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研究テーマ 遺伝子欠失DT40細胞株を用いたRNA結合蛋白Sam68の機能解析

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表　(有)・無　(学会名・内容)

1.芳賀　泉、李　慶華、房木ノエミ、藤澤順一：
RNA結合蛋白Sam68の細胞周期特異的な機能発現の解析
第20回日本分子生物学会年会　1997, 12月　京都

2.李　慶華、芳賀　泉、藤澤順一：トリSam68遺伝子の解析
第21回日本分子生物学会年会　1998, 12月　横浜

3.李　慶華、芳賀　泉、伊藤道恭、藤澤順一：
遺伝子欠失DT40細胞株を用いたRNA結合蛋白Sam68の機能解析
第22回日本分子生物学会年会　1999, 12月　福岡

(2) 学会誌等に発表した論文　有・(無)　(雑誌名・論文名)

3．今後の研究計画

これまでの研究でSam68が細胞周期のS期の進展に関与していることが示唆されたことから、同時期に発現調節を受けているmRNAの性質とその調節機構に興味が持たれる。そこで、Sam68によってその安定性、局在性或いは翻訳レベルでの調節を受けている標的mRNAの同定と、そのコードする蛋白の細胞増殖制御における役割を明らかにすることを今後の目標にする。そのために、今回単離したSam68遺伝子欠失細胞特異的に発現が減少あるいは増大しているmRNAをRNA differential display法で単離・同定する。発現量に差の見られるmRNAに関しては、Sam68蛋白との結合性を確認し、さらに細胞周期および細胞増殖刺激に対応した動態を解析する。また、Sam68の有無によって発現が変動する蛋白に関しても２次元の蛋白電気泳動法を用いて同定を試み、有意な差が確認される蛋白に関しては単離・精製し、アミノ酸配列決定、遺伝子クローニングまで進める。

4．研究指導者の意見

李慶華氏は、関西医科大学大学院入学以来、２年間という短い期間の間に、分子生物学および細胞生物学における基本的な手技を殆どマスターし、また、教室のセミナーにも積極的に参加されるなど、その能力と勤勉さが高く評価されます。これまでの研究に対する取り組み方から判断して、あと２年間の在学期間中に本研究をさらに展開し、優秀な学位論文を作成していただけるものと確信しております。研究生活を経済的に支えて下さいました日中友好協会のご厚意に心から感謝いたしますとともに、学位取得後は、その知識と技術を中国の医学と医療の発展に役立てていただくことを念願しております。

研究指導者氏名 藤澤順一 ㊞

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

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遺伝子欠失 DT40 細胞株を用いた RNA 結合蛋白 Sam68 の機能解析

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要旨

Sam68 はチロシンキナーゼ Src により細胞周期のM期特異的にリン酸化される主要な分子として同定された分子量 68 kda の RNA 結合蛋白質であり、これまでに Src, Fyn 等多くのシグナル伝達分子を会合することが示されている。また、RNA 結合ドメインを欠失した分子種の強制発現が細胞増殖を抑制することから、細胞増殖制御における RNA 動態調節との関連が注目されている。

細胞増殖制御における Sam68 の機能を明らかにする目的で、トリ B 細胞株 DT40 を用いて細胞レベルでの遺伝子ターゲッティングを行い Sam68 蛋白の発現を欠失した DT40 細胞株を得た。

Sam68 欠失 DT40 細胞の細胞増殖速度は、野性株と比較してわずかながら減少する傾向にあり、細胞周期の解析と合わせ、これは主に S 期の伸長に原因すると考えられた。また、血清濃度減少にともない、S 期の伸長と細胞のアポトーシスの増加が顕著に観察されることから、Sam68 蛋白は S 期或いはその準備期（G1 期）における RNA 機能の調節に関与する可能性が示唆された。

細胞内シグナル伝達との関連を明らにする目的で、B 細胞受容体刺激にともなう蛋白質のチロシンリン酸化を解析したところ、B 細胞シグナル伝達において主要な役割を果たす 80 kda の蛋白 BLANK のリン酸化が、Sam68 欠失 DT40 細胞において有意に減少していた。この結果は、Sam68 が部分的ながら B 細胞シグナル伝達に関与することを示している。

今後、Sam68 欠失細胞において動態の変化が観察される mRNA 分子種の有無と RNA 動態調節における細胞内シグナル伝達系の関与に興味が持たれる。

KEY WORDS
Sam68　チロシンキナーゼ RNA 結合蛋白　遺伝子ターゲッティング　細胞周期 シグナル伝達

研究報告

< Purpose >

The RNA binding protein, Sam68 is the only known Src mitotic target and tyrosine phosphorylated specifically during mitosis. Since the Sam68 variant molecule lacking its RNA-binding motif (KH motif) by alternative splicing produced in the stage of contact inhibition has been shown to regulate the cell growth negatively, the functional role(s) of RNA binding in cell growth control was implicated. Sam68 was identified to associate with various signaling molecules (through its SH3 and SH2 binding domains) such as Fyn, Lck, Grb2, PI3K and so on, suggesting its involvement in signal transduction pathway. However, the physiological function or impact of Sam68 in cell growth control and signal transduction events remains to be elucidated. To address the functional role of Sam68 in cell growth control and/or signaling transduction pathways, we established DT40 B cell deficient in Sam68 by gene targeting method and analyzed its functional role in cell growth control and signaling transduction pathways.

< Method >

Cells and Antibodies

Wild type DT40 cell and its derivative mutant cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1 % chicken serum, 50μM 2-mercaptoethanol, and antibiotics. Anti-Sam68 (P62GAP) antibody and anti-chicken IgM antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Southern Biotechnology Associates, Inc., respectively. Antibody to phosphotyrosine (Tyr-P, 4G10) was a generous gift from Dr. Nariuchi (University of Tokyo).

Isolation of Sam68 deficient DT40 cells

A 0.7 Kb chicken Sam68 cDNA fragment was cloned from DT40 total RNA by RT-PCR using synthetic DNA oligonucleotides corresponding to the KH region of human Sam68 cDNA (5':GGATTTATTTTCTCACAAGAATATG, 3':TGGATTTGCATGTCTTCATTGAAGTG). This fragment was then used as a probe for screening λ ZAP DT40 cDNA library and a 2.4 kbp cDNA of chicken Sam68 was obtained. A 8Kb genomic clones were obtained by LA-PCR using primers to SGS homology and 3'non-coding regions. Targeting vectors, CSG-bsr and CSG-hisD, were constructed by replacing the genomic fragment containing exons that correspond to chicken Sam68 amino acids residues 157-257, 348-671 with a bsr or his-D cassettes (gifts from Dr. Kurosaki, Kansai Medical University). 50μg of CSG-bsr was linearized by SalI restriction cleavage and transfected into DT40 cells by electroporation (550V, 25μF). After selection of clones in the presence of blasticidin S (50μg/ml), genomic DNAs were prepared and screened by Southern blot analysis. 50μg of linearized CSG-his DNA was further transfected into the clone with deletion of Sam68 gene on a haploid genome and selected with both bsr (50μg /ml) and L-histidinol (1mg/ml).

Southern and RNA blot analysis

DNA was prepared from wild-type and Sam68 deficient DT40 cells using DNA_{ZOL}™ reagent, 10μg of DNA was digested by EcoRI, separated in 0.8% TAE agarose gel, transferred to Hybond™-N+ Nucleic acid transfer membranes (Amersham) and probed with P^{32} labeled DNA probe. RNA was also prepared from wild-type and Sam68 deficient DT40 cells using TRI_{ZOL}^{R} reagent. Total RNA (20μg) was separated in 1.2% formaldehyde gel, transferred to Hybond™-N+ membrane (Amersham) and probed with P^{32}-labeled chicken Sam68 cDNA probe.

Protein blot analysis

Cells were lysed in solubilization buffer (1% Nonidet P40, 150mM Tris-HCl, pH 7.4, 50 units/ml Trasylol (Bayer, Leverkusen, Germany), and 1mMNa_3vo_4) at 4°C. The lysates were cleared by centrifugation, size fractionated by SDS-PAGE, and transferred onto an Immmobilon™ Transfer membrane (Millipore) and probed with antiserum against human Sam68.

Phosphorylation analysis

DT40 cells and Sam68 deficient cells were stimulated for 5 and 10 min at $2.5x10^5$ cells/ml in RPMI1640 with M4 (4μg /ml) at 37°C. Cells were lysed in 80μl SDS-sample buffer and the

proteins with phosphorylated tyrosine were detected by protein blotting with anti-phosphotyrosine antibody (4G10).

Flow-cytometric analysis

For DNA content analysis, cells were pelletted and resuspended in the Cycle TEST PLUS DNA reagent A, B, C in succession according to the manufacturer's protocol. For expression of cell surface receptor, cells were washed, subsequently incubated with FITC-conjugated anti-chicken IgM (Bethyl Laboratoriees, InC., Montgomery, TX), and analyzed by FACSCan with FACSort program.

< Results >

Isolation and structural analysis of chicken Sam68 gene

In order to disrupt Sam68 gene in a chicken B cell line, DT40 by homologous recombination, we first isolated the chicken Sam68 cDNA. Since the antiserum against the KH domain of human Sam68 could recognize chicken Sam68 in DT40 cell (data not shown), it was expected that the KH domain is highly conserved between chicken and human. Thus RT-PCR of DT40 RNA was performed by using synthetic DNA oligonucleotides corresponding to the KH region of human Sam68 cDNA as PCR primers. As a result a 0.7 kbp fragment was obtained and the nucleotides sequence of this fragment was indicated to encode the KH region of chicken Sam68.

Then using the 0.7 kbp RT-PCR fragment as a probe a full-length chicken Sam68 cDNA was isolated by screening the DT40 cDNA library. Sequence analysis of the full-length cDNA revealed that the structure of Sam68 is conserved by 75% and 78% in the levels of amino-acid and nucleotide sequences, respectively, between human and chicken. A stretch of 138 amino acids including the KH domain was found to be identical between the two species (Fig.1).

From the information of cDNA sequence several PCR primers corresponding chicken Sam68 gene were synthesized and used for isolating the genomic DNA fragment by PCR using DT40 genomic DNA as a template. An 8 kbp genomic fragment was amplified by using primers to a region 5' to KH domain and a 3' non-coding sequence. Structural analysis of the genomic fragment showed that functional motifs such as tyrosine-rich motif, proline-rich motif and nuclear localization signal were located as separate exons in the genome structure.

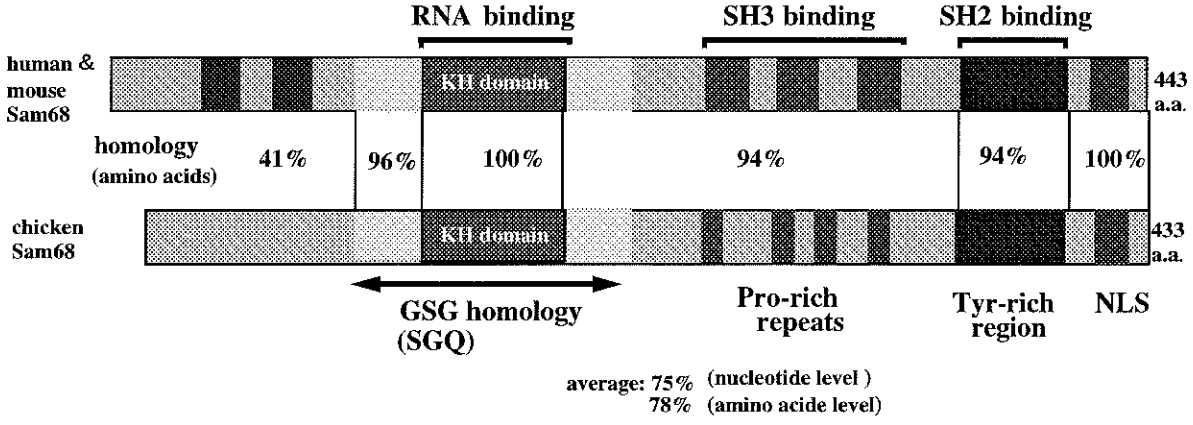

Fig.1 Structure of human and chicken Sam68 protein

Targeted disruption of Sam68 gene

We constructed the targeting vectors with the isolated genomic DNA fragment (Fig. 2b) and transfected the vectors into DT40 cells (Fig. 2 a). The deletions of two Sam68 alleles in DT40 cell were introduced by sequential transfection of two different targeting vectors containing drug resistant genes against puromycin and histidinol, respectively. Cell clones with homologous recombination were screened by Southern blot analysis of genomic DNA and eleven independent clones were identified (Fig. 2 c).

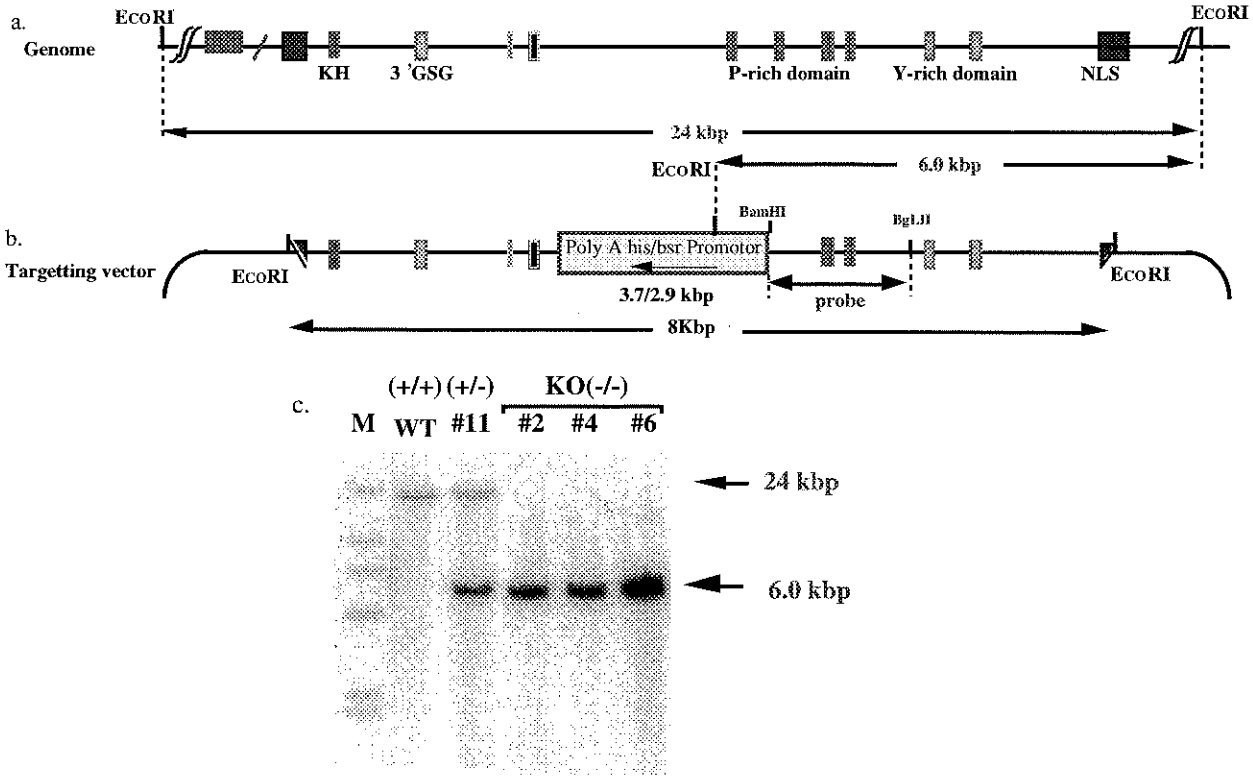

Fig.2 Structure of genomic chicken Sam68 (a) and targeting vector (b)
Southern analysis of genomic DNA (c).

To verify null mutations, RNA and protein blot analysis using specific probes were carried out. RNA blot analysis identified a 2.3 Kb RNA species in Sam68 knocked-out DT40 cells instead of 3.0 kb wild type Sam68 mRNA (Fig. 3 left pattern). As a 40 kDa protein observed in protein blotting analysis of knocked-out DT40 cells was suggested to be encoded by the 2.3 kb mRNA, cDNA fragment from this short RNA was cloned by RT-PCR using primers in KH domain and NLS. Sequence analysis of the cDNA clearly showed that the 2.3 kb mRNA was generated by alternative splicing, which skipped the RNA sequence corresponding to drug resistant genes in targeting vector (Fig.4). Since the amount of 40 kda product expressed in knocked-out cells, however, was ranged between 1% (in clone#6) to 10% (in clone#2) of wild type Sam68 (Fig.3 right pattern), we further analyzed the characteristics of these knocked-out cells.

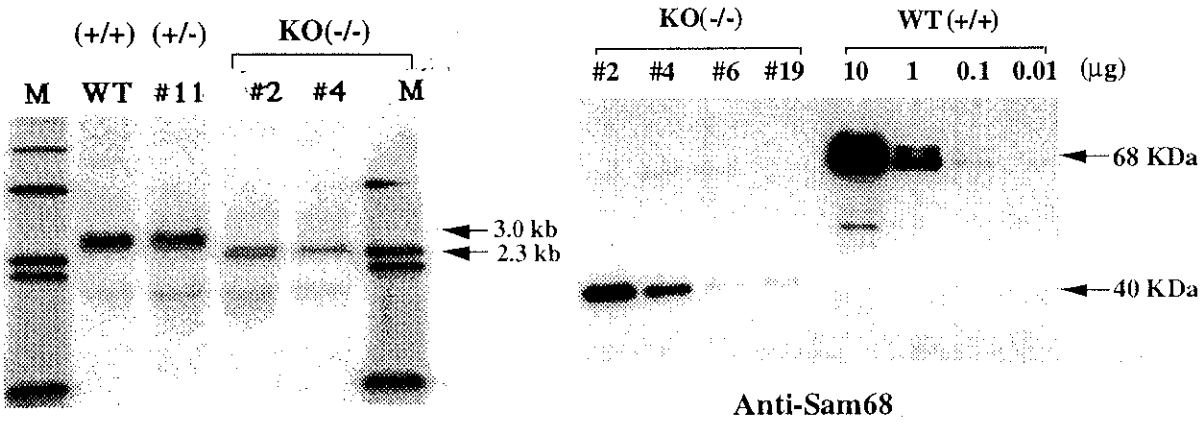

Fig.3 RNA (left) and protein (right) analysis of Sam68 knocked-out DT40 cell

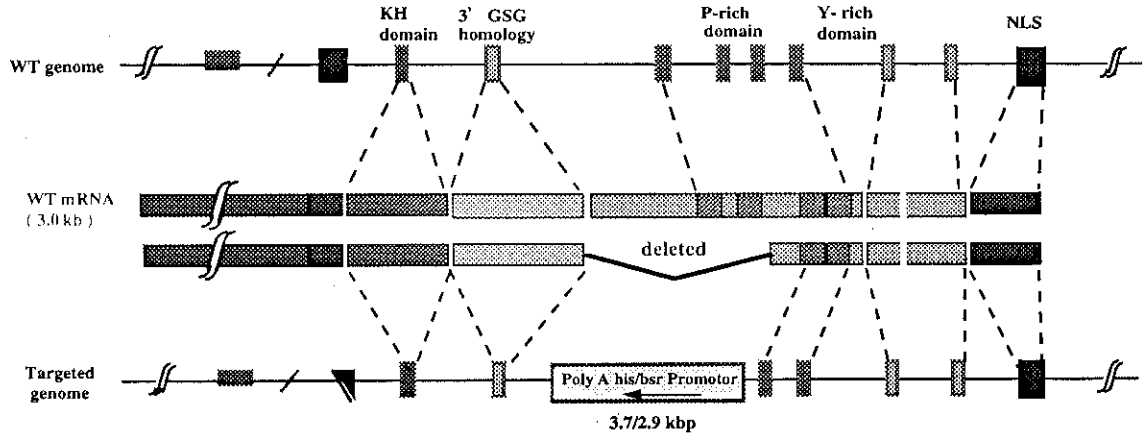

Fig.4 Structure of truncated chicken Sam68 mRNA

Growth retardation and elongation of S phase in cell cycle of Sam68 deficient cells

To search the functional role of Sam68 in cell growth control, we first analyze the growth rate in wild type and Sam68 deficient DT40 cells. When numbers of cell in normal growth condition were monitored every 12 hours, the growth rate of Sam68 deficient cells was found to be slower than that of wild type (Fig. 5 left pattern).

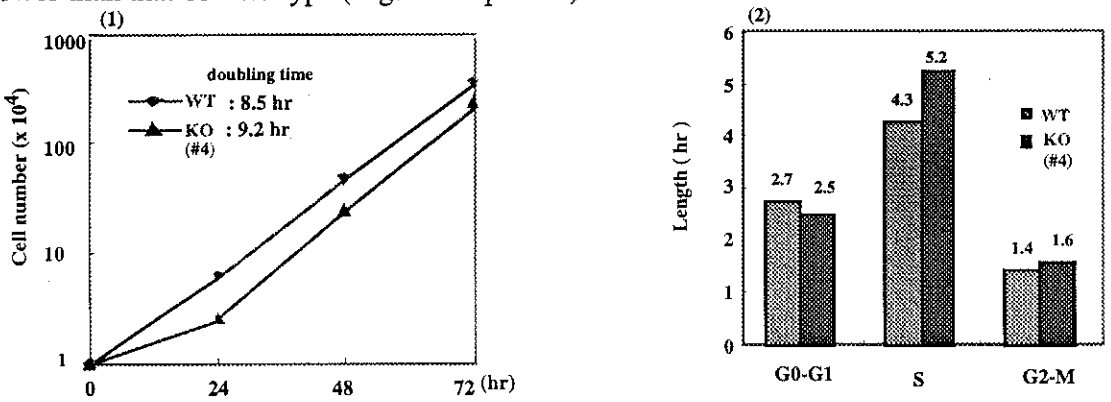

Fig. 5 Analysis of cell growth rate (left) and cell cycle (right)

To further clarify the points in cell cycle that were affected by the deletion of Sam68 gene the flow-cytometoric analysis was carried out. Cell cycle pattern of each cell clones demonstrated that the retardation of cell growth of Sam68 deficient cells was mainly due to the elongation of S phase in cell cycle(Fig. 5 right pattern). Effect of Sam68 depletion on the S phase was further supported by the serum depletion experiment in which the expansion of S phase in Sam68 deficient cells was observed (Fig. 6), whereas little effect on S phase was seen in the wild type DT40 cells). Together with evidences that RNA binding activity is essential for functions of other KH-domain containing proteins as well as Sam68 those results implicated that Sam68 involves in cell growth control probably by modulating the function of mRNAs in S phase or early.

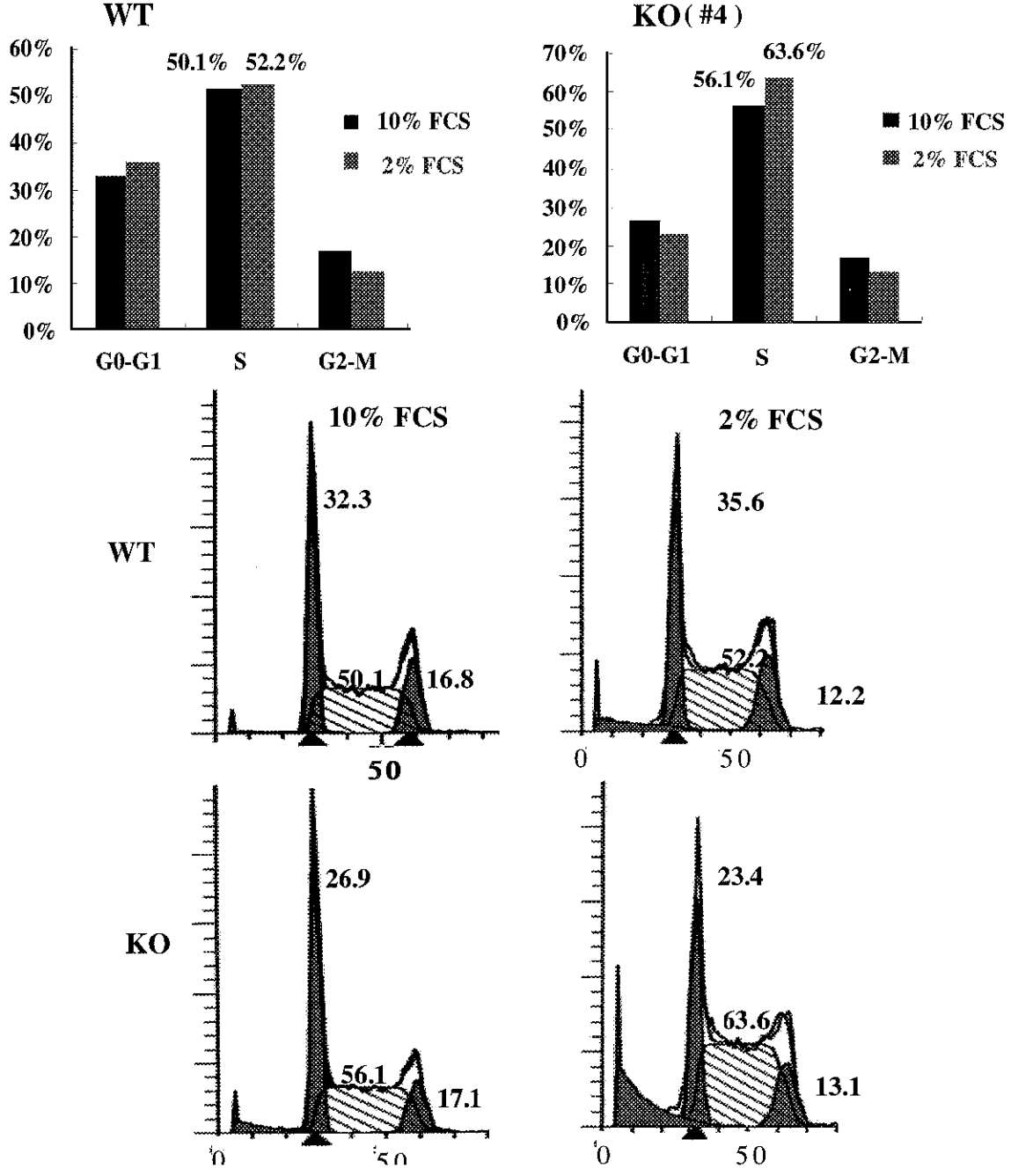

Fig.6 Analysis of cell cycle after serum depletion

Reduced tyrosine-phosphorylation of BLNK in Sam68 deficient cells

Interaction of Sam68 with various signaling molecule indicates the involvement of Sam68 in signal transduction cascade. To assess the role of Sam68 in signal transduction, tyrosine phosphorylation of cellular proteins was analyzed. After cross-linking of B cell receptor on DT40 cells by anti-chicken IgM monoclonal antibody, M4, tyrosine phosphorylation of cellular proteins was analyzed by protein blotting with anti-phosphotyrosine antibody. The result demonstrated that the overall-pattern of phosphorylated protein in Sam68 deficient cells was similar to that in wild type DT40 cells. However, the maximal level of tyrosine phosphorylation on BLNK, a prominent signaling molecule in B cell, has been observed significantly lower in Sam68 deficient cells (Fig. 7). Since the expression levels of sIgM on the Sam68 deficient clones were essentially same as that of parental DT40 cells (data not shown) it was suggested that deletion of Sam68 resulted in the reduced B cell signaling although it is partial.

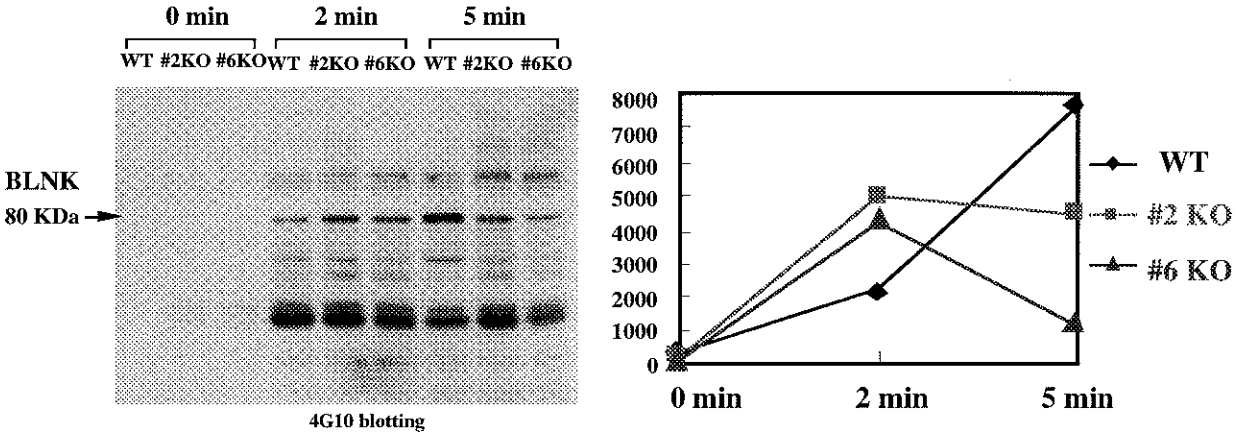

Fig.7 Tyrosine phosphorylation of cellular proteins after cross-linking of BCR

< Discussion >

Sam68 is able to bind RNA and contains a KH domain and a region similar to a RGG box(Gibson et al., 1993), (Dryfuss et al., 1993), characteristic for a distinct family of RNA binding proteins(Ebersole et al., 1996). Sam68 is therefore a putative regulator of RNA metabolism and it could give means for a rapid pathway to regulate protein expression by modifying the mRNA stability and/or mRNA translation (Brawerman et al., 1989, Morley et al., 1991).

In addition to the RNA binding activity, tyrosine-phosphorylation by Src during mitosis and interaction with signal-transducing proteins indicated that Sam68 plays some important role(s) in cell cycle regulation. More direct evidence for a role of Sam68 in cell cycle control is that expression of a splice variant of Sam68 in which part of the KH domain is deleted inhibits serum-stimulated progression into S phase of the cell cycle (Barlat et al., 1997).

To elucidate the functional role of Sam68 in cell cycle control, we attempted to isolate the chicken DT40 cell-clone lacking intact Sam68 gene. Sam68 deficient DT40 cells were obtained by serial homologous recombination of Sam68 genome with targeting vectors and characteristics in cell growth of these cells were analyzed. The slower growth rate and elongated S phase in Sam68

deficient cells were observed and these results raised the possibility that Sam68 plays a specific role in mRNA metabolism and/or translational control in S phase or earlier. Additional observations in which serum depletion on Sam68 deficient cells resulted in the expansion of S phase and the increase of apoptotic cell also supported this hypothesis.

The RNA binding activity of Sam68 is inhibited by either Src-mediated tyrosine phosphorylation (Wang et al., 1995) or by the binding of an individual Src SH3 domain (Taylor et al., 1995). It is assumed, therefore, that tyrosine phosphorylation and the following protein association is closely associated with the control of RNA metabolism. In addition Sam68 binds a wide variety of different signal-transducing proteins including Grb2, PLγ1 (Richard et al., 1995), Grap (Trub et al., 1997), the p85 subunit of PI3-kinase, Crk (Taylor et al., 1995), Nck (lwe et l., 1997) and rasGAP (Wong et al., 1992), as well as the protein tyrosine kinases Src and ItK (Andrderotti et al., 1997). These interactions which are mediated throughout phosphorylated tyrosines and proline-rich (SH3-binding) sequences in Sam68 suggested that Sam68 may act also as an adaptor molecule in signal transduction pathway.

BLNK is a B cell specific protein that is phosphorylated by Syk after BCR ligation and interacts with variety of downstream effector proteins including PLCγ2, Vav, Grb2 and Nck (Fu and Chan, 1997, Fu et al.,1998, Wienands et al., 1998). Our present data showed that upon B cell receptor stimulation the maximal level of tyrosine phosphorylation on BLNK has been observed significantly lower in Sam68 deficient cells. We therefore hypothesized that Sam68 functions as an adaptor molecule to link the interaction of BCR with Syk to downstream effectors and this adaptor function may involve in the control of cell cycle progression through modulating RNA metabolism and /or translationability.

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