Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 337 (2005) 191–194
BBRC
www.elsevier.com/locate/ybbrc

The mammalian homolog of the *Drosophila* discs large tumor suppressor protein up-regulates expression of the ELR$^+$ CXC chemokine *Scyb5*

Tomoiki Aiba [a,b], Kazuyoshi Kohu [a,1], Takefumi Ishidao [a,2], Naomi Yasui [a], Akira Horii [b], Hiroyuki Aburatani [c], Tetsu Akiyama [a,*]

[a] *Laboratory of Molecular and Genetic Information, Institute for Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*
[b] *Department of Molecular Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan*
[c] *Genome Science Division, Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan*

Received 3 September 2005
Available online 15 September 2005

Abstract

The mammalian homolog of the *Drosophila* discs large tumor suppressor protein Dlg functions as a scaffolding protein that facilitates the transmission of diverse signals. In the present study, we attempted to identify the downstream target genes of Dlg, and found that Dlg up-regulates expression of the ELR$^+$ CXC chemokine *Scyb5*, which has been implicated in the immune system. Our finding suggests that Scyb5 may play an important role in the tumor suppressor function of Dlg.

Keywords: Tumor suppressor; Discs large; Dlg; Scaffolding protein; MAGUK; Scyb5; Microarray

Dlg was originally identified as a *Drosophila* tumor suppressor gene. In *Drosophila* imaginal disc epithelial cells, *dlg* loss-of-function mutations result in the neoplastic overgrowth, which is associated with abnormalities of septate junction, apicobasal polarity, and cell adhesion [1–3]. In addition to epithelial cells, *dlg* is also implicated in cell polarity of *Drosophila* neuroblasts [4,5].

The four best characterized human homologs of the *dlg* gene are *hDlg* (*SAP97*), *Chapsyn-110* (*PSD-93*), *NE-Dlg* (*SAP102*), and *PSD-95* (*SAP90*) [6,7]. The products of these genes share a distinctive domain structure, including three PDZ (PSD-95/DLG/ZO-1) domains, an SH3 (src homology 3) domain, and a guanylate kinase-like (GK) domain, which is characteristic of the membrane-associated guanylate kinase homolog (MAGUK) family of proteins [8]. Despite its name, the GK domains of these proteins do not possess functional guanylate kinase activity, since the amino acid residues required for ATP binding are not conserved. The PDZ domains of these proteins mediate protein–protein interactions by binding to the carboxyl-terminal S/TXV motif of their target proteins [9]. hDLG localizes to the cytoplasm and to cell–cell adhesion sites in epithelial cells. We previously reported that hDlg has a nuclear localization signal (NLS) between the SH3 and GK domains, and localizes to the nucleus only when the intramolecular SH3–GK interaction is abrogated [10]. The physiological role, however, remains to be elucidated.

hDlg interacts via its PDZ domain with the tumor suppressor adenomatous polyposis coli (APC) [11] and several viral oncoproteins [12,13]. Additionally, hDlg inhibits cell cycle progression from the G0/G1 to S phase in NIH3T3

* Corresponding author.
E-mail address: akiyama@iam.u-tokyo.ac.jp (T. Akiyama).
[1] Present address: Department of Molecular Immunology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan.
[2] Present address: Laboratory of Molecular Genetics, RIKEN Tsukuba Institute, Tsukuba, Ibaraki 305-0074, Japan.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.09.037

cells [14]. Furthermore, expression of the *hDlg* gene is down-regulated in both premalignant cervical neoplasias [15] and breast cancers [16]. In particular, in breast cancers, eight missense mutations and one nonsense mutation have been identified; two of these cases with *hDlg* mutation have been shown to be associated with LOH, corresponding to Knudson's two-hit hypothesis. These findings raise the possibility that the human homolog of *Dlg* is also involved in tumorigenesis.

Mice carrying a gene trap insertion within the *Dlg* locus were previously developed. The resulting mutation generated a truncated Dlg protein containing the first three PDZ domains [17,18]. Mice homozygous for the gene trap insertion were reported to display perinatal lethality, growth retardation in utero, craniofacial dysmorphogenesis, and dysregulation of cell proliferation in the lens epithelium. Recently, we have generated Dlg-null mice using gene targeting and found that homozygous mutant mice exhibit the phenotype similar to those of the above-mentioned mutant mice (unpublished data). Approximately 12% of Dlg heterozygous mice developed B-NK lymphomas in 15–20 months of age, suggesting that Dlg is involved in lymphomagenesis. In the present study, to clarify the role of mammalian Dlg in lymphomagenesis, we performed Affymetrix microarray analysis and obtained a Dlg-regulated target gene, *SCYB-5*/*LIX*.

Materials and methods

Mouse embryonic fibroblast culture and immortalization protocol. Primary mouse embryonic fibroblast (MEFs) were obtained from Day 13.5 embryos, essentially as described [19]. To establish immortalized MEFs, primary MEFs were passaged according to the defined 3T3 protocol; 3×10^5 cells were plated per 60-mm dish every 3 days and cultured until growth rates resumed the rapid rates seen in primary MEFs. Primary MEFs and immortalized MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Microarray and RT-PCR analyses. Total RNA was prepared using ISOGEN (NIPPON GENE). For microarray analysis, target RNA was prepared as described [20]. The Affymetrix MG-U74v2 GeneChips were used to compare gene expression profiles of Dlg-null and wild-type MEFs according to the manufacturer's instructions. Briefly, biotinylated cRNAs were synthesized and hybridized to the GeneChip probe arrays, which were then washed in washing solution, stained with streptavidin–phycoerythrin, and scanned. The data obtained were analyzed with Affymetrix GeneChip software. For RT-PCR analysis, total RNA was converted to cDNA using SuperScript III reverse transcriptase essentially according to the manufacturer's protocol. Primer sequences (from 5′ to 3′) were as follows: mouse actin, forward—GGGGTCACCCACACTGTGCCCATCTACGAG, reverse—ACTCCTGCTTGCTGATCCACATCTGCTGGA; mouse IL1rn, forward—AGAGGCAGCCTGCCGCCCTTCTGGGAAAAG, reverse—TTGGTCTTCCTGGAAGTAGAACTTCGTGAC; mouse Igfbp3, forward—ACATCAGTGAGTCCGAGGAGGAGCACAATG, reverse—CTACTGGCTCTGCACGCTGAGGCAATGTAC; mouse Scyb14, forward—ATGAGGCTCCTGGCGGCCGCGCTGCTCCTG, reverse—TTCGTAGACCCTGCGCTTCTCGTTCCAGGC; mouse Scyb5, forward—AGCTGCCCCTTCCTCAGTCATAGCCGCAAC, reverse—TATTCCGGAGACAATGCAATAGTCACCCTC. The PCR cycling parameters are pretreatment 94 °C for 2 min; then followed by 26 cycles (for actin) or 30 cycles (for IL1rn, Igfbp3, Scyb14, and Scyb5) at 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min.

Enrichment of Dlg-transfected cells by auto-MACS cell sorting. MEFs were cotransfected with the pMACS K^k.II vector (Miltenyi Biotec) expressing a surface marker, truncated mouse H-$2K^k$, and pMkitneo or pMkitneo-hDlg using Lipofectamine and PLUS reagent (Invitrogen). Cells were harvested 48 h after cotransfection, resuspended in PBE (PBS with 5 mM EDTA) buffer, and incubated with MACSelect K^k microbeads (Miltenyi Biotec) followed by magnetic separation.

Immunoblotting. Cells were lysed in a lysis buffer containing 1% Triton X-100, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. Proteins were separated by 8% SDS–polyacrylamide gel electrophoresis and transferred onto an Immunobilon-P membrane (Millipore). Immunoblots were probed with primary antibodies against Dlg (BD Transduction Lab.) and α-tubulin (Oncogene), then with alkaline phosphatase-conjugated mouse anti-rabbit IgG antibodies (Promega), and visualized using NBT/BCIP (Promega).

Results and discussion

Affymetrix MG-U74Av2 GeneChips, which contained probe sets interrogating approximately 36,000 genes and ESTs from the UniGene database, were used to analyze gene expression in murine embryonic fibroblasts (MEFs) that were generated from DLG-null and wild-type mice, respectively. MG-U74Av2 array analysis revealed that 15 genes were up-regulated and 36 genes were down-regulated more than 1.5-fold in Dlg-null MEFs compared with control wild-type MEFs. Representative genes are listed in Table 1.

To validate the array data, we performed RT-PCR analysis using MEFs #33 and #44, which were generated from independent two lines of DLG-null mice, respectively. We also used an immortalized cell line established from MEFs #33. We found that expression of 4 of the 36 down-regulated genes identified in the array analysis, *IL-1ra*, *SCYB-5*, *SCYB-14*, and *IGF-BP3*, was markedly decreased in Dlg-null MEFs (Fig. 1). On the other hand, down-regulation of the other 32 genes was not detected. Additionally, none of the 15 up-regulated genes in the array data displayed differential expression. These results suggest that Dlg regulates expression of *IL1rn*, *Scyb5*, *Scyb14*, and *Igfbp3*.

To further confirm this possibility, we examined the effect of exogenous expression of hDLG on the expression of these genes in Dlg-null MEFs. Since transfection efficiency of the immortalized MEFs #33 was very low, we utilized Auto-MACS cell sorting to enrich hDlg-transfected MEFs. As shown in Fig. 2, expression of *Scyb5*, but not other three genes, was increased significantly in hDlg-transfected Dlg-null MEFs compared with empty vector-transfected Dlg-null MEFs. These results suggest that DLG regulates *Scyb5* expression in MEFs.

Scyb5 is one of the CXC chemokines, which constitute a large family of chemotactic cytokines. The CXC chemokines, which contain one residue (X) between the first two conserved cystein residues, have been shown to attract and activate neutrophils in inflammatory processes [21]. Also, CXC chemokines are implicated in the recruitment of T cells to sites of inflammation [22]. Furthermore, it has been shown that CXC chemokine signaling is stimulated by T-cell receptor (TCR) signaling. In this regard, it

Table 1
List of representative genes differentially expressed in Dlg-null #33 MEFs compared with wild-type #33 MEFs

Unigene	Gene name	Description	Fold change[a]	Sort score[a]
Mm.382	*Dlgh1*	Discs, large homolog 1 (*Drosophila*)	−5.8	−2.52
Mm.882	*IL1rn*	Interleukin 1 receptor antagonist	−18.8	−5.79
Mm.29254	*Igfbp3*	Insulin-like growth factor binding protein 3	−3.1	−0.50
Mm.30211	*Scyb14*	Chemokine (CXC motif) ligand 14	−2.7	−0.96
Mm.4660	*Scyb5*	Chemokine (CXC motif) ligand 5	−2.7	−0.82

[a] (−) indicates a decrease in expression.

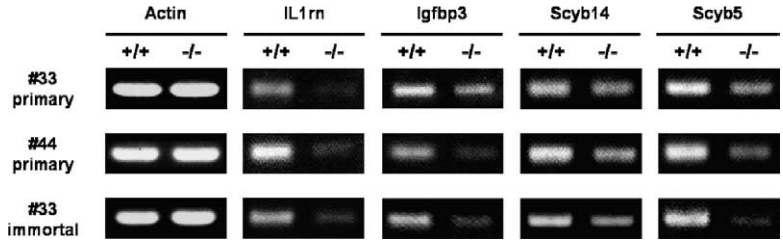

Fig. 1. Semi-quantitative RT-PCR analysis. Expression of *IL-1ra*, *IGF-BP3*, *Scyb14*, and *Scyb5* in two lines of Dlg-null primary MEFs (#33 and #44) and immortalized MEFs #33 were examined.

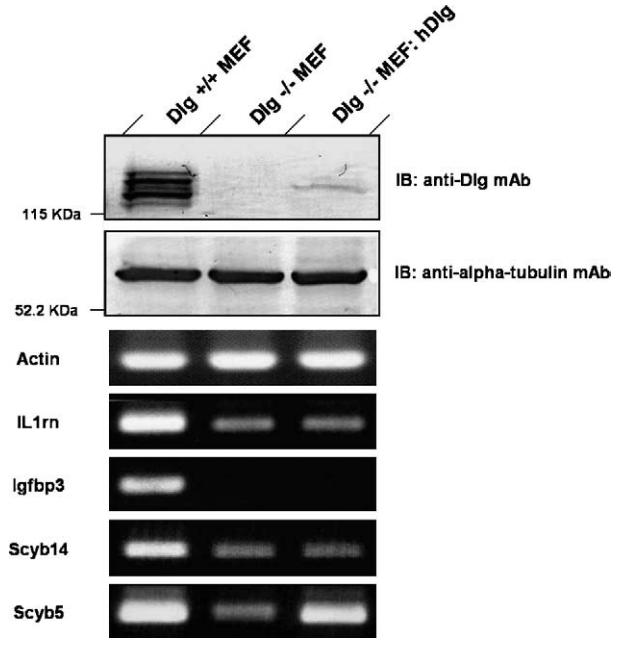

Fig. 2. Effect of exogenous expression of hDlg. MEFs indicated were transfected with hDlg and expression of the indicated genes was examined by semi-quantitative RT-PCR analysis. The results of immunoblotting analysis using anti-Dlg antibody are also shown.

is interesting that Dlg have been shown to play an important role in the activation of T cells [23,24]. We therefore speculate that Scyb5 may be transactivated downstream of Dlg-dependent TCR signaling and contribute to T-cell function. Moreover, we found that approximately 12% of Dlg heterozygous mice developed B-NK lymphomas. Since it is known that some of the genes that influence the development or functions of T cells are involved in the pathogenesis of B-cell lymphomas [25], it is possible that up-regulation of Scyb5 may contribute to lymphomagenesis. Further elucidation of the significance of Scyb5 in the tumor suppressor function of Dlg is underway in our laboratory.

Acknowledgments

We thank K. Matsuura for reading the manuscript. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas and the Organization for Pharmaceutical Safety and Research.

References

[1] D.F. Woods, P.J. Bryant, The Discs-Large tumor suppressor gene of *Drosophila* encodes a guanylate kinase homolog localized at septate junctions, Cell 66 (1991) 451–464.
[2] D.F. Woods, C. Hough, D. Peel, G. Callaini, P.J. Bryant, Dlg protein is required for junction structure, cell polarity, and proliferation control in *Drosophila* epithelia, J. Cell Biol. 134 (1996) 1469–1482.
[3] D. Bilder, M. Li, N. Perrimon, Cooperative regulation of cell polarity and growth by *Drosophila* tumor suppressors, Science 289 (2000) 113–116.
[4] T. Ohshiro, T. Yagami, C. Zhang, F. Matsuzaki, Role of cortical tumour-suppressor proteins in asymmetric division of *Drosophila* neuroblast, Nature 408 (2000) 593–596.
[5] C.Y. Peng, L. Manning, R. Albertson, C.Q. Doe, The tumour-suppressor genes lgl and dlg regulate basal protein targeting in *Drosophila* neuroblasts, Nature 408 (2000) 596–600.
[6] R.A. Lue, S.M. Marfatia, D. Branton, A.H. Chishti, Cloning and characterization of hdlg: the human homologue of the *Drosophila* discs large tumor suppressor binds to protein 4.1, Proc. Natl. Acad. Sci. USA 91 (1994) 9818–9822.
[7] K.O. Cho, C.A. Hunt, M.B. Kennedy, The rat brain postsynaptic density fraction contains a homolog of the *Drosophila* discs-large tumor suppressor protein, Neuron 9 (1992) 929–942.
[8] S.D. Dimitratos, D.F. Woods, D.G. Stathakis, P.J. Bryant, Signaling pathways are focused at specialized regions of the plasma membrane by scaffolding proteins of the MAGUK family, Bioessays 21 (1999) 912–921.

[9] B.Z. Harris, W.A. Lim, Mechanism and role of PDZ domains in signaling complex assembly, J. Cell Sci. 114 (2001) 3219–3231.
[10] K. Kohu, F. Ogawa, T. Akiyama, The SH3, HOOK and guanylate kinase-like domains of hDLG are important for its cytoplasmic localization, Genes Cells 7 (2002) 707–715.
[11] A. Matsumine, A. Ogai, T. Senda, N. Okumura, K. Satoh, G.H. Baeg, T. Kawahara, S. Kobayashi, M. Okada, K. Toyoshima, T. Akiyama, Binding of APC to the human homolog of the *Drosophila* discs large tumor suppressor protein, Science 272 (1996) 1020–1023.
[12] T. Kiyono, A. Hiraiwa, M. Fujita, Y. Hayashi, T. Akiyama, M. Ishibashi, Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the *Drosophila* discs large tumor suppressor protein, Proc. Natl. Acad. Sci. USA 94 (1997) 11612–11616.
[13] T. Suzuki, Y. Ohsugi, M. Uchida-Toita, T. Akiyama, M. Yoshida, Tax oncoprotein of HTLV-1 binds to the human homologue of *Drosophila* discs large tumor suppressor protein, hDLG, and perturbs its function in cell growth control, Oncogene 18 (1999) 5967–5972.
[14] T. Ishidate, A. Matsumine, K. Toyoshima, T. Akiyama, The APC-hDLG complex negatively regulates cell cycle progression from the G0/G1 to S phase, Oncogene 19 (2000) 365–372.
[15] R.A. Watson, T.P. Rollason, G.M. Reynolds, P.G. Murray, L. Banks, S. Roberts, Changes in expression of the human homologue of the *Drosophila* discs large tumour suppressor protein in high-grade premalignant cervical neoplasias, Carcinogenesis 11 (2002) 1791–1796.
[16] T.J. Fuja, F. Lin, K.E. Osann, P.J. Bryant, Somatic mutations and altered expression of the candidate tumor suppressors CSNK1 epsilon, DLG1, and EDD/hHYD in mammary ductal carcinoma, Cancer Res. 64 (2004) 942–951.
[17] G. Caruana, A. Bernstein, Craniofacial dysmorphogenesis including cleft palate in mice with an insertional mutation in the discs large gene, Mol. Cell. Biol. 21 (2001) 1475–1483.
[18] M.M. Nguyen, M.L. Nguyen, G. Caruana, A. Bernstein, P.F. Lambert, A.E. Griep, Requirement of PDZ-containing proteins for cell cycle regulation and differentiation in the mouse lens epithelium, Mol. Cell. Biol. 23 (2003) 8970–8981.
[19] G.J. Todaro, H. Green, Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines, J. Cell Biol. 17 (1963) 299–313.
[20] M. Takasato, K. Osafune, Y. Matsumoto, Y. Kataoka, N. Yoshida, H. Meguro, H. Aburatani, M. Asashima, R. Nishinakamura, Identification of kidney mesenchymal genes by a combination of microarray analysis and Sall1-GFP knockin mice, Mech. Dev. 121 (2004) 547–557.
[21] A. Zlotnik, O. Yoshie, Chemokines: a new classification system and their role in immunity, Immunity 12 (2000) 121–127.
[22] C. Nakajima, T. Mukai, N. Yamaguchi, Y. Morimoto, W.R. Park, M. Iwasaki, P. Gao, S. Ono, H. Fujiwara, T. Hamaoka, Induction of the chemokine receptor CXCR3 on TCR-stimulated T cells: dependence on the release from persistent TCR-triggering and requirement for IFN-gamma stimulation, Eur. J. Immunol. 32 (2002) 1792–1801.
[23] M.J. Ludford-Menting, J. Oliaro, F. Sacirbegovic, E.T. Cheah, N. Pedersen, S.J. Thomas, A. Pasam, R. Iazzolino, L.E. Dow, N.J. Waterhouse, A. Murphy, S. Ellis, M.J. Smyth, M.H. Kershaw, P.K. Darcy, P.O. Humbert, S.M. Russell, A network of PDZ-containing proteins regulates T cell polarity and morphology during migration and immunological synapse formation, Immunity 22 (2005) 737–748.
[24] R. Xavier, S. Rabizadeh, K. Ishiguro, N. Andre, J.B. Ortiz, H. Wachtel, D.G. Morris, M. Lopez-Ilasaca, A.C. Shaw, W. Swat, B. Seed, Discs large (Dlg1) complexes in lymphocyte activation, J. Cell Biol. 166 (2004) 173–178.
[25] R. Kuppers, Mechanisms of B-cell lymphoma pathogenesis, Nat. Rev. Cancer 5 (2005) 251–262.