Retardation of the G2-M phase progression on gene disruption of RNA binding protein Sam68 in the DT40 cell line[1]

Qing-Hua Li[a], Izumi Haga[a], Toshiki Shimizu[a,b], Michiyasu Itoh[a], Tomohiro Kurosaki[c], Jun-ichi Fujisawa[a,*]

[a] *Department of Microbiology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan*
[b] *First Department of Internal Medicine, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan*
[c] *Institute for Liver Research, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan*

Received 2 July 2002; accepted 16 July 2002

First published online 25 July 2002

Edited by Giulio Superti-Furga

Abstract Sam68 is an RNA binding protein that is tyrosine-phosphorylated by Src during mitosis and has been postulated to have a role in cell cycle control by modulating RNA metabolism. To elucidate the function of this protein, we isolated a Sam68-deficient DT40 cell line by gene disruption. The Sam68-deficient cells exhibited markedly decreased growth and the growth retardation was due to elongation of the G2-M phase, however, the kinase activity associated with Cdc2 remained unaltered. Our results indicate that Sam68 may play a critical role in G2-M progression in a manner independent of the control of Cyclin/Cdc2 kinase activity.

Key words: Sam68; RNA binding protein; Gene targeting; DT40 cell; Cell cycle

1. Introduction

Sam68 belongs to a family of RNA binding proteins called the STAR (signal transduction and activation of RNA metabolism) family [1], whose members retain a stretch of a conserved KH (hnRNP K homology) domain embedded in a larger GSG (GRP33-Sam68-GLD-1) homology region [2]. These domains have been shown to be responsible for RNA binding [3,4] and dimer formation [5,6]. There is considerable genetic evidence, obtained for various species, supporting a physiological role of the KH domain [6–8].

Sam68 was originally identified as a M phase-specific target of c-Src [9,10], and shown to interact with various cellular molecules such as Src family protein kinases [11–13], Grb2 [12,13], PLCγ1 [12–14], PI3K [13,15], Nck [16], Jak3 [13] and STAT3 [17]. Furthermore, since tyrosine phosphorylation of Sam68 in vitro decreases its RNA binding properties [18], a functional role of Sam68 in linking signal transduction to RNA metabolism is indicated.

It has been demonstrated that over-expression of a Sam68 isoform with the deletion of its KH domain by alternative splicing resulted in the suppression of cell growth [19]. In addition, the reduction of Sam68 expression by means of a retroviral-based antisense RNA strategy resulted in neoplastic transformation of NIH3T3 cells [20]. These results strongly indicate a functional role of Sam68 in the control of cell growth.

On the other hand, Sam68 was recently shown to function as a cellular homologue of the HIV-1 Rev protein by transporting unspliced viral RNA into the cytoplasm [21]. However, the mechanism of nuclear export of viral RNA by Sam68 as well as the biological role, if any, of Sam68 in the nuclear export of cellular RNA remained to be elucidated.

We report here the isolation of Sam68-deficient cells from the chicken DT40 cell line by the gene disruption procedure. The growth rate of the Sam68-deficient cells was significantly reduced compared with that of the wild-type and the growth retardation was mainly due to elongation of the G2-M phase, although the Cdc2 kinase activity, the key for G2-M progression, was not affected by the loss of Sam68 expression. These results constitute evidence of the involvement of Sam68 in G2-M progression through a mechanism not involving modulation of Cdc2 kinase activity.

2. Materials and methods

2.1. Isolation of cDNA and genomic DNA of chicken Sam68

A 0.7 kb chicken Sam68 cDNA fragment was amplified from DT40 total RNA by reverse transcription polymerase chain reaction (RT-pCR) using synthetic oligonucleotides corresponding to the KH region of human Sam68 cDNA (sense primer, 5′-GGATTTATTTTCTCATAAGAACATG-3′, antisense primer, 5′-TGGATCTGCATGTCTTCATTGAAGTC-3′) and used as a probe for screening of a λ ZAP DT40 cDNA library. A plasmid clone with a 2.4 kb cDNA fragment was obtained from hybridized bacteria by means of super-infection with helper M13 phage. Using synthetic oligonucleotides corresponding to the 5′- and 3′-non-coding sequences (5′-GTCCTTCCGGCCCTCACTTCC-3′ and 5′-CCTGTTGCTTTGCCCACACCCAGACAAGTAA-3′, respectively), a 9.2 kb genomic DNA fragment was PCR-amplified and sub-cloned into the pGEM-T vector (Stratagene, San Diego, CA, USA).

2.2. Cells and gene disruption

DT40 cells and mutant clones of them were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% chicken serum (Sigma), and 50 mM 2-mercaptoethanol at 37°C under 5% CO_2.

*Corresponding author. Fax: (81)-6-6993 1668.
E-mail address: fujisawa@takii.kmu.ac.jp (J.-i. Fujisawa).

[1] The chicken Sam68 cDNA nucleotide sequence has been submitted to GenBank database under accession number AY057837.

Abbreviations: Sam68, src-associated substrate in mitosis of 68 kDa; STAR, signal transduction and activation of RNA metabolism; hnRNP, heterogeneous nuclear ribonucleoprotein; KH, K homology; GLD-1, germline defective-1; GSG, GRP33-Sam68-GLD-1; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis

0014-5793/02/$22.00
PII: S0014-5793(02)03103-4

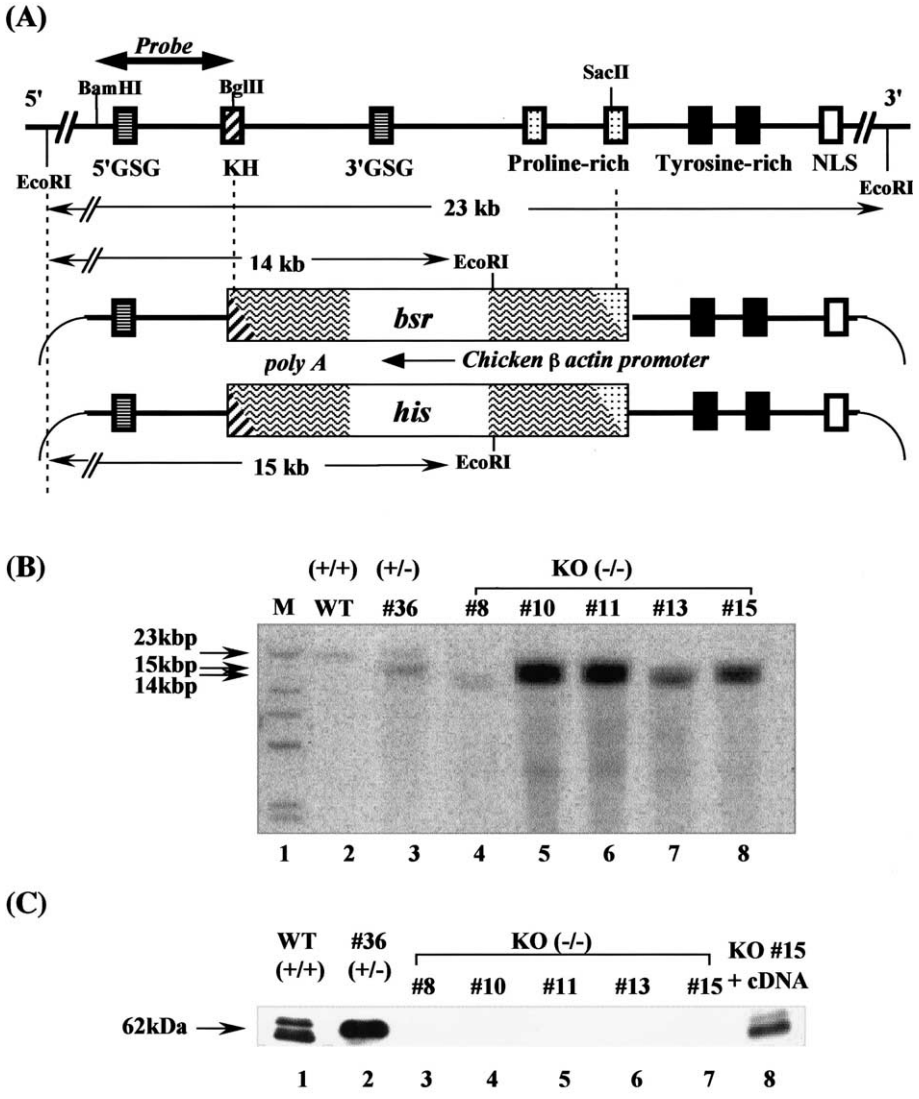

Fig. 1. Disruption of the Sam68 gene in chicken DT40 cells. A: Structures of the chicken Sam68 gene (upper panel) and targeting vectors (middle and lower panels). Each exon contains functional motifs such as the GSG motif, KH motif, proline-rich motif, tyrosine-rich motif, and nuclear localization signal (NLS). B: Southern blot analysis. Genomic DNAs from wild-type cells (lane 2), mutant cells with recombination in a haploid allele (#36) (lane 3), and knocked-out clones #8, #10, #11, #13 and #15 (lanes 4–8) were digested with *Eco*RI, and probed with a ^{32}P-labeled 1 kb genomic fragment encompassing the 5′-GSG and KH exons. C: Protein analysis of chicken Sam68. Proteins prepared from wild-type and mutant cells were subjected to a 7.5% SDS–PAGE and then analyzed by Western blotting with an anti-Sam68 antibody.

Targeting vectors, CSG-bsr and CSG-hisD, were constructed by replacing the genomic fragment containing exons encoding amino acid residues 179–321 of chicken Sam68 with a bsr or hisD expression cassette. 25 μg of CSG-bsr was linearized by *Sal*I restriction cleavage and then transfected into DT40 cells by electroporation (550 V, 25 μFD). After selection of clones in the presence of blasticidin S (Funakoshi, Tokyo, Japan) at a concentration of 50 μg/ml, genomic DNAs were prepared from drug-resistant cell clones and screened by Southern blot analysis. 25 μg of linearized CSG-hisD DNA was further transfected into the clone with deletion of the Sam68 gene on the haploid genome, and selected with both blasticidin S and L-hisdinol (Sigma) at concentrations of 50 μg/ml and 1 mg/ml, respectively.

2.3. Transduction of chicken Sam68 cDNA

An amphotropic retrovirus vector expressing chicken Sam68 cDNA was isolated from a culture supernatant of PT67 packaging cell line that had been infected with an ecotropic retrovirus produced by BOSC23 cells transfected with a vector plasmid, pRchicken-Sam68-puro. DT40 cells with gene disruption were infected with the amphotropic retrovirus and then selected with puromycin at a concentration of 0.5 μg/ml in the culture medium.

2.4. Southern blot analyses

Genomic DNA was prepared from wild-type and Sam68-deficient cells using DNA$_{ZOL}$™ reagent (Gibco-BRL). 10 μg of DNA was digested with *Eco*RI, separated in a 0.8% agarose gel, transferred to a Hybond™-N+ transfer membrane (Amersham), and then probed with a ^{32}P-labeled *Bam*HI–*Bgl*II genomic fragment of 1 kb long.

2.5. Protein analysis

Cells were lysed in solubilization buffer (1% Nonidet P40, 150 mM Tris–HCl, pH 7.4, 50 U/ml Trasylol (Bayer, Leverkusen, Germany), and 1 mM Na_3VO_4 (Sigma)) at 4°C. The lysates were cleared by centrifugation, and then 10 μg aliquots of lysates were separated by 7.5% SDS–PAGE, electro-blotted onto an Immobilon™ transfer membrane (Millipore), and then probed with rabbit antiserum against human Sam68 (Santa-Cruz, San Diego, CA, USA). Chemiluminescence obtained with horseradish peroxidase-conjugated protein A (ECL System; Amersham) was used for protein detection.

2.6. Cell cycle analysis by flow cytometry

5×10^5 cells were fixed with 50% methanol at 4°C overnight, washed with phosphate-buffered saline, and then suspended in Na_2HPO_4–citrate buffer at room temperature. After RNase treatment at 37°C for 30 min, the cells were stained with a propidium iodide solution for 30 min on ice. Cell samples were filtered into 12×75 mm tubes and subjected to analysis with a FACScan (Becton Dickinson), and then cell cycle analysis was performed with the ModFit program (Verity Software House).

2.7. Histone H1 kinase assay

25×10^6 cells were collected and protein extracts were prepared as previously described. 25 μg of a cell lysate was incubated with 0.2 μg of anti-Cdc2 monoclonal antibody (Santa Cruz) on ice for 2 h, followed by incubation with protein A-Sepharose for 1 h at 4°C. The immunoprecipitate was washed twice with lysis buffer and once with assay buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and 1 mM dithiothreitol), and then suspended in 10 μl of assay buffer. The reaction was started by the addition of ATP at the final concentration of 1 μM with 5 μCi [γ^{32}P]ATP and 0.5 mg/ml H1 histone (Alexis® Biochemicals), and continued at 30°C for 5 min. The samples were analyzed on a 12% SDS–polyacrylamide gel and exposed to X-ray film for 12 h.

3. Results

3.1. Targeted disruption of the chicken Sam68 gene

In order to disrupt the Sam68 gene in the chicken DT40 B cell line by homologous recombination, we first isolated chicken Sam68 cDNA as described in Section 2. The overall sequence homology at the amino acid and nucleotide levels was 75% and 78%, respectively, between human Sam68 and the isolated cDNA. Since a stretch of a 138 amino acid sequence including the KH domain was identical and the putative functional domains, such as the proline-rich domain, tyrosine-rich domain, and nuclear localization signal, were almost completely conserved, we concluded that this gene was the chicken orthologue of Sam68 (GenBank accession number AY057837).

Then, we PCR-amplified a 9.2 kb genomic fragment from DT40 genomic DNA using primers corresponding to the sequences of the 5′- and 3′-non-coding regions in the cDNA and constructed targeting vectors by replacing a 4.1 kb genomic fragment covering the 3′ half of the KH exon and a part of the 3′ GSG domain with an expression cassette of a drug resistance gene, *bsr* and *his* (Fig. 1A). After sequential transfection of targeting vectors into DT40 cells, cell clones with homologous recombination in both alleles were selected by cell culture in the presence of selection drugs, blasticidin and histidinol, and further screened by Southern blot analysis of genomic DNA. *Eco*RI digestion of genomic DNA prepared from drug-resistant cell clones gave two bands of 14 kb and 15 kb (cell clones #8, #10, #11, #13 and #15, lanes 4–8, respectively, in Fig. 1B) instead of a 23 kb band, which was observed for wild-type cells (lane 2, Fig. 1B) and a singly knocked-out clone, #36 (lane 3, Fig. 1B).

Protein blot analysis with antiserum against human Sam68 demonstrated that five clones, #8, #10, #11, #13 and #15, lacked expression of the Sam68 protein (lanes 3–7, Fig. 1C). Northern analysis with a probe for the KH domain also confirmed the loss of a 3 kb mRNA in knocked-out cells, which was observed for the wild-type and a mutant clone with recombination in a single allele (data not shown).

3.2. Growth retardation of Sam68-deficient DT40 cells

When the growth rates of Sam68-deficient and wild-type DT40 cells were monitored, the Sam68-deficient cells were found to grow significantly more slowly than the wild-type and the doubling times of two independent Sam68-deficient clones (#8 and #15) were 13.7 and 13.2 h, respectively, whereas the parent DT40 cells multiplied once in 9.9 h at 37°C (Fig. 2).

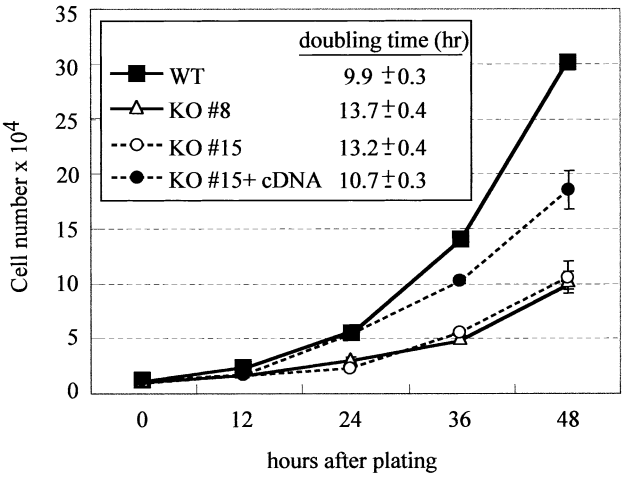

Fig. 2. Growth curves for chicken Sam68-deficient cells. 10^4 cells/well of the wild-type (■), two lines of Sam68-deficient cells, #8 (△) and #15 (○), and the Sam68 cDNA-transduced #15 mutant cells (●) were plated in a six well culture dish, and the cell number was determined in triplicate every 12 h. Data represent the mean ± S.E.M. The doubling time shown in the inset is the average of three independent experiments.

To confirm that the retarded growth of knocked-out cells was due to the loss of Sam68 expression, chicken Sam68 cDNA was introduced into one of the knocked-out cell clone, #15, by means of a retrovirus vector. Although the amount of Sam68 protein expressed from the retrovirus vector was less than half of that in the case of the wild-type (lane 8, Fig. 1C), the growth rate of cDNA-transduced cells recovered substantially and the doubling time was reduced to 10.7 h (Fig. 2). Therefore, we concluded that the abolition of Sam68 expression led to retardation of cell growth.

3.3. The G2-M phase was elongated in Sam68-deficient cells

To clarify the points in the cell cycle that were affected by deletion of the Sam68 gene, flow-cytometric analysis was carried out. The cell cycle pattern of each cell clone demonstrated that the ratio of the G2-M phase to G1-S phase was significantly increased in Sam68 knocked-out cells (Fig. 3A). The lengths of the phases of the cell cycle were determined by multiplying the doubling time (Fig. 2, inset) by the percentage of each phase, and the results indicated that the G2-M phase was specifically affected in knocked-out cells (Fig. 3B). While the G0-G1 and S phases of two knocked-out cell clones remained substantially unchanged, the G2-M phase in mutant clones was elongated more than two-fold. Again, re-expression of chicken Sam68 in the knocked-out cells shortened the length of the G2-M phase, indicating an effect of Sam68 gene disruption on G2-M progression.

3.4. Cdc2 kinase activity in Sam68-deficient cells

It has been well demonstrated that the kinase activity of the cyclin B–Cdc2 complex drives progression of the G2-M phase in the cell cycle. Therefore, the Cdc2 kinase activity in Sam68

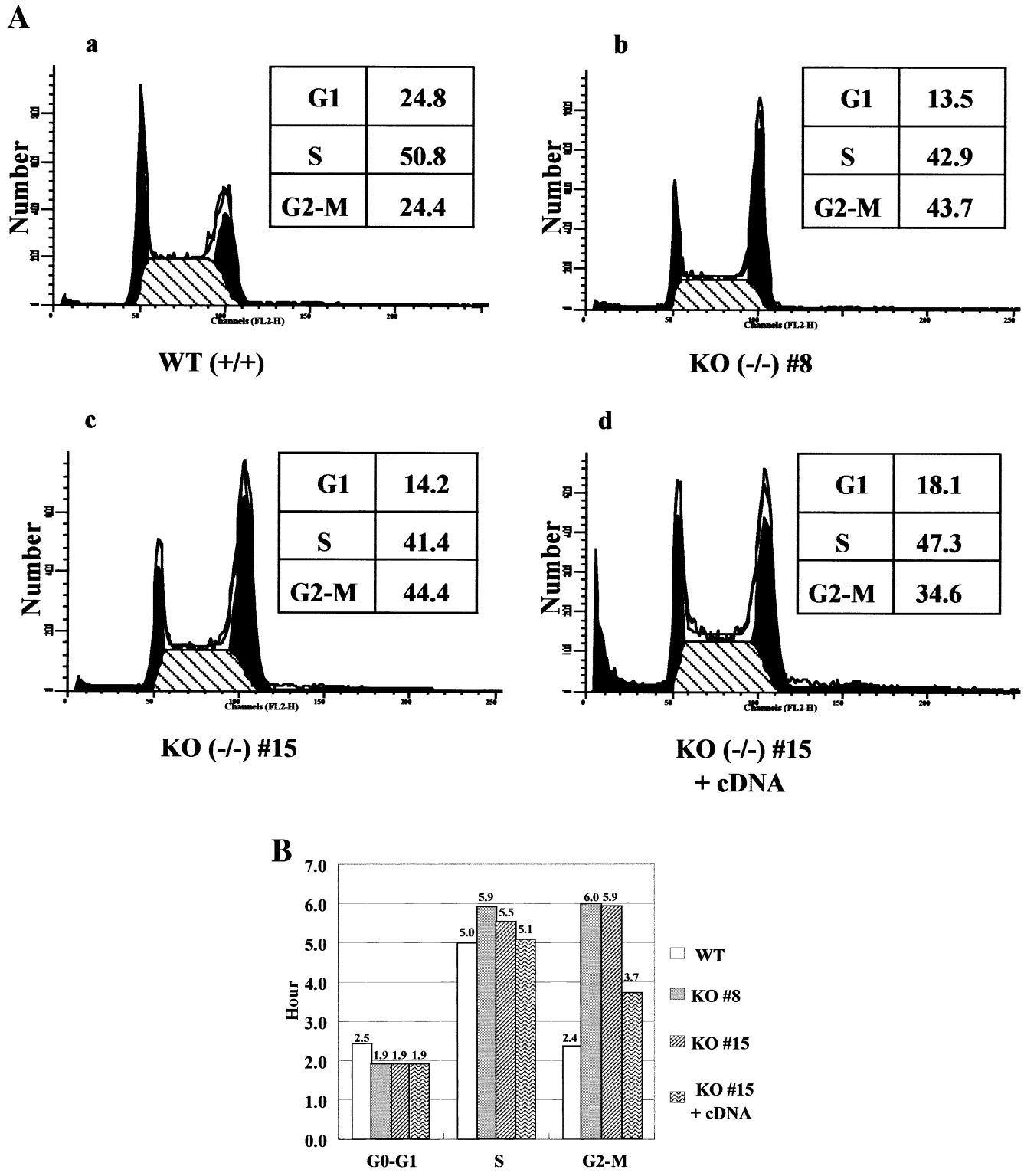

Fig. 3. Flow-cytometric analysis of Sam68-deficient DT40 cells. A: Sam68 knocked-out cells (b, #8; c, #15) showed an increased G2-M phase compared to wild-type (a) and cDNA-transformed (d) cells. B: Phase lengths of the cell cycle in wild-type, Sam68 knocked-out #8 and #15 and cDNA-transformed knocked-out #15 cells.

mutant cells was examined in comparison with that in wild-type DT40 cells. Cdc2 kinase was immunoprecipitated in cell extracts prepared from wild-type and mutant cells with an anti-Cdc2 antibody, and the immune complexes were incubated with [γ-^{32}P]ATP and histone H1 as substrates. As shown in Fig. 4, similar kinase activities, as indicated by the ^{32}P intensity with histone H1, were demonstrated in Sam68-deficient cells. These results strongly indicated that the retardation of G2-M progression observed in Sam68-deficient DT40 cell was not due to a change in Cdc2 kinase activity.

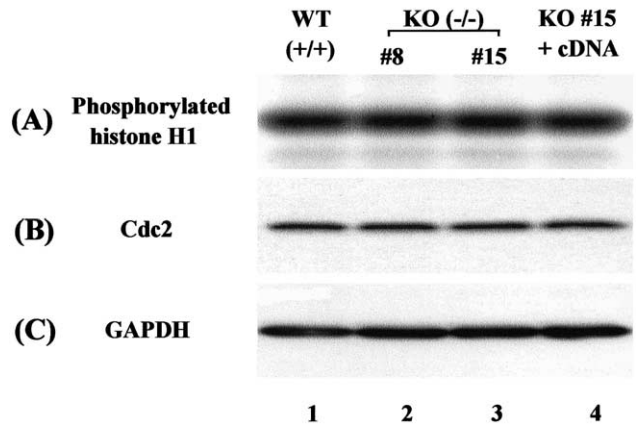

Fig. 4. Analysis of Cdc2 kinase activity. A: Histone H1 kinase assay. Lane 1: wild-type cells, lanes 2 and 3: mutant clones, #8 and #15, respectively, and lane 4: a mutant clone transformed with chicken Sam68 cDNA, #15+cDNA. B: Expression of Cdc2. 10 μg of each protein was separated by 12% SDS–PAGE and Cdc2 was detected with an anti-Cdc2 antibody. C: GAPDH expression on the same blot as in B was monitored as a control.

4. Discussion

We demonstrated here that deletion of the Sam68 gene in chicken DT40 cells resulted in growth retardation due to elongation of the G2-M phase in the cell cycle. Since exogenous expression of Sam68 cDNA in the knocked-out cells could reverse the growth defect, it was strongly suggested that Sam68 is involved in G2-M progression in the cell cycle.

On the other hand, Barlet et al. [19] documented that the expression of a splicing variant of Sam68, in which part of KH is deleted, inhibited serum-stimulated progression to the S phase of the cell cycle. The reason for the discrepancy between the two experiments is uncertain. However, the mutant Sam68 protein lacking the RNA binding domain could interact with signaling molecules in a dominant-negative fashion, leading to inhibition of signal transduction irrespective of the control of RNA metabolism. Therefore, over-expression of the variant Sam68 molecule could result in a different consequence in cell growth control from the depletion of the natural form of Sam68. In this respect, it should be noted that Sam68 is tyrosine-phosphorylated and Src-associated only in mitosis, and that the RNA binding property of Sam68 is impaired when it is phosphorylated [18]. The mitotic phosphorylation of Sam68 at threonine residues by Cdc2 has also been demonstrated [22]. Thus, a major part of the Sam68 function could be closely linked to M phase progression. In fact, the treatment of Src-transformed fibroblasts with a Src-tyrosine kinase inhibitor, radicicol, blocked the mitosis-specific phosphorylation of Sam68 and retarded the exit of cells from mitosis [23].

In murine NIH3T3 fibroblasts, homozygous functional inactivation of Sam68 by means of a retrovirus-based antisense RNA strategy has been shown to lead to neoplastic transformation [20]. This indicated the possible role of Sam68 as a tumor suppressor, as indicated in the case of another STAR protein, GLD-1, in *Caenorhabditis elegans* [24]. However, as re-expression of Sam68 did not uniformly reverse phenotypic abnormalities, the authors suggested that some neoplastic progression had occurred during antisense-induced Sam68 deficiency through some indirect mechanism. In this context, it would be interesting to postulate that the alteration of G2-M progression observed in Sam68-deficient DT40 cells may reflect some defect(s) in the G2-M checkpoint, which could lead to neoplastic transformation.

Although an activity of Sam68 in the enhancement of Rev-mediated nuclear export of unspliced HIV-1 mRNA has been demonstrated [21], the function was inhibited by olomoucine, an inhibitor of Cdc2 kinase [25]. The result also strongly indicates the close association of Sam68 function with mitotic control.

Wang et al. [26] have reported a defect in cell growth due to the loss of an RNA binding protein, HuR, in a colorectal carcinoma cell line. Suppression of HuR expression resulted in instability of the mRNAs encoding cyclin A and cyclin B1 and thus the reduction of cyclin/Cdc2 kinase activity was attributed to the blockade of cell cycle progression mostly in the S and G2-M phases. In the case of Sam68-deficient cells, however, the Cdc2 kinase activity did not change although substantial retardation of cell growth was observed in the G2-M phase. Therefore some machinery, yet unknown, other than the cyclin B/Cdc2 kinase complex should go out of control with the loss of Sam68 expression in DT40 cells.

In this sense, it is of note that Hill et al. [27] have reported the co-localization of Sam68 with β-actin mRNA in the peripheral regions on cell motility in response to serum stimulation. Thus, the metabolism of mRNAs for proteins involved in cytokinesis or cell motility such as β-actin could be modulated by Sam68 during G2-M progression in the cell cycle.

To understand the mechanism(s) responsible for the G2-M retardation, RNA species whose functions are modulated by Sam68 should be found, but so far little is known as to the target RNA molecules for Sam68. We are currently identifying target mRNA species for Sam68. Combined with the information on the target molecules, the Sam68-deficient cell line obtained in this report should provide a novel reagent that should prove useful for further studies elucidating the possible mechanism of cell growth control though the RNA metabolism.

Acknowledgements: This study was supported by a grant from the Japanese Private School Foundation of the Ministry of Education. We also thank Mr. N.J. Halewood for the language assistance.

References

[1] Vernet, C. and Artzt, K. (1997) Trends Genet. 13, 479–484.
[2] Zorn, A.M., Grow, M., Patterson, K.D., Ebersole, T.A., Chen, Q., Artzt, K. and Krieg, P.A. (1997) Gene 188, 199–206.
[3] Wong, G., Muller, O., Clark, R., Conroy, L., Moran, M.F., Polakis, P. and McCormick, F. (1992) Cell 69, 551–558.
[4] Lin, Q., Taylor, S.J. and Shalloway, D. (1997) J. Biol. Chem. 272, 27274–27280.
[5] Chen, T., Damaj, B.B., Herrera, C., Lasko, P. and Richard, S. (1997) Mol. Cell. Biol. 17, 5707–5718.
[6] Chen, T. and Richard, S. (1998) Mol. Cell. Biol. 18, 4863–4871.
[7] Siomi, H., Choi, M., Siomi, M.C., Nussbaum, R.L. and Dreyfuss, G. (1994) Cell 77, 33–39.
[8] Zorn, A.M. and Krieg, P.A. (1997) Genes Dev. 11, 2176–2190.
[9] Fumagalli, S., Totty, N.F., Hsuan, J.J. and Courtneidge, S.A. (1994) Nature 368, 871–874.
[10] Taylor, S.J. and Shalloway, D. (1994) Nature 368, 867–871.
[11] Weng, Z., Thomas, S.M., Rickles, R.J., Taylor, J.A., Brauer, A.W., Seidel-Dugan, C., Michael, W.M., Dreyfuss, G. and Brugge, J.S. (1994) Mol. Cell. Biol. 14, 4509–4521.
[12] Richard, S., Yu, D., Blumer, K.J., Hausladen, D., Olszowy,

M.W., Connelly, P.A. and Shaw, A.S. (1995) Mol. Cell. Biol. 15, 186–197.
[13] Fusaki, N., Iwamatsu, A., Iwashima, M. and Fujisawa, J. (1997) J. Biol. Chem. 272, 6214–6219.
[14] Maa, M.C., Leu, T.H., Trandel, B.J., Chang, J.H. and Parsons, S.J. (1994) Mol. Cell. Biol. 14, 5466–5473.
[15] Sanchez-Margalet, V. and Najib, S. (1999) FEBS Lett. 455, 307–310.
[16] Lawe, D.C., Hahn, C. and Wong, A.J. (1997) Oncogene 14, 223–231.
[17] Sanchez-Margalet, V. and Martin-Romero, C. (2001) Cell. Immunol. 211, 30–36.
[18] Wang, L.L., Richard, S. and Shaw, A.S. (1995) J. Biol. Chem. 270, 2010–2013.
[19] Barlat, I., Maurier, F., Duchesne, M., Guitard, E., Tocque, B. and Schweighoffer, F. (1997) J. Biol. Chem. 272, 3129–3132.
[20] Liu, K., Li, L., Nisson, P.E., Gruber, C., Jessee, J. and Cohen, S.N. (2000) J. Biol. Chem. 275, 40195–40201.
[21] Reddy, T.R., Xu, W., Mau, J.K., Goodwin, C.D., Suhasini, M., Tang, H., Frimpong, K., Rose, D.W. and Wong-Staal, F. (1999) Nat. Med. 5, 635–642.
[22] Resnick, R.J., Taylor, S.J., Lin, Q. and Shalloway, D. (1997) Oncogene 15, 1247–1253.
[23] Pillay, I., Nakano, H. and Sharma, S.V. (1996) Cell Growth Differ. 7, 1487–1499.
[24] Jones, A.R. and Schedl, T. (1995) Genes Dev. 9, 1491–1504.
[25] Reddy, T.R., Tang, H., Xu, W. and Wong-Staal, F. (2000) Oncogene 19, 3570–3575.
[26] Wang, W., Caldwell, M.C., Lin, S., Furneaux, H. and Gorospe, M. (2000) EMBO J. 19, 2340–2350.
[27] Hill, M.A., Schedlich, L. and Gunning, P. (1994) J. Cell Biol. 126, 1221–1229.