Involvement of Fyn Tyrosine Kinase in Progression of Cytokinesis of B Lymphocyte Progenitor

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Abstract. We analyzed the role of Fyn tyrosine kinase in cell cycle progression of B lymphocyte progenitor (pro B cell). Whereas there were no substantial defects in the intramarrow B cell genesis in the *fyn(−)* mouse, and long-term proliferation of *fyn(−)* pro B cells was maintained in vitro under a serum containing culture condition, the cell cycle was arrested at G2/M upon serum deprivation. Morphological analyses demonstrated that the cytokinesis of *fyn(−)* pro B cells was retarded in the presence of serum and that the entry of *fyn(−)* pro B cells into late telophase was completely blocked under the serum-free condition. In contrast, the earlier phases of mitosis of *fyn(−)* pro B cells proceeded normally without FCS. This failure to initiate late telophase resulted in the accumulation of elliptical binucleated cells that might be the outcome of the nuclear division without cytokinesis. Consistent with this defect in the progression of cytokinesis, Fyn was localized in the midspace of dividing pro B cells at anaphase. These results suggested that Fyn localizes at the midspace of dividing pro B cells and regulates the progression of cytokinesis.

FYN is a member of *src* family protein tyrosine kinase (PTK)[1] that is widely expressed in various cell types including lymphocytes (25, 32). Biochemical studies demonstrated that Fyn is associated with antigen receptor complexes of both T and B lymphocytes and that it is activated by antigen stimulation (6, 29). Consistent with these findings, overexpression of the T cell–specific splice form of Fyn renders T cells hyperreactive to T cell receptor–mediated stimulation (7, 13). Moreover, Fyn has been shown to couple with interleukin (IL)-2 or IL-7 receptors on the lymphocyte surface and to be phosphorylated by the stimulation by these cytokines (18, 31, 40). The functional role of Fyn has been directly tested using gene knockout technology (1, 15, 35, 42–43). According to these studies, while disruption of *fyn* gene has little effect on the development of $CD4^+CD8^+$ immature T cells or B cells, the T cell receptor–induced activation of mature T cells was impaired (1, 35). Thus, as predicted from the biochemical evidence (6, 29), the interaction of Fyn with T cell receptor is an essential step in triggering antigen-induced T cell activation. However, no substantial defect has been identified in the cytokine-induced response of the lymphocytes of the *fyn*-deficient (*fyn[−]*) mice (1, 15, 35, 43). Thus, while Fyn might be able to associate with cytokine receptors under some conditions, it may not have any functional role in transmitting the signal, or it may be substituted by other *src* family PTK.

Whereas previous studies on lymphocytes have focused on the role of Fyn in the signal transduction pathway from surface receptors to transcriptional regulators (5, 37), several lines of evidence indicate that Fyn plays a role in remodeling the cytoskeletal structure. Fyn associates with α-tubulin in human T lymphocytes (17, 21). Neural cell adhesion molecule–mediated neurite outgrowth or myelin-associated glycoprotein-mediated myelination is inhibited in the cells from the *fyn(−)* mouse (3, 39), though it remains to be determined whether or not Fyn directly regulates the remodeling of cytoskeletal component in these situations. In fact, *fyn(−)* mice show various symptoms of a disturbed central nervous system (15, 42–43). The immunohistochemical study has shown that Fyn is concentrated in the growth cone of neurons (2). In this respect, it is important to note the observation of Ley et al. that Fyn colocalizes with the mitotic spindle fiber and centrosome of human T cells (20). This finding implies that the role of

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1. *Abbreviations used in this paper*: IL, interleukin; IL-7R, interleukin-7 receptor; KL, the ligand for c-Kit; pro B cell, B lymphocyte progenitor; PTK, protein tyrosine kinase.

Fyn in the regulation of cytoskeletal component is generalized to many cell types, including lymphocytes.

We have investigated the molecular mechanisms underlying the cell cycle progression of normal B lymphocyte progenitors (pro B cell). As we and others have shown, IL-7 is a requisite signal for intramarrow B lymphopoiesis (14, 26, 36). Moreover, we showed that pro B lines are readily established from fresh bone marrow cells under a defined culture condition that contains IL-7, the ligand of c-Kit (KL), and transferrin (44). To our knowledge, only pro B cells have been successfully propagated in vitro in an IL-7–dependent manner. Thus, our long-term culture of pro B cells should consist of the best available tool with which to study the role of IL-7 signal in cell cycle progression. IL-7 appears to be the major signal regulating G1/S transition of pro B cells in the primary culture (44). Thus, the finding that Fyn is expressed in pro B cells and that it can associate with the IL-7 receptor (IL-7R) is important, even though intramarrow B cell production was not affected in the *fyn(−)* mice. Given that Fyn is functional but also redundant in pro B cells, in vitro culture would be more effective for detecting a subtle activity of Fyn. Indeed, this has been exemplified by Beggs et al., who showed that the effect of *fyn* gene disruption was readily manifested as a defect in neural cell adhesion molecule-induced neurite outgrowth in vitro (3). Thus, we attempted to establish pro B cell lines from a strain of the *fyn(−)* mouse (43), using the chemically defined culture condition capable of supporting the proliferation of normal pro B cells (44). Despite repeated attempts, however, we failed to establish primary cultures of pro B cells from *fyn(−)* mice under the serum-free defined condition, whereas it was easily established in the presence of serum. Whatever the mechanism behind this is, this finding clearly indicates that Fyn plays a role in the proliferation of pro B cells, though its activity is apparent only under the serum-free condition. Thus, this study aims at determining which process in the cell cycle of pro B cells is dependent upon Fyn. The results showed that Fyn plays a role in the final step of cytokinesis and that it could be compensated by a signal induced by yet unknown factors present in the serum.

Materials and Methods

Cell Lines and Culture

The chemically defined medium in which pro B cell lines were established and maintained was mSFO2 (Sanko Pure Chemical Co., Ltd., Chiba, Japan) containing KL (50 ng/ml), IL-7 (20 U/ml), and 0.1% BSA (44). Pro B cell lines established from C57BL/6 mice and from the mice whose *RAG2* gene was disrupted (*RAG2[−]*; 34) were used as control cells (44). *Fyn(−)* pro B cell lines were established by adding 5% FCS to this defined medium. For the growth factor starvation experiment, pro B cells in the cultures were washed three times, resuspended at the concentration of 5×10^5 cells/ml, and cultured in the presence or absence of KL, IL-7, or FCS (Hyclone Laboratories, Inc., Logan, UT). The frequency of colonogenic B cell precursors that proliferated on the PA6 stromal cell layer in the presence of IL-7 was determined by a limiting dilution assay (16). In some experiments, growth of pro B cells was assayed using the ST2 stromal cell line that can support long-term culture of pro B cells with the chemically defined medium (4).

Thymidine Incorporation Assay and Cell Cycle Analysis

Pro B cells (5×10^4) suspended in 100 μl medium were placed in the wells of 96-well cluster dishes. The cells were cultured for 24 h under various conditions, and 0.5 μCi [^{3}H]thymidine (Amersham Intl., Little Chalfont, UK) was added to each well 16 h before harvest. After incubation, the cells were lysed on glass fiber filters, washed, and the radioactivity level on the filters was counted. Flow cytometer analysis of the cell cycle of pro B cells was performed as described (44).

Reagents for Histochemistry

Production of a rat anti–murine Fyn mAb will be described elsewhere (Yasuda and Yagi, in preparation). The specificity of this mAb is shown in Fig. 6. Anti-α-tubulin antibody and rhodamine-conjugated phalloidin was purchased from Sigma Chemical Co. (St. Louis, MO) and anti-β-tubulin mAb SMI62 was from Sternberger Monoclonal, Inc. (Baltimore, MD). FITC-labeled rabbit anti–mouse IgG (Organon Teknika-Cappel Co., Malvern, PA), FITC-labeled goat anti–rat IgG (American Qualex Antibodies & Immunochemicals Co., La Mirada, CA) and FITC-labeled goat anti–rat IgG (Chemicon Intl. Inc., Temecula, CA) were applied in indirect immunohistochemistry. Antibodies used for Western blotting were peroxidase-labeled goat anti–rat IgG and peroxidase-labeled rabbit anti–mouse IgG (Zymed Labs, Inc., South San Francisco, CA).

Immunofluoresence Staining

The cultured cells were collected, centrifuged for 1 min, resuspended in 3.7% paraformaldehyde in PBS, and fixed at 37°C for 20 min. After fixation, the cells were washed twice with PBS and cytospun onto gelatin-coated glass slides. The specimens were permeabilized in PBS containing 0.2% Triton X-100 (Nacalai Tesque, Inc., Kyoto, Japan) for 5 min and incubated with PBS containing 1% albumin (Sigma Chemical Co.) and 0.5% fish gelatin (Sigma Chemical Co.) for 10 min to block nonspecific staining.

For three-color staining of β-tubulin, F-actin, and chromosome, the specimens were incubated with anti-β-tubulin antibody (SMI62) for 1.5 h at room temperature, washed three times with PBS (spaced apart with 5-min incubations), incubated with a mixture of the FITC anti–mouse-IgG (1:50 dilution), rhodamine-conjugated phalloidin (1:80 dilution), and either Hoechst 33258 (1 μg/ml bisbenzimide) or TOTO3 iodide (1 μM; Molecular Probes, Inc., Eugene, OR) for 30 min at room temperature, and washed three times with PBS. To immunostain Fyn, FITC anti–rat-IgG was the second antibody. The stained cells were mounted with GEL/MOUNT™ (Biomeda Corp., Foster City, CA) and observed using either an epifluorescence microscope (Axiophot; Zeiss Inc., Oberkochen, Germany) or a confocal laser scan microscope (MRC-1000; Bio-Rad Laboratories, Richmond, CA).

Western Blotting

The cultured cells were lysed in the lysis buffer (25 mM TrisHCl, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.5% deoxycholic acid, 1% SDS, 5 mM aprotinin, 5 mM leupeptin, 5 mM pepstatin A) on ice for 20 min. The lysates were centrifuged at 13,000 rpm for 20 min, size fractionated by SDS-PAGE (10%), and transferred onto a polyvinyldifluoride membrane (Millipore Corp., Bedford, MA). The membranes were blocked with PBS containing 3% skim milk and 1% BSA at 4°C overnight, incubated with the first antibody at room temperature for 2 h, washed three times with PBS, and incubated with the second antibody for 1 h. After washing the membrane three times with PBS containing 0.05% Tween 20 and once with PBS, the proteins stained by the antibodies were visualized by chemiluminescence using the ECL system (Amersham Intl.) with exposure to medical x-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Results

Serum Dependence of the Proliferation of Pro B Cells Cultured from the Bone Marrow of the fyn(−) Mice

We described a serum-free and chemically defined culture condition that supports the long-term growth of IL-7R$^+$c-Kit$^+$ pro B cells from various strains of mice (44). Despite extensive efforts, however, we failed to obtain pro B cell lines from the *fyn(−)* mice (43) under this serum(−) condition (not shown). In contrast, no substantial defect in the intramarrow production of B lymphocytes was de-

tected in this strain of mouse (not shown). When we measured the frequency of pro B cells that are clonogenic in cultures with the PA6 stromal cell layer, IL-7, and 5% FCS, the frequency in the bone marrow of *fyn(−)* mice was nearly the same as that of normal mice (Fig. 1 *A*). Moreover, *fyn(−)* pro B cells normally proliferated in this culture, generating visible colonies. This finding indicated that long-term culture of pro B cells can be established from the *fyn(−)* mouse in the presence of serum. We thus attempted to establish *fyn(−)* pro B cell lines using a medium containing 5% calf serum which was otherwise the same as our defined condition (serum[+] condition).

Using bone marrow cells from individual *fyn(−)* mice, we established primary cultures of IL-7R$^+$c-kit$^+$sIgM$^-$ pro B cells and maintained them for more than 6 mo (not shown). Likewise, primary cultures of C57BL/6 pro B cells were established using the serum(+) condition.

The growth requirements of these cell lines were examined by exposure to various culture conditions. As shown in Fig. 1 *C*, the proliferation of *fyn(−)* pro B cells was entirely dependent upon the presence of serum, while that of C57BL/6 pro B cells was maintained in the absence of serum. Moreover, like pro B cells from the normal mouse, KL and IL-7 were required for their growth. Hence, the signals triggered by these cytokines can be efficiently transmitted in the absence of Fyn to drive the cell cycling when FCS is present in the culture. The same cells proliferated under the serum(−) condition if the stromal cell layer was provided (Fig. 1 *B*). This indicates that the molecular cue driving the cell cycle of *fyn(−)* pro B cells is

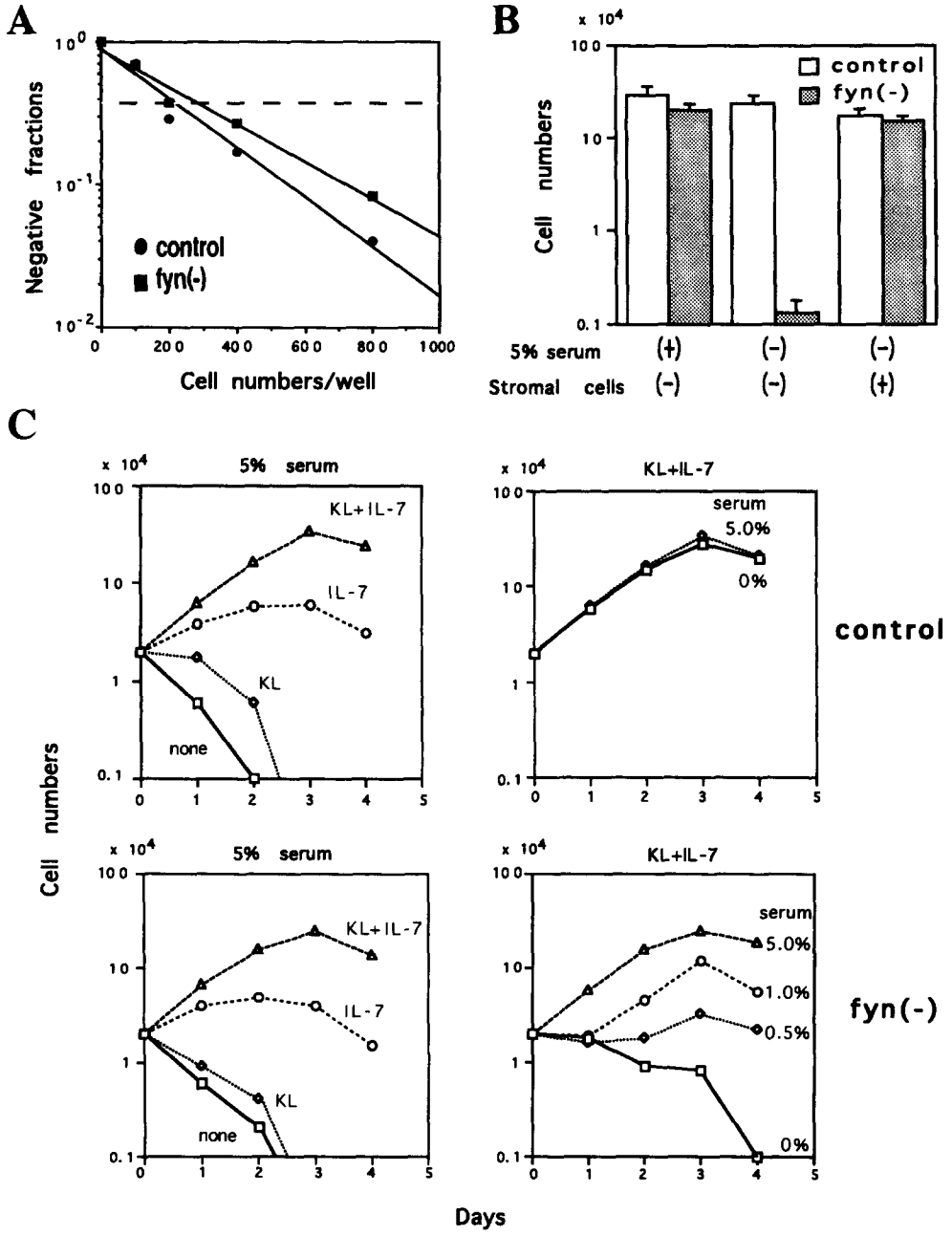

Figure 1. In vitro analyses of the proliferation of *fyn(−)* pro B cells. (*A*) Bone marrow cells were harvested from *fyn(−)* or control littermate. Varying numbers of the cells were cultured for 10 d in 96-well cluster dishes in the presence of a PA6 stromal cell layer and IL-7. Generation of B lineage cells in each well was scored as described (34). 96 wells were set for each cell number, and the proportion of negative wells was calculated. The frequency of the in vitro clonable B cell progenitors calculated from this chart was 1/210 and 1/280 for the control and *fyn(−)* mice, respectively. (*B*) Pro B cells (1×10^4) established from the *fyn(−)* or C57BL/6 mice were stimulated with KL+IL-7 in the presence or absence of 5% FCS or the PA6 stromal cell layer. 4 d later, the cells were harvested and counted. Each bar represents the arithmetic mean and standard deviation of quadruplicate cultures. (*C*) Pro B cells (2×10^4) established from *fyn(−)* or C57BL/6 (*control*) mice under the serum(+) condition were cultured with various combinations of KL, IL-7, and FCS. In the presence of KL and IL-7, *fyn(−)* pro B cells proliferated only when >1% of FCS was included in the medium. In the medium containing 5% FCS, both KL and IL-7 are required for the proliferation of *fyn(−)* pro B cells. Each point represents the arithmetic mean of duplicate cultures.

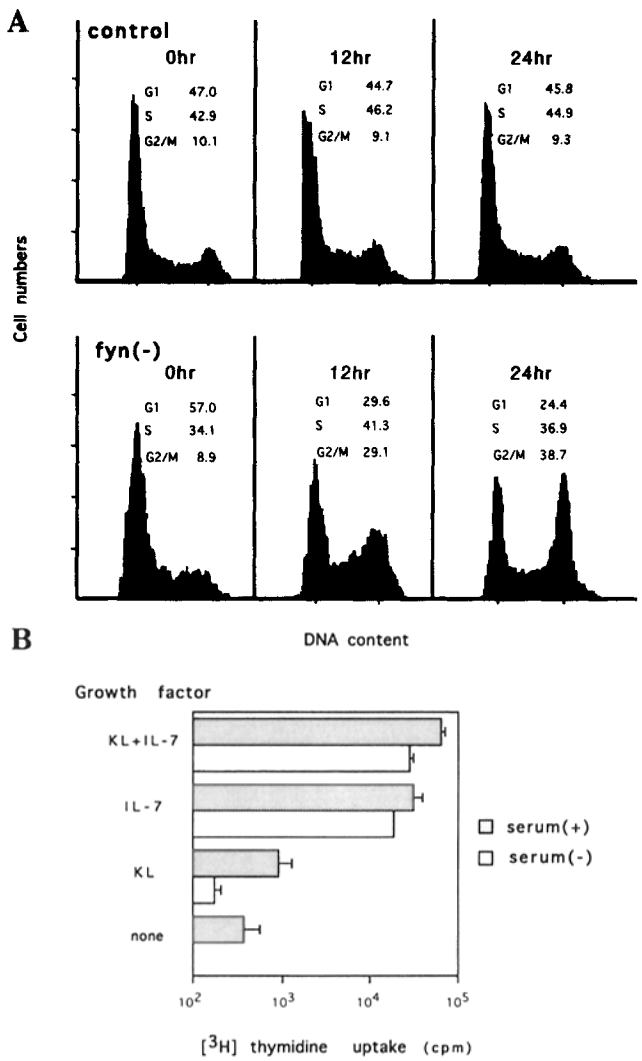

Figure 2. Cell cycle of *fyn(−)* pro B cells is arrested at G2/M upon serum deprivation. (*A*) 10^6 pro B cells that were established from *fyn(−)* or C57BL/6 mice under the serum(+) condition were transferred to the serum(−) condition. After 12 or 24 h, the cells were harvested, and the proportion of each cell cycle stage was assessed by propidium iodide staining followed by flow cytometry. *Fyn(−)* pro B cells progressively accumulated at G2/M-phase upon serum starvation. (*B*) 5×10^4 *fyn(−)* pro B cells were cultured in the presence or absence of KL, IL-7, or 5% FCS for 24 h. At 16 h, 0.5 μCi [^{3}H]thymidine was added to each well. Each bar represents the arithmetic mean and standard deviation of triplicate cultures.

not specific to FCS. Lysophosphatidic acid, insulinlike growth factor, IL-3, IL-6, hepatocyte growth factor, platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor, phorbol-ester, or cAMP could not substitute for FCS in the proliferation of *fyn(−)* pro B cells under the serum(−) condition.

Involvement of Fyn in the M-phase Progression of Pro B Cells Revealed under the Serum(−) Condition

We investigated which stage of the cell cycle is affected in *fyn(−)* pro B cells cultured under the serum(−) condition. 10^6 *fyn(−)* pro B cells that had been maintained under the serum(+) condition were transferred into the serum(−) condition, and the proportion of each cell cycle stage was measured 12 or 24 h later. During this short-term incubation in the serum(−) condition, the proportion of viable cells as assessed by trypan blue exclusion assay was >90%, whereas that of apoptotic cells as detected by propidium iodine staining followed by flow cytometry was ~20% (not shown). As shown in Fig. 2 *A*, *fyn(−)* pro B cells progressively accumulated at the G2/M stage upon deprivation of FCS, whereas the cell cycle profile of the control cells did not change. When IL-7 was also removed from the culture, this remarkable increase in the G2/M stage did not occur, as most cells were arrested at G1 (not shown). On the other hand, despite the absence of cell growth, DNA synthesis as assessed by [^{3}H]thymidine incorporation was maintained for 24 h after transferring the cells into the serum(−) condition (Fig. 2 *B*). This suggested that S-phase progression proceeded normally in the *fyn(−)* pro B cells before reaching the G2/M stage. A cytological examination of the same samples revealed that ~40% of the cells were binucleated 24 h after serum starvation (Fig. 3 *B*). Though rare, some cells had four nuclei (Fig. 3 *C*). Morphology of *RAG2(−)* pro B cells cultured in the serum(−) condition was also shown (Fig. 3 *A*). These findings indicated that Fyn plays a role in the progression of M-phase, whereas other stages of cell cycle can progress without Fyn even under the serum(−) condition.

Final Step of Cytokinesis Is Impaired in fyn(−) Pro B Cells

To further specify the Fyn-dependent process in the M-phase of pro B cells, we analyzed the structure of two major cytoskeletal components, F-actin and tubulins, in proliferating pro B cells. Pro B cells from the C57BL/6 mice were used as the control. The cells were first maintained under the serum(+) condition, then maintained in the same medium or transferred to the serum(−) condition. The cells were harvested 24 h later and stained with FITC-labeled anti-β-tubulin, rhodamine-labeled phalloidin, and Hoechst 33258. The cells at prophase, prometaphase, metaphase, anaphase, telophase, and late telophase were counted according to the criteria described by Brinkley et al. (4).

Although cytokinesis and nuclear division in the control pro B cells proceeded as defined by these criteria, the process of cytokinesis in the *fyn(−)* pro B cells proceeded slower than that of nuclear division (not shown). In this case, stage determination for *fyn(−)* pro B cells was made on the basis of the cell shape. The proportion of each mitotic phase was counted at 4, 8, 16, or 24 h after transfer into the serum(−) condition.

The series of photographs in Fig. 4 presents the cytoskeletal structures of the late M-phase of control pro B cells in the serum(−) condition, and *fyn(−)* pro B cells in the serum(+) or serum(−) condition 24 h after the initiation of culture. The shift from the serum(+) to serum(−) condition had little effect on the proliferation of the control pro B cells, and all stages of M-phase were observed (for cells representing anaphase, early and late telophases, see Fig. 4, *A–D*). On the other hand, in *fyn(−)* pro B cells, cytokinesis was significantly retarded even in the presence of

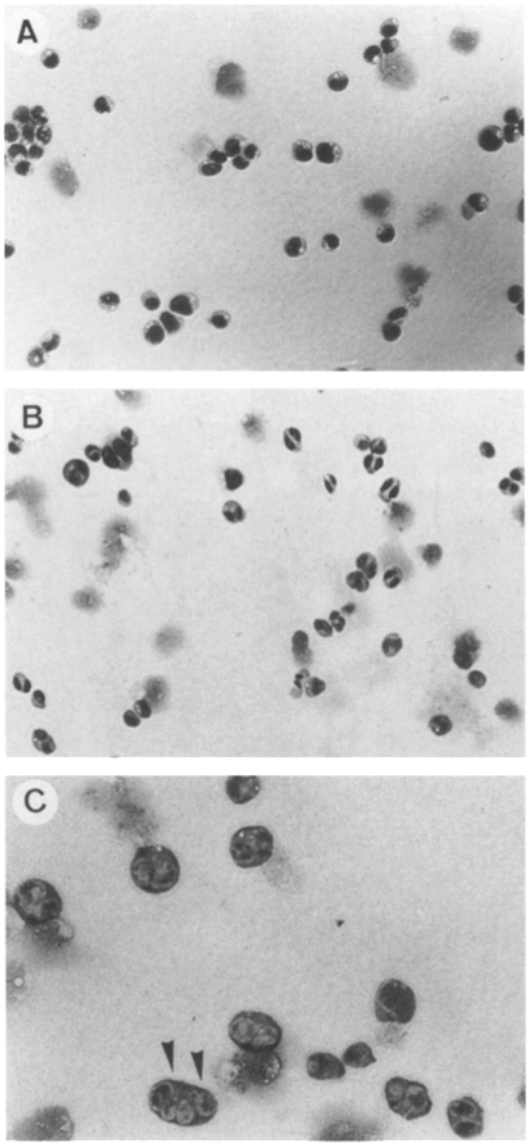

Figure 3. Morphology of *fyn(−)* pro B cells 24 h after serum starvation. *RAG2(−)* pro B cells (*A*) or *fyn(−)* pro B cells (*B* and *C*) cultured for 24 h under the serum(−) condition were cytospun and stained with May-Grünwald-Giemsa. A large proportion of *fyn(−)* pro B cells were binucleated after 24 h of serum starvation (*B*), while no such cells were found in *RAG(−)* pro B cells (*A*). Arrowheads indicate a representative cell containing four nuclei that is often found in the serum-starved *fyn(−)* pro B cell culture (*C*).

serum (for cells representing anaphase, early and late telophases, see Fig. 4, *E–G*). When the cells at late telophase were compared, the chromosomes were still segregated in the control pro B cell (Fig. 4 *C*), whereas complete daughter nuclei were formed during telophase of the *fyn(−)* pro B cell (Fig. 4 *G*). Another characteristic of *fyn(−)* pro B cells was the presence of binucleated cells that constituted 2% of the cells proliferating under the serum(+) condition (Fig. 4 *H*). In contrast, virtually no binucleated cells were detectable in cultures of the control pro B cells (<0.1%). Upon serum deprivation of *fyn(−)* pro B cells, binucleated cells increased in a time-dependent manner and reached a level of 40% within 24 h (Fig. 5). In this cell population, cells at early telophase with cleavage furrows were present as well as other earlier mitotic phases (Fig. 4, *I–K*). This indicated that the microtubule dynamics, like shortening of the kinetochore microtubules and growth of the polar microtubules, proceed in the absence of Fyn. On the other hand, the late telophase was absent in this cell population (Fig. 5), as no cells with deep cleavage furrows were present. This showed that the cell cycle of *fyn(−)* pro B cells was arrested before entering late telophase upon transfer into the serum(−) condition, whereas earlier phases proceeded normally. In some cells at telophase, the F-actin staining was concentrated at the cleavage furrow (Fig. 4 *J*). We also found cells in which the microtubules started to degrade and the cleavage furrow started to relapse, as the F-actin staining disappeared from the cleavage furrow (Fig. 4 *K*). Those cells might be in the process of forming the elliptical binucleated cells after failing to initiate the late telophase. In a large proportion of the binucleated cells (Fig. 4 *I*) and also in the tetranucleated cells (Fig. 4 *L*), β-tubulin staining remained concentrated in the internuclear space, while F-actin staining was redistributed over the cytoplasm.

Subcellular Localization of Fyn during Mitosis of Pro B Cells

The above results indicated a role of Fyn in the progression of telophase of pro B cells. Thus, we investigated whether this defect is correlated with the localization of Fyn in the pro B cells. For this purpose, we stained the *fyn(−)* and control pro B cells with an anti-murine Fyn mAb. As shown in Fig. 6 *A*, this mAb detects a single p59 band in the lysate of *RAG2(−)* pro B cells, whereas it did not react with any proteins in *fyn(−)* pro B cells (Fig. 6 *B*). In most interphase cells, Fyn is diffusely localized in the submembranous cytoplasm (Fig. 6 *C*). About 30% of the cells showed colocalization of Fyn with the pericentrosomal region of pro B cells at the interphase (Fig. 6 *E*), and most cells at mitosis did also (Fig. 6, *F* and *G*). Upon entering anaphase, the fluorescence intensity of Fyn in the cytoplasm decreased, whereas the midspace separating the dividing cell was intensely stained (Fig. 6, *C* and *F*). While the mitotic pole and spindle was stained, its staining is weaker than that of the midspace structures (Fig. 6 *F*). Upon entering the telophase, Fyn again redistributed homogeneously over the submembranous cytoplasm (Fig. 6, *H* and *I*).

Discussion

While a role of the *src* family molecules in the cell cycle progression has been suspected, much attention has been focused on its role in the progression of the early phase of cell cycle (9, 38). This study demonstrated for the first time that Fyn plays a functional role in the late phase of mitosis.

This function of Fyn became apparent as a result of a combination of several conditions. We established a chemically defined culture condition in which cell cycle progression of normal pro B cells can be maintained (44), whereas that of *fyn(−)* pro B cells cannot. Thus, even a subtle difference between normal and *fyn(−)* pro B cells in cell cy-

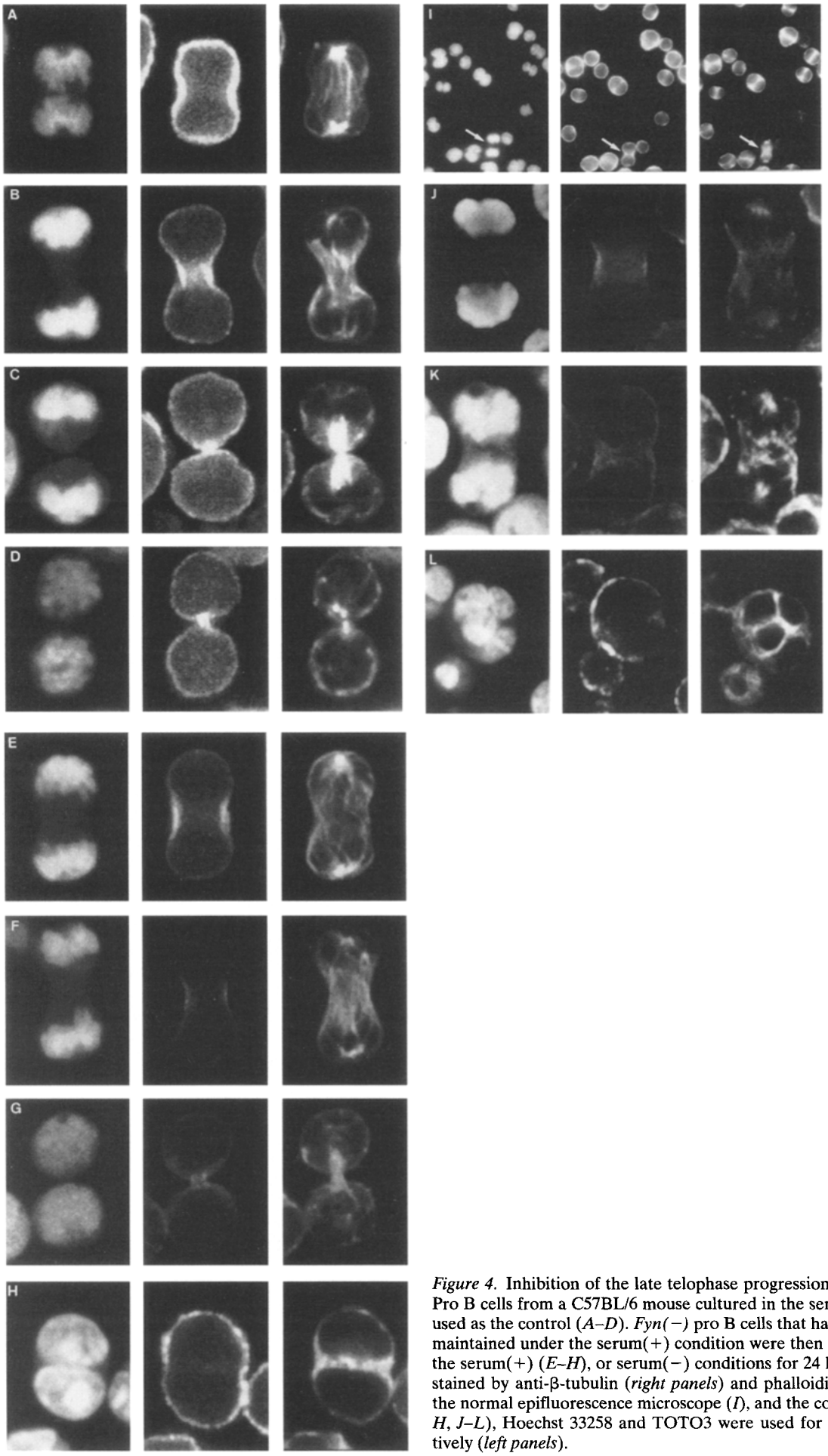

Figure 4. Inhibition of the late telophase progression of *fyn(−)* pro B cells. Pro B cells from a C57BL/6 mouse cultured in the serum(−) condition were used as the control (*A–D*). *Fyn(−)* pro B cells that had been established and maintained under the serum(+) condition were then incubated either under the serum(+) (*E–H*), or serum(−) conditions for 24 h (*I–L*). The cells were stained by anti-β-tubulin (*right panels*) and phalloidin (*middle panels*). For the normal epifluorescence microscope (*I*), and the confocal microscope (*A–H, J–L*), Hoechst 33258 and TOTO3 were used for staining DNA, respectively (*left panels*).

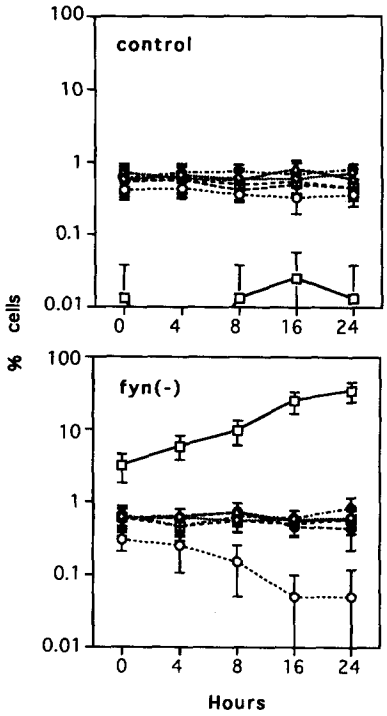

Figure 5. Proportion of each mitotic phase in *fyn(−)* pro B cells after transfer to the serum(−) condition. C57BL/6 or *fyn(−)* pro B cells (10^6) that were maintained under the serum(+) condition were transferred to the serum(−) condition. The cells were stained in the same manner as described in the legend to Fig. 4. The proportion of each mitotic phase and binucleated cells were determined under an epifluorescence microscope. Each point represents the arithmetic mean and standard deviation of quadruplicate cultures. −□−, binucleated cells; ···◇···, prophase cells; ···△···, prometaphase cells; ···⊞···, metaphase cells; ···◆···, anaphase cells; ···⊕···, early telophase cells; −○−, late telophase cells.

cle progression was able to be detected under our serum(−) culture condition. However, it is important to note that primary cultures of *fyn(−)* pro B cells were established under the serum(+) condition. This enabled us to carry out an experiment in which the cell cycle of the *fyn(−)* pro B cell that was maintained in the serum(+) condition was arrested by transferring into the serum(−) conditions. In this experiment, the function of Fyn in the cytokinesis was compensated to keep the cells cycling on the one hand, and manifested as cell cycle arrest on the other. Moreover, the progression of other cell cycle phases of *fyn(−)* pro B cells was maintained even under the serum(−) condition in the presence of IL-7. Thus, the cell cycle proceeded until it reached the telophase when Fyn plays an essential role. A combination of these conditions eventually specified the functional role of Fyn as regulating the progression of telophase of pro B cells.

At present, it is not clear whether the regulatory role of Fyn in the telophase progression is specific to pro B cells or generalized to other cell types. The findings of Ley et al., that Fyn colocalized with centrosomes and mitotic spindle fibers of T cells (20), suggest a role in the M-phase progression of T lymphocytes. Moreover, this study showed that Fyn colocalized with mitotic spindle fibers and centrosomes in pro B cells at the mitotic phase. However, microtubule dynamics as the formation of spindle fibers, shortening of kinetochore microtubules, and growth of polar microtubules occurs normally in the absence of Fyn. It is noteworthy that there was a clear difference in the localization of Fyn in human T and pro B cells, the diffuse submembranous localization of Fyn is rarely identified in human T cells (20). On the other hand, Fyn was found in the submembranous cytoplasm of all pro B cells at interphase. Moreover, the staining was more intense in the cleavage site during anaphase of the pro B cells. Concomitant with the entry into telophase, Fyn disappears from the cleavage site. The mAb used in this study is very specific, as it detected a single 59-kD band in the Western blotting and did not react to *fyn(−)* pro B cells. Whereas the study of Ley et al. used a polyclonal antibody against the peptide fragments of Fyn, the specificity control of the antibodies was carefully presented (20). Thus, this difference in the Fyn staining profile suggests that the function and subcellular localization of Fyn differs among T and B lymphocytes. Nevertheless, the dynamic behavior of Fyn during the mitotic phase of pro B cells correlates well with its function in progressing to the telophase of these cells. It would be of interest to know the stage at which the mitosis of *fyn(−)* T cells is arrested.

Our results show that the proportion of the cells progressing from prophase to telophase remained constant upon transferring *fyn(−)* pro B cells to the serum(−) condition, while that of late telophase bearing a deep cleavage furrow decreased along with the accumulation of binucleated cells. Thus, the generation of the binucleated cells may well be the outcome of a series of processes wherein nuclear division of the cells proceeds normally but the cleavage furrow, which was once formed as a shallow fold upon entering telophase, dissociates due to the failure to form deep furrows. There are several situations in which binucleated cells were generated by cell cycle arrest (8, 11, 19, 22, 28, 30). Among these, two are of particular interest in terms of the present results. Cool et al. have shown that transfection of a constitutive active T cell–specific phosphatase resulted in cell cycle arrest with the accumulation of binucleated cells (8, 22). Though the target molecules of the transfected phosphatase are unclear, this finding indicates the involvement of tyrosine phosphorylation in cytokinesis. In fact, the total amount of tyrosine-phosphorylated proteins was markedly reduced in *fyn(−)* pro B cells as compared with the pro B cells from other strains, and some of the bands found in the normal pro B cells were absent (Yasunaga et al., unpublished results).

Cytochalasin B treatment induces the cell cycle arrest of cultured fibroblasts and generates binucleated cells, although the treated cells could form deep cleavage furrows (19, 30). Time-lapse recording of this process revealed that the deep furrow formed between the nuclei relapsed to form elliptical binucleated cells. Since cytochalasin B blocks both the association and dissociation of actin subunits, this finding indicated that early telophase and nuclear division are independent of actin function, whereas late telophase, particularly cytokinesis, requires it. On the other hand, *fyn(−)* pro B cells showed defects in the formation of the deep furrow. Therefore, polar microtubules degrade without being bundled in the midbody. Thus, it is likely that Fyn is involved in the process before the cyto-

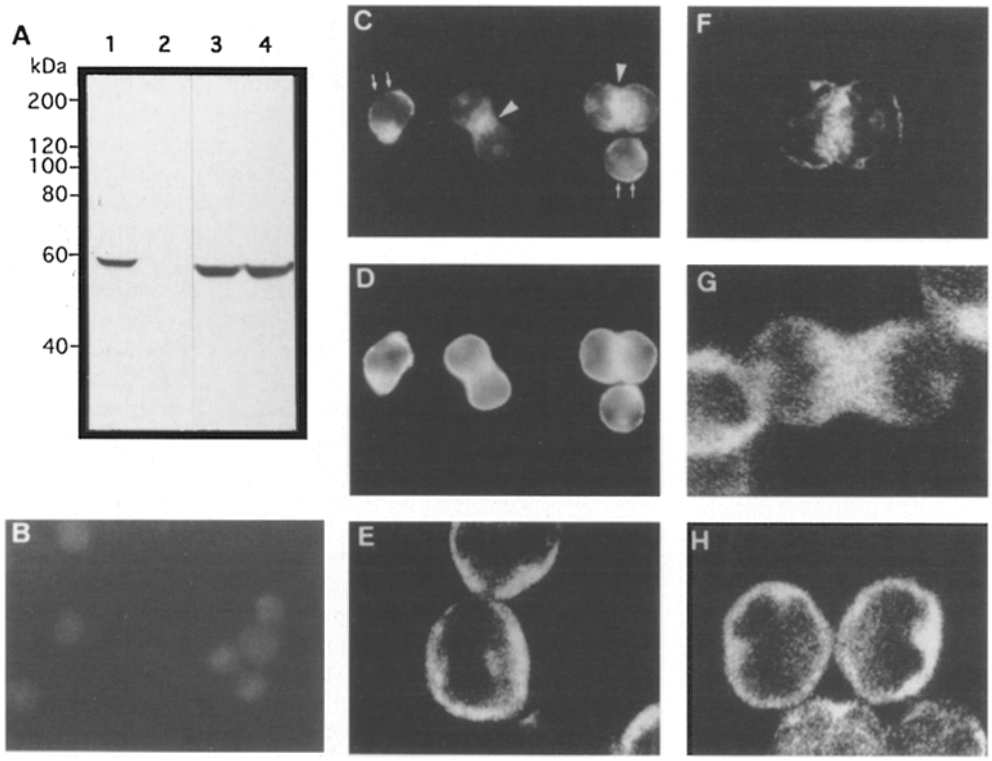

Figure 6. Immunolocalization of Fyn in control or *fyn(−)* pro B cells. (*A*) Western blots of Fyn (lanes *1* and *2*) and β-tubulin (lanes *3* and *4*) expression. Lysates from *RAG2(−)* pro B cells (lanes *1* and *3*) and *fyn(−)* pro B cells (lanes *2* and *4*) were analyzed. This anti-Fyn mAb does not stain *fyn(−)* pro B cells (*B*), whereas it stains cytoplasm of the cells at interphase (*C* and *E*) and pericentrosomal regions, spindle fibers, and midspace of cells at anaphase (*C* and *F*). In the cells at telophase, Fyn is redistributed throughout the cytoplasm (*G* and *H*). Photographs from *B* to *D* are the normal epifluorescence microscope, and those from *E* to *H* are the confocal microscope. Photographs *C* and *D* are *RAG2(−)* pro B cells stained either by anti-Fyn (*C*) or phalloidin (*D*), and those from *E* to *H* are C57BL/6 pro B cells stained by anti-Fyn. Arrowheads indicate cells at anaphase to early telophase, and arrows indicate cells at interphase (*C*).

chalasin B–sensitive step. Consistent with this notion, Fyn concentrated in the midspace of the cells at anaphase and redistributed after entering telophase. Hence, it is likely that Fyn in the midspace of the cells at anaphase functions to initiate the deep furrow formation by transmitting the signal to the contractile machinery including actin and myosin.

Despite the demonstration of its involvement, how Fyn regulates the formation of deep cleavage furrow remains unknown. In fact, many molecules localize in the midspace of cleaving cells. Among them, tubulins, calmodulin, and a GAP-associated protein p62 were shown to be potential targets of the *src* family PTK (10, 12, 23, 27, 41). Thus, it would be of interest to know if these molecules play a role in the downstream of Fyn. However, it was also shown that the *src* family PTK has little specificity for phosphorylation substrates, particularly in vitro (24). Therefore, further functional study is required for determining the molecules in the downstream of Fyn. As the cytokinesis of pro B cells is absolutely dependent upon Fyn, and both normal and *fyn(−)* pro B cell lines are available, the experimental system described here will be useful for investigating the tyrosine kinase–based intermolecular interactions during cytokinesis.

While Fyn was shown to associate directly with IL-7R (31, 40), it remains to be elucidated whether Fyn involved in the cytokinesis of pro B cells is activated by IL-7 signals or by other molecular cues. Consistent with other reports (31, 40), tyrosine phosphorylation of Fyn was induced by IL-7 in our pro B cell lines (Yasunaga et al., unpublished results). Moreover, IL-7 starvation results in cell cycle arrest both at G1 and G2/M (44). While G2/M arrest by IL-7 starvation appears consistent with the notion that IL-7 signal activates Fyn to function in cytokinesis, we could not detect an increase in the number of binucleated cells in IL-7–starved *RAG2(−)* pro B cells (Yasunaga et al., unpublished observations). Hence, IL-7 signal might be required for earlier phase of G2/M so that the cell cycle is arrested before reaching the late telophase where the effect of Fyn becomes apparent. Alternatively, Fyn involved in the late telophase is activated by different signals. A number of reports indicate that $p34^{cdc2}$ can phosphorylate Src at mitosis (9, 33, 38). If the same is also true for Fyn, it could be autonomically activated in the mitotic pro B cells.

In conclusion, the data presented here demonstrates for the first time that Fyn plays a functional role in the progression of cytokinesis, particularly in the formation of deep cleavage furrow. Several lines of genetic evidence suggest that the function of *src*-family molecules are redundant due to expression of several members of this family in particular cell types (9, 38). Indeed, intramarrow B cell genesis is normal in *fyn(−)* mouse, and *fyn(−)* pro B cells can grow under the culture condition containing FCS, IL-7, and KL. However, the present results imply that there are conditions wherein Fyn manifests its unique role despite the presence of molecules that can compensate for its function. Thus, our defined culture condition should be useful for investigating the function of other molecules

that are expressed at the pro B cell stage but their functional significance remains unclear even from the gene knockout experiments. Moreover, *fyn(−)* pro B cells, the cell cycle progression of which can be controlled at the telophase by manipulating culture conditions, will provide a useful target for gene transfection, so that the functional hierarchy of *src* family molecules or other molecular mechanisms regulating telophase may be investigated.

We would like to thank Drs. M. Nakasato and S. Nishikawa for the serum-free mSFO2 medium, and Mr. H. Imai for his help in preparing confocal images.

This work was supported in part by grants from the Ministry of Education, Culture, and Science of Japan (07CE2005, 06283283, and 07457085), and by a grant from the Institute of Physical and Chemical Research (RIKEN).

Received for publication 12 July 1995 and in revised form 16 October 1995.

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