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Rabbit Antibody Raised against Murine Cyclin D3 Protein Overexpressed in Bacterial System

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Since the commercially available rabbit anti-cyclin D3, generated from c-terminal 16 amino acid residues which are common to human and murine cyclin D3, is highly cross-reactive with many other cellular proteins of mouse, a new rabbit polyclonal anti-cyclin D3 has been raised by using murine cyclin D3 protein expressed at a high level in *Escherichia coli* as the immunogen. To express murine cyclin D3 protein in *E. coli*, the cyclin D3 cDNA fragment encoding c-terminal 236 amino acid residues obtained by polymerase chain reaction (PCR) was inserted into the *Nco*I/*Bam*HI site of protein expression vector, pET 3d. Molecular mass of the cyclin D3 overexpressed in the presence of IPTG (Isopropyl β-D-thiogalactopyranoside) was approximately 26 kDa as calculated from the reading frame on the DNA sequence, and the protein was insoluble and mainly localized in the inclusion bodies that could be easily purified from the other cellular soluble proteins. When renaturation was performed following denaturation of the insoluble cyclin D3 protein in the inclusion bodies using guanidine hydrochloride, 4.4 mg of soluble form of cyclin D3 protein was produced from the transformant cultured in 100 ml of LB media under the optimum conditions. Four-hundred micrograms of the soluble form of cyclin D3 protein was used for each immunization of a rabbit. When the antiserum obtained 2 weeks after tertiary immunization was applied to Western blot analysis, it was able to detect 33 kDa cyclin D3 protein in both murine lymphoma cell line BW5147.G.1.4 and human Jurkat T cells at 3,000-fold dilution with higher specificity to murine cyclin D3, demonstrating that the new rabbit polyclonal anti-murine cyclin D3 generated against c-terminal 236 amino acid residues more specifically recognizes murine cyclin D3 protein than does the commercially available rabbit polyclonal antibody raised against c-terminal 16 amino acids residues.

Cell cycle progression is governed by a highly conserved regulatory network involving the sequential formation, activation and subsequent inactivation of a series of cyclin and cyclin-dependent kinase (cdk) complexes (1, 9, 21, 22). Since there are at least ten different cyclins and seven cdks, phosphorylation of the appropriate substrate (s) by the specific cyclin/cdk complex may allow the cells to enter or traverse a particular phase of the cell cycle.

In mammalian cells the G_2/M transition requires the catalytically active $p34^{cdc2}$ kinase (cdk1)/cyclin B complex (2, 3, 17-19, 23, 26) whereas the progression through the early phase of G_1 is regulated by catalytic complexes formed with D-type G_1 cyclins and principally cdk4 and cdk6 and late in G_1 by cyclin E and cdk complexes (22, 23).

In a previous study, we demonstrated that murine G_0 T cells following activation by immobilized anti-CD3 homogenously traverse G_1 and enter S phase (5). Under the same conditions cyclin D2 and D3 were differentially expressed. The accumulation of cyclin D2-specific mRNA reached a maximum by 5 h and was sustained at least until G_2/M boundary, whereas the level of cyclin D3-specific mRNA detectable in G_0 T cells declined by 5 h and then increased until G_2/M boundary, suggesting that the precise role and the catalytic subunit of the D cyclins during the activation of T cells might be different. However, the transcriptional upregulation mechanism for both D cyclins in G_1 of T cells appeared to be similar in that the tyrosine phosphorylation of a 97 kDa protein and p56lck kinase activation correlated with the IL-2-induced proliferation of T cells were apparently required for the expression of cyclin D2- and cyclin D3-specific mRNA (7).

For further understanding of the expressional regulation mechanism as well as the functional role of cyclin D3 during the activation of murine T cells, anti-murine cyclin D3 antibody is required. Since the polyclonal anti-cyclin D3 generated from c-terminal 16 amino acid resi-

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dues which are common to human and murine cyclin D3 is commercially available, it has been applied to Western analysis and immunostaining of murine cyclin D3 in T cells. However, the antibody appeared to be highly cross-reactive to many other cellular proteins of mouse.

In the present study, we have developed a rabbit antiserum against the murine cyclin D3 protein that is corresponding to C-terminal 236 amino acid residues overexpressed in the *Escherichia coli* system using pET vector (25). The antiserum obtained two weeks after the tertiary immunization is able to specifically recognize murine cyclin D3 in the order of 3,000-fold dilution, when it is applied to Western analysis.

MATERIALS AND METHODS

Bacterial Strains and Vector Plasmid

Escherichia coli BL21(DE3)pLysS [*hsdS gal* (λcI*ts*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-*T7gene*1) *pLysS*] and the protein expression vector pET 3d were provided by Dr. Joel Shaper (The Johns Hopkins University, Baltimore, MD, U.S.A.). The pBluescript SK plasmid harbouring murine cyclin D3 was kindly supplied by Dr. Hitoshi Matsushime (University of Tokyo, Tokyo, Japan). The recombinant pET 3d plasmid hobouring a murine cyclin D3 cDNA fragment encoding C-terminal 236 amino acids in the *Nco*I/*Bam*HI site was designated pET 3d-CYL3.

PCR Procedure and Purification of PCR Product

In order to obtain the cyclin D3 cDNA fragment for insertion into the *Nco*I/*Bam*HI site of pET 3d vector, polymerase chain reaction (PCR) was performed in the presence of cyclin D3 cDNA as well as both *Nco*I-forward primer (5'-AATGACCATGGGGAAGCTGCTGGCATACTG-3') and *Bam*HI-backward primer (5'-ATAGAGGATCCTCACAGGTGAATGGCTGTGAC-3'). The target DNA was amplified in 50 μl of reaction mixture containing 5 μl of 10× buffer (100 mM Tris-HCl, 15 mM KCl, 1 mg/ml gelatin, pH 8.3), 4 μl of 1.25 mM dNTP, 1 μl of cyclin D3 cDNA (100 ng), 50 pmol of each of forward and backward primer, and 1 unit of Taq polymerase (Promega, Medison, WI, U.S.A.). PCR was carried out according to the following procedure: 25 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C.

For the purification of PCR product, the reaction mixture was extracted with equal volume of buffer-saturated phenol and chloroform sequentially, and precipitated with 2.5 volume of cold ethanol. The PCR product was further purified on an Elutip-D column (Schleicher & Schuell, Keene, NH, U.S.A.) according to the manufacturer's instruction.

DNA Sequence Analysis

DNA sequence analysis was performed by the dideoxy chain termination method (Sequenase™ version 2.0, USB, OH, U.S.A.) using synthetic oligonucleotide primers designed according to the sequence of cyclin D3 cDNA (14).

Expression of Cyclin D3 Gene in *E. coli*

Induction of the expression of the cyclin D3 gene inserted into pET-3d vector in *E. coli* was performed essentially as described by Studier *et al.* (25). Briefly, the transformant was cultured with shaking in LB media containing ampicillin (50 μg/ml) and chloramphenicol (20 μg/ml) at 37°C, and when $O.D_{600nm}$ reached to 0.4, 0.3 mM IPTG was added and the cells were further cultured for an additional 4 h.

To identify and localize the cyclin D3 protein produced by the *E. coli* transformant, the bacterial culture was fractionated into three portions as described in Fig. 1 (27) and equivalent amounts of each fraction were electrophoresed on 11% SDS polyacrylamide gel. The presence of cyclin D3 protein was determined by staining with Coomassie brilliant blue.

Immunization of Rabbit with Cyclin D3 Protein

The overexpressed cyclin D3 protein localized in the inclusion bodies was denatured by guanidine hydrochloride and then renatured into a soluble form. The protein concentration of the soluble form of cyclin D3 was quantitated with a BCA microassay kit (Pierce, Rockford, IL, USA).

One milliliter of cyclin D3 protein solution (400 μg) was mixed with an equal volume of Complete Freund's Adjuvant, and 0.8 ml of the mixture was injected intramuscularly at the thigh muscle of two rear legs and 1.2 ml was injected subcutaneously at 4 different sites on the back of a rabbit. For secondary and tertiary immunization, cyclin D3 protein mixed with Incomplete Fruends's Adjuvant was injected into the rabbit in the same manner every four weeks. The bleeding was done two weeks after each immunization.

Mammalian Cell Culture

For the propagation of human Jurkat T cell line and

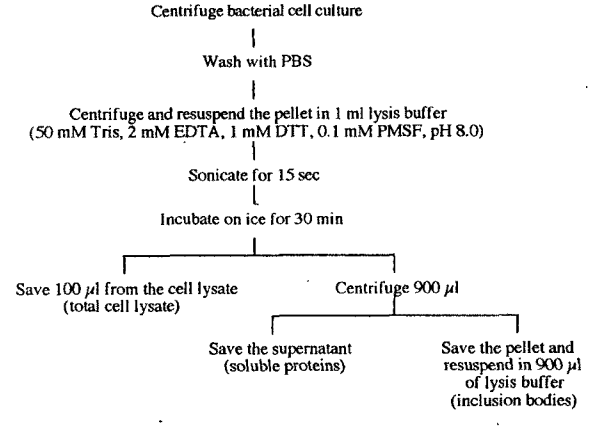

Fig. 1. Procedure for the fractionation of bacterial culture.

murine lymphoma cell line BW 5147.G.1.4 in culture, RPMI 1640 (GIBCO BRL, Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum (UBI, Lake Placid, NY, U.S.A.) and DMEM (GIBCO BRL) supplemented with 10% fetal calf serum (UBI), respectively were used. To obtain cell lysate, equivalent cultures were centrifuged and extracted in lysis buffer (20 mM Tris, 137 mM NaCl, 1 mM Na3VO4, 1 mM PMSF, 10 g of aprotinin per ml, 1% Nonidet P-40, pH 8.0) for 30 min at 4°C.

Western Analysis

Equivalent amounts of cellular protein were subjected to electrophoresis on 8-16% SDS gradient polyacrylamide gel and electrotransferred to an Immobilon-P membrane (Millipore Corporation, Bedford, MA, U.S.A.). The membrane was probed with either commercially available polyclonal anti-cyclin D3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or rabbit antiserum raised against murine cyclin D3 protein in this study and then with ^{125}I-goat anti-rabbit IgG (New England Nuclear, Boston, MA, U.S.A.). The presence of cyclin D3 was detected by autoradiography after exposure at -70°C.

RESULTS

Construction of Recombinant Plasmid pET 3d-CYL3

To produce murine cyclin D3 in *E. coli*, a cyclin D3 cDNA fragment corresponding to C-terminal 236 amino acids was inserted into the *NcoI*/*Bam*HI site of an expression vector pET 3d and designated pET 3d-CYL3. The cyclin D3 cDNA fragment required to construct pET 3d-CYL3 was obtained by PCR. When PCR was done with cyclin D3 cDNA in the presence of both *Nco* I-forward primer and *Bam*HI-backward primer, 730 bp of the PCR product was amplified (Fig. 2).

After the PCR product was purified as described in Materials and Methods, 60 ng of the PCR product was treated with *NcoI*/*Bam*HI and then ligated with 0.1 μg of pET 3d plasmid that was linearized by the same restriction enzymes. When the ligation mixture was used for the transformation of *E. coli* BL21(DE3)pLysS, transformants were obtained with a frequency of 10^{-6}. Among twelve transformants randomly selected, nine appeared to contain the recombinant plasmid pET 3d-CYL 3. Subsequently, from a transformant, pET 3d-CYL3 was purified and its DNA sequence was analyzed. As shown in Fig. 3, the sequence analysis employing eight different oligo DNA primers including both *Nco*I-forward primer and *Bam*HI-backward primer revealed that the reading frame of the cyclin D3 cDNA fragment inserted in the cloning site in pET 3d was correct. The transformant containing pET 3d-CYL3 was designated *E. coli* pET CYL3-7.

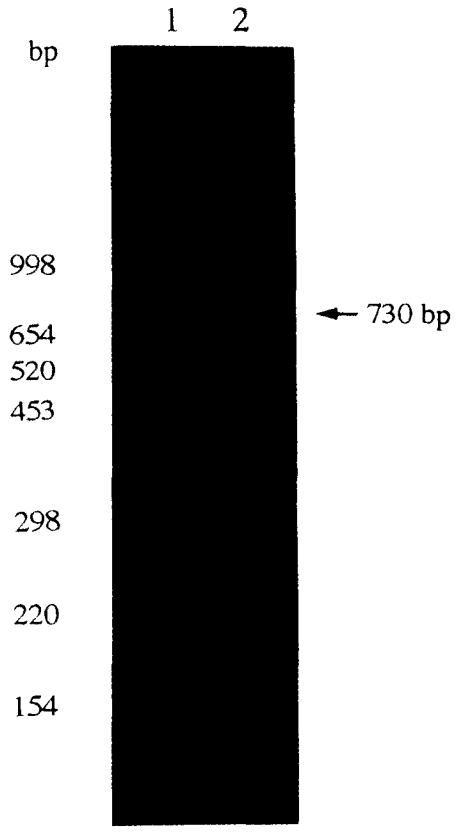

Fig. 2. Electrophoresis of cyclin D3 cDNA fragment encoding c-terminal 236 amino acids amplified by PCR.
PCR was done with cyclin D3 cDNA in the presence of both *Nco*I-forward primer and *Bam*HI-backward primer. The PCR product amplified (lane 2) was electrophoresed with sizer markers (lane 1) on 2.5% NuSieve and 1% agarose gel.

Identification and Localization of Cyclin D3 Protein in *E. coli*

For the identification and localization of cyclin D3 protein produced in *E. coli* pET 3d-CYL3-7, the strain was cultured with shaking in LB media containing ampicillin (50 μg/ml) and chloramphenicol (20 μg/ml) at 37°C, and when growth O.D at 600 nm reached to 0.4, 0.3 mM IPTG was added and cultivation was continued for additional 4 h. The bacterial culture was fractionated into three portions to identify the cyclin D3 protein produced and to address its localization in the host. While cyclin D3 protein induced in the presence of 0.3 mM IPTG was mainly localized in the portion of the inclusion body and its molecular mass was approximately 26 kDa as expected from the reading frame on DNA sequence, the protein was undetectable in the portion of the cellular soluble proteins (Fig. 4).

Factors Affecting Production of Cyclin D3 Protein in *E. coli*

Optimum conditions for the production of cyclin D3

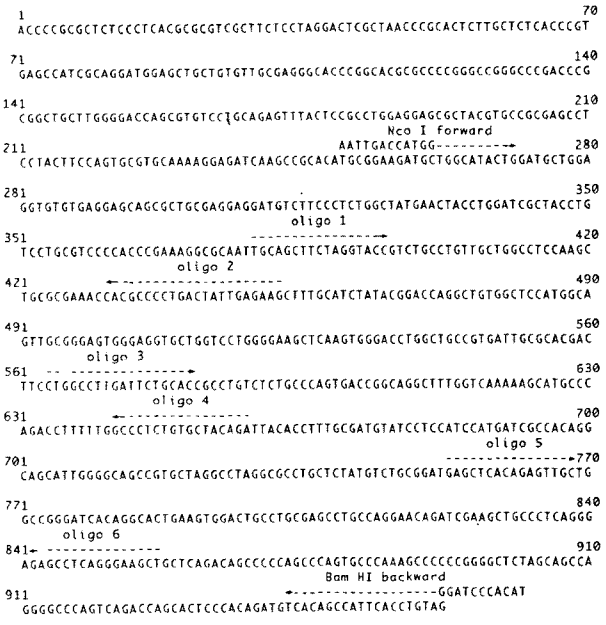

Fig. 3. Nucleotide sequence of the PCR-amplified cyclin D3 cDNA fragment and location of oligonucleotide primers employed for the DNA sequencing.

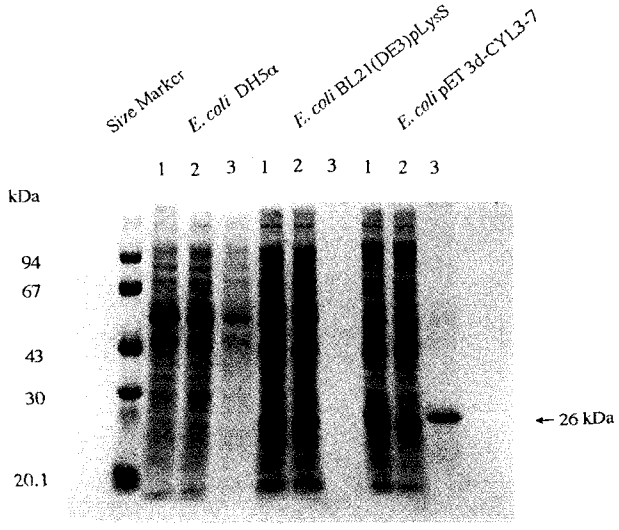

Fig. 4. Identification of cyclin D3 protein expressed in *E. coli*.

The transformant *E. coli* pET CYL3-7 harboring pET 3d-CYL3 was able to express a 26 kDa protein as expected from c-terminal 236 amino acid residues of murine cyclin D3, whereas none of *E. coli* DH5 and *E. coli* BL21(DE3)pLysS as controls expressed the 26 kDa protein. In *E. coli* pET CYL3-7 the 26 kDa protein was detectable in the portions of total cell lysate (lane 1) and inclusion body (lane 3), but not dectectable in cellular soluble protein-portion (lane 2).

protein in *E. coli* pET 3d-CYL3-7 were determined in terms of IPTG concentration and the period of IPTG-induction as well as the temperature of IPTG-induction.

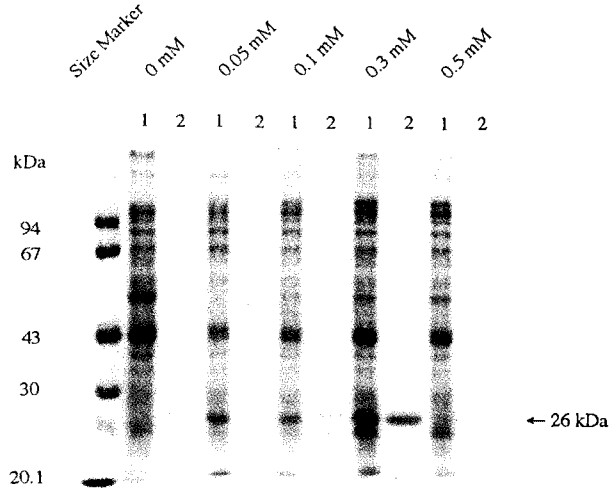

Fig. 5. Effect of IPTG concentration on the production of cyclin D3 protein.

The amount of cyclin D3 protein in *E. coli* pET CYL3-7 induced by various concentration of IPTG ranging from 0.05 to 0.5 mM was investigated. For detection of the cyclin D3 protein, both total cell lysate (lane 1) and the inclusion body-portion (lane 2) of the bacterial cells were electrophoresed on 11% SDS polyacrylamide gel.

When the effect of IPTG concentration ranging from 0.05 to 0.5 mM on the production of cyclin D3 protein was investigated, 0.3 mM IPTG appeared to be the optimum concentration (Fig. 5). In the presence of 0.3 mM IPTG, the effect of IPTG-induction on the production of cyclin D3 protein reached maximum 4 h after induction (Fig. 6). By employing 0.3 mM IPTG and 4 h of induction period, the effect of temperature on the production of cyclin D3 was also determined. When IPTG-induction was performed at 37°C, the production of cyclin D3 was a maximum. However, the level of cyclin D3 protein was declined at either 30°C or 40°C, and was not detectable at 20°C (Fig. 7).

Solubilization of Cyclin D3 Protein in Inclusion Body

In order to obtain cyclin D3 protein in soluble form, the cyclin D3 protein contained in the portion of the inclusion bodies was recovered, after *E. coli* pET 3d-CYL 3-7 was cultured in a volume of 100 ml under optimum conditions. The inclusion bodies containing cyclin D3 protein were denatured in a denaturation buffer (50 mM Tris, 5 M guanidine hydrochloride, 5 mM EDTA, pH 8.0) for 1 h at 4°C and centrifuged to save the supernatant as described elsewhere (13). For renaturation the supernatant was mixed with 10 ml of a renaturation buffer (50 mM Tris, 1 mM dithiothreitol, 20% glycerol, 0.2 mM phenylmethylsulfornyl fluoride, pH 8.0) and incubated at 4°C with stirring overnight. After the mixture was centrifuged at 14,000 g for 20 min, 11 ml of supernatant containing a soluble form of cyclin D3 protein was obtained. The soluble form of cyclin D3 protein in the supernatant

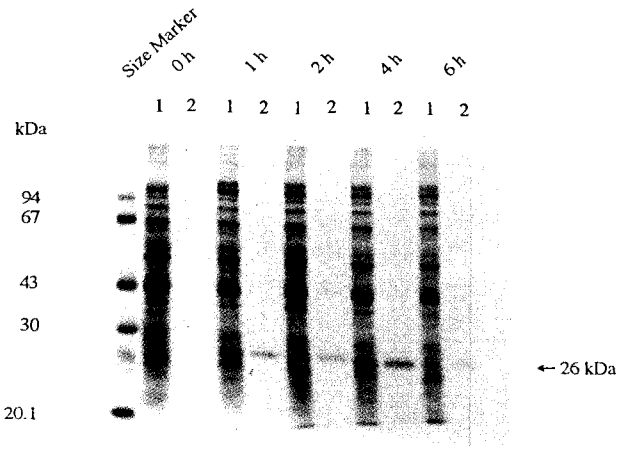

Fig. 6. Optimum period of IPTG-induction for the production of cyclin D3 protein.

Induction of cyclin D3 protein in *E. coli* pET CYL3-7 by 0.3 mM IPTG was performed for various periods. For detection of the cyclin D3 protein, both total cell lysate (lane 1) and inclusion body-portion (lane 2) of the bacterial cells were electrophoresed on 11% SDS-polyacrylamide gel.

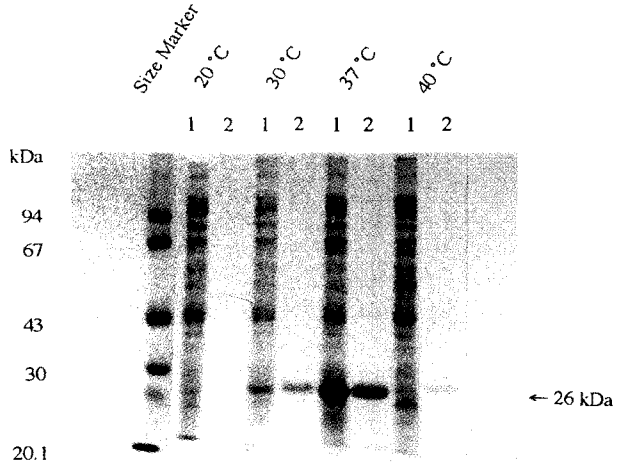

Fig. 7. Optimum temperature of IPTG-induction for the production of cyclin D3 protein.

Induction of cyclin D3 protein in *E. coli* pET CYL3-7 by 0.3 mM IPTG was performed for 4 h at various temperature. For detection of the cyclin D3 protein, both total cell lysate (lane 1) and inclusion body-portion (lane 2) of the bacterial cells were electrophoresed on 11% SDS-polyacrylamide gel.

could be identified after electorphoresis on 11% SDS polyacrylamide gel (Fig. 8). The protein concentration of the supernatant was 0.4 mg/ml.

Rabbit Antiserum Raised against Cyclin D3 Protein

For production of rabbit polyclonal antibody against cyclin D3 protein, 400 μg of the soluble form of recombinant cyclin D3 protein mixed with Adjuvant was in-

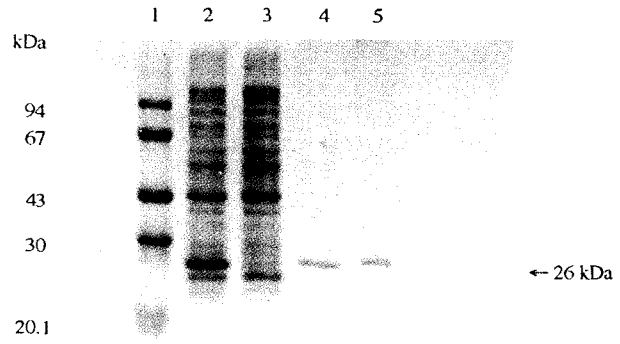

Fig. 8. Renaturation of insoluble cyclin D3 protein contained in the portion of inclusion body into soluble form.

The insoluble cyclin D3 protein contained in the portion of inclusion body was recovered after *E. coli* pET 3d-CYL3-7 was cultured in a volume of 100 ml with optimum condition. The protein was denatured in 1 ml of denaturation buffer for 1 h at 4°C and subsequently renatured by mixing with 10 ml of a renaturation buffer and stirring overnight at 4°C. The mixture was centrifuged at 14000 g for 20 min to obtain soluble form of cyclin D3 protein. The 26 kDa protein detectable in the portions of total cell lysate (lane 2) and inclusion body (lane 4) was identified as a soluble form in the supernatant (lane 5), but not dectectable in cellular soluble protein-portion (lane 3), after electorphoresis on 11% SDS-polyacrylamide gel.

Table 1. Quantitation of anti-cyclin D3 antibody by ELISA.

Sample	Dilution ratio				
	1:250	1:500	1:1,000	1:2,000	1:3,000
preserum	–	–	–	–	–
1° serum	++	+	–	–	–
2° serum	+++	+++	++	+	–
3° serum	+++	+++	+++	+++	++

The titer of rabbit polyclonal antibody raised against recombinant cyclin D3 protein expressed in *E. coli* pET 3d-CYL3-7 was assayed by ELISA method. At a 3,000-fold dilution, the antiserum obtained after tertiary immunization was able to detect 5 μg of antigen previously coated onto 96-well plate. Symbols: +++, $O.D_{405}=1.0\simeq0.7$; ++, $O.D_{405}=0.6\simeq0.3$; +, $O.D_{405}=0.2\simeq0.1$; –, $O.D_{405}<0.1$.

jected intramuscularly and subcutaneously for each primary, secondary and tertiary immunization. To evaluate the antibody titer of antiserum by the ELISA method, bleeding from the rabbit was done two weeks after each immunization.

Since in the order of upto 3,000-fold dilution the antiserum obtained 2 weeks after tertiary immunization appeared to possess a high enough titer to recognize the antigen (Table 1), the animal was sacrificed and antiserum was recovered. By Western analysis, the antibody produced was further tested as compared to commercially available rabbit polyclonal cyclin D3 antibody which is raised against the carboxy terminal 16 amino acids of human cyclin D3. As shown in Fig. 9, the antibody more specifically recognized murine cyclin D3 protein, whereas

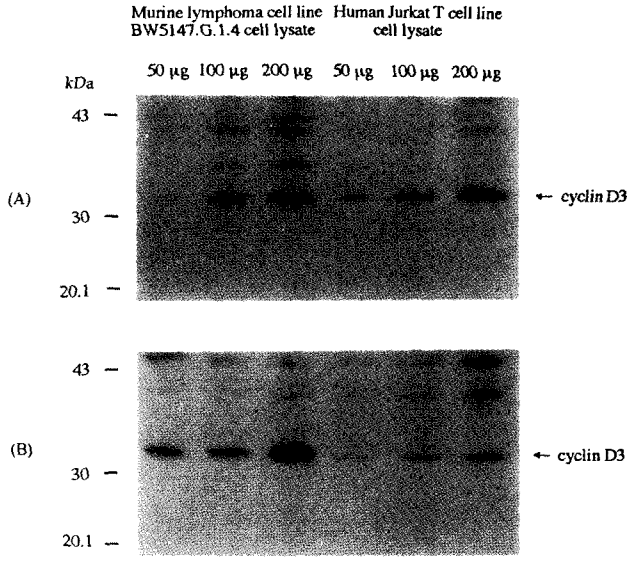

Fig. 9. Western analysis of both murine and human cyclin D3 protein using commercially available anti-cyclin D3 (A) or rabbit polyclonal anti-cyclin D3 (B) raised in this study against recombinant murine cyclin D3 protein. Molecular sizes are shown in kDa.

commercially available anti-cyclin D3 antibody recognized both human and murine cyclin D3 proteins similarly with several redundant nonspecific binding bands.

DISCUSSION

Cyclin D is among the novel types of mammalian G_1 cyclin isolated by screening human cDNA library to complement CLN-deficient yeast and designated cyclin C, D, and E (28, 12, 10). *PRAD* 1 was cloned as a gene rearranged in a parathyroid tumor and is identical to human cyclin D1 (15). A murine homologue of cyclin D1 (CYL 1) was independently isolated from a cDNA library prepared from mouse macrophages synchronously progressing through G1 in response to colony-stimulating factor 1 (14). The murine cyclin D1 cDNA probe was used to identify two related genes, murine cyclin D2 (CYL2) and murine cyclin D3 (CYL3). Unlike other types of cyclin, cyclin D1, D2, and D3 have unique cell and tissue-specific patterns of expression. While cyclin D2 gene was ubiquitously expressed, cyclin D1 and D3 were initially known to be macrophage-specific and T cell-specific D-type cyclin, respectively (14). These D-type cyclins are known to regulate early G_1 phase by principally complexing with cdk4 and cdk6, and several emerging evidences suggest that cyclin D may also be involved in cell differentiation, apoptosis, and oncogenesis (4, 11, 16).

Our interest in murine cyclin D3 expressed in T cells originated from our observation that the expression of cyclin D3 is most likely dependent on c-Myc expression induced by the interaction of IL-2 with high affinity IL-2 receptor (6), and that the expression level of cyclin D3-specific mRNA during the activation of murine T cells decreases with advancing age (8). It was highly likely that the acquisition of a good quality anti-murine cyclin D3 is prerequisite for elucidating its transcriptional regulation mechanism as well as its functional role during the activation and subsequent early phase of cell cycle progression of G_0 T cells, and for understanding the age-related change in its expression. When commercially available rab bit polyclonal anti-cyclin D3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied to Western analysis and immunohistochemistry, it recognized nonspecifically several other cellular proteins of murine T cells and turned out to be not applicable to the immunoassay of murine cyclin D3. Thus, we decided to generate a new rabbit polyclonal anti-murine cyclin D3.

Recently, an *E. coli* system employing pET vectors was developed not only to overexpress target DNAs under control of a T7 promoter and T7 RNA polymerase but also to regulate the expression of target DNAs by placing a T7 RNA polymerase gene under control of *lac*UV5 promoter, which is inducible by IPTG (26). In the absence of T7 RNA polymerase, transcription of target DNAs by *E. coli* RNA polymerase was low enough that very toxic genes could be cloned in these vectors. In addition, the expression system was constructed to express target DNAs from its own translation start and not as a fusion protein. By using the *E. coli* system with pET 3d vector, murine cyclin D3 protein was expressed at a high level and adapted to raise the rabbit polyclonal anti-cyclin D3 antibody. For expressing the cyclin D3 protein in the *E. coli* system, a murine cyclin D3 cDNA fragment encoding c-terminal 236 amino acid residues, which has a methionine codon at the 5' end and a translation stop codon at 3' end was inserted into the *Nco*I/*Bam*HI site of pET 3d vector. When the recombinant plasmid was introduced into *E. coli* BL21(DE3)pLysS which can express T7 RNA polymerase in the presence of IPTG, the transformant isolated was able to overexpress a 26 kDa protein by IPTG-induction. Since the DNA sequence encoding C-terminal 236 amino acids of cyclin D3 inserted in the cloning site of pET 3d was correct and the molecular mass of a protein overexpressed by the transformant was 26 kDa as expected from the amino acid sequence, the protein overexpressed was considered to be cyclin D3.

The cyclin D3 protein overexpressed in the *E. coli* system by IPTG-induction was mainly localized in the portion of the inclusion bodies, whereas in the absence of IPTG the protein was not produced so that it was not detectable either in the portion of cellular soluble proteins or in the inclusion bodies. Since it was thought that immunization with a soluble form of cyclin D3 protein

would be better than using the insoluble form in the inclusion bodies for producing an antibody which can recognize the intact cyclin D3 protein, and since it is generally accepted that an overexpressed protein that tends to be localized in the inclusion bodies can be produced as a soluble form by changing the culture conditions, the effect of culture conditions on production as well as localization of cyclin D3 protein in the host was examined. However, regardless of culture conditions tested, cyclin D3 protein produced was localized mainly in the portion of the inclusion bodies.

Because the inclusion bodies are easily separated from the majority of the contaminating *E. coli* proteins by centrifugation at the appropriate g force and also they contain highly concentrated expressed proteins, the localization of an expressed protein in the inclusion bodies can provide an important purification step. In addition, an efficient procedure converting the insoluble eukaryotic proteins localized in the inclusion bodies into the active form has been reported (13). By taking advantage of these previous data, the cyclin D3 protein overexpressed under the optimum conditions was purified as the inclusion bodies and converted into a soluble form of cyclin D3 protein. When direct conversion of the insoluble form of cyclin D3 protein in the inclusion bodies into soluble form by denaturation and subsequent renaturation procedure was performed, 4.4 mg of the soluble form of cyclin D3 protein could be recovered using the insoluble form of cyclin D3 contained in the inclusion bodies harvested from 100 ml culture of the transformant.

To produce rabbit polyclonal anti-cyclin D3 antibody, the soluble form of the cyclin D3 protein was used as the immunogen. The antiserum obtained 2 weeks after tertiary immunization was able to detect not only the antigen at 3,000-fold dilution when it was applied to ELISA, but also both intact human and murine cyclin D3 protein by Western analysis. Since the commercially available rabbit polyclonal anti-cyclin D3 is raised against the c-terminal 16 amino acid residues which are common to both mouse and human cyclin D3, the antibody could similarly detect a 33 kDa of the intact cyclin D3 protein contained in the cell lysates of murine lymphoma BW5147.G.1.4 and human Jurkat T cells as expected. However, the antibody detected nonspecifically several other proteins particularly in the murine cell lysate. In contrast, the newly generated rabbit polyclonal anti-cyclin D3 could much more specifically detect murine cyclin D3 than did the commercial antibody. The new antibody appeared to detect human cyclin D3 by cross-reaction because murine cyclin D3 shares 94.5% homology with human cyclin D3 on the amino acid level.

Together these results demonstrate that the *E. coli* system employing pET vector is very successful for overexpression of murine cyclin D3 protein, and that the rabbit polyclonal anti-murine cyclin D3 generated against c-terminal 236 amino acid residues much more specifically recognizes murine cyclin D3 protein than does the commercially available rabbit polyclonal antibody raised against c-terminal 16 amino acid residues, and may suggest that a protein localized in the inclusion body when overexpressed has the advantage as an immunogen since it can be easily purified and renatured into the soluble form.

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