Cyclin D3-Cdk6 complex is immune to CDK inhibitors and uniquely controls cell's growth competence

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Mammalian cells require a D cyclin-dependent kinase for the cell cycle start[1-3], yet many mesenchymal cells express three seemingly redundant D cyclins[3-4] and similarly, seemingly redundant Cdk4 and Cdk6 (ref 5,6) as their kinase partner. Here we show that cyclin D3-Cdk6 complex is unique among the D cyclin and kinase combinations in the ability to promote the cell cycle start. In an anchorage-minus G_1-arrested rat fibroblast, only D3-Cdk6 complex retains kinase activity due mainly to its ability to evade inhibition by $p27^{KIP1}$(ref 7,8) and $p21^{CIP1}$(ref 9-11) CKIs, strikingly resembling viral cyclin-bound Cdk6 (ref 12). Rodent fibroblasts engineered to overexpress both Cdk6 and cyclin D3 highly resist serum starvation- or cell-cell contact-imposed G_1-arrest. In BALB/c 3T3 cells, D3 is constitutively expressed, but Cdk6 is markedly induced with concomitant activation by a growth competence factor. We propose a role for D3-Cdk6 complex in regulating cell's growth competence in response to external factors.

During studies on the mechanisms starting the cell cycle in response to oncogenic stimulation, we found that unlike Cdk4, Cdk6 kinase retained activity at G_1-

arrest imposed by anchorage deprivation, at which $p21^{CIP1}$ and $p27^{KIP1}$ CKIs were abundantly expressed. The cells used were clones of the NRK-F49 cell line 3-5 fold overexpressing Cdk4 or Cdk6, obtained by transfection with an expression vector harboring the corresponding cDNA. Several independent overexpressors for each construct were initially examined with similar results and, therefore, one representing clone for each construct [Cdk6 overexpressor K6-2 and Cdk4 overexpressor K4-m5 (ref 13)] was chosen for in-depth analysis. Just like the original NRK cells, these overexpressors could not start the cell cycle without anchorage unless stimulated with oncogenic growth factors, typically with EGF plus TGF-β. When embedded in anchorage-free methylcellulose semisolid medium, they came to arrest in G_1. But when stimulated with EGF plus TGF-β, the arrested cells could start the cell cycle and form colonies in methylcellulose medium (data not shown, described later). The anchorage-minus G_1-arrested K6-2 and K4-m5 cells were stimulated with EGF plus TGF-β for various times and lysed. From the corresponding overproducer cell lysates, Cdk4 and Cdk6 were immunoprecipitated with specific antibodies and their kinase activities were assayed. In parallel, the amounts of the kinases and D cyclins in the lysates and the immunoprecipitates were determined by western blot. As shown in Fig. 1***a***, Cdk4 was almost inactive at 0 and 3 h post-stimulation despite being associated with D cyclins and free from inhibitory tyrosinephosphorylation[2], and became activated at 6 and 9 h. By contrast, Cdk6 was already active at 0 h and there was only a mild increase in activity thereafter. All the three D cyclins D1, D2, and D3 are expressed in NRK cells and these overproducers though they differ from one another in amount and expression

pattern (Fig. 1*a*). The cyclin partner in the active Cdk6 was D3. When cyclins D1, D2 and D3 were immunoprecipitated from the K6-2 lysates and assayed for bound Rb kinase activity, the D3, but not D1 or D2, immunoprecipitate at 0 and 3 h showed activity. When similar assays were performed for the lysate of K4-m5 cells, again only D3 immunoprecipitate had activity albeit it was much reduced. Because Cdk4 kinase activity was virtually none in this lysate (Fig. 1*a*), this residual D3-associated kinase activity was attributable to the Cdk6 that was present in this immunoprecipitate.

These results were fully confirmed by the analysis of anchorage-minus G_1-arrested K6-2 cells that were additionally overexpressing cyclin D3 or D1. K6-2/D1-3 and K6-2/D3-5 are representative K6-2 clones overexpressing cyclin D1 two-fold and D3 five-fold, respectively (Fig. 1*b*). Perhaps due to a higher susceptibility of D1 to proteolytic degradation, we could not obtain any clones overexpressing D1 more than 2-fold. The cells were arrested in G_1 by incubation in methylcellulose medium and similarly stimulated to start the cell cycle with every 3 h harvests. Cyclin D3 or D1 was immunoprecipitated from the lysates, and their associated Rb kinase activities and the amount of associated Cdk6 were assayed. The D3-associated kinases from K6-2/D3-5 cells were already fully activated at 0 and 3 h, and had virtually no increase during subsequent stimulation (Fig. 1*b*). By contrast, the D3-associated kinase activity from K6-2/D1-3 at these time points was nearly undetectable due to a marked reduction in D3-Cdk6 complex by competition with overexpressed D1, as shown by western blot of the immunoprecipitates. Furthermore, confirming the Fig. 1*a* data, the D1-associated kinases from K6-2/D1-3 were inactive at these time points and significantly activated

during stimulation. We thus concluded that only D3-Cdk6 complex among other combinations retained Rb kinase activity at anchorage-minus G_1-arrest point.

During anchorage-minus G_1-arrest, $p27^{KIP1}$ is highly induced[14]. Similarly, in K4-m5 and K6-2 cells, it was highly expressed at the G_1-arrest point and gradually decreased during stimulation (Fig. 2). $p21^{CIP1}$ and $p18^{INK4}$ (ref 11) were expressed constantly. Interestingly, the level of $p21^{CIP1}$ was significantly higher in K4-m5 than in K6-2 cells for unknown reasons. We therefore investigated a possible role for $p27^{KIP1}$ and $p21^{CIP1}$ in the selective inhibition of D cyclin-dependent kinases. To this end, D1 and D3 were immunoprecipitated from the same amounts of the K6-2 and K4-m5 cell lysates obtained after stimulation for various times, and the amounts of the associated kinases and the inhibitors were quantified by western blot. As shown in Fig. 2, D1 co-precipitated with both Cdk4 and Cdk6 from K6-2 cell lysates whereas D3 coprecipitated only with Cdk6 from the same cell lysates, indicating that D3 has a higher binding affinity to Cdk6 than D1 does. The D3 immunoprecipitates from both overexpressors contained much lower amounts of $p27^{KIP1}$ relative to Cdk4 or/and Cdk6 irrespective of the kind of the bound kinases. On the other hand, $p21^{CIP1}$ was nearly undetectable in the D3 immunoprecipitates from K6-2 cells, in which Cdk6 was the major associated kinase. Thus, D3-Cdk6 complex poorly bound these CKIs. To confirm this finding, we performed an indirect kinase inhibition assay. Lysates of rapidly growing K6-2/D3-5 and K4-m5 cells, which contained Cdk6-D3 and Cdk4-D1 as the major complex, respectively, were mixed with various amounts of $p27^{KIP1}$, and Cdk6 and Cdk4 were then immunoprecipitated and assayed for activity. By addition of $p27^{KIP1}$, Cdk4 was

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strongly inhibited, but Cdk6 was not (Fig. 3b). We concluded that D3-Cdk6 is immune to the CKIs, strikingly resembling viral cyclin-bound Cdk6 (ref 12), and that this perhaps largely if not exclusively accounts for the complex being active at the anchorage-minus G_1-arrest point despite the presence of large amounts of CKIs.

The ability of D3-Cdk6 complex to evade inhibition by CKIs led us to examine the growth properties of the Cdk6 single, Cdk6/D3 double, and Cdk6/D1 double overexpressors in comparison to parental NRK cells. Their doubling times during exponential growth were similar, but their cell cycle arrest ability varied greatly. In growth medium, parental NRK ceased growth at a density of $5x10^4$ cells per 1.5 cm dish due to cell contact. K6-2/D3-5 was highly insensitive to contact inhibition and overgrew to a 3-4 fold higher density with still a large population continuing cell cycling but coming to death due to detachment from the dish. K6-2 overgrew to a 50% higher density (Fig. 3***a***). By contrast, additional overexpression of D1 strongly counteracted the effect of Cdk6 overexpression, and K6-2/D1-32 cells grew to a slightly less density than NRK cells. Overexpression of Cdk4 significantly facilitated density-imposed G_1-arrest and K4-m5 cells ceased growth at half the density of NRK cells, perhaps due to a reduction in D3-Cdk6 complex by competition for D3 with overproduced Cdk4 (Fig. 1*a*). These overexpressors behaved similarly to serum starvation. K6-2/D3-5 almost completely evaded serum starvation-imposed G_1-arrest. During a 6 h labeling in 0.05% FCS, 40% of K6-2/D3-5 entered S phase, a value 5 times higher than of NRK and nearly comparable to 55% of log phase NRK (Fig. 3***b***). In higher FCS concentrations, such phenotype of K6-2/D3-5 became less clear, but % S phase cells of the

overexpressors proportionally correlated with their attainable maximum cell densities on a culture dish and their D-dependent kinase activities at the anchorage-minus G_1-arrest point, as shown already. Interestingly, despite the striking growth ability, K6-2/D3-5 cells were unable to grow without anchorage unless given oncogenic stimulation (Fig. 3*c*). When stimulated with EGF plus TGF-β, they proliferated very rapidly and formed gigantic colonies in methylcellulose medium. BALB/c 3T3 and NIH3T3 fibroblasts engineered to overexpress both Cdk6 and D3 showed similar properties (data not shown). Thus, an elevated amount of D3-Cdk6 complex made cells highly competent to growth.

The ability of D3-Cdk6 to evade inhibition by CDK inhibitors and make cells growth-competent may put this kinase complex in a unique position in growth control, and raises the possibility that Cdk6 or/and D3 might mediate an external signal to regulate cell's growth competence. Quiescent BALB/c 3T3 and C3H10T1/2 mouse fibroblasts require two types of growth factor to resume DNA synthesis[15,16]. One is called a "competence factor" and may be platelet-derived growth factor (PDGF) and 12-O-tetradecanoyl phorbol-13 acetate (TPA) a tumor promoter. The other is called a " progression factor" and may be epidermal growth factor. Accordingly, we examined a possible role for Cdk6/D3 in factor-induced growth competence. BALB/c 3T3 cells were grown to confluence and stimulated with PDGF for varying times up to 24 h. The amounts of Cdk6, Cdk4, D1, D3, $p27^{Kip1}$ and $p21^{CIP1}$ expressed at each time point were quantified by western blot. In parallel, Cdk4 and D3-associated kinase activities were assayed. Cdk4 and D3 were constitutively expressed. By contrast, Cdk6 was highly

induced with concomitant activation, as indicated by the appearance of D3-associated Rb kinase activity (Fig. 4*a*). D1 was also induced with concomitant Cdk4 activation, but to a much lesser extent perhaps due to inhibition by $p27^{KIP1}$ and $p21^{CIP1}$, which were repressed and reciprocally induced, respectively, during the treatment. PDGF induced Cdk6 also in the C3H10T1/2 mouse fibroblast (Fig. 4*b*). Moreover, TPA also induced Cdk6 albeit weakly in both C3H10T1/2 and BALB/c 3T3 cells (Fig. 4*b*, data not shown). We thus conclude that Cdk6-D3 can regulate growth competence in the presence of CKIs and that Cdk6 itself mediates external stimuli that influence growth competence in some fibroblasts.

Mammals contain many redundant factors. But the most of them are functionally similar and differ only in cell type or developmental stage for expression. By contrast, D cyclins and their kinase partners are unlike such situations. D3-Cdk6 complex is unique in function and controls growth competence in fibroblasts despite that unlike Cdk4, Cdk6 is non-essential for the onset of S phase at least in NRK[2]. This unique function of D3-Cdk6 complex largely owes to its ability to evade inhibition by CKIs in a striking resemblance to viral cyclin-associated Cdk6 (ref 12). In fibroblasts, Cdk6 itself is an end effector of external stimuli that influence growth competence. Such role of Cdk6 (and D3) would be particularly important under the circumstances where CKIs are present sufficiently enough to inhibit induced D1-Cdk4 complex. The act of Cdk6 as an effector of external stimuli does not seem to be specific to fibroblasts. In lymphocytes Cdk6 is induced upon stimulation with antigen or interleukin[17, 18].

Methods

Cell culture and transfection

The normal rat kidney cell line NRk-49F cells (American type Culture Collection) were maintained in DMEM with 5% FCS. Synchronization to G_1 by anchorage deprivation was carried out as described[19]. NRK cell clones overexpressing Cdk6 alone, both Cdk6 and cyclin D3 or both Cdk6 and D1 were constructed by transfection with the pEF1-neo expression vector[20] harboring a human Cdk6 cDNA followed by G418 (400 $\mu g\ ml^{-1}$) selection and identification of overexpressors, and by subsequent transfection of one Cdk6 overexpressor (K6-2) with pEF1-hygro harboring a rat D1 or mouse D3 (gift of J. Kato) cDNA followed by hygromycin (100 $\mu g\ ml^{-1}$) selection.

Immunoprecipitation, kinase assay and western blot

For each time point, $4x10^6$ cells were embedded in 50 ml of methylcellulose-DMEM medium with 5% FCS and incubated for 36 h to arrest in G_1 followed by stimulation with EGF + TGF-β[19]. The cells were harvested every 3 h and lysed with 0.5 ml of ice-cold IP buffer composed of 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 1mM PMSF, 4 $\mu g\ ml^{-1}$ each of leupeptin, pepstatin and aprotinin, 1 mM NaF, 0.1 mM $NaVO_4$ and 10 mM β-glycerophosphate as described[2]. Cdk6, Cdk4, and Cyclins D1, D2 and D3 were immunoprecipitated from the lysates with specific antibodies as described[2, 21.] A 15 µl suspension of anti-rabbit or anti-mouse IgG-bound agarose beads (Sigma) was then added, and incubation continued for another 1 h. The beads were then collected by a

brief centrifugation and washed twice with ice-cold glycerol-free IP buffer. Kinase assay was carried out with a truncated Rb protein (QED) as a substrate followed by detection of Ser780-phosphorylated Rb with the antibody (MBL) [21]. Western blot analysis was performed as described (Jinno 1999).

BrdU incorporation assay

Cells were incubated in DMEM containing 0.5%, 0.25% or 0.05% FCS for 42 h . BrdU was then added to the culture at a concentration of 1 ng ml^{-1} and incubated for another 6 h. Cells were then fixed with 70% ethanol for 30 min, washed with phosphate-buffered saline (PBS) twice, treated with 6N HCl for 20 min and neutralized with 0.1M $Na_2B_4O_7$. The fixed cells were washed with 0.25% Triton X-100, then with PBS containing 5% FCS for 30 min and incubated with anti-BrdU (1:1000 diluted) in a room temperature for 1 h followed by incubation with Texas red linked 2nd anti-mouse (1:100 diluted) for another 1 h to visualize cells in S phase.

Indirect kinase inhibition assay

NRK cells were arrested in G_1 by incubating in methylcellulose DMEM for 36 h and lysed with IP buffer. The lysate was heat-inactivated and briefly centrifuged as described[22]. The supernatant was used as the $p27^{KIP1}$ preparation. The susceptibility of Cdk4-D1 and Cdk6-D3 complexes to $p27^{KIP1}$ was assayed by incubation of the lysates from rapidly growing K4-m5 and K6-2/D3-5 cells with various amounts of the$p27^{KIP1}$-enriched solution followed by immunoprecipitation of Cdk4 or Cdk6 and assay of Rb

kinase[22].

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Figure Legends

Figure 1 Cdk6-cyclin D3 complex retains activity at G_1-arrest induced by anchorage deprivation. **a**. Rb kinase activities in Cdk4, Cdk6 or D cyclin immunoprecipitates from the Cdk4 or Cdk6 overproducers. K6-2 and K4-m5 (ref 13) cells were arrested in G_1 in methylcellulose DMEM and stimulated with EGF + TGF-β. The cells were harvested every 3 h and lysed. Cdk4, Cdk6 and/or D cyclins were immunoprecipitated with αCdk4 (c-22), αCdk6 (c-21), αcyclin D1 (H295, 92-13G), αcyclin D2 (M-20) and αcyclin D3 (C-16) antibodies (Santa Cruz), and the associated Rb kinase activities and cyclin or kinase partners were assayed. **b**. Comparison of Rb kinase activities in Cdk6 or cyclin D3 or D1 immunoprecipitates from Cdk6 single, Cdk6/D3 or Cdk6/D1 double overproducers. K6-2/D1-3, K6-2 and K6-2/D3-5 cells were arrested in G_1, stimulated, harvested and lysed as in **a**. Similarly, associated Rb kinase activities and cyclin and kinase partners were assayed.

Figure 2 Cyclin D3-Cdk6 complex is immune to $p27^{Kip1}$ and $p21^{CIP1}$. ***a***. D3-Cdk6 complex poorly binds $p27^{Kip1}$ and $p21^{CIP1}$. K4-m5 and K6-2 cells were arrested in G_1 by anchorage deprivation, stimulated, harvested and lysed as in Fig. 1. $P27^{KIP1}$, $p21^{CIP1}$, and $p18^{INK4}$ in the lysates were quantified by western blot. Cyclins D1 and D3 were immunoprecipitated from the lysates, and associated Cdk6, Cdk4, $p21^{CIP1}$ and $p27^{KIP1}$ were quantified by western blot. ***b***. $p27^{KIP1}$ inhibits Cdk4, but not Cdk6-D3. The susceptibility of Cdk4 and Cdk6-D3 to inhibition by $p27^{KIP1}$ was assayed as in Methods.

1x of $p27^{KIP1}$ defines the amount of $p27^{KIP1}$ contained in 800 μg protein of the G_1-arrested NRK cell lysate. Lysates (800 μg protein) prepared from rapidly growing K4-m5 and K6-2/D3-5 cells were incubated with 0.2x, 1x or 2x of the $p27^{KIP1}$ preparation. Cdk4 and Cdk6 were then immunoprecipitated and assayed for Rb kinase activity.

Figure 3 Overexpression of Cdk6 and cyclinD3 blocks contact inhibition- or serum starvation-induced G_1-arrest of NRK cells. ***a.*** Cells overexpressing both Cdk6 and D3 resists density-imposed growth arrest. NRK, K4-m5, K6-2, K6-2/D3-5 and K6-2/D1-3 cells were plated into 12 well culture dishes at a density of $5x10^4$ cells per well, cultured in DMEM with 5% FCS and counted everyday. The data shown are values averaged from 3 independent experiments. Thin lines are standard errors. ***b.*** Cells overexpressing both Cdk6 and D3 resist serum starvation-imposed G_1-arrest. Semi-confluent NRK, K6-2, K6-2/D3-5, and K6-2/D1-3 cells were cultured in DMEM with 0.05%, 0.25% or 0.5% FCS for 42 h, then labeled with BrdU for 6 h. The cells in S phase were determined by nuclear staining with anti-BrdU antibody. As a reference, rapidly growing NRK cells were similarly determined for % S phase cells. The data shown are values averaged from 3 experiments. Thin lines are standard errors. ***c.*** Overexpression of Cdk6 and D3 does not induce anchorage-independent growth, but markedly promotes the anchorage-independent growth induced by EGF + TGF-β. NRK and K6-2/D3-5 cells were incubated in 5% FCS methylcellulose DMEM in the presence or absence of EGF + TGF-β for 2 weeks.

Figure 4 A growth competence factor induces Cdk6 in mouse fibroblasts. ***a***. PDGF induces Cdk6 with concomitant activation in BALB/c 3T3 cells. BALB/c 3T3 cells were grown to confluence and then stimulated with PDGF (40 ng ml^{-1}) for various times. Cells were lysed, and Cdk6, Cdk4, D1, D3, p27 and p21 were quantified by western blot. In parallel, D3 and Cdk4 were immunoprecipitated and the associated Rb kinase activity was assayed. ***b***. PDGF and TPA induce Cdk6 in C3H10T1/2 cells. C3H10T1/2 cells were arrested in G_1 by contact inhibition and serum starvation[15], and then stimulated with PDGF (40 ng ml^{-1}) or TPA (100 ng ml^{-1}) for 24 h. Cell lysates were prepared, and Cdk6, Cdk4, D1, D3, p27 and p21 were quantified by western blot.