Suppression of fibroblast cell growth by overexpression of LIM-kinase 1

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Abstract LIM-kinase 1 (LIMK1) is a serine/threonine kinase containing two LIM motifs at the N-terminus. The functional role of LIMK1 has remained unknown. In this study, we examined the role of LIMK1 in cell growth of fibroblasts. Induced expression of LIMK1 in NIH3T3 cells led to growth retardation. Transfection of LIMK1 sense cDNA into NIH3T3 and H-ras-transformed FYJ10 fibroblasts significantly suppressed colony formation of these cells. In contrast, transfection with LIMK1 antisense cDNA strongly stimulated colony formation of the NIH3T3 cells. These findings suggest that LIMK1 functions as a negative regulator of fibroblast cell growth, and may play a role in tumor suppression.

Key words: Growth suppression; Protein kinase; LIM motif; DHR/PDZ domain; LIMK

1. Introduction

We recently identified a novel class of closely related protein kinases, termed LIM-kinases (LIMKs), composed of LIMK1 and LIMK2 [1–4]. These kinases contain characteristic structural features of the two N-terminal LIM motifs, the internal DHR (Dlg homology region) (also called PDZ or GLGF) domain, and the unusual C-terminal protein kinase domain. The LIM motif, a structural motif composed of two adjacent zinc fingers separated by a 2-amino-acid linker, is found in diverse proteins, including homeodomain-containing transcription factors, cytoskeletal proteins and other signaling molecules [5,6]. The DHR domain is a 90–100-amino-acid motif, previously found in various cell-junction proteins and enzymes [7,8]. Since both of these domains are thought to function as the binding surfaces of protein-protein interactions [9–13], they are likely to be involved in the regulation of kinase activity and subcellular localization of LIMK family proteins. The C-terminal kinase domains of LIMKs contain a consensus sequence of protein kinases, but are unique in that they have an unusual sequence motif (DLNSHN) in the kinase catalytic loop in subdomain VIB and a highly basic kinase insert between subdomains VII and VIII [1–4].

LIMK1 mRNA is highly expressed in the developing nervous system, heart and gut, and in adult brain and spinal cord [1–4,14–16], and a high level of expression in trophoblast giant cells has also been described [15]. LIMK1 has serine/threonine kinase activity and is mainly localized in the cytoplasm [3]. The LIMK1 gene was assigned on human chromosome 7q11.23 [3], and a recent report suggests that hemizygosity of the LIMK1 gene leads to impaired visuospatial cognition in patients of Williams' syndrome [17]. The unique structural features of LIMK family kinases suggest their specific roles in previously uncharacterized signaling pathways, but the cellular functions of these kinases remain to be determined. We have now examined the effects of overexpression of LIMK1 on cell proliferation of NIH3T3 and Ras-transformed FYJ10 fibroblasts. Using an induced expression system and colony formation assay, we found that ectopic expression of LIMK1 significantly suppressed cell growth of NIH3T3 and Ras-transformed fibroblasts. Transfection of the antisense LIMK1 cDNA stimulated colony formation of NIH3T3 cells. The evidence that LIMK1 has an anti-proliferative activity on fibroblasts represents the first demonstration of cellular functions of LIMK family kinases.

2. Materials and methods

2.1. Cell lines and culture conditions

NIH3T3, 208F [18] and FYJ10 (H-Ras transformed derivative of rat 208F fibroblast) [18] cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Rat-1A cells [19] were maintained in DMEM with 20% FBS. 208F and FYJ10 cells were provided by Dr. R. Schäfer and Rat-1A cells by Dr. Y. Nakabeppu.

2.2. Plasmid construction

The 3.0-kb *Apa*I-*Hin*dIII fragment of human LIMK1 cDNA [1] containing the full-size coding region was inserted into pBluescript SKII$^-$ (Stratagene) with a *Not*I linker, and then subcloned into the *Not*I-digested expression vectors, pRc-RSV (Invitrogen) and pMAMneo (Clontech), both of which contain the neomycin resistance gene. Transcription of the cDNA insert is driven by a Rous sarcoma virus long terminal repeat (RSV-LTR) promoter in pRc-RSV and by a dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV)-LTR promoter in the pMAMneo vector.

2.3. Immunoprecipitation and immunoblotting

Cells were washed twice with phosphate-buffered saline, suspended in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% SDS, 10 mM NaF, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride), and incubated on ice for 30 min. Cell lysates from 10^7 cells were preadsorbed with Protein A-Sepharose (Pharmacia) and the supernatants were incubated with anti-LIMK1 antibodies and Protein A-Sepharose. Anti-LIMK1 antibodies were raised against the C-terminal 10 amino acids of human LIMK1 and purified as described previously [3]. The immunoprecipitates were washed three times with washing buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM dithiothreitol) and used for immunoblot analysis. Immunoblot analysis was carried out as described previously [3].

2.4. Cell growth assay

NIH3T3 cells were transfected with pMAMneo and pMAMneo-LIMK1 plasmids by the calcium phosphate precipitation method. Cells were replated at 1:20 dilution 24 h after transfection, and cul-

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Abbreviations: LIMK, LIM-containing protein kinase; DHR, Dlg homology region; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Dex, dexamethasone; APC, adenomatous polyposis coli protein; BrdU, bromodeoxyuridine; MBP, myelin basic protein.

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tured in DMEM in the presence of 600 μg/ml G418 (Sigma). After 2 weeks, several independent G418-resistant clones (M5, M19 and M20 from pMAMneo mock-transfectants and L7, L16 and L18 from pMAMneo-LIMK1 transfectants) were isolated. For cell growth assay, 10^5 cells of L7 and M19 clone were seeded in a 6-well culture plate and cultured in DMEM with or without 2 μM Dex. Cell numbers were counted at daily intervals after plating, using a hemocytometer.

2.5. Microinjection

Microinjection assays were performed as described elsewhere [20]. Expression plasmids containing LIMK1, APC (adenomatous polyposis coli protein) or β-galactosidase (βGal) cDNAs were injected into NIH3T3 cells, and 24 h later 50 μM bromodeoxyuridine (BrdU) was added. After 18 h, cells were fixed and BrdU incorporation was detected, as described [20].

2.6. Colony formation assay

NIH3T3 and FYJ10 cells were transfected by the calcium phosphate method with 10 μg of the pRc-RSV expression plasmids containing LIMK1 cDNA in a sense (pRc-RSV-LIMK1(S)) or an antisense (pRc-RSV-LIMK1(AS)) orientation. Cells were replated at a 1:20 dilution 24 h after transfection and cultured for 10–14 days in DMEM containing 10% FBS and 400–600 μg/ml G418. Drug-resistant colonies were fixed in methanol and visualized by staining with 0.2% crystal violet.

3. Results

3.1. Growth phase-dependent expression of LIMK1 protein

Expression of LIMK1 protein during different growth phases in Rat-1A fibroblasts was monitored by immunoblot

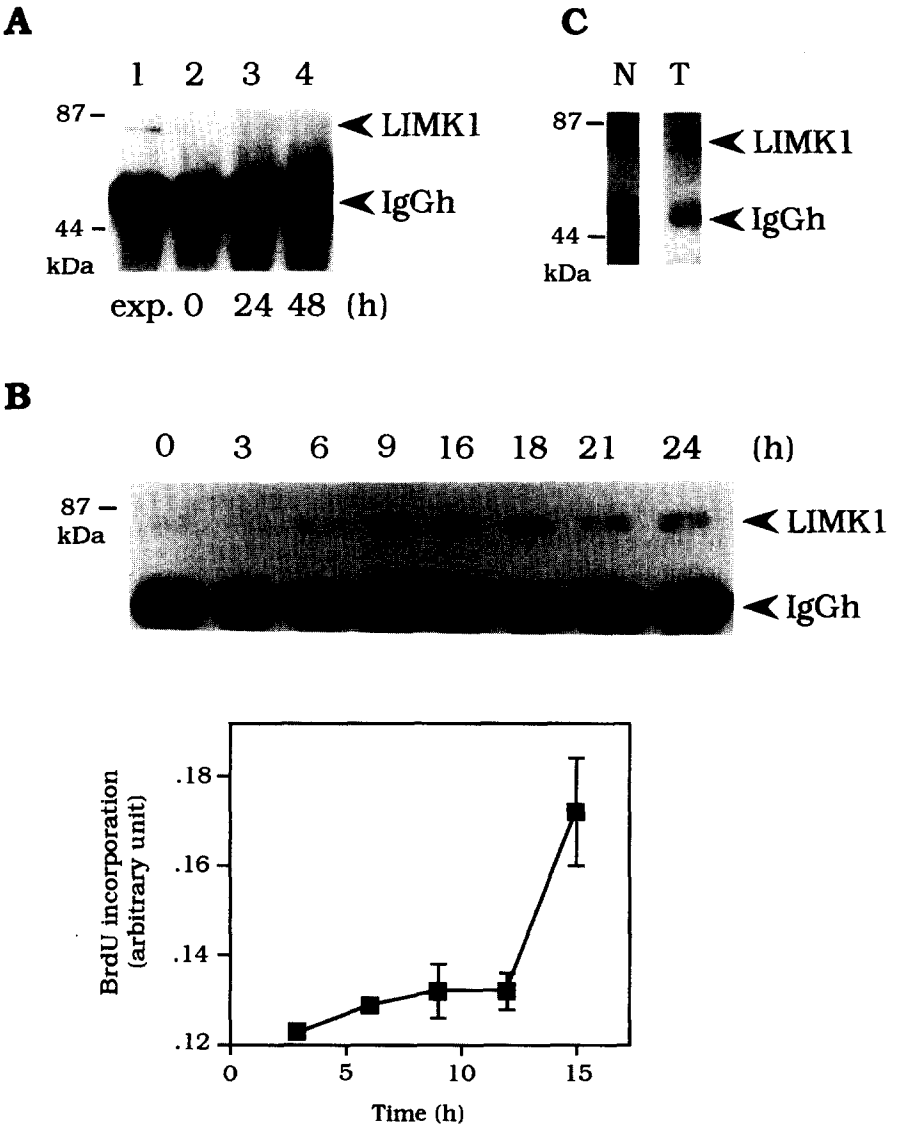

Fig. 1. Growth phase-dependent expression of LIMK1 protein. (A) Expression of LIMK1 protein in exponentially growing or growth-arrested cells. Rat-1A cells were cultured in DMEM supplemented with 20% FBS, grown to confluence, and growth-arrested by serum starvation for 48 h. Cell densities in lanes 1–4 were 0.75, 2.7, 2.0, 1.9×10^5 cells/cm^2, respectively. Lysates prepared from 10^7 cells, in exponentially growing (lane 1) or cultured to confluence (lane 2) and serum-starved for 24 and 48 h (lanes 3,4), were immunoprecipitated with anti-LIMK1 antibodies, run on SDS-PAGE, and immunoblotted with the same antibody. (B) Expression of LIMK1 protein in response to serum stimulation. Rat-1A cells were grown to confluence, serum-starved for 48 h and then stimulated to re-enter the cell cycle by addition of 20% FBS. Lysates from 10^7 cells cultured for the indicated number of hours after serum stimulation were analyzed as in (A). Lower panel shows the level of DNA synthesis, as measured by incorporation of BrdU. (C) Expression of LIMK1 protein in 208F (N) and H-Ras-transformed 208F (T) cell lines. Lysates from 10^7 cells cultured to confluency were analyzed as in (A). Arrows indicate the predicted elution positions of LIMK1 (74 kDa) and immunoglobulin heavy chain (IgGh) (50 kDa).

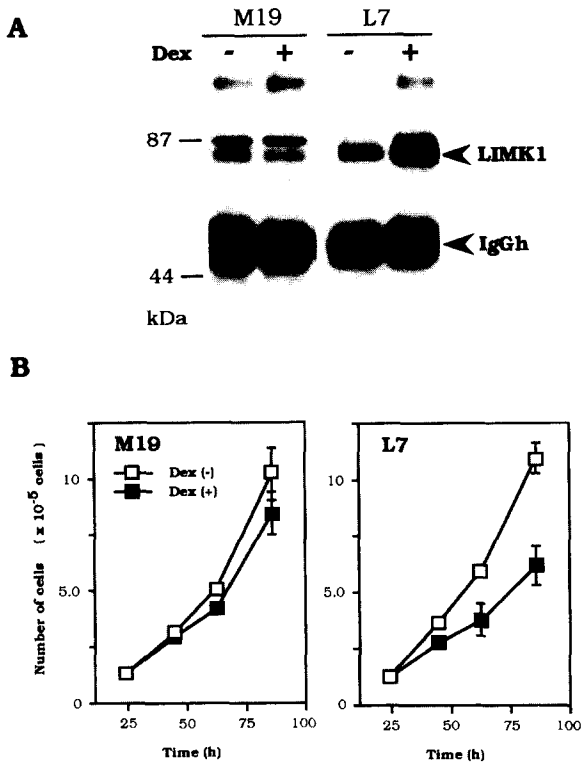

Fig. 2. Induced expression of LIMK1 suppresses growth of NIH3T3 cells. (A) Expression of LIMK1 protein in response to Dex treatment. NIH3T3 cell lines transfected with pMAMneo (M19) or pMAMneo-LIMK1 (L7) were cultured with or without 2 μM Dex for 48 h and the expression of LIMK1 protein was evaluated by immunoblot analysis after immunoprecipitation, using anti-LIMK1 antibodies. Arrows indicate the predicted elution positions of LIMK1 (74 kDa) and IgGh (50 kDa). (B) Growth curves of M19 and L7 cells. Cells were seeded on 6-well culture plates at a density of 10^5 cells/well and cultured in DMEM containing 10% FBS with closed squares) or without (open squares) 2 μM Dex. Cell numbers were counted at the indicated time.

analysis, using anti-LIMK1 antibodies. Asynchronized, log-phase cultures were grown to confluence and then serum-starved and left for an additional 2 days to arrest growth completely, and to attain a quiescent state. Expression of LIMK1 protein was detected in asynchronized, exponentially growing cells, but was barely evident in growth-arrested cells either cultured to confluency or serum-starved for 1–2 days (Fig. 1A). Rat-1A cells cultured to confluency and serum-starved for 48 h were stimulated with 20% serum to re-enter the cell cycle, and the cell cycle-regulated expression of LIMK1 protein was analyzed (Fig. 1B). Immunoblot analysis revealed that expression of LIMK1 protein was induced at 9 h after serum stimulation and remained at maximal level for up to 24 h. BrdU incorporation analysis indicated that DNA synthesis began at 12 h after serum stimulation. Thus, expression of LIMK1 protein was induced by serum stimulation prior to entry to DNA synthesis, and was retained during cell cycle progression. Expression of LIMK1 protein was not detectable in 208F fibroblasts cultured to confluency, but was detectable in H-Ras-transformed 208F cells cultured under similar conditions (Fig. 1C). In some experiments (Figs. 1B and 2A), LIMK1 protein was detected as a doublet, but not in others (Fig. 1A,C), the reason for which has remained unclear. Based on the predicted molecular mass (74 kDa) of LIMK1 protein and the elution positions of the doublet bands, the lower and upper band might be an intact and a phosphorylated form of LIMK1 protein, respectively.

3.2. Growth suppression of NIH3T3 cells by induced expression of LIMK1

The growth phase-dependent expression of LIMK1 protein in Rat-1A cells suggested the possible involvement of LIMK1 in the regulation of growth of fibroblasts. To examine the effect of LIMK1 expression on cell growth, we first attempted to generate NIH3T3 cell lines which constitutively overexpressed LIMK1, using a pRc-RSV expression vector, however, this approach was not rewarding, presumably because LIMK1 suppressed the growth (see below and Fig. 3). We therefore generated NIH3T3 cell clones that could inducibly overexpress LIMK1. The pMAMneo-LIMK1 expression plasmid we constructed has LIMK1 cDNA under the control of a Dex-inducible MMTV-LTR promoter, and a neomycin resistance gene for selection of transformant cells. NIH3T3 cells were transfected with pMAMneo-LIMK1 or pMAMneo vector and cultured for 2 weeks in the presence of G418. Several independent drug-resistant clones were picked up and analyzed for Dex-inducible expression of LIMK1 protein. As shown in Fig. 2A, L7 cells, obtained by transfection with pMAMneo-LIMK1, inducibly expressed LIMK1 protein by the addition of Dex, while M19 cell lines, mock-transfected with pMAMneo vector, did not induce LIMK1 expression. The growth curves for L7 and M19 cells cultured with or without Dex indicate significant growth retardation of L7 cells due to treatment with Dex (Fig. 2B). Treatment with Dex reduced cell growth by approx. 40% in LIMK1-transfected L7 cells, but by only about 20% in mock-transfected M19 cells. Similar results were obtained in other independent LIMK1- (L16 and L18) or mock-transfected (M5 and M20) cell lines. The doubling time of L7 cells was extended from 26.7 to 32.5 h by culturing with Dex, while that of M19 cells was unchanged (22.9 to 23.1 h). No significant change was observed in the cell cycle profiles of L7 cells cultured for 72 h with or without Dex, as measured by flow cytometric analysis of the DNA content (data not shown). Thus, the overexpression of LIMK1 retarded cell growth, but did not cause cell cycle arrest at any specific stage. In addition, exposure of L7 cells to Dex for up to 86 h did not cause cell death, based on gross visual inspection.

3.3. Inhibition of colony formation of fibroblasts by LIMK1

To determine further the potential of LIMK1 to inhibit cell growth, we made use of a colony formation assay. The pRc-RSV expression plasmids containing LIMK1 cDNA in either sense or antisense orientation were constructed. These plasmids are under the control of RSV LTR promoter for high-level and constitutive transcription and contain a neomycin resistance gene for selection of transformant cells. NIH3T3 cells were transfected with the plasmids and cultured in the presence of G418 and drug-resistant colonies were visualized after 10 days of this transfection. Cells transfected with the LIMK1 sense cDNA expression plasmid formed fewer colonies than did those transfected with the control pRc-RSV vector (Fig. 3A,B). In contrast, the number of G418-resistant colonies recovered after transfection with antisense LIMK1 cDNA was significantly (3–4 times) higher than seen with the control vector (Fig. 3C). Therefore, LIMK1 apparently

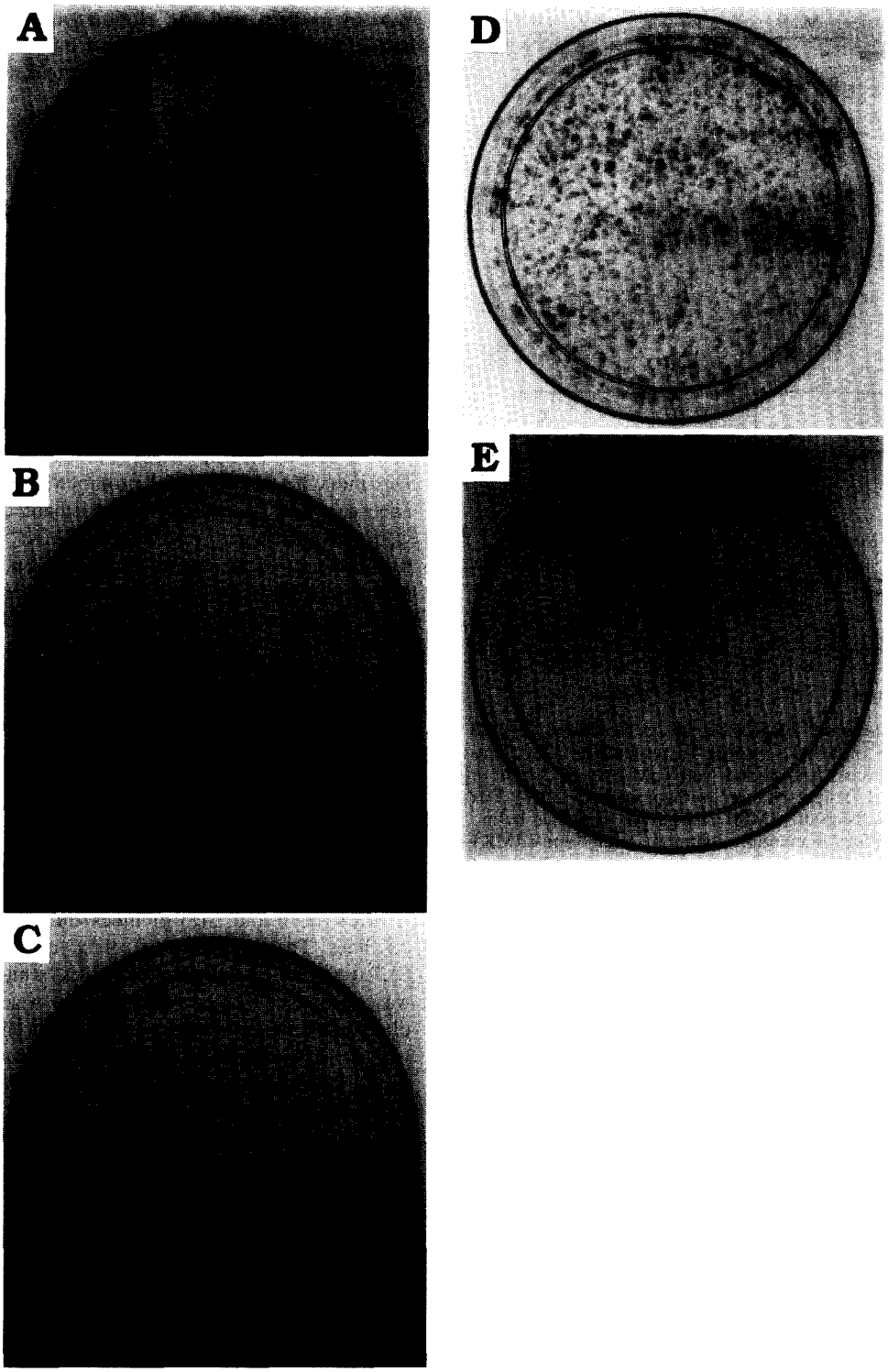

Fig. 3. G418-resistant colony formation assay. LIMK1 cDNA was inserted into the pRc-RSV expression vector in a sense (S) or an antisense (AS) orientation. Left panels (A–C); NIH3T3 cells were transfected with pRc-RSV vector (A), pRc-RSV-LIMK1(S) (B), or pRc-RSV-LIMK1(AS) (C), and grown in DMEM containing 10% FBS and G418 (600 μg/ml). After 10 days, drug-resistant colonies were fixed with methanol and stained with crystal violet. Right panels (D,E); FYJ10 cells were transfected with pRc-RSV vector (D) or pRc-RSV-LIMK1(S) (E), and grown in DMEM containing 10% FBS and G418 (400 μg/ml). After 14 days, drug-resistant colonies were fixed and stained. Results were reproducible in triplicate experiments repeated twice.

functions as a negative regulator of growth of NIH3T3 cells. Morphologic transformation (focus formation in monolayer cultures) was not observed in any of the transfectants. In a similar manner, we also examined the effect of LIMK1 on growth of activated Ras-transformed rat embryonic fibroblast FYJ10 cells. The number of colonies recovered after transfection with sense LIMK1 cDNA was markedly reduced, when compared with the case of transfection with the control vector

Table 1
Effects of LIMK1 microinjection on BrdU incorporation

Plasmid	Number of $BrdU^+$ cells/number of injected cells	$BrdU^+$ cells (%)
pME-βGal	36/41	88
pRc-RSV-LIMK1	42/52	81
pME-APC	9/54	17

NIH3T3 cells were microinjected with the plasmids, and BrdU incorporation was detected as described in Section 2.

(Fig. 3D,E). Thus, overexpression of LIMK1 significantly suppressed the growth of both NIH3T3 and FYJ10 fibroblast cells.

.4. Microinjection assays

To elucidate the mechanism by which LIMK1 inhibits cell growth, microinjection experiments were carried out. Injection of the plasmid containing LIMK1 cDNA into NIH3T3 cells did not inhibit DNA synthesis, as determined by BrdU incorporation, whereas under similar conditions, injection of APC DNA significantly inhibited BrdU incorporation (Table 1). Thus, growth suppression induced by LIMK1 is not due to the cell cycle arrest at the G1 phase.

4. Discussion

Using inducible expression experiments and colony formation assays, we obtained evidence that LIMK1 suppressed the growth of fibroblasts. Our finding that transfection of LIMK1 antisense cDNA increased the number of drug-resistant colonies of NIH3T3 cells suggests that endogenously expressed LIMK1 normally functions as a negative regulator in cell growth. However, the mechanisms by which LIMK1 inhibits cell growth seem to differ from those of other typical growth suppressors, such as p53, pRB (retinoblastoma protein) and cyclin-dependent kinase inhibitors [21,22]. Overexpression of LIMK1 retarded cell growth but, in contrast to the aforementioned growth suppressors, did not arrest the cell cycle at any specific stage, as determined by flow cytometry. Microinjection of LIMK1 cDNA did not prevent cells from entering the S phase. Thus, LIMK1 appears to have growth inhibitory effects not by directly blocking the cell cycle progression machinery, but rather by affecting other processes distally related to cell proliferation, such as the regulation of protein and RNA synthesis and cytoskeletal organization. The patterns of expression of LIMK1 protein (higher expression in growing phase than in resting phase, and higher expression in Ras-transformed fibroblasts than in parental cells) suggest that LIMK1 functions in the growing stage and may play a role of slowing the pace of the cell cycle by regulating certain events in this phase. Further studies are required to resolve the question of how LIMK1 retards cell growth. In particular, identification of the upstream regulators and downstream targets of LIMK1 will be important.

LIMK1 contains a unique extracatalytic domain composed of two LIM motifs at the N-terminus and a DHR domain at the internal region. These structural motifs likely have roles in the cellular functions of LIMK1. The LIM motif was identified in diverse proteins, some of which were found to be involved in growth regulation and oncogenesis [5,6]. As the LIM motifs are thought to be involved in protein-protein interactions, the LIM motifs in LIMK1 probably function as binding modules to interact with other signaling proteins. LIMK1-associated proteins were detected by co-immunoprecipitation analysis, but the molecular properties of these proteins have remained to be identified [3,14]. Further identification and characterization of LIMK1-associated proteins will serve to elucidate the mechanisms of anti-proliferative activity of LIMK1. Additionally, LIMK1 contains a DHR domain in the internal region of the molecule. *Drosophila* Dlg (Disc large) protein, after which the DHR domain was named, is localized to septate junctions in imaginal disc epithelia and is known as a tumor suppressor protein, since its mutation causes neoplastic proliferation of epithelial cells [23]. Recently, a human homolog of Dlg was found to bind through its DHR domain to APC protein, a product of the tumor suppressor gene mutated in familial adenomatous polyposis and sporadic colorectal tumors [13]. Thus, it is likely that in both vertebrates and invertebrates Dlg protein functions as a tumor suppressor by the DHR-mediated association with APC. While the mechanism by which LIMK1 suppresses cell growth is unclear, the DHR domain of LIMK1 may be involved in growth suppressing activity by forming a complex with APC or related proteins.

The growth inhibitory activity of LIMK1 raises the possibility that genes of LIMK1 and its relative LIMK2 may function as novel tumor suppressors. The chromosomal localization of human LIMK1 and LIMK2 genes was assigned to 7q11.23 and 22q12, respectively [3]. The LIMK1 gene was recently suggested to be one of the causal genes of Williams' syndrome [17]. The locus of the LIMK2 gene is close to that of the tumor suppressor gene NF2 (neurofibromatosis 2) [24]. Since loss on 22q near to but outside the NF2 locus was detected in some cases of menigiomas, gliomas, ovarian carcinoma and colorectal cancers [25–27], inactivation of the LIMK2 gene may be linked to the tumorigenesis of these cancers.

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