The p58\textsuperscript{PITSLRE} is a p34\textsuperscript{cdc2}-related protein kinase that plays an important role in normal cell cycle progression. Elevated expression of p58\textsuperscript{PITSLRE} in eukaryotic cells prevents them from undergoing normal cytokinesis and appears to delay them in late telophase. To investigate the molecular mechanism of p58\textsuperscript{PITSLRE} action, we used the yeast two-hybrid system, screened a human fetal liver cDNA library, and identified cyclin D3 as an interacting partner of p58\textsuperscript{PITSLRE}. In vitro binding assay, in vivo coimmunoprecipitation, and immunofluorescence cell staining further confirmed the association of p58\textsuperscript{PITSLRE} with cyclin D3. This binding was observed only in the G2/M phase but not in the G1/S phase of the cell cycle; meanwhile, no interaction between p110\textsuperscript{PITSLRE} and cyclin D3 was observed in all the cell cycle. The overexpression of cyclin D3 in 7721 cells leads to an exclusively accumulation of p58\textsuperscript{PITSLRE} in the nuclear region, affecting its cellular distribution. Histone H1 kinase activity of p58\textsuperscript{PITSLRE} was greatly enhanced upon interaction with cyclin D3. Furthermore, kinase activity of p58\textsuperscript{PITSLRE} was found to increase greatly in the presence of cyclin D3 using a specific substrate, \(\beta\)-1,4-galactosyltransferase 1. These data provide a new clue to our understanding of the cellular function of p58\textsuperscript{PITSLRE} and cyclin D3.

The eukaryotic cell division cycle is tightly regulated by the activation and deactivation of the cyclin-dependent kinases (CDKs).\textsuperscript{1} Active CDK serves as a protein kinase subunit, the kinase activity of which is dependent on its association with a regulatory cyclin subunit (1–3). In mammalian cells both the CDKs and cyclins consist of numerous members, including cyclin A-H and at least nine different p34\textsuperscript{cdc2}-related kinases (4, 5). Among them, the CDKs 4 and 6 are first activated by cyclin binding is required for the activation of different CDK subunits with different cyclin subunits (1–3, 13, 14). Although cyclin binding is required for the activation of the CDK subunit of the complex, other means of modulating the activity of CDKs also exist, such as phosphorylation and dephosphorylation of the key residues on the CDK subunit and the binding of cyclin-dependent kinase inhibitors (2, 3, 14, 15).

The PITSLRE protein kinases are parts of the large family of p34\textsuperscript{cdc2}-related kinases whose functions appear to be linked with cell cycle progression, apoptotic signaling, and tumorigenesis (16–25). The PITSLRE homologues exist in human, mouse, chicken, Drosophila, and Xenopus, suggesting that their functions may be well conserved (16, 19, 26, 27). The small p58\textsuperscript{PITSLRE} isoform was originally isolated from a human liver cDNA library and has a 299-amino acid region with 68% homology to the p34\textsuperscript{cdc2} protein kinase (16). During the study of p58\textsuperscript{PITSLRE}, 10 isoforms of the p58\textsuperscript{PITSLRE} subfamily of protein kinases including p110\textsuperscript{PITSLRE} have been isolated by molecular cloning (19). The discovery of multiple p58\textsuperscript{PITSLRE} isoforms has led to the renaming of these kinases according to an established nomenclature system, which is based on the single amino acid codon designation of the conserved PSTAIRE box region of p34\textsuperscript{cdc2} (17). The p110\textsuperscript{PITSLRE} isoform can be detected in all phases of the cell cycle, whereas the p58\textsuperscript{PITSLRE} isoform is mainly expressed in G2/M phase (28). Ectopic expression of p58\textsuperscript{PITSLRE} in Chinese hamster ovary fibroblasts leads to a late telophase delay, abnormal cytokinesis, and a reduced rate of cell growth (16). Conversely, the diminished expression of p58\textsuperscript{PITSLRE} mRNA is found to increase DNA replication and enhance cell growth (17). Further analysis of the Chinese hamster ovary cells ectopically expressed of p58\textsuperscript{PITSLRE} demonstrated that the reduced cell growth was due to apoptosis (20). In addition, it was shown that the p58\textsuperscript{PITSLRE} and p110\textsuperscript{PITSLRE} isoforms were cleaved by caspase proteases to generate smaller 46–50-kDa proteins that could also phosphorylate histone H1 during tumor necrosis factor \(\alpha\)- and Fas-mediated apoptosis (21–23). Because of its ultimate function in cell growth control, the p58\textsuperscript{PITSLRE} and its family have been a target for alteration, translocation, and deletion during tumorigenesis (18, 24, 25).

Although the p58\textsuperscript{PITSLRE} plays an important role in cell cycle progression, little is known about its interaction proteins. Meanwhile, study of the p110\textsuperscript{PITSLRE} isoform showed that it could interact with the RNA-binding protein RNP51, RNA...
polymerase II, and multiple transcriptional elongation factors, regulating some aspects of RNA splicing or transcription in proliferating cells (29, 30). Thus, the identification of the cellular proteins that interact with p58PITSLRE is a useful approach for defining the cellular function and regulatory mechanism of p58PITSLRE. To investigate this issue, a two-hybrid screening from human fetal liver cDNA library was carried out using the full length of p58PITSLRE as bait. As a result, cyclin D3 was identified as a p58PITSLRE-associated protein. This interaction between p58PITSLRE and cyclin D3 is specific, as demonstrated by the inability of the other D-type cyclins to associate with p58PITSLRE using in vitro binding assays and yeast two-hybrid assays and the inability of the p110PITSLRE to associate with cyclin D3 using immunofluorescence cell staining and immunoprecipitation. More importantly, we showed that the p58PITSLRE was associated with the cyclin D3 in vitro at G2/M phase by coimmunoprecipitation and immunofluorescence. Interestingly, the elevated expression of cyclin D3 affected p58PITSLRE cellular distribution. Moreover, kinase activity of p58PITSLRE was greatly enhanced upon cyclin D3 deletion mutants of p58PITSLRE were constructed by PCR with pLexA-histone H1 were purchased from Roche Molecular Biochemicals. Bovine Signal Transduction Laboratories. Protein G-agarose, glutathione secondary antibody, and the goat anti-mouse-rhodamine secondary antibody, the goat anti-rabbit-fluorescein isothiocyanate antibody were purchased from Sigma (St. Louis, MO). The mouse monoclonal anti-cyclin D3 antibody was purchased from Santa Cruz Biotechnology, and the mouse monoclonal anti-cyclin D3 antibody was purchased from Signal Transduction Laboratories. Protein G-agarose, glutathione-Sepharose beads, the mouse monoclonal anti-HA (12CA5) antibody, and histone H1 were purchased from Roche Molecular Biochemicals. Bovine β-1,4-galactosyltransferase 1, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride were purchased from Sigma. [-35S]methionine, Hybond polyvinylidene difluoride membrane, goat anti-mouse-heralds peroxidase secondary antibody, goat anti-rabbit-horseradish peroxidase secondary antibody, and the enhanced chemiluminescence (ECL) assay kit were purchased from Amersham Biosciences.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents—**7721 cells, a human hepatocarcinoma cell line, were obtained from the Institute of Cell Biology, Academia Sinica. The 7721 cells ectopically expressed of p58PITSLRE (7721/p58 cells) were constructed and confirmed in our previous work (31). The rabbit polyclonal anti-PITSLRE antibody, the goat anti-rabbit-fluorescein isothiocyanate secondary antibody, and the goat anti-mouse-rodhamine secondary antibody were purchased from Santa Cruz Biotechnology, and the mouse monoclonal anti-cyclin D3 antibody was purchased from Signal Transduction Laboratories. Protein G-agarose, glutathione-Sepharose beads, the mouse monoclonal anti-HA (12CA5) antibody, and histone H1 were purchased from Roche Molecular Biochemicals. Bovine β-1,4-galactosyltransferase 1, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride were purchased from Sigma. [-35S]methionine, Hybond polyvinylidene difluoride membrane, goat anti-mouse-heralds peroxidase secondary antibody, goat anti-rabbit-horseradish peroxidase secondary antibody, and the enhanced chemiluminescence (ECL) assay kit were purchased from Amersham Biosciences.

**In Vivo Association of p58PITSLRE with Cyclin D3**

- **Cell Culture and Synchronization—**All the cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) bovine calf serum, 100 units/ml penicillin, and 50 µg/ml streptomycin at 37 °C under 5% CO2 in humidified air. G1/S phase-arrested 7721 cells were obtained by sequential thymidine treatment. First, the cells were treated with 2.5 mM thymidine for 24 h then changed to the fresh medium for another 24 h and replaced with the 2.5 mM thymidine medium for 24 h. To block cells in G2/M phase, cells were seeded in RPMI 1640 medium with 10% fetal bovine serum and 2.5 mM thymidine. After 24 h, the cells were washed twice with PBS and fed with medium containing camptothecin (0.5 µM). One hour later, the cells were washed twice with PBS and fed with complete medium for an additional 24 h.

- **Immunoprecipitation, Immunoblot Assays, and Cellular Fractionation—**The 7721 cells grown in RPMI 1640 medium supplemented with 10% (v/v) bovine calf serum were plated in 60-mm dishes (Nunc) at a concentration of 6 × 105 cells/dish the day before transfection. Plasmid DNA (4 µg) was transfected into 7721 cells with a calcium phosphate precipitation method. Two days after transfection, cells were washed three times with ice-cold PBS and solubilized with 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5 mM orthovanadate, 0.1 mM NaF, 0.1 mM benzamide, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Detergent-insoluble materials were removed by centrifugation at 13,000 rpm for 15 min at 4 °C. The whole cell lysates were incubated with mouse normal IgG or anti-HA monoclonal antibody at 4 °C for 2 h. Pre-equilibrated protein G-agarose beads were then added, and after 4 h of incubation, they were collected by centrifugation and then gently washed three times with the lysis buffer. The bound proteins were eluted by boiling in SDS sample buffer and resolved on a 10% SDS-PAGE gel. The proteins were transferred onto a polyvinylidene difluoride membrane and probed with a 1:1000 dilution of a monoclonal anti-cyclin D3 antibody. Proteins were detected using the ECL kit.

The coimmunoprecipitation in 7721 cells under normal physiological situations was conducted with the normal 7721 cells and the 7721 cells synchronized at a different cell cycle phase. The method was the same as above except that the antibody used for immunoprecipitation was monoclonal anti-cyclin D3 antibody and for immunoblot was rabbit polyclonal anti-PITSLRE antibody. The coimmunoprecipitation for the HeLa cells was the same as that of the 7721 cells.

The cellular fractionation was performed as below. G1/M phase-arrested cells (8 × 105) were suspended for 5 min on ice in 500 µl of buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 25 mM KCl, 250 mM sucrose, 1× complete protease inhibitors, 0.3% Nonidet P-40). After gentle mixing, the lysate fraction was centrifuged at 1000 rpm for 2 min at 4 °C. The resulting supernatants constituted the cytoplasmic fractions with the
In Vitro Interactions between p58\textsuperscript{PITSLRE} and D-type Cyclins—The fact that cyclin D3 was identified by two-hybrid screening using p58\textsuperscript{PITSLRE} as bait raised the question of whether p58\textsuperscript{PITSLRE} interacted preferentially with this D-type cyclin or it also interacted with the other two D-type cyclins. To answer this question, we used a direct two-hybrid experiment to compare cyclin D1, cyclin D2, and cyclin D3 for their ability to bind to p58\textsuperscript{PITSLRE}. As a positive control, cyclin D3 was included in this experiment. As the negative control, p58\textsuperscript{PITSLRE} alone did not permit growth of the yeast on nutrient-deficient medium. Subsequent transformation with either of the D-type cyclin constructs showed that neither of the other two D-type cyclins permitted activation of the reporter genes, whereas cyclin D3, in the presence of p58\textsuperscript{PITSLRE}, did activate the two reporter genes (data not shown). These data indicated that only cyclin D3 but not cyclin D1 or cyclin D2 interacted with p58\textsuperscript{PITSLRE} in the yeast two-hybrid system (Fig 1).
positive controls and GST incubated with cyclin D3 as a negative control. The protein mixtures were bound to glutathione-Sepharose beads, washed, and subjected to SDS-PAGE. The resulting gel was then exposed. Only the GST-p58PITSLRE band was observed when GST-p58PITSLRE was incubated with cyclin D1 or cyclin D2. A strong cyclin D3 signal was observed after incubation of GST-p58PITSLRE with cyclin D3 (Fig. 2). For the positive control and negative control, cyclin D1, cyclin D2, and cyclin D3 were observed after incubation with GST-CDK4, and no cyclin D3 was observed after incubation with GST. These data showed that p58PITSLRE interacted preferentially with the cyclin D3.

Mapping of the p58PITSLRE Region That Interacted with Cyclin D3—In addition to the conserved p34cdc2-related Ser/Thr protein kinase catalytic domain, p58PITSLRE also contains a unique 74-amino acid NH₂-terminal region with a putative calmodulin binding site, nuclear localization sequence, and three tandem PEST sequences (16). During Fas- and tumor necrosis factor α-induced cell death, its NH₂-terminal region is cleaved by multiple caspases (21–23). Furthermore, ectopic expression of its NH₂-terminal deletion mutant, which resembles the final caspase-modified product, has also been shown to induce apoptosis (20). To investigate the region in p58PITSLRE responsible for binding to cyclin D3, we constructed two p58PITSLRE mutants (Fig 1), one containing NH₂-terminal 100 amino acids (NH₂-p58PITSLRE) and the other lacking NH₂-terminal 74 amino acids (ΔNH₂-p58PITSLRE) (20). These two mutant constructs were co-transformed either with the empty pB42AD plasmid or with pB42AD-cyclin D3 into yeast cells. Co-transformants were tested for growth in the absence of leucine and production of β-galactosidase. No growth occurred in all the co-transformants (data not shown), which indicated that neither p58PITSLRE nor p58PITSLRE mutants interacted with cyclin D3. This result suggests that the full-length of p58PITSLRE might be necessary for its binding to cyclin D3 (Fig. 1), which will be further described below.

Binding of p58PITSLRE with Cyclin D3 at G₂/M Phase in Mammalian Cells—To further investigate the interaction of p58PITSLRE with cyclin D3, we tested whether they associated in mammalian cells. The p58PITSLRE protein kinase was tagged at its amino terminus with an HA epitope and transiently expressed in 7721 cells, a human hepatocarcinoma cell line. The expression of p58PITSLRE was confirmed by a monoclonal antibody against HA epitope. HA-p58PITSLRE and cyclin D3 proteins, were immunoprecipitated with normal mouse IgG or anti-HA monoclonal antibody followed by immunoblot analysis using an anti-cyclin D3 monoclonal antibody. As shown in Fig. 3B, cyclin D3 was coimmunoprecipitated with HA-p58PITSLRE, whereas no cyclin D3 was detected in the control mouse IgG immunoprecipitation.

The ectopic expression of p58PITSLRE is not cell cycle-regulated, whereas in vivo, p58PITSLRE is produced almost exclusively in G₂/M. To investigate whether p58PITSLRE and cyclin D3 can interact in a normal physiological situation, we synchronized the 7721 cells and did immunoprecipitation in different stages of the cell cycles. After sequential thymidine treatment, there were 91.25% cells in G₁ phase, 3% cells in S phase, and no cells in G₂/M phase (Fig. 3C). To arrest cells in G₂/M phase, we incubated cells first with thymidine (2.5 mM), then with camptothecin (0.5 μM). Finally, there were 72.75% of the cells arrested in G₂/M phase (Fig. 3C). After synchronization, much more p58PITSLRE protein was found in the G₂/M phase-arrested cells than in the G₁/S phase-arrested cells (Fig. 3D).

Cell lysates from different cell cycles were subjected to immunoprecipitation with anti-cyclin D3 antibody followed by immunoblot analysis using a rabbit anti-PITSLRE polyclonal antibody. As shown in Fig. 3E, p58PITSLRE coimmunoprecipitated with cyclin D3 in G₂/M phase but not in G₁/S phase. For normal 7721 cells, there were about 15% cells in G₂/M phase, so the interaction could still be observed. However, the amount of the p58PITSLRE that coimmunoprecipitated with cyclin D3 in the normal 7721 cells was much less than that in the G₂/M phase-arrested 7721 cells. In addition, we also detected this association between p58PITSLRE and cyclin D3 in HeLa cells with communoprecipitation (data not shown).

To further address the subcellular interaction of p58PITSLRE with cyclin D3, we did coimmunoprecipitation after crude fractionation of the G₂/M phase-arrested 7721 cell lysates into nuclear and cytoplasmic components (Fig. 3F). The results showed that p58PITSLRE and cyclin D3 interacted mostly in the nuclear fraction but not in the cytoplasmic fraction.

The rabbit polyclonal anti-PITSLRE antibody used for immunoblotting was raised against a COOH-terminal peptide, PITSLRE, which is conserved in all the PITSLRE isoforms (19). Therefore, it can recognize all the PITSLRE isoforms in the 7721 cells, including p58PITSLRE and p110PITSLRE (Fig. 3A), with the expression of p110PITSLRE much more than that of p58PITSLRE. Cyclin D3 coimmunoprecipitated only with p58PITSLRE in the G₂/M phase but not with p110PITSLRE in all the cell cycle (shown in Fig. 3E). Thereby it demonstrated that only p58PITSLRE isoform could interact with cyclin D3.

Immunofluorescence Analysis of the p58PITSLRE and Cyclin D3—To determine whether cyclin D3 colocalized with p58PITSLRE in mammalian cells, we examined the subcellular localization of p58PITSLRE and cyclin D3. The 7721 cells syn-
Fig. 3. Association of p58<sup>PITSLRE</sup> with cyclin D3 at G<sub>s</sub>/M phase in vivo. A, expression of cyclin D3 (upper panel) and HA-p58<sup>PITSLRE</sup> (lower panel) in transiently transfected 7721 cells. B, interaction between p58<sup>PITSLRE</sup> and cyclin D3 in the p58<sup>PITSLRE</sup> transiently transfected 7721 cells.

In Vivo Association of p58<sup>PITSLRE</sup> with Cyclin D3
Fig. 4. **Immunofluorescence analysis of p58\(^{\text{PITSLRE}}\) and cyclin D3 in 7721 cells.** The 7721 cells were fixed after synchronization and reacted with a mouse monoclonal anti-cyclin D3 antibody and a rabbit polyclonal anti-PITSLRE antibody. The secondary antibodies were anti-mouse IgG-conjugated to fluorescein isothiocyanate and anti-rabbit IgG-conjugated to rhodamine red. The images were captured with a Leica confocal microscope and software provided by Leica. A, the 7721 cells synchronized in G\(_1\)/S phase were observed. I, the cyclin D3 image captured. II, the PITSLRE image of the same frame as in I. III, the merge of I and II. B, the 7721 cells synchronized in G\(_1\)/M phase were observed. I, the cyclin D3 image captured. II, the PITSLRE image of the same frame as in I. III, the merge of I and II.

In Vivo Association of p58\(^{\text{PITSLRE}}\) with Cyclin D3

A 7721 cells in G\(_1\)/S phase

| | | |
|---|---|---|
| I | Anti-cyclinD3 | II | Anti-PITSLRE | III | Merge |

B 7721 cells in G\(_2\)/M phase

| | | |
|---|---|---|
| I | Anti-cyclinD3 | II | Anti-PITSLRE | III | Merge |

Lysates of the transiently transfected 7721 cells were immunoprecipitated (IP) using an anti-HA monoclonal antibody or a control mouse IgG. The immunoprecipitates were immunoblotted (WB) with an anti-cyclin D3 antibody (upper panel) or an anti-HA antibody (lower panel). C, flow cytometry analysis of the 7721 cells synchronized at different cell cycle. The 7721 cells were synchronized as described under "Experimental Procedures." The cells at each time point were harvested, fixed, stained with propidium iodide, and analyzed by quantitative flow cytometry with standard optics of FACScan flow cytometer (BD PharMingen FACStar) and the Cell Quest program. I, normal 7721 cells. II, 7721 cells synchronized in G\(_1\)/S phase. III, 7721 cells synchronized in G\(_2\)/M phase. D, expression of the PITSLRE isoforms in the 7721 cells (right lane) G\(_2\)/M phase-arrested 7721 cells (middle lane), G\(_1\)/S phase-arrested 7721 cells (left lane). E, cell cycle-specific interaction between p58\(^{\text{PITSLRE}}\) and cyclin D3. Lysates of the 7721 cells arrested in G\(_2\)/M phase, normal 7721 cells, and G\(_1\)/S phase-arrested 7721 cells were immunoprecipitated using an anti-cyclin D3 antibody and immunoblotted with an anti-PITSLRE antibody from left to right. F, interaction of the cyclin D3 with p58\(^{\text{PITSLRE}}\) in the nucleus and cytoplasm of 7721 cells in G\(_2\)/M phase. Coimmunoprecipitations of the cyclin D3 and p58\(^{\text{PITSLRE}}\) were done in the cytoplasmic fraction and the nuclear fraction of the G\(_2\)/M phase-arrested 7721 cells, respectively.
**DISCUSSION**

For a long time, β-1,4-galactosyltransferase 1 was considered the only protein that could interact with p58\textsuperscript{PITSLRE} (16). Through this binding, p58\textsuperscript{PITSLRE} phosphorylates β-1,4-galactosyltransferase 1 and enhances its activity (16, 31, 32). Actually, β-1,4-galactosyltransferase 1 serves as a substrate for p58\textsuperscript{PITSLRE}. As a p54\textsuperscript{RbE2}–related protein kinase, p58\textsuperscript{PITSLRE} plays an important role in cell cycle control by leading to a late mitotic delay in response to minimal overexpression of this protein kinase (16, 20). In addition, expression of p58\textsuperscript{PITSLRE} is G2/M phase-specific, resulting from translation controlled by an internal ribosome entry site (29). Based on its sequence homology and function, p58\textsuperscript{PITSLRE} might be considered a CDK in G2/M phase, but its partner cyclin and substrates other than β-1,4-galactosyltransferase 1 remain unknown. In this study, we demonstrate that cyclin D3 interacts with p58\textsuperscript{PITSLRE} in vitro and in vivo, and this interaction is found only in G2/M phase but not in the G1/S phase of the cell cycle. The elevated expression of cyclin D3 leads to an exclusively accumulation of p58\textsuperscript{PITSLRE} in the nuclear region. Moreover, kinase activity of p58\textsuperscript{PITSLRE} is greatly decreased without cyclin D3 binding. All of these suggest that cyclin D3 may function as a regulatory partner of p58\textsuperscript{PITSLRE}.

The human cyclin D3 gene was cloned from a placental cDNA library by cross-hybridization with cyclin D1 probe (35). Compared with cyclin D1 and cyclin D2, little is known about the function of cyclin D3 (36). Cyclin D1 knockout mice are slightly smaller and exhibit a lack of normal mammary gland development in adult female mice as well as retinopathy (37, 38), whereas mice lacking cyclin D2 are infertile due to lack of development of ovarian granulosa cells (39). Successful disruption of the cyclin D3 gene in mice has not been reported. The overexpression of cyclin D3 in fibroblast cells leads to accelerated passage through G1 phase with no effect on the overall doubling time (36). Moreover, cyclin D3 is found to not only play a crucial role in progression through the G1 phase but also to regulate apoptosis induced by T cell receptor activation in leukemic T cell lines (40). As cells enter cell cycle from quiescence, one or more D-type cyclins (cyclins D1, D2, D3) are induced and subsequently expressed throughout the cell cycle in response to mitogen stimulation, whereas cyclin A, B, and E (mitotic cyclins) are expressed periodically (3, 6, 7). Considerable attention has been paid to the role of D-type cyclins in controlling the G1 phase progression by regulating CDKs 4 and 6 activation and Rb function (3, 7, 41). There is currently little evidence of a role for them in the later cell cycle. Here, we show that cyclin D3 may function in G2/M phase, serving as an interaction partner of p58\textsuperscript{PITSLRE} and regulating some parts of its function. This interaction linked a G1 cyclin (cyclin D3) with a G2/M CDK (p58\textsuperscript{PITSLRE}). No interaction between the p58\textsuperscript{PITSLRE} protein kinase and the other two D-type cyclins was observed in direct two-hybrid assay and GST pull down experiments. This indi-
cates that the binding between p58PITSLRE and cyclin D3 might be specific. The high homology between the three D-type cyclins has suggested redundancy in their functions. However, there is more and more evidence that the three D-type cyclins are not equivalent in many ways, such as the tissue-specific expression patterns (7), different affinities to CDKs (42), different inductions by various signals in a cell lineage-specific manner (3, 7), and different phenotypes of the knock-out mice (37–39) (homozygous disruption of cyclin D3 is not obtained by now). Given our results, it is likely that the interaction with p58PITSLRE plays a distinct role of cyclin D3 in cell cycle control.

The p58PITSLRE belongs to a large family that contains many isoforms. Among them the p58PITSLRE and p110PITSLRE are mostly studied and described. The p110PITSLRE protein kinase was shown to participate in a signaling pathway that potentially regulates transcription and RNA-processing events, whereas the p58PITSLRE plays an important role in the cell cycle progression control. Although the p110PITSLRE isoform contains the entire p58PITSLRE sequence, it did not associate with cyclin D3 by immunoprecipitation (Fig. 3) and immunofluorescence cell staining (Fig. 4). This suggests that the NH2 terminus of p110PITSLRE may interfere or block the conformation of the COOH terminus so that the p58PITSLRE sequence in the p110PITSLRE could not reach and interact with cyclin D3. These data are in agreement with the different functions of the two PITSLRE isoforms.

Our studies have demonstrated that cyclin D3 interacted and colocalized with p58PITSLRE at G2/M phase, and the elevated expression of cyclin D3 affected p58PITSLRE cellular distribution. Addition, we speculate that this interaction and colocalization mainly existed in the nucleus for the biochemical fractionation study, which showed that p58PITSLRE and cyclin D3 interacted mostly in the nuclear fraction but not in cytoplasmic fraction (Figs. 4 and 5). When co-transfected with a control plasmid, p58PITSLRE was shown to localize predominantly in the nucleus, with a little cytoplasmic distribution (Fig. 5). This is consistent with the protein structure and function of p58PITSLRE, which contains a nuclear localization sequence in its NH2-terminal region (16). For the p110PITSLRE, it primarily localized in the nucleus (19, 29, 30). Upon co-transfection with cyclin D3, p58PITSLRE appeared completely nucleus-localized without any signal de-

![Fig. 6. Activation of the p58PITSLRE kinase activity on histone H1 by cyclin D3 association.](image)

**A.** Immunoblot analysis of cyclin D3 immunodepletion efficiency. After immunodepletion, the precipitates were immunoblotted with anti-cyclin D3 antibody. More than 90% depletion was achieved by cyclin D3 immunodepletion. **B.** Anti-HA monoclonal antibody (lanes 3 and 4) and anti-PITSLRE polyclonal antibody (lanes 1 and 2) were used to precipitate p58PITSLRE from 200 µg cell lysates of 7721p58. After immunodepletion of cyclin D3 (−cyclin D3) or directly (+cyclin D3), kinase activity of the precipitates was measured with histone H1 as the substrate. The figure is representative of three independent experiments performed. **C.** Anti-HA precipitates (B) or anti-PITSLRE precipitates (C) in the presence of cyclin D3 (−cyclin D3) or absence of cyclin D3 (−cyclin D3). The measurements are representative of three independent experiments performed. The relative phosphorylation activity is presented as percent where kinase activity of HA precipitates (A) or anti-PITSLRE precipitates (B) in the presence of cyclin D3 is arbitrarily set at 100%.

![Fig. 7. Phosphorylation of β-1,4-galactosyltransferase 1 by p58PITSLRE.](image)

**A.** Anti-HA monoclonal antibody (A) or anti-PITSLRE polyclonal antibody (B) was used to precipitate p58PITSLRE in the presence of (+cyclin D3) or absence of cyclin D3 (−cyclin D3). Kinase activity of the precipitates was measured with β-1,4-galactosyltransferase 1 as the substrate as described under “Experimental Procedures.” The measurements are representative of three independent experiments performed. The relative phosphorylation activity is presented as percent where kinase activity of HA precipitates (A) or anti-PITSLRE precipitates (B) in the presence of cyclin D3 is arbitrarily set at 100%.
ected in the cytoplasm (Fig. 5). However, it is preliminary to say that cyclin D3 can enhance p58<sup>PITSLRE</sup> nuclear translocation, because many factors can make increased nuclear accumulation. This issue is currently under investigation in our lab. From Fig. 4 and Fig. 5, we found that there were still plenty of cyclin D3 that did not interact with p58<sup>PITSLRE</sup>, because cyclin D3 acts as a regulatory subunit of CDKs 4 and 6 as well as an interaction partner of two distinct types of transcription factors, estrogen receptor and DMP1 (43, 44). Through direct binding, cyclin D3 can enhance the growth-promoting activity of the estrogen receptor and inhibit the growth-restraining activity of the DMP1 (43, 44). The other issue raised from Fig. 4A is that cyclin D3 does not show any tendency toward nuclear localization in G<sub>S</sub>/S phase-arrested cells, which might be due to the different abundance or affinities of the D-type cyclins to the CDKs in 7721 cells (3, 7, 42). The other two D-type cyclins may occupy most of the CDKs so that cyclin D3 distributes all over the cells instead of a tendency toward the nucleus. From Fig. 5B, we can also observe the cytoplasmic distribution of the cyclin D3 in control cells, but upon co-expression with p58<sup>PITSLRE</sup>, cyclin D3 localizes exclusively in the nucleus.

The in vitro immune complex kinase assay showed that kinase activity of p58<sup>PITSLRE</sup> was significantly decreased when the binding between p58<sup>PITSLRE</sup> and cyclin D3 was abrogated by immunodepletion with a monoclonal anti-cyclin D3 antibody. We used two different antibodies for immunoprecipitation in this assay; one is the mouse anti-HA monoclonal antibody, and the other is the rabbit polyclonal anti-PITSLRE antibody. The observed slight decrease in the kinase activity of the anti-PITSLRE immunoprecipitates in the absence of cyclin D3 could be due to its low specificity for p58<sup>PITSLRE</sup>. All together, it is speculated that cyclin D3 may function as a regulatory partner of p58<sup>PITSLRE</sup> in G<sub>M</sub> phase, which is a good explanation for the results of Herzinger and Reed (36). In their study, they found that the overexpression of cyclin D3 in fibroblast cells led to accelerated passage through G<sub>1</sub> phase with no effect on the overall growth rate, which suggested that the accelerated passage through G<sub>1</sub> phase might be compensated for by expanding subsequent cell cycle phases. Here we partly confirmed their postulation and demonstrated that p58<sup>PITSLRE</sup> might be the target molecule for the subsequent expanding G<sub>M</sub> phase cell cycle.

In summary, this study demonstrates that cyclin D3, a G<sub>1</sub> cyclin, specifically interacted with p58<sup>PITSLRE</sup>, a G<sub>2</sub>/M CDK. This binding happened in G<sub>M</sub>/ phase instead of G<sub>S</sub>/S phase and resulted in enhanced kinase activity of p58<sup>PITSLRE</sup>. Therefore, cyclin D3 functioned not only in G<sub>1</sub> phase as a regulatory subunit of CDKs 4 and 6 but also in G<sub>M</sub>/ phase as a partner of p58<sup>PITSLRE</sup> during cell cycle progression. Further analysis of this interaction along with past studies might result in a much more generalized understanding of the regulation and function of cyclin D3 and p58<sup>PITSLRE</sup>, thereby providing new insights into the control of G<sub>M</sub>/M phase cell cycle progression.

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