representative L-type Ca$^{2+}$ channel currents. Currents were elicited by a 500-ms depolarizing pulse to $+30$ mV from a holding potential of $-80$ mV. B, representative T-type Ca$^{2+}$ channel currents. Currents were evoked by a depolarizing pulse to $+10$ mV from a holding potential of $-80$ mV in the presence of 1 $\mu$M nifedipine. C, current density-voltage relationships for L-type (closed circle) and T-type (open circle) Ca$^{2+}$ channels.

**Fig. 3.** Current densities of L- and T-type Ca$^{2+}$ channels of the control Swiss 3T3 cells and Ha-Ras effector domain mutant-expressing cells. The current density of each cell line was calculated from the results shown in Fig. 2 and presented as mean ± S.E. The statistical significance was analyzed using the Student's t test. The decrease of T-type Ca$^{2+}$ currents in 12V and 12V35S Ras mutant-expressing cells is statistically significant ($p < 0.05$) as indicated by asterisks.
The 3T3/MEK1 and 2 cell lines were tested electrophysiologically. Similar to 12V and 12V3SS-expressing cell lines, the 3T3/MEK1 cells exhibited an L-type current density of 3.3 ± 0.39 pA/pF (n = 11) and a T-type density of 0.005 ± 0.003 pA/pF (n = 8). The latter was significantly less (p < 0.001) than control cells (Fig. 6D). We obtained similar recordings in the 3T3/MEK2 cells (data not shown). To further show that activation of the MAPK pathway is responsible for inhibition of T-type channels in ras-transformed cells, Swiss 3T3 cells expressing the 12V Ras mutant were pretreated with the MEK-specific inhibitor, PD98059 (57, 58) for 24 h. We found that the T-type Ca²⁺ channel currents recovered to the levels similar to that of control Swiss 3T3 cells (Fig. 6, column B) with a current density of 0.67 ± 0.2 pA/pF (n = 6) after PD98059 treatment (Fig. 6D). Thus, we conclude that activation of the MAPK pathway is responsible for the T-type Ca²⁺ channel depression in ras-transformed cells.

Inhibition of T-type Ca²⁺ Channels Induces Morphological Transformation—Activation of the MAPK pathway is common in cells transformed by many oncogene products and frequently observed in cancer cells (most recently reviewed in Ref. 59). Accordingly, we examined whether inhibition of the T-type Ca²⁺ channel by the MAPK pathway would have a role in cellular transformation. It has been shown that Swiss 3T3 cells are transformed efficiently with oncogenically activated Ras (Ras 12V) when co-transfected with mutant p53 (60, 61). Moreover, overexpression of oncogenically activated Ras induces cell cycle arrest and cell death in a p53-dependent manner (62–64). As expected, 12V-transfected cells showed a spindle morphology (Fig. 7, panel a). The transfected cells were treated with 1 μM nifedipine. Nifedipine treatment (inhibition of L-type Ca²⁺ channels) did not affect cell morphology; 12V-transfected cells showed spindle morphology (panel b), whereas cells transfected with 12V37G, 12V40C, or 12V37G + 12V40C retained a flat
Calcium plays an important role in a variety of fundamental cellular events, including muscle contraction, hormone secretion, gene expression, cell cycle control, and cell morphology (65). The levels of intracellular Ca\(^{2+}\) are differentially regulated depending on the cell type. In fibroblasts, L- and T-type Ca\(^{2+}\) channels are present. Fluctuations or oscillations of membrane potential is generally low enough to activate T-type but not L-type channels. Thus, the T-type channel likely plays a predominant role in regulating intracellular concentration of Ca\(^{2+}\) in fibroblasts (13). It has been shown that the T-type Ca\(^{2+}\) channel current, but not the L-type, are inhibited in cells transformed by various oncogenes including ras (12). In this study, we show that activation of the MAPK pathway is responsible for the inhibition of T-type channels in ras-transformed cells. Because MAPK activation is a converging point of the transforming functions of various oncogene products (reviewed in Refs. 59 and 66), specific inhibition of T-type channels in cells transformed by various oncogenes, shown by Chen et al. suggesting that mere cell cycling does not allow for morphological transformation even if the T-type channel is blocked (Fig. 9, panel b). Because Ras targets multiple effectors, in addition to the inhibition of the T-type channels by the MAPK pathway, one or more of those downstream effectors of Ras other than the pathways examined in this study appear to be required for induction/maintenance of morphological transformation.

**Suppression of T-type Ca\(^{2+}\) Channel Activity by Activation of the MAPK Pathway Is Essential for Morphological Transformation**—By use of MEK-specific inhibitor PD98059, we directly examined whether the suppression of the T-type Ca\(^{2+}\) channel by activation of the MAPK pathway is important for maintenance of a transformed morphology. Swiss 3T3 cells stably transfected with oncogenically activated Ras (12V Ras) were treated with 75 \(\mu\)M PD98059 for 24 h and examined under a light microscope. PD98059 treatment (thus inactivation of the MAPK pathway) resulted in reversion of the transformed morphology to a flat morphology similar to normal Swiss 3T3 cells (Fig. 10, panel b). Together with the findings that PD98059 treatment recovers the T-type Ca\(^{2+}\) channel activity (Fig. 6, column b), our observations demonstrate that inhibition of T-type Ca\(^{2+}\) channels by activation of the MAPK pathway is critical for maintaining morphological transformation.

**DISCUSSION**

**Requirement of Other Ras Effectors in Addition to the MAPK Pathway for Morphological Transformation**—Under a serum-starved condition, normal cells become arrested at resting state (G\(_0\)). It has been shown that expression of 12V, 12V35S, 12V37G, and 12V40C Ras mutants are spindle-shaped (Fig. 8, panels a and c), whereas cells expressing transfected 12V37G and 12V40C mutants show a flat morphology (panels e and g). By treatment of 12V37G- and 12V40C-transfected cells with 3 \(\mu\)M mibebradil, cells acquired a spindle like morphology (panels f and h). These results demonstrate that inhibition of the T-type channel, but not the L-type channel, is essential for induction and/or maintenance of morphological transformation.

**FIG. 7. Photomicrographs of 3T3/p53(V143A) cells transiently transfected with ras mutants.** The 3T3/p53(V143A) cells were transfected with 12V, 12V37G, 12V40C, and 12V37G + 12V40C. Two days after transfection, cells were split into four culture dishes and fed with media containing 1% FBS. Plates from each transfection were treated with 1 \(\mu\)M nifedipine (panels b, f, j, and n), 3 \(\mu\)M mibebradil (panels g, k, and o), and mibebradil (1 \(\mu\)M) + mibebradil (3 \(\mu\)M) (panels d, h, i, and p). Five days after cell splitting, cells were fixed, Giemsa-stained, and examined under a microscope. Magnification, 400×.
MAPK-mediated Suppression of T-type Ca$^{2+}$ Channel

(12), is likely mediated by the activation of the MAPK pathway.

One of the major transformation-associated phenotypes induced by activation of the MAPK pathway is change of cell shapes (from flat to round-up or spindle-shaped morphology). For example, ras-transformed cells show a marked morphological reversion from spindle-shaped to flattened upon exposure to the MEK-specific inhibitor. Such morphological changes are believed to play a critical role in malignant transformation. By use of the T-type channel blocker, mibefradil, we investigated the role of T-type Ca$^{2+}$ channel inhibition in cellular transformation. We found that loss of T-type Ca$^{2+}$ channel activity is essential for induction and/or maintenance of morphological transformation. However, abrogation of T-type Ca$^{2+}$ channel activity alone does not appear to be sufficient for inducing morphological changes, because treatment of either serum-starved or actively proliferating nontransformed cells with the T-type channel blocker fails to induce morphological transformation. Thus, in addition to inhibition of the T-type channels, activation of another downstream effector(s) of Ras, which is activated by all the Ras effector domain mutants examined in this study, appears to be required for induction of morphological transformation.

The actin cytoskeleton is known to play a major role in regulation of cell morphology. It has recently been shown that Rho and Rac proteins, both of which are targeted by activated Ras, are involved in actin organization. Rho is required for regulation of the assembly of actin stress fibers and focal adhesions (67), and Rac1 is necessary for the formation of membrane ruffles (68). Our present findings of the involvement of the MAPK pathway in blocking T-type Ca$^{2+}$ channel activity, which results in the induction of a spindle-shape morphology,
demonstrates that morphological transformation by Ras is achieved by an integration of events imposed by multiple downstream pathways of Ras.

It remains to be elucidated how activation of the MAPK pathway inhibits T-type Ca²⁺ channels. The several regulatory mechanisms of L-type channel activity have been proposed. For example, direct phosphorylation of the pore-forming subunit of the L-type channel by protein kinase A, protein kinase C, as well as calmodulin kinase II have been reported (69–71) and is predicted to modulate the activity of L-type channels (72, 73). Accessory subunits also regulate the activity of L-type channels via an allosteric mechanism as well as by chaperoning the pore-forming subunit (Ref. 74, also reviewed in Ref. 75). However, little is known about regulatory mechanisms of T-type channels, and in fact, cDNA of the T-type channel has just recently been cloned (56, 76). There are several potential mechanisms that may explain how activation of the MAPK pathway inhibits T-type channels. MAPK is known to regulate the transcrip- tion of a variety of genes through direct phosphorylation of major transcription factors, including ELK-1, Sap1, bZIP, ATF4, ATF2, Net/Erp/Sap2, AP-1, c-Jun, Fos, Fra1, Fra2 and c-Myc (for reviews, see Refs. 59 and 66). Thus, MAPK may inhibit T-type Ca²⁺ channels in Swiss 3T3 cells by modulating the expression of the T-type channel itself or accessory proteins (at present, it is not known whether the T-type channel is a multisubunit complex or a single subunit). Alternatively, MAPK may directly inactivate T-type channels through phosphorylation. Indeed, there are two MAPK phosphorylation consensus sites in the C-terminal region of the T-type channel. These questions are currently under investigation in our laboratory. Because alteration of cell morphology is a critical event for cells to acquire a metastatic phenotype, the protocols that inhibit T-type Ca²⁺ channels in Swiss 3T3 cells by modulating MAPK may directly inactivate T-type channels through phosphorylation. Indeed, two MAPK phosphorylation consensus sites in the C-terminal region of the T-type channel. These questions are currently under investigation in our laboratory. Because alteration of cell morphology is a critical event for cells to acquire a metastatic phenotype, the protocols that inhibit T-type Ca²⁺ channels in Swiss 3T3 cells by modulating MAPK may directly inactivate T-type channels through phosphorylation.