Oncogenic stimulation recruits cyclin-dependent kinase in the cell cycle start in rat fibroblast

Shigeki Jinno, Shih-Chieh Hung, Hanako Yamamoto, Jie Lin, Akihisa Nagata, and Hiroto Okayama*

Department of Biochemistry and Molecular Biology, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

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The rat fibroblast NRK cells are transformed reversibly by a combination of growth factors. When stimulated with serum, NRK cells rely on cyclin-dependent kinase 4 (Cdk4) for their S phase entry. However, when stimulated with serum containing oncogenic growth factors, they come to rely on either Cdk4 or Cdk6, and their S phase entry cannot be blocked unless both Cdk4 and Cdk6 are immunodepleted. Such change of dependence does not occur in the NRK cell mutants defective in an oncogenic signal pathway and, therefore, deficient in anchorage-independent cell cycle start ability, correlating Cdk6 dependence with this remarkable, cancer-associated phenotype. However, both Cdk4 and Cdk6 are activated upon serum stimulation, and neither the amounts of Cdk6, Cdk4, cyclin D1, and cyclin-dependent kinase inhibitors nor the activities or subcellular localization of Cdk6 and Cdk4 are significantly influenced by oncogenic stimulation. Thus, oncogenic stimulation invokes Cdk6 to participate in a critical step of the cell cycle start in a rat fibroblast, but by a mechanism seemingly unrelated to the regulation of the kinase. Given that many hematopoietic cells employ predominantly Cdk6 for the cell cycle start and perform anchorage-independent growth by nature, our results raise the possibility that the oncogenic stimulation-induced anchorage-independent cell cycle start of NRK is elicited by a mechanism similar to the one used for hematopoietic cell proliferation.

NRK | G1 | Cdk4 | transformation

In mammals, D-type cyclin-dependent kinases (Cdks) are essential for the cell cycle start. At early G1, Cdk4 and Cdk6 associate with induced D-type cyclins and phosphorylate retinoblastoma (Rb) protein (1–4). The phosphorylated Rb is then inactivated by further phosphorylation performed by the second G1 cyclin-dependent kinase Cdk2-cyclin E, leading to the activation of the E2F-DP1 transcriptional factor complex, which, in turn, induces genes essential for S phase onset and progression (5, 6). Cdk4 and Cdk6 are highly related to each other and thought to perform a similar function though they differ in expression cell type. In general, Cdk4 is expressed preferentially in mesenchymal cells whereas Cdk6 is expressed preferentially in all hematopoietic cells and exclusively in some (2, 7). The activities of these kinases are negatively regulated by various protein inhibitors generally called CKIs (cyclin-dependent kinase inhibitor; Cdk, cyclin-dependent kinase). For example, p15, p16, p18, p21, and p27 are responsible for the activity in fibroblasts and epithelial cells (13–15). Depending on their amount, p21 and p27 also function as a positive regulator by promoting the assembly of these kinases with D-type cyclins (16, 17). Consequently, the D-type cyclin-dependent kinases serve as a key target for cell growth regulation. In fact, constitutive activation of the kinases by overexpression of a D-type cyclin or deletion of a CKI gene, or expression of a CKI-evading viral cyclin renders mesenchymal cells to highly resist growth arrest (18–20). However, such cells are not malignantly transformed.

One important characteristic that distinguishes transformed from untransformed cells is the ability to proliferate without anchorage (21, 22). Mesenchymal cells in adult mammals require attachment to intercellular matrix or a mimicking artificial solid surface for their G1/S transition. When deprived of anchorage by being embedded in soft agar or methylcellulose, they cannot start the cell cycle and arrest in G1 (23, 24). Upon neoplastic transformation, they acquire the ability to grow without anchorage. The anchorage-independent growth ability in soft agar appears to be a fundamental attribute of cancer cells because it correlates well with their in vivo tumorigenicity (25, 26).

The rat normal kidney fibroblast NRK-49F is typical of untransformed cells and unable to grow in soft agar. But, these cells undergo reversible transformation when treated with epidermal growth factor (EGF) combined with transforming growth factor β (TGF-β) or with oncogenes (27, 28). Establishment and analysis of several recessive NRK cell mutants refractory to transformation by EGF and TGF-β showed that a cascade shared by EGF and platelet-derived growth factor signals is present in this cell and serves as a major target for oncogene action for transformation. The analysis also implied that this cascade contains Ras small G protein and Raf-1 kinase as key components and branches into mitogenic and oncogenic signals, the latter of which is essential for growth in soft agar (28, 29). Subsequently, the oncogenic stimuli-induced anchorage-independent cell cycle start was found to be the basis for growth in soft agar (30). The properties of the NRK mutant 23, which was later isolated and subsequently identified as CrkII for its mutated gene, supported this conclusion (31).

One critical question regarding malignant transformation, therefore, is how oncogenic signals induce the anchorage-independent cell cycle start. During studies on cell cycle start factors, we recently found that oncogenic stimulation specifically invokes Cdk6 to participate in a critical step of the cell cycle start in NRK cells and that this Cdk6 participation is closely associated with oncogenic stimuli-induced anchorage-independent cell cycle start ability. In this communication, we describe the experimental data that led to this finding and discuss the biological implication of the finding and possible mechanisms for oncogenic stimulation-induced Cdk6 participation.

Materials and Methods

Cells and Chemicals. The normal rat kidney line NRK-49F was maintained as described (32). The antibodies αCdk4 (C-22), αCdk6 (C-21), αCdk2 (M2), αCyclin D1 (72–13G), αp15 (M-20), αp16 (M-156), αp18 (M-20), αp21 (C-19), αp27 (F-8), αDp1 (K-20), and αE2F-1 (C-20) were purchased from Santa Cruz Biotechnology, rRb (G3–245) was purchased from PharMingen, αHistone H1 (clone 10) was purchased from Transduction Laboratories (Lexington, KY), and αHistone H1 was purchased from Leinco Technology (Ballwin, MO).

Microinjection (32). NRK or K6D1 cells were seeded in 3.5-cm dishes at a concentration of 104 cells per dish and were arrested in G1 by incubating in DMEM containing 0.05% FCS for 48 hr.

Abbreviations: EGF, epidermal growth factor; TGF-β, transforming growth factor β; Rb, retinoblastoma; CKI, cyclin-dependent kinase inhibitor; Cdk, cyclin-dependent kinase.

*To whom reprint requests should be addressed.

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Cells then were stimulated to start the cell cycle with DMEM containing 5% FCS or 5% FCS and 5 ng/ml each of EGF and TGF-β. One hour later, the cells were microinjected with an antibody solution by using the A1S microinjector (Zeiss) and incubated for 20 hr in the corresponding growth medium containing 5 μM BrdUrd. Cells were fixed with 70% ethanol, treated with 4 M HCl for 20 min, neutralized with 1 M sodium tetraborate, and rinsed with PBS containing 0.5% Triton X-100 and 5% FCS. They were then incubated with the mouse anti-BrdUrd antibody ZBU30 (diluted 1:1,000) (Seikagaku Kogyo, Tokyo) and stained with FITC-conjugated anti-mouse IgG (diluted 1:100) (Amersham-Pharmacia). Microinjected cells were identified by staining the injected antibodies with Texas Red-conjugated anti-rabbit IgG (diluted 1:100) (Amersham-Pharmacia).

Immunoblot Detection (13, 32). Cells (5 × 10⁵) were lysed with 0.5 ml of ice-cold RIPA buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM PMSF, 4 mg/ml each of leupeptin, pepstatin, and aprotinin, 0.1 M NaF, 2 mM sodium orthovanadate, and 10 mM β-glycerophosphate. Cell lysates (20 μg protein per lane) were electrophoresed on 10% SDS-polyacrylamide gels, transferred to PVDF membrane filters, probed with the antibodies, and detected with the enhanced chemiluminescence system (Amersham-Pharmacia).

In Vitro Kinase Assay (33). NRK (5 × 10⁵) and 5 × 10⁵ K6d1 cells were lysed with 2 ml and 0.5 ml of ice-cold immunoprecipitation buffer consisting of 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 21 mM DTT, 0.1% Tween-20, 10% glycerol, 1 mM PMSF, 4 mg/ml each of leupeptin, pepstatin, and aprotinin, 0.1 M NaF, 2 mM sodium orthovanadate, and 10 mM β-glycerophosphate, respectively. Lysates were incubated at 4°C for 2 hr with 0.3 μg of αCdk4, αCdk6, or αCdk2. Protein G-Sepharose suspension (15 μl) (Amersham-Pharmacia) then was added and incubated at 4°C for an additional 1 hr. Immune complexes bound to Protein G-Sepharose were precipitated by centrifugation and washed with ice-cold, glycerol-free immunoprecipitation buffer. The immunopurified Cdk4, Cdk6, and Cdk2 were incubated at 30°C for 30 min in 10 μl of reaction buffer (50 mM Hepes, pH 7.5/1 mM EGTA/10 mM KCl/10 mM MgCl₂/1 mM DTT) containing 5 μg of truncated Rb (QED Bioscience, San Diego) and 10 mM ATP. The reaction products were electrophoresed on 10% SDS-polyacrylamide gels and transferred to poly(vinylidene difluoride) membrane filters. The Rb molecules phosphorylated by Cdk4 or Cdk6 were immunodetected with the anti-Ser811-phosphorylated Rb antibody (New England Biolabs).

Immunofluorescent Staining. NRK cells were arrested in G₁ by serum depletion and stimulated with DMEM containing 5% FCS alone or 5% FCS and 5 ng/ml EGF and TGF-β. At 9 hr poststimulation, cells were fixed with 70% ethanol and treated with PBS containing 0.5% Triton X-100 and 5% FCS. They then were incubated with αCdk4 or αCdk6 (diluted 1:200) and stained with FITC-conjugated anti-rabbit IgG.

Results

In the rat fibroblast line NRK-49F, Cdk4 is constitutively expressed and essential for starting the cell cycle (15). Cdk6 is also expressed but dispensable for the cell cycle start. Microinjection of an anti-Cdk4 (αCdk4), but not anti-Cdk6 (αCdk6), antibody effectively blocked the onset of S phase though both antibodies recognize the same C-terminal region of the corresponding molecules and are similar in immunoprecipitation potency. To investigate the effect of oncogenic stimulation on the utilization of cell cycle start factors, we carried out a similar microinjection experiment. NRK cells were arrested in G₁ by serum starvation, stimulated with culture medium containing FCS, microinjected with αCdk4 and/or αCdk6, and assayed for their entry into S phase by BrdUrd incorporation. As shown in Fig. 1, microinjection of αCdk4 (marked as dilute in the figure) blocked the S phase onset of approximately 50% of the cells. A 10-fold concentrated αCdk4 (unmarked) was slightly more effective and blocked the S phase onset of 60% of the cells. Microinjection of both αCdk4 and αCdk6 was no better in blocking than microinjection of αCdk4 alone. The inhibition of S phase onset by αCdk4 was epitope-specific, and pretreatment of the antibody with the epitope peptide completely abrogated its blocking ability.

When NRK cells were stimulated with growth medium containing FCS, EGF, and TGF-β instead of FCS alone, there was a dramatic change in results. αCdk4, even being concentrated 10-fold, could not block the S phase onset of the cells any longer. Needless to say, αCdk6 had no effect. However, comicroinjection of both αCdk4 and αCdk6 was found to effectively inhibit S phase onset. This blocking effect was eliminated when the antibodies were neutralized with the epitope peptides before use, indicating that the blocking of S phase onset indeed was effected by immunodepletion of Cdk4 and Cdk6. These results indicate that the addition of EGF plus TGF-β invokes Cdk6 to participate in a critical step of the cell cycle start in NRK cells.

To investigate whether this effect indeed was elicited by the EGF-induced oncogenic signals, we examined the significance of TGF-β alone and the behavior of two independent, transformation-deficient NRK mutants, 39–1 and 23, chosen as representatives (28, 31). The role of TGF-β in the growth factor-induced NRK transformation is to up-regulate the transcription of the EGF receptor gene, thereby counteracting EGF-induced down-regulation of EGF receptor (35). In fact, NRK cells overexpressing EGF receptor can be transformed reversibly by EGF alone, and the addition of TGF-β rather inhibits their transformation (S. Kizaka-Kondoh, M. Noda, and H.O., unpublished results). Consistent with this finding, the addition of TGF-β alone did not recruit Cdk6 in the cell cycle start. When NRK cells were stimulated with FCS containing TGF-β, their S phase onset was blocked by αCdk4. The slight decrease in the S phase cell population seen in this experiment was due largely to growth inhibition by TGF-β.

The NRK mutants 23 and 39–1 are refractory to transformation by EGF plus TGF-β and by various oncogenes. Their defects have been mapped between EGF receptor and Ras and between Ras and Raf-1, respectively, in the signal cascade shared by EGF and platelet-derived growth factor, which serve as targets for the action of major oncogenes (28, 29, 31). The gene mutated in 23 is CrkII whereas that in 39–1 has not been identified. When the mutants were stimulated with FCS containing EGF plus TGF-β, αCdk4 effectively blocked their S phase onset in an epitope-specific manner, and coinjection of αCdk6 did not enhance blocking. Thus, the mutants stimulated with the oncogenic growth factors behaved just as the NRK stimulated with FCS. These results strongly indicate that oncogenic signals, but not other biological effects of EGF or TGF-β, recruited Cdk6 in the cell cycle start in NRK cells.

To gain insights into the mechanism underlying this recruitment, we compared the levels of Cdk4, Cdk6, cyclin D1, and various CKIs expressed between NRK cells stimulated with FCS and those stimulated with FCS containing the oncogenic growth factors. As reported previously (15), both Cdk4 and Cdk6 were expressed constitutively, and their levels were unchanged by the addition of oncogenic stimulation (Fig. 2). On the other hand, cyclin D1 was induced at 6 hr and peaked at 9–12 hr. Again, their level and the time course of induction were basically unchanged. p27 decreased after 6 hr poststimulation, and, reciprocally, p21
was induced. p18, a specific Cdk4 inhibitor, was expressed constitutively whereas p15 and p16 were undetectable in NRK cells. Again, the levels of these CKIs were not influenced significantly by oncogenic stimulation.

We next examined possible differences in the level of the activities, and the time course of the activation, of Cdk4 and Cdk6. NRK cells were arrested by a combination of serum starvation and contact inhibition followed by growth stimulation by replating at a low cell density in FCS or FCS containing EGF plus TGF-β. The cells were harvested every 3 hr until the onset of S phase and gently lysed with the Triton-free lysis buffer. Cdk4 and Cdk6 then were immunoprecipitated and assayed for kinase activities with a truncated Rb as a substrate followed by immunodetection of Ser-780-phosphorylated Rb (15, 33). Because both Cdk4 and Cdk6 specifically phosphorylate the Ser-780 residue (33), immunodetection of the phosphorylated Rb reduces backgrounds and, thereby, increases the reliability of assay results. As shown in Fig. 3A, Cdk4 was activated at 6–9 hr postrelease, coinciding with cyclin D1 induction (Fig. 2), in the same time course and to the same extent irrespective of the presence or absence of oncogenic stimulation. Contrary to our anticipation, Cdk6 behaved similarly. Cdk6 was activated by FCS alone, and the level of the activation was similar if not identical in the presence or absence of oncogenic stimulation. These results show that both Cdk4 and Cdk6 were activated to the same extent irrespective of the presence or the absence of oncogenic stimulation, yet Cdk6 participated in the cell cycle start only in the presence of oncogenic stimulation.

To confirm these results, we constructed NRK cell clones overexpressing both Cdk6 and cyclin D1 and examined their behavior. One clone named K6D1 expressed a 10-fold higher amount of Cdk6 (Fig. 3B) and a 2- to 3-fold higher amount of cyclin D1. The level of Cdk6 kinase activity in K6D1, when assayed with 10-fold less cells, roughly matched that in the original NRK cells (Fig. 3A and C), indicating that the overexpressor had a 10-fold higher Cdk6 kinase activity. Again, Cdk6 kinase was activated to the same extent with or without oncogenic stimulation (Fig. 3C). The overexpressor then was tested for its behavior particularly to serum stimulation in the same immunodepletion assay as in Fig. 1. When stimulated with FCS alone, the overexpressor was blocked for S phase onset by immunodepletion of Cdk4 alone (Fig. 3D) despite the presence of a 10-fold higher Cdk6 activity. The slight reduction in the blocking effect was attributable to an increase in the number of cells that failed to arrest in G1 because of overexpressed Cdk6 and cyclin D1, which accounted for 20% of the total cells. When stimulated with FCS containing EGF plus TGF-β, the cells were not blocked for S phase onset by immunodepletion of Cdk4 any longer, as expected. Quite reasonably, coinjection of both αCdk4 and αCdk6, which effectively blocked the S phase onset of the original NRK cells (Fig. 1), failed to block because of the presence of an excessive amount of Cdk6 protein in the cells. Similar results were obtained with other overexpressor clones. These results led us to conclude that oncogenic stimulation invoked Cdk6 to participate in a critical step of the cell cycle start, not via the regulation of its catalytic activity.
Rb and its family member, p130, have been considered to be major biological effectors of these kinases and Cdk2 (6). Therefore, the time course and extent of phosphorylation of these factors during the cell cycle start were investigated by Western blot analysis (Fig. 2). Coinciding with the onset of the activation of Cdk4 and Cdk6, Rb shifted to the phosphorylated position. p130 behaved similarly though its shift was delayed slightly. As is known, the amount and the electrophoretic positions of E2F and DP1, the effectors of the Rb family, were unchanged during the cell cycle start. Again, oncogenic stimulation did not induce any significant changes in the time course and extent of phosphorylation of these molecules.

We examined the subcellular localization of Cdk4 and Cdk6 molecules by immunohistochemical staining as well as by Western blotting after fractionation. In quiescent cells, most of Cdk4 and Cdk6 proteins were localized diffusely in the cytoplasm and there was no significant change in the localization after stimulation with and without oncogenic stimulation (Fig. 4A). This result was confirmed by fractionation of disrupted cells into the nucleus and the cytoplasm followed by immunoblot detection (Fig. 4B). Again, there was no obvious change in subcellular localization of Cdk4 and Cdk6 proteins upon oncogenic or nononcogenic stimulation. Consistent with ours, in T cells, the majority of at least Cdk6 molecules are localized in the cytoplasm (36). All these results show that oncogenic stimulation involved Cdk6 to participate in the cell cycle start, but via a mechanism perhaps unrelated to the regulation of its kinase activity or subcellular localization, and that this Cdk6 participation was closely associated with the anchorage-independent cell cycle start activity.

Both Cdk6 and Cdk4 phosphorilate Rb protein at the same sites (33). In addition, homozygous RB gene knockout is insufficient for the induction of the anchorage-independent cell cycle start though it dispenses cyclin D1 and markedly accelerates the growth rate of the cells (37, 38). Therefore, Rb (and probably its family members) is unlikely to be the Cdk6 effector specifically employed for the anchorage-independent cell cycle start. This led us to tentatively build the working model that shows that oncogenic stimulation makes available a factor (or system) that differs from the one used for the anchorage-dependent cell cycle start, requires the action of Cdk6 (or Cdk4) for its function, and promotes the anchorage-independent cell cycle start. This model was correct, mere activation of these Cdns and phosphorylation of Rb would be insufficient for inducing the anchorage-independent cell cycle start.

To test this prediction, we carried out the following analysis. In this analysis, NRK cell clones overexpressing Cdk4 or Cdk6 were used for ease in kinase assays. Overexpression of these kinases did not influence the properties of NRK cells regarding oncogenic responses to EGF plus TGF-β. The overexpressors were arrested in G1 by a combination of serum starvation and contact inhibition, stimulated with medium containing FCS for...
transformed reversibly by a combination of growth factors, understanding this remarkable phenomenon. NRK cells are arrested in G1 by serum depletion, stimulated for Cdk4, Cdk6, and Cdk2 activities as well as for the level of p27 reduction in the p27 amount and phosphorylation of Rb occurrence. Moreover, reduction in the p27 amount and phosphorylation of Rb occurred in these cells to the levels similar to those in the cells entering the S phase by stimulation with EGF plus TGF-β. However, the majority of the cells stimulated with ECS even for 7 hr failed to enter S phase. By contrast, more than 70% of the NRK cells stimulated with EGF plus TGF-β in methylcellulose growth medium for 24 hr entered S phase and reached G2 phase. These results indicate that a commitment step to the anchorage-independent cell cycle start indeed resides after the activation of G1 cyclin kinases, which is consistent with the model.

**Discussion**

Oncogenic stimuli induce the anchorage-independent cell cycle start. Our data presented here provide a possible clue to understanding this remarkable phenomenon. NRK cells are transformed reversibly by a combination of growth factors, typically by EGF plus TGF-β, and come to grow in soft agar. When stimulated to grow nononcogenically, they absolutely depended on Cdk4 for the cell cycle start. But when stimulated to grow oncogenically, they came to depend on Cdk4 or Cdk6 for the cell cycle start, and immunodepletion of both Cdk4 and Cdk6 was required to block their cell cycle start. This shows that oncogenic stimulation specifically invokes Cdk6 to participate in a critical step of the cell cycle start in the rat fibroblast.

The oncogenic stimulation-induced Cdk6 participation is unlikely to occur via the regulation of the kinase activity. We failed to detect any significant changes in the amount, activity, and subcellular localization of Cdk6 and its relevant regulators upon oncogenic stimulation. To explain these results, we tentatively built the model that demonstrates that oncogenic stimulation makes available a distinct factor (or system) that requires the action of Cdk6 (or Cdk4) for its function and promotes the anchorage-independent cell cycle start because there was a close correlation between oncogenic stimulation-induced Cdk6 participation and this remarkable cancer phenotype as shown with the NRK cell mutants. This model predicted that commitment to the anchorage-independent cell cycle start would depend also on the availability of this hypothetical system and, consequently, would not simply be made by mere activation of Cdk6 or Cdk4. This predication was supported by the Fig. 5 data and is remarkably consistent with recent related findings. Human herpes virus 8 encodes the novel cyclin called cyclin K, and Cdk6 and Cdk4, associated with this cyclin, are highly active and evade...
inhibition by CKI. Consequently, cells expressing cyclin K ignore and, therefore, proliferate despite cell–cell contacts and growth factor starvation, but still cannot grow without anchorage (20).

Two mechanisms are conceivable for oncogenic stimulation, making the hypothetical factor (or system) available for the cell cycle start. One is that oncogenic stimulation induces the factor (or system) or promotes its processing to readiness to use, and the other is that oncogenic stimulation alters the substrate specificity of Cdk6 in such a way that Cdk6 can activate the hypothetical factor that is already present. The alteration of the substrate specificity of Cdk6 might be achieved either by a change in associated cyclin D partners among three subtypes or by its association with some accessory proteins. These two mechanisms are equally compatible with our data.

The nature of this hypothetical factor (or system) is unknown, but it may be closely related to the one used in hematopoietic cells because many hematopoietic cells express almost exclusively Cdk6 and perform anchorage-independent growth by nature (7). The molecular role that the hypothetical factor would play in the anchorage-independent cell cycle start is totally unknown and even beyond speculation. However, its major role is unlikely to be to inactivate Rb protein because homozygous Rb gene knockout is insufficient for the induction of the anchorage-independent cell cycle start though it dispenses cyclin D1 and markedly accelerates the growth rate of the cells (37, 38). As already shown, the anchorage-dependent cell cycle start system absolutely relies on Cdk4, at least in NRK cells. By contrast, this hypothetical system appears to rely on Cdk6 or Cdk4 because the anchorage-independent cell cycle start of NRK cells also can be induced in the absence of Cdk6 activity (S.J. and H.O., unpublished observation). This is consistent with the recent observations suggesting that there seems to be no absolute requirement for Cdk6 in the ability to grow without anchorage (2, 39).

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