DISCUSSION

Although it is generally believed that an alteration in claudin expression is involved in tumorigenesis, the role of individual claudins in the regulation of cancer-related cell functions, such as invasion and migration and regulating the intercellular barrier function of TJs, has remained unclear. This is because various types of cells, some of which lack the ability to form functional TJs, have been used in different studies. Therefore, in this study, we systematically examined the effect of overexpression of various claudins on Caco-2 cell invasion and migration, as well as on the intercellular barrier function of TJs, thereby allowing all these parameters to be investigated in a single system.

Overexpression of claudin-4 or claudin-2 either increased or deceased, respectively, TER in Caco-2 cells, results that are consistent with those obtained in other types of cells.[27,40,41] Overexpression of claudin-4 or claudin-2 also decreased or increased, respectively, the paracellular permeability of FD4 in these cells, suggesting that these claudins can exert an effect on cancer development by modulating the accessibility of nutrients and growth factors. As each of these claudins localizes at TJs under our experimental conditions, their differing effects on TJ barrier function appear to be due to their differing activities at these sites rather than differences in localization.

Overexpression of claudin-4, but not the other claudins, stimulated the invasive activity of Caco-2 cells. A similar effect has been observed in ovarian cancer cells (HOSE), whereas the opposite effect was observed in pancreatic cancer cells (SUIT-2).[16,25] Despite stimulating cell invasion, overexpression of claudin-4 inhibited the migration of Caco-2 cells, although it specifically increased the expression and

activity of MMP-2 and MMP-9. Thus, MMP activity rather than cell migration appears to represent the mode of action by which claudin-4 stimulates cell invasion. It is known that claudins affect cell physiology through recruiting signal transduction-related molecules at TJs.[42] Furthermore, claudin-1, -2, -3 and –5 have been suggested to recruit and activate pro-MMP-2.[26,43] However, since overexpressed claudin-4 exists ubiquitously in cells under our culture conditions, claudin-4 could be affecting the expression and activity of MMPs either directly or by modulating signal transduction in the cytoplasm. Supporting this notion, the co-localization of claudins with MMP-2 is not limited to TJs but is also observed in the cytoplasm.[43]

Overexpression of claudin-2 or claudin-3 and -4 stimulated or inhibited, respectively, the migration of Caco-2 cells. We also observed actin stress fibers in Caco-2 cells expressing claudin-2, and found that each of these claudins delocalized from the cell surface to intracellular compartments after wound formation (activation of migration activity), an event that has previously been reported only for claudin-3 [44]. Thus, migration-stimulating signals induce delocalization of claudins into intracellular compartments, with some of these claudins having a positive effect on cell migration whereas some of others exert the opposite effect. At present, the mechanism by which the different claudins influence cell migration remains unclear.

In summary, the results of this study suggest that overexpression of claudin-2 stimulates cancer development by decreasing the intercellular barrier function of TJs and increasing cell migration. On the other hand, the overall effect of overexpression of claudin-4 on cancer development remains unclear, as it increases both the intercellular barrier function of TJs and cell invasion. Furthermore, we found that the subcellular localization of TJs differs between cells cultured at high density (cells contacting each

other) and those grown at low density (migrating and growing cells). Thus, it seems that claudin expression affects the invasion and migration activities and the intercellular barrier function of TJs independently, with both effects being important for cancer development.

ACKNOWLEDGEMENTS

Grants-in-Aid of Scientific Research from the Ministry of Health, Labour, and Welfare of Japan, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Grants-in-Aid of the Japan Science and Technology Agency.

REFERENCES

1. Hamazaki, Y., Itoh, M., Sasaki, H., Furuse, M., and Tsukita, S., *J Biol Chem*, **277**, 455-461 (2002)
2. Itoh, M., Furuse, M., Morita, K., Kubota, K., Saitou, M., and Tsukita, S., *J Cell Biol*, **147**, 1351-1363 (1999)
3. Anderson, J. M., and Van Itallie, C. M., *Am J Physiol*, **269**, G467-475 (1995)
4. Tsukita, S., Furuse, M., and Itoh, M., *Nat Rev Mol Cell Biol*, **2**, 285-293 (2001)
5. Gonzalez-Mariscal, L., Betanzos, A., Nava, P., and Jaramillo, B. E., *Prog Biophys Mol Biol*, **81**, 1-44 (2003)
6. Van Itallie, C. M., and Anderson, J. M., *Annu Rev Physiol*, **68**, 403-429 (2006)
7. Furuse, M., Sasaki, H., Fujimoto, K., and Tsukita, S., *J Cell Biol*, **143**, 391-401 (1998)
8. Tsukita, S., and Furuse, M., *J Cell Biol*, **149**, 13-16 (2000)
9. Morin, P. J., *Cancer Res*, **65**, 9603-9606 (2005)
10. Oliveira, S. S., and Morgado-Diaz, J. A., *Cell Mol Life Sci*, **64**, 17-28 (2007)
11. Swisshelm, K., Macek, R., and Kubbies, M., *Adv Drug Deliv Rev*, **57**, 919-928 (2005)
12. Rangel, L. B., Agarwal, R., D'Souza, T., Pizer, E. S., Alo, P. L., Lancaster, W. D., Gregoire, L., Schwartz, D. R., Cho, K. R., and Morin, P. J., *Clin Cancer Res*, **9**, 2567-2575 (2003)
13. Vermeer, P. D., Einwalter, L. A., Moninger, T. O., Rokhlina, T., Kern, J. A., Zabner, J., and Welsh, M. J., *Nature*, **422**, 322-326 (2003)
14. Soler, A. P., Miller, R. D., Laughlin, K. V., Carp, N. Z., Klurfeld, D. M., and

Mullin, J. M., *Carcinogenesis*, **20**, 1425-1431 (1999)

15. Nakajima, M., Katayama, K., Tamechika, I., Hayashi, K., Amano, Y., Uehata, M., Goto, N., and Kondo, T., *Clin Exp Pharmacol Physiol*, **30**, 457-463 (2003)
16. Michl, P., Barth, C., Buchholz, M., Lerch, M. M., Rolke, M., Holzmann, K. H., Menke, A., Fensterer, H., Giehl, K., Lohr, M., Leder, G., Iwamura, T., Adler, G., and Gress, T. M., *Cancer Res*, **63**, 6265-6271 (2003)
17. Miwa, N., Furuse, M., Tsukita, S., Niikawa, N., Nakamura, Y., and Furukawa, Y., *Oncol Res*, **12**, 469-476 (2001)
18. Iacobuzio-Donahue, C. A., Maitra, A., Shen-Ong, G. L., van Heek, T., Ashfaq, R., Meyer, R., Walter, K., Berg, K., Hollingsworth, M. A., Cameron, J. L., Yeo, C. J., Kern, S. E., Goggins, M., and Hruban, R. H., *Am J Pathol*, **160**, 1239-1249 (2002)
19. Hough, C. D., Sherman-Baust, C. A., Pizer, E. S., Montz, F. J., Im, D. D., Rosenshein, N. B., Cho, K. R., Riggins, G. J., and Morin, P. J., *Cancer Res*, **60**, 6281-6287 (2000)
20. Hewitt, K. J., Agarwal, R., and Morin, P. J., *BMC Cancer*, **6**, 186 (2006)
21. Mima, S., Tsutsumi, S., Ushijima, H., Takeda, M., Fukuda, I., Yokomizo, K., Suzuki, K., Sano, K., Nakanishi, T., Tomisato, W., Tsuchiya, T., and Mizushima, T., *Cancer Res*, **65**, 1868-1876 (2005)
22. Mima, S., Takehara, M., Takada, H., Nishimura, T., Hoshino, T., and Mizushima, T., *Carcinogenesis*, (2008)
23. Oku, N., Sasabe, E., Ueta, E., Yamamoto, T., and Osaki, T., *Cancer Res*, **66**, 5251-5257 (2006)
24. Dhawan, P., Singh, A. B., Deane, N. G., No, Y., Shiou, S. R., Schmidt, C., Neff,

J., Washington, M. K., and Beauchamp, R. D., *J Clin Invest*, **115**, 1765-1776 (2005)

25. Agarwal, R., D'Souza, T., and Morin, P. J., *Cancer Res*, **65**, 7378-7385 (2005)
26. Miyamori, H., Takino, T., Kobayashi, Y., Tokai, H., Itoh, Y., Seiki, M., and Sato, H., *J Biol Chem*, **276**, 28204-28211 (2001)
27. Van Itallie, C. M., Fanning, A. S., and Anderson, J. M., *Am J Physiol Renal Physiol*, **285**, F1078-1084 (2003)
28. Blackman, B., Russell, T., Nordeen, S. K., Medina, D., and Neville, M. C., *Breast Cancer Res*, **7**, R248-255 (2005)
29. Boireau, S., Buchert, M., Samuel, M. S., Pannequin, J., Ryan, J. L., Choquet, A., Chapuis, H., Rebillard, X., Avances, C., Ernst, M., Joubert, D., Mottet, N., and Hollande, F., *Carcinogenesis*, **28**, 246-258 (2007)
30. Tavelin, S., Hashimoto, K., Malkinson, J., Lazorova, L., Toth, I., and Artursson, P., *Mol Pharmacol*, **64**, 1530-1540 (2003)
31. Corral, R. S., Iniguez, M. A., Duque, J., Lopez-Perez, R., and Fresno, M., *Oncogene*, **26**, 958-969 (2007)
32. Taraboletti, G., Sonzogni, L., Vergani, V., Hosseini, G., Ceruti, R., Ghilardi, C., Bastone, A., Toschi, E., Borsotti, P., Scanziani, E., Giavazzi, R., Pepper, M. S., Stetler-Stevenson, W. G., and Bani, M. R., *Exp Cell Res*, **258**, 384-394 (2000)
33. Bradford, M. M., *Anal Biochem*, **72**, 248-254 (1976)
34. Larkins, T. L., Nowell, M., Singh, S., and Sanford, G. L., *BMC Cancer*, **6**, 181 (2006)
35. Biganzoli, E., Cavenaghi, L. A., Rossi, R., Brunati, M. C., and Nolli, M. L., *Farmaco*, **54**, 594-599 (1999)

36. Pellegrin, S., and Mellor, H., *J Cell Sci*, **120**, 3491-3499 (2007)

37. Egeblad, M., and Werb, Z., *Nat Rev Cancer*, **2**, 161-174 (2002)

38. Deryugina, E. I., and Quigley, J. P., *Cancer Metastasis Rev*, **25**, 9-34 (2006)

39. Fu, X., Parks, W. C., and Heinecke, J. W., *Semin Cell Dev Biol*, **19**, 2-13 (2008)

40. Furuse, M., Furuse, K., Sasaki, H., and Tsukita, S., *J Cell Biol*, **153**, 263-272 (2001)

41. Van Itallie, C., Rahner, C., and Anderson, J. M., *J Clin Invest*, **107**, 1319-1327 (2001)

42. Matter, K., and Balda, M. S., *Nat Rev Mol Cell Biol*, **4**, 225-236 (2003)

43. Ichiyasu, H., McCormack, J. M., McCarthy, K. M., Dombkowski, D., Preffer, F. I., and Schneeberger, E. E., *Am J Respir Cell Mol Biol*, **30**, 761-770 (2004)

44. Matsuda, M., Kubo, A., Furuse, M., and Tsukita, S., *J Cell Sci*, **117**, 1247-1257 (2004)

FIGURE LEGENDS

Fig. 1. Overexpression of claudins and their localization in Caco-2 cells.

Caco-2 cells stably transfected with claudin-1, -2, -3, -4 or -15 expression plasmid (Cldn-1, -2, -3, -4 or -15) and mock transfectant control cells (Mock) were cultured and whole cell extracts (10 μg protein) were prepared and analyzed by immunoblotting with an antibody against each claudin or actin (A). These cells (2 x 10^5 (B) or 2 x 10^4 (C) cells/well) were cultured for 7 days (B) or 24 h (C) and samples were incubated with antibodies against each claudin and/or ZO-1 or occludin. After incubation with the respective secondary antibody, cells were inspected using fluorescence microscopy (B, C).

Fig. 2. Effect of overexpression of each claudin on the barrier function of TJs.

Caco-2 cells stably transfected with claudin-1, -2, -3, -4 or -15 expression plasmid (Cldn-1, -2, -3, -4 or -15) and mock transfectant control cells (Mock) were cultured for 7 days. The TER (A) and permeability of FD4 (B) were examined as described in the Materials and Methods. Values are mean ± S.D. (n=3). **$P<0.01$ (A, B).

Fig. 3. Effect of overexpression of each claudin on cell invasion.

Caco-2 cells stably transfected with claudin-1, -2, -3, -4 or -15 expression plasmid (Cldn-1, -2, -3, -4 or -15) and mock transfectant control cells (Mock) were cultured for the indicated periods and cell numbers were determined by direct cell counting (A). These cells were cultured on matrigel-coated transwells for 48 h and

invasion activity was measured as described in the Materials and Methods. The results are expressed relative to the control (B). Values are mean ± S.D. (n=3). $^{**}P<0.01$ (A, B).

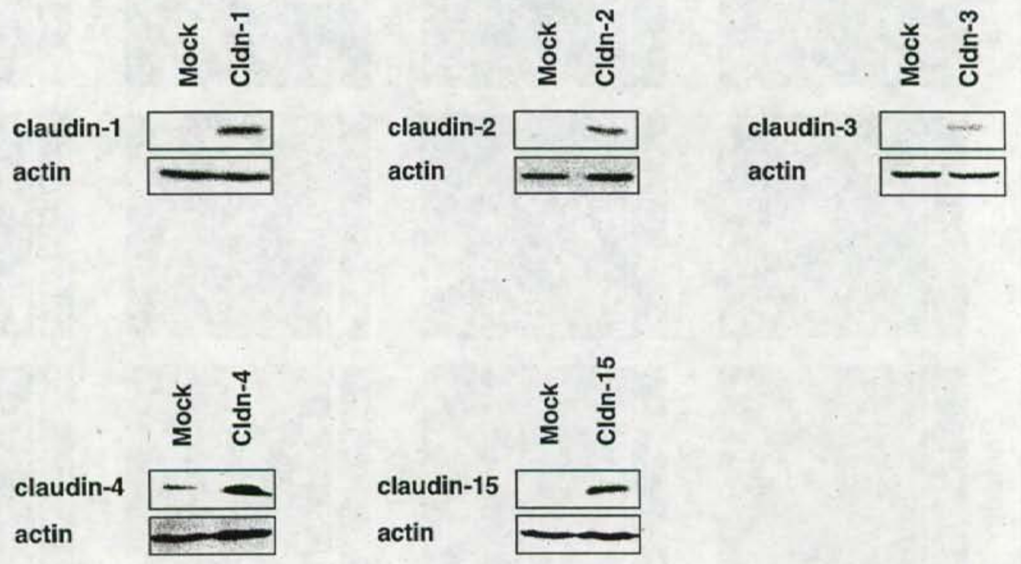
Fig. 4. Effect of overexpression of each claudin on cell migration.

Caco-2 cells stably transfected with claudin-1, -2, -3, -4 or -15 expression plasmid (Cldn-1, -2, -3, -4 or -15) and mock transfectant control cells (Mock) were cultured in transwell chambers for 48 h. Cell migration activity was measured as described in the Materials and Methods and is expressed relative to the control. Values are mean ± S.D. (n=3). $^{**}P<0.01$ (A). These cells were cultured for 7 days, then wounded, and cultured for a further 48 h (B, C). Actin stress fibers were observed by immunostaining (B). The localization of each claudin was monitored as described in the legend of Fig. 1. Wounded sides are shown by broken lines (C).

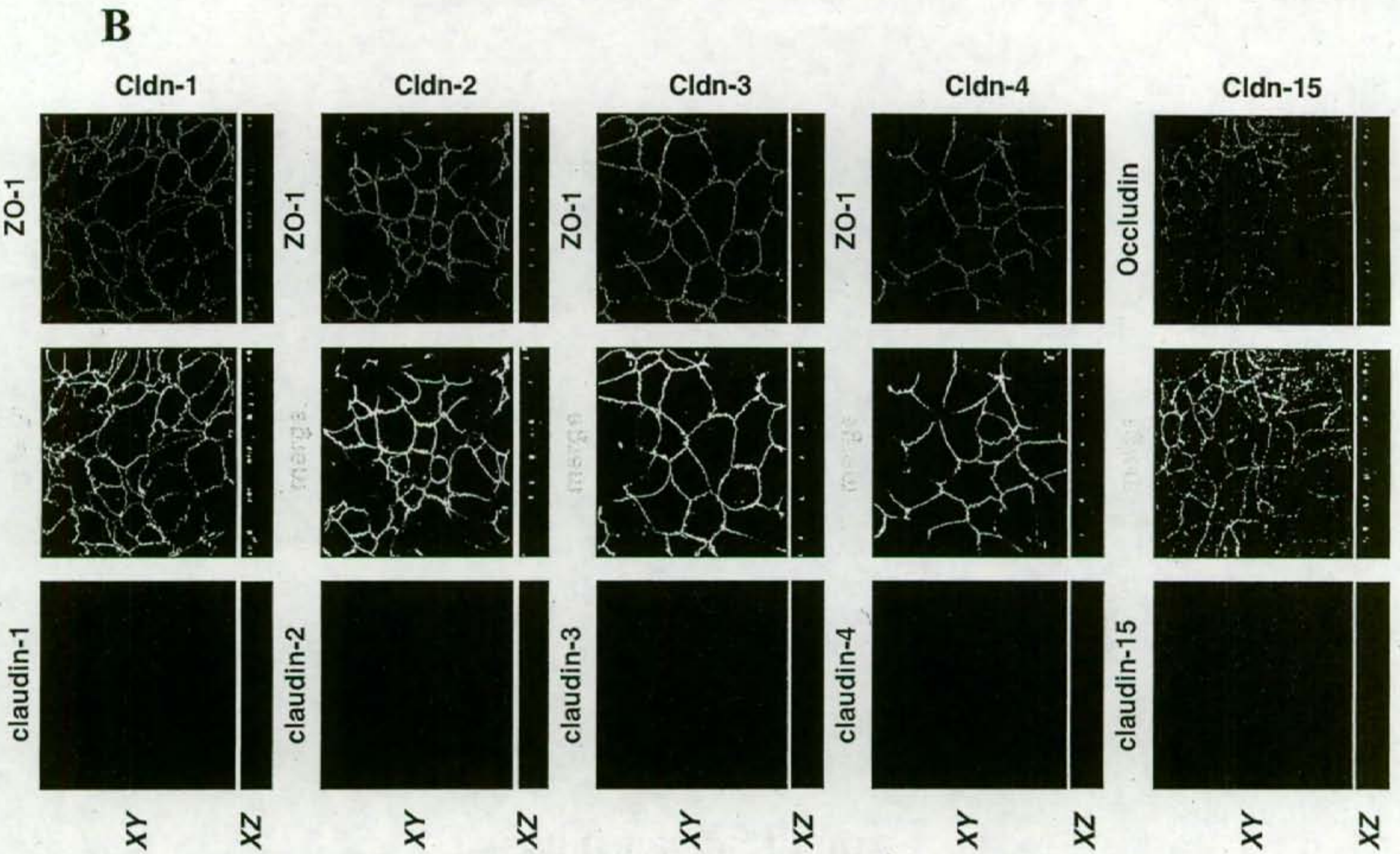
Fig. 5. Effect of overexpression of each claudin on the activity and expression of MMPs.

Caco-2 cells stably transfected with claudin-1, -2, -3, -4 or -15 expression plasmid (Cldn-1, -2, -3, -4 or -15) and mock transfectant control cells (Mock) were cultured for 24 h (A, B). MMP activity in the culture medium was measured as described in the Materials and Methods (A). The mRNA expression of *MMP-2* and *MMP-9* was estimated by real-time RT-PCR as described in Materials and Methods. Values are mean ± S.D. (n=3). $^{**}P<0.01$ (B).

Takehara *et al.*, Figure 1

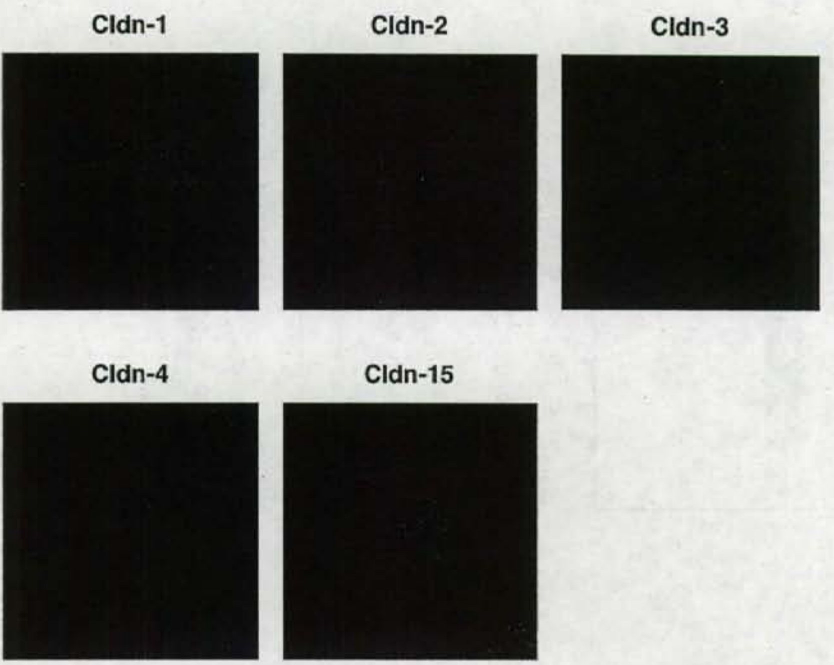


Takehara *et al.*, Figure 1



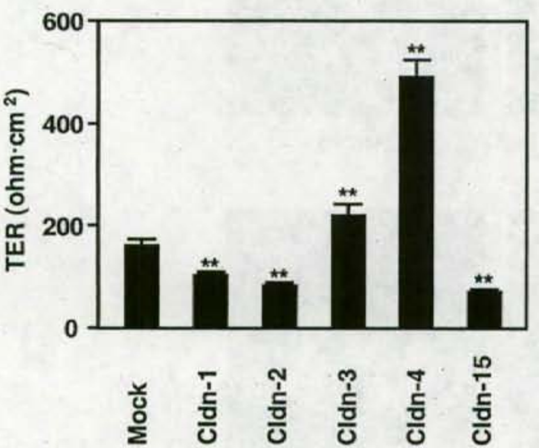
Takehara *et al.*, Figure 1

C

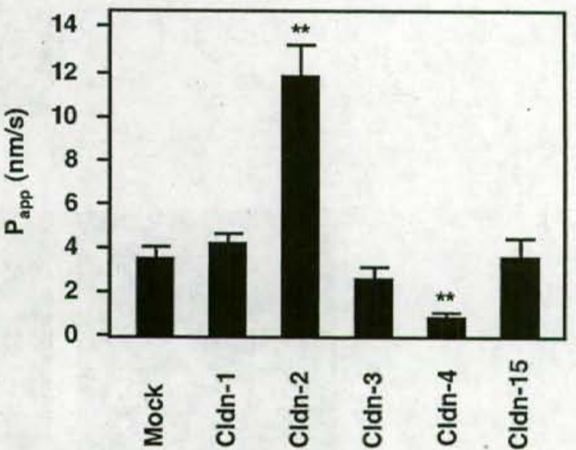


Takehara *et al.*, Figure 2

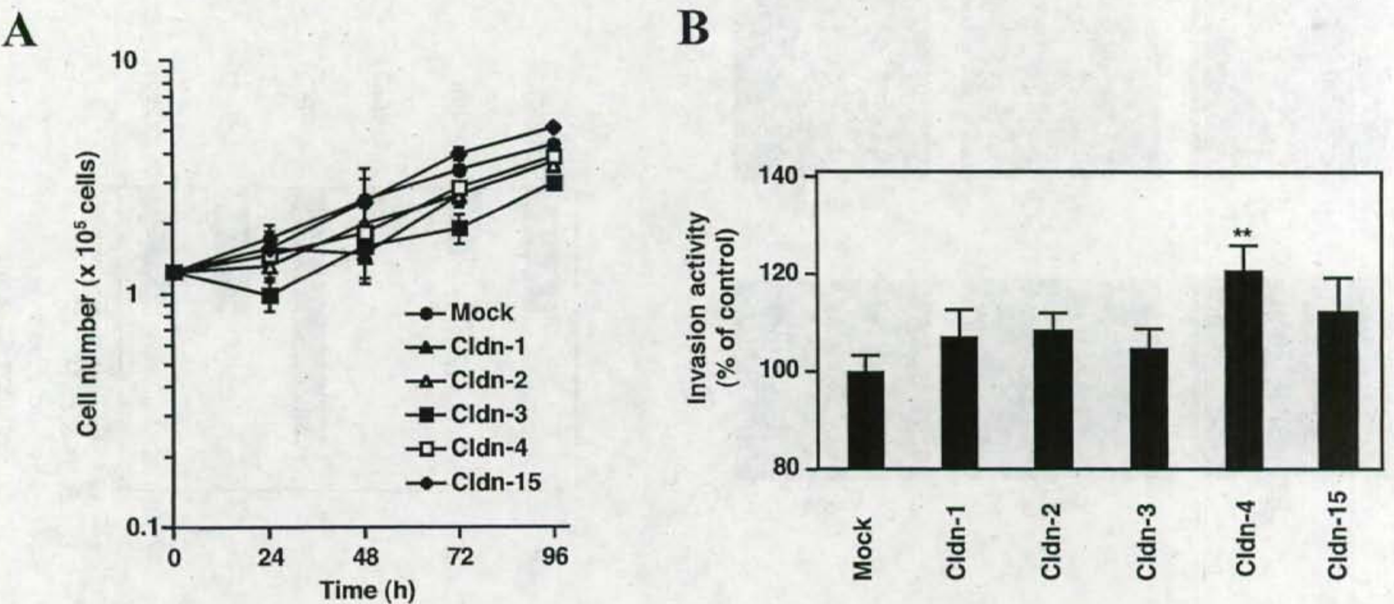
A



B

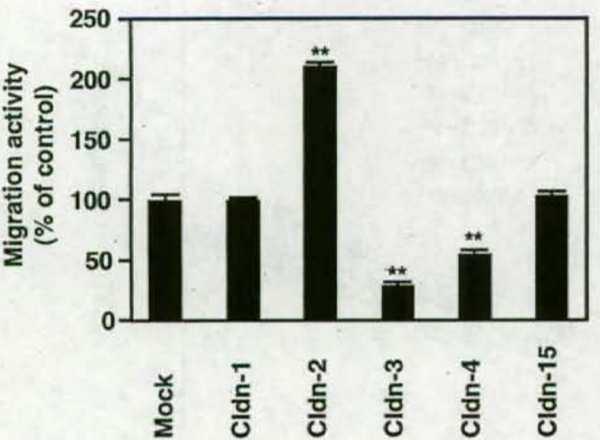


Takehara *et al.*, Figure 3

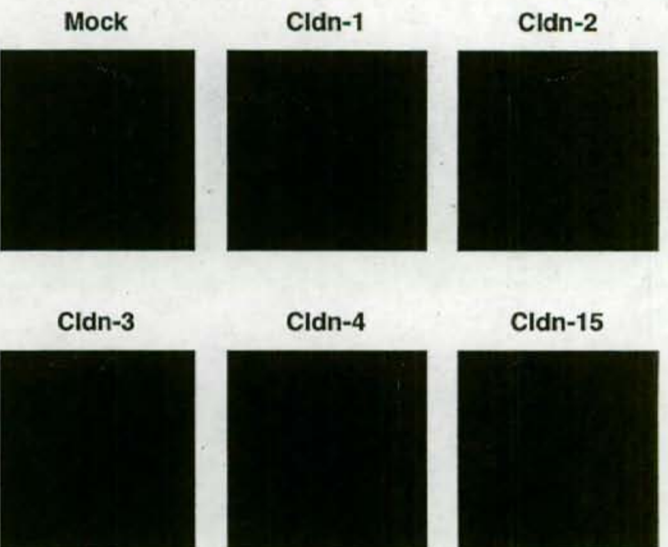


Takehara *et al.*, Figure 4

A

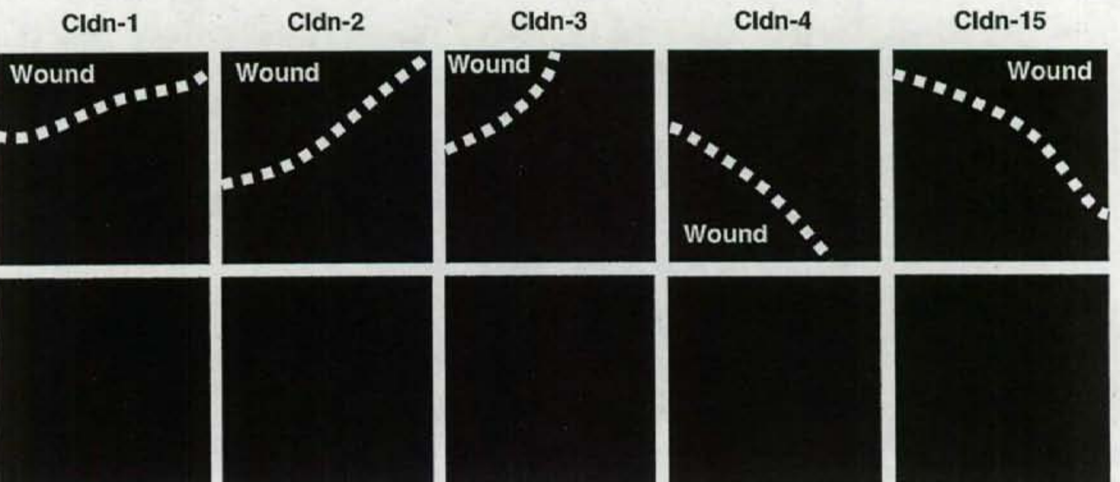


B



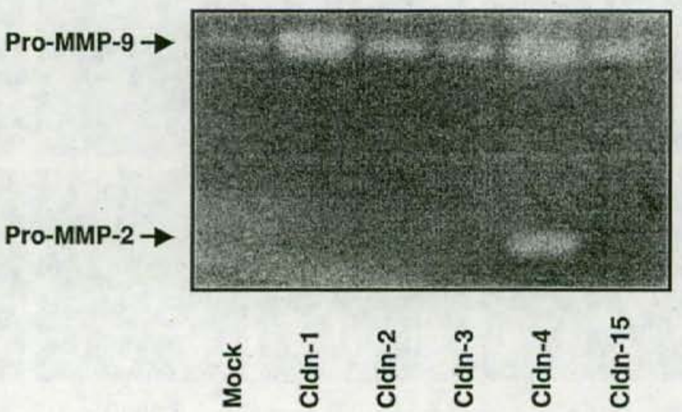
Takehara *et al.*, Figure 4

C



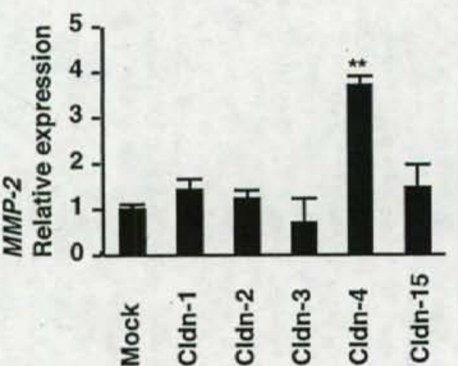
Takehara *et al.*, Figure 5

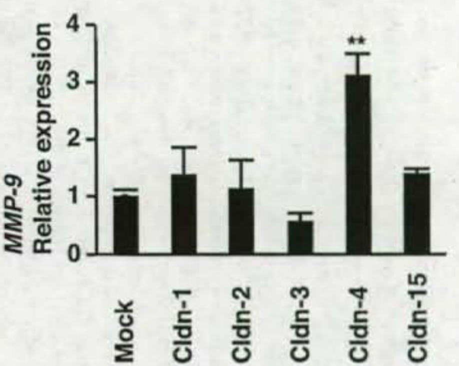
A



Takehara *et al.*, Figure 5

B





THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 6, pp. 3396–3407, February 6, 2009
© 2009 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Linkage between Phosphorylation of the Origin Recognition Complex and Its ATP Binding Activity in *Saccharomyces cerevisiae**[S]

Received for publication, June 4, 2008, and in revised form, December 3, 2008 Published, JBC Papers in Press, December 8, 2008, DOI 10.1074/jbc.M804293200

Masaki Makise, Masaya Takehara, Akihiko Kuniyasu, Nanako Matsui, Hitoshi Nakayama, and Tohru Mizushima[1]
From the Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan

The initiation of chromosomal DNA replication is tightly regulated to achieve genome replication just once per cell cycle and cyclin-dependent kinase (CDK) plays an important role in this process. Adenine nucleotides that bind to the origin recognition complex (ORC) are also suggested to be involved in this process. Of the six subunits of the *Saccharomyces cerevisiae* ORC (Orc1–6p), both Orc1p and Orc5p have ATP binding activity, and both Orc2p and Orc6p are phosphorylated by CDK in cells. In this study we constructed a series of yeast strains expressing phospho-mimetic mutants of Orc2p or Orc6p and found that expression of a Ser-188 mutant of Orc2p (Orc2-5Dp) delays G_1-S transition and S phase progression and causes the accumulation of cells with 2C DNA content. Using antibody that specifically recognizes Ser-188-phosphorylated Orc2p, we showed that Ser-188 is phosphorylated by CDK in a cell cycle-regulated manner. Expression of Orc2-5Dp caused phosphorylation of Rad53p and inefficient loading of the six minichromosome maintenance proteins. These results suggest that the accumulation of cells with 2C DNA content is due to inefficient origin firing and induction of the cell cycle checkpoint response and that dephosphorylation of Ser-188 of Orc2p in late M or G_1 phase may be involved in pre-RC formation. *In vitro*, a purified mutant ORC containing Orc2-5Dp lost Orc5p ATP binding activity. This is the first demonstration of a link between phosphorylation of the ORC and its ability to bind ATP, which may be important for the cell cycle-regulated initiation of DNA replication.

The initiation of chromosomal DNA replication is tightly regulated to replicate the genome just once per cell cycle. To achieve this, both induction of initiation at the G_1-S boundary and inhibition of initiation in other phases of the cell cycle are required. The mechanisms governing this regulation in eukaryotes have been studied the most extensively in budding yeast (*Saccharomyces cerevisiae*), and we describe mostly events in budding yeast in this paper otherwise noticed. Cyclin-dependent protein kinases (CDKs)[2] play essential roles in both the induction and inhibition of initiation; low CDK activity in late M and G_1 phases is required to prepare for initiation of DNA replication, and high CDK activity in S, G_2, and early M phases is required for suppression of re-initiation of DNA replication before cell division. This high CDK activity is also involved in initiation of DNA replication at the G_1-S boundary (1–4).

Cell cycle-regulated formation of protein complexes on origins of chromosomal DNA replication is a key step in regulation of the initiation of DNA replication. In G_1 phase (under low CDK activity), a protein complex called the "pre-replication complex (pre-RC)" is formed on each origin. The pre-RC contains several proteins including the origin recognition complex (ORC), Cdc6p, Cdt1p, and the six minichromosome maintenance proteins (MCM), Mcm2–7p. The ORC was originally identified as a six-protein complex that specifically bound to *S. cerevisiae* origins of DNA replication (5), and its homologues have been found in various eukaryotic species, including human (3). In this manuscript, "ORC" refers to *S. cerevisiae* ORC. The ORC is bound to chromatin at the origins of chromosomal DNA replication throughout the cell cycle and is thought to function as a "landing pad" for the assembly of pre-RC. At the G_1-S boundary, CDK and another kinase (Cdc7p-Dbf4p) activate the pre-RC to initiate chromosomal DNA replication. After initiation, re-formation of the pre-RC is strictly prohibited to suppress re-initiation of DNA replication, and high CDK activity is essential for this process; artificial inhibition of CDK activity in G_2 phase resulted in re-formation of pre-RC and re-initiation of DNA replication (6–8). The B type cyclin-CDK complex affects initiation of DNA replication through two distinct mechanisms, phosphorylation of, or direct binding to replication-related proteins (9, 10). Therefore, identification of the components of the protein complex present on origin DNA that are phosphorylated by CDK and an understanding of the role of this phosphorylation are important for understanding the mechanisms which ensure that replication occurs just once per cell cycle.

It has been suggested that Orc2p, Orc6p, Cdc6p, and MCM are phosphorylated by CDK in a cell cycle-regulated manner.

* This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

[1] To whom correspondence should be addressed: 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. Tel. and Fax: 81-96-371-4323; E-mail: mizu@gpo.kumamoto-u.ac.jp.

[2] The abbreviations used are: CDK, cyclin-dependent protein kinase; ORC, origin recognition complex; pre-RC, pre-replicative complex; MCM, minichromosome maintenance proteins; 5-FOA, 5-fluoroorotic acid; HA, hemagglutinin; α-factor, α-mating factor; ChIP, chromatin immunoprecipitation; *ARS1*, autonomously replicating sequence 1; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter.