Role of IL-7 and KL in activating molecules controlling the G₁/S transition of B precursor cells

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Keywords. CDK4, cell cycle, cyclin D3, pro-B cell

Abstract

While chemically defined conditions for culturing normal tissue have been attained for only a few cell types, the sustained proliferation of B precursor cells expressing IL-7 receptor and c-Kit can be supported under chemically defined conditions containing recombinant IL-7 and the ligand for c-Kit (KL). To understand the biochemical basis of the cell cycle progression of B precursor cells proliferating under these conditions, we investigated the correlation between growth factor stimulation and CDK4 activity. Consistent with our findings that IL-7 regulates the G₁/S transition, while KL has only a little role in this process, the kinase activity of CDK4 was related closely with IL-7 stimulation but not KL stimulation. We investigated the mechanism underlying CDK4 activation in the IL-7-stimulated B precursor cells. Our results showed that (i) CDK4 and cyclin D3 are the G₁/S regulators in B precursor cells; (ii) their expression levels are unchanged between the cells in G₁ arrest and cycling cells; and (iii) they are present in an associated form even when the cell cycle stage is arrested at G₁. Thus, the regulation of the expression of CDK4 and cyclin D3 or regulation of their assembly are not the mechanisms for activating CDK4 in the B precursor cells. On the other hand, a number of molecules co-immunoprecipitated with CDK4 were enhanced in the lysate of IL-7-stimulated B precursor cells. Thus, we present a possibility that CDK4 activation might be regulated by molecules associated with the CDK4–cyclin D3 complex in an IL-7-dependent manner.

Introduction

Over the last decade, our understanding of the biochemical basis of cell cycle progression of mammalian cells has remarkably progressed, mostly due to the discovery that all eukaryotes share basically the same machinery for controlling the cell cycle. Indeed, some cyclins and their catalytic subunits, cyclin-dependent kinases (CDK), have been cloned and shown to be not only homologous to their yeast counterparts but also able to function in yeast (1,2). Furthermore, a number of studies have demonstrated that inhibition of those cyclin–CDK complexes results in cell cycle arrest, indicating that those molecules play a master role in cell cycle progression (3,4). Studies on mammalian cells, however, have shown that the basal cell cycle machinery of mammalian cells is integrated in a network of molecules that regulates the activity of CDK both positively and negatively (5). Some of these are molecules that have been cloned or identified later as tumor suppresser genes (6,7). The discovery of p35, a neuron-specific positive regulator (8,9), suggests that the regulatory networks vary depending on the context of cell lineages. Moreover, the repertoire of cyclin–CDK complexes is more complicated in mammalian cells than in yeast and only a portion are expressed in a given tissue (5). As the proliferation of each cell type should be regulated differently to form a multicellular organism, this complexity of cell cycle molecules might be a natural consequence of developing complex organisms such as mammals, that consist of >200 cell types, the proliferation of which is regulated distinctly, yet in coordination with other cell types. Hence, an important issue
in the control of the mammalian cell cycle is to determine how the cell-type-specific control of cell cycle progression is achieved by different cell lineages.

We studied the molecular mechanisms by which intramarrow stromal cells control B cell genesis. By generating two mAb that recognize the IL-7 receptor (IL-7R) and c-Kit, we found that the B precursor cells with high in vitro clonogenic activity are IL-7R"c-Kit", suggesting that two receptors function as essential signaling pathways for the proliferation of B precursor cells (10). Consistent with this, we showed that a subset of B precursors can proliferate in a chemically defined condition where IL-7, KL, transferrin and BSA are contained as protein components (11,12). This model system for investigating the cell cycle progression of B precursors has been further improved using cultured RAG-2" mouse B precursor cells, in which differentiation is arrested at the IL-7R"c-Kit" stage, thereby providing a population that stably expresses the two receptors (12). These maintained B precursors require both IL-7 and KL, and the former plays a more direct role in the G1/S progression (11).

The aim of this study was to elucidate the biochemical basis of the cell cycle progression of B precursors regulated by IL-7 and KL. Our results showed that the activation of CDK4 and CDK2 present in the B precursor cells is under the direct control of the IL-7 signal, whereas the signal from c-Kit enhanced their total activity. These characteristics of the G1/S checkpoint of B precursor cells are discussed with respect to previous observations of these molecules in other cell lines.

**Methods**

**Cytokines and cell culture**

Recombinant IL-7 and KL were prepared as described (11,13). RAG-2" mouse B precursor cells were established from the RAG-2 gene knockout mouse (14) and maintained in a chemically defined culture medium (mSFO2: Sanko Pure Chemical Co.) including IL-7 and KL (12). A CYG/SCID7 B precursor (pro-B) lymphoma cell line from the SCID mouse was maintained in the same culture medium without IL-7 and KL (15).

**Analysis of cell cycle and apoptosis by FACS**

B precursor cells (1×10⁶) cultured under various conditions were harvested, fixed in cold 70% ethanol, then resuspended in PBS containing 50 µg/ml propidium iodide, 100 U/ml RNase A and 0.1% glucose. The cell suspension was incubated at room temperature for 2 h and the proportion of each cell cycle stage and apoptotic cells was measured by flow cytometry (EPICS XL). The data were processed using a multi-cycle program.

**Antibodies**

Polyclonal antibodies against cyclin D1, D2, D3, CDK2 and CDK4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Western blotting**

Cells were harvested by centrifugation, washed once with cold PBS, suspended in TBS (10⁶ cell/ml) again, sonicated for 30 s, lysed with SDS sample buffer and size-separated by SDS-PAGE. Proteins were transferred to Immobilon-P (Millipore, Bedford, MA) and the membrane was blocked in TBS containing 3% non-fat dried milk and 2% BSA. After incubating with the antibodies, the reactive bands were detected using peroxidase-labeled anti-rabbit IgG antibody and visualized with enhanced chemical luminescence.

**Immunoprecipitation and metabolic labeling**

Cells were harvested by centrifugation, washed once with cold PBS and lysed in NP-40 buffer (1 ml/10⁷ cell pellets) at 4°C. NP-40 buffer consisted of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 10 µg/ml of leupeptin, 10 µg/ml of aprotinin, 50 mM NaF, 0.2 mM sodium orthovanadate and 0.5 mM DTT. The cell lysate was cleared once using Protein A-Sepharose beads, incubated with 1–2 µg of polyclonal antibodies and precipitated by 30 µg Protein A-Sepharose beads. For metabolic labeling, 1×10⁶ cells were incubated in methionine-free mSFO2 medium for 30 min. Trans[^35]S label (ICN, Irvine, CA) was then added at a final concentration of 0.1 µCi/ml and the cells were incubated for a further 4 h at 37°C. Metabolically labeled cell extracts were cleared twice by incubations with Protein A-Sepharose beads for 40 min at 4°C. Immunoprecipitation and SDS-PAGE proceeded as described. Labeled proteins were detected using a Bioimage analyzer (BAS 2000).

**Kinase assay of CDK**

In vitro kinase activities of CDK4 and CDK2 were measured as described by Matsushime et al. (16). Phosphorylated proteins were detected by a Bioimage analyzer (BAS 2000).

**Results**

**Role of IL-7 and KL in cell survival and cell cycle progression of B precursor cells**

We established several B precursor cell lines under fully chemically defined conditions, and analyzed the signals required for their sustained proliferation in vitro (12). In this study, we used a B precursor cell line established from the RAG-2" mouse because its differentiation is arrested at the stage where IL-7R and c-Kit are stably expressed. As the proliferation of B precursor cells is regulated by minimum signals under our culture conditions, this culture is useful for investigating the biochemical basis of growth factor regulated cell proliferation. However, compared with other transformed cell lines, growth-factor-dependent B precursor cells have an obvious problem in that they enter the apoptosis process upon deprivation of either of the growth factors. This was shown by starving the RAG-2" pro-B cells of IL-7 and/or KL for 24 or 48 h and measuring the proportion of apoptotic cells and each cell cycle stage by flow cytometry (Fig. 1). Consistent with our previous report that cells in the absence of IL-7 were not labeled by BrdU (11), IL-7 starvation resulted in the prompt arrest of the B precursor cells in G1, though KL starvation had little effect on the proportion of each cell cycle stage. In contrast, more apoptotic cells were generated by 24 h of KL than IL-7 starvation (Fig. 1B and C). The resting phase during IL-7 starvation appeared to be at G1 rather than G0 and G2/M.
Growth factor deprivation induces apoptosis of B precursor cells. The DNA content of RAG-2^{+} B precursor cells cultured in the presence of IL-7 plus KL (A), only IL-7 (KL starvation) for 24 h (B), only KL (IL-7 starvation) for 24 h (C), in the absence of growth factors for 24 h (D), in the presence of IL-7 for 48 h (E) and only KL for 48 h (F) was analyzed by FACS. The transverse line in the top of each figure indicates apoptotic cells (left) and viable cells (right). Data were expressed as the ratio of apoptotic cells among all cells.

CDK4 activation by IL-7 in B precursor cells

Correlation of kinase activities of CDK4 and CDK2 with IL-7 stimulation

CDK4 and CDK2 are the major molecules regulating G_1/S transition in many cell types (5). As both kinases are expressed in our B precursor cells (Fig. 2), we investigated whether the IL-7-stimulation that is required for G_1/S progression of B precursor cells is correlated with the kinase activities of CDK4 and CDK2.

Fig. 2. The expression levels of CDK4, CDK2 and cyclin D3 are unchanged between the cells in G_1 arrest and cycling cells. Whole cell lysates of RAG-2^{+} B precursor cells with anti-CDK4, CDK2 and cyclin D3 antibodies in the presence of IL-7 plus KL (lane 1), only IL-7 (lane 2), only KL (lane 3) and in the absence of growth factors (lane 4) were Western blotted. Starvation was for 24 h. Each lane contained the lysate from 1X10^6 viable cells.

Fig. 3. IL-7 signal induces the kinase activity of CDK4 and CDK2. In vitro kinase activities of immunoprecipitates of the lysates of RAG-2^{+} and CYG/SCID B precursor cells with anti-CDK4 and CDK2 antibodies were measured (upper figures). Each lane contained 3X10^6 viable cells and was designed as in Fig. 2. Lower figures display Western blots of the immunoprecipitates of cell lysates with the same antibodies. This shows the amount of CDK4 and CDK2 that phosphorylated the substrate.

The B precursor cell line was cultured with or without either IL-7 or KL for 24 h and the cell lysates were prepared. CDK4 and CDK2 were immunoprecipitated from each cell lysate and the kinase activities of the immunoprecipitates were assessed using a recombinant RB protein as a substrate (16) (Fig. 3). Kinase activities were detected only in the immunoprecipitates from the IL-7-stimulated B precursor cells irrespective of the presence of KL in the cultures. While KL
alone had little effect on the activation of the CDK4 or CDK2, it markedly augmented their kinase activities induced by IL-7. As no other phosphorylated proteins were detected in the immunoprecipitates, no endogenous substrate of CDK4 or CDK2 was co-immunoprecipitated with these molecules. Nevertheless, this result implied that the kinase activities of CDK4 and CDK2 are direct or indirect indicators of IL-7 stimulation. As CDK4 activation precedes that of CDK2 (5,17), only CDK4 activity was analyzed in subsequent experiments.

Cell-cycle-independent expression of cyclin D3 and CDK4 in B precursor cells

To determine the mechanism by which CDK4 is activated in IL-7-stimulated B precursor cells, we verified several possibilities. First, we examined whether or not activity of CDK4 in the B precursor cells was regulated through the level of expression of CDK4 or D-type cyclins. As CDK4 was associated with only cyclin D3 among the D-type cyclins in this B precursor cell line (Fig. 4), we measured the expression of cyclin D3 and CDK4 in the B precursor cells incubated without IL-7 and/or KL by means of Western blotting (Fig. 2). The amount of CDK4 expression was similar among the cells cultured with IL-7 plus KL, IL-7 alone or KL alone. Although the expression of cyclin D3 was highest in the B precursor cells cultured with IL-7 plus KL, we could not detect any significant difference between the B precursor cells cultured with IL-7 or KL alone. As IL-7 starvation results in prompt arrest of the cells at G1, the expression level of CDK4 and cyclin D3 did not correlate with the cell cycle stage in the B precursor cells. Considering the fact that CDK4 is activated only in the B precursor cells stimulated with IL-7, this indicated that the regulation of the amount of CDK4 or cyclin D expression is not the mechanism responsible for the activity of CDK4 in the B precursor cells.

Constitutive assembly of cyclin D3 and CDK4 in B precursor cells

It has been suggested that regulation of the assembly of cyclin Ds and CDK4 is one mechanism that activates CDK4 (18). Thus, we examined if this is the case for CDK4 activation in the B precursor cells. The assembly of cyclin D3 and CDK4 was detected by immunoprecipitation of the lysate of B precursor cells cultured either in the presence or absence of IL-7 or KL with the anti-CDK4 antibody followed by Western blotting (Fig. 2). While some reduction in the amount of cyclin D3 was detectable in the B precursor cells incubated without IL-7 or KL, cyclin D3 co-precipitated with CDK4 in the B precursor cells whether or not the cell cycle progression was maintained by IL-7 stimulation. This result suggested that the assembly control of cyclin D3 and CDK4 is not the mechanism that regulates the G1/S progression of B precursor cells.

Molecules associated with cyclin D3-CDK4 in a cell-cycle-dependent manner

The possibility remained that CDK4 activity in the B precursor cells is regulated by negative or positive regulators. In fact, CDK inhibitory proteins (CKI) or CDK activating kinases (CAK) are associated with cyclin-CDK complexes (19). Thus we next investigated whether any of the molecules were present in the B precursor cells cultured for 24 h in the presence of IL-7 and/or KL, then labeled with [35S]methionine. After 4 h incubation, the cell lysate was prepared, immunoprecipitated with anti-CDK4 antibody and size-fractionated by SDS-PAGE (Fig. 5). We also analyzed the growth factor-independent B precursor cell line CYG/SCID7 in the same manner, in which CDK4 and CDK2 were constitutively activated in the absence of growth factors. As shown in Fig. 5, a number of distinct bands co-immunoprecipitated with anti-CDK4 antibody. The most dense band at 34 kDa should correspond to cyclin D3 and CDK4 (20). A band at p21 that was present in all lysates may correspond to CIP1 (19), p48, p67, p97 and p105 bands were enhanced compared with cyclin D3 and CDK4 in the immunoprecipitates from cells stimulated with IL-7. All these bands were present in the CYG/SCID7 cell line that cycled without IL-7 stimulation. Moreover, a p58 band was detected specifically in this transformed cell line.
whether the consequences of growth factor stimulation of B precursor cells are reflected in the functional state of the G1/S stage. Indeed, stimulating T cells with an antigen that induces the G0 to G1 transition of the resting T cells is a signal inducing the increase of cyclin D3 expression (24,25). Cyclin D2 is induced by anti-CD40 antibodies and Staphylococcus aureus in resting B cells (26). As another way of regulating CDK4 kinase activity, Kato et al. showed that the assembly of cyclin D and CDK4 in fibroblasts is induced by adding serum to the culture (18). However, this could not be the mechanism of G0/S progression in the B precursor cells, because cyclin D3 was present in association with CDK4 regardless of the activity of CDK4. This study, however, could not exclude the possibility that this assembly in B precursor cells was also under the control of extracellular signals, as the signal regulating the assembly of cyclin D and CDK4 would be difficult to detect in cells with no G0 stage.

Thus, the mechanism by which CDK4 is activated in the B precursor cells may be control of the cyclin D3-CDK4 complex through negative or positive regulatory proteins, including those that phosphorylate or dephosphorylate CDK4 and which associate or dissociate with this complex in a cell-cycle-dependent manner. In fact, anti-CDK4 antibody precipitated a number of proteins in the B precursor cells that were enhanced when they were stimulated by IL-7. On the other hand, the p21 protein appeared to associate with CDK4 regardless of the cell cycle stage. In this study, we could not detect bands that associated with CDK4 only in the cells starved of IL-7. Thus, IL-7-induced cell cycle progression of the B precursor cells might not be regulated in the same manner as IL-2-stimulated T lymphocytes (24) and a macrophage colony stimulating factor-dependent macrophage line (27) that enters the cell cycle upon dissociation of the inhibitory p27 protein induced by growth factor stimulation. It is possible that CDK4 activation in the B precursor cells is regulated through positive regulators except CAK that assemble into CDK4-cyclin D3 complexes upon IL-7 stimulation. However, the results do not necessarily exclude the involvement of a regulatory mechanism through CKI, because CKI can associate with active and inactive CDK in normal cells (28). Also, positive regulators may be counterbalanced by the continuous presence of CKI (17,19). Anti-CDK4 antibody may not necessarily immunoprecipitate CAK. Moreover, metabolic labeling of cells does not necessarily detect all proteins.
expressed in vivo. Since our experimental conditions could be regarded as containing only the minimum growth signals for the B precursor cells, it remains obscure whether or not the proliferation of B precursor cells in the actual bone marrow in vivo is under the control of the same mechanisms.

As IL-7 is an essential factor for B precursor cell cycle progression, it is conceivable that CDK4 and CDK2 were constitutively activated in the transformed B precursor cells cultured in the absence of IL-7. Positive CDK regulators play roles in tumorigenesis (29,30). How this transformed cell acquired growth factor-independent mechanisms for activating CDK4 is of interest. Particularly, whether or not the p58 protein that was specifically detected in the transformed B precursor cells plays a role in the process of the transformation is important.

Although many CDK inhibitors have been identified, only one, other than D-type cyclins, is a known activator of CDK4. The CAK that can phosphorylate and activate CDK4 consists of cyclin H and CDK7, with molecular mass of 37 and 39 kDa respectively (31,32). The CDK4-associated proteins in the cycling B precursor cells, however, appeared to be distinct from cyclin H or CDK7 because of a difference in their molecular sizes. These results implied that B precursor cells may bear a specific set of cell cycle control molecules in addition to the specificity of growth factor receptors. Indeed, several lines of evidence suggest an enormous complexity in the combined expression of cell cycle molecules among cell types, particularly those regulating the G1/S transition (5,33). Differentiation may also alter the cycle machinery, even in cells of the same lineages (34,35). With respect to B lineage differentiation, IL-7-dependent early pre-B cells express cyclin D2 (36). If so, the differentiation of the B precursor cells studied here into the next pre-B cell stage should be accompanied by a shift of cyclin D usage and that might play a significant role in the change of the mode of cell proliferation (10,12). Studies on B lineage differentiation have demonstrated that the mode of cell growth changes dramatically at the pivotal steps of B lineage differentiation from pro-B to pre-B, pre-B to early B, or before and after antigenic stimulation. Thus, it is conceivable that the cell cycle regulating molecules in B lineage cells are as diverse as the extracellular receptors regulating cell proliferation. Nevertheless, the most important issue is to determine the nature of the molecules that play a role in activating CDK4 in the B precursor cells. We hope that they might include some of the bands that were shown to be associated with CDK4 in the cycling B precursor cells in this paper. Purification of these molecules from the activated B precursor cells is currently in progress.

Acknowledgements

We thank the Chemo-Sero-Therapeutic Research Institute and Dr Sudo (TORAY Industries) for providing recombinant KL and IL-7 respectively. We also thank Dr Akiyama for the generous gift of recombinant RB protein. This work was supported by grants from the Japanese Ministry of Education, Science and Technology (nos 06253213, 05272102 and 05454210), a grant from Japanese Ministry of Science and Technology, and a grant from RIKEN.

Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>CAK</td>
<td>CDK activating kinase</td>
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<td>CDK</td>
<td>cyclin-dependent kinase</td>
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<td>CKI</td>
<td>CDK inhibitory proteins</td>
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<td>IL-7R</td>
<td>IL-7 receptor</td>
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<td>KL</td>
<td>c-KIT ligand</td>
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References

CDK4 activation by IL-7 in B precursor cells


