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ARTICLES

Post-Proliferative Cyclin E-Associated Kinase Activity in Differentiated Osteoblasts: Inhibition by Proliferating Osteoblasts and Osteosarcoma Cells

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Abstract Spontaneous differentiation of normal diploid osteoblasts in culture is accompanied by increased cyclin E associated kinase activity on (1) the retinoblastoma susceptibility protein pRB, (2) the p107 RB related protein, and (3) two endogenous cyclin E-associated substrates of 78 and 105 kD. Activity of the *diff*erentiation-related cyclin *E c*omple*x*es (*diff.ECx*) is not recovered in cdc2 or cdk2 immunoprecipitates. Phosphorylation of both the 105 kD endogenous substrate and the p107 exogenous substrate is sensitive to inhibitory activity (*diff.ECx-i*) present in proliferating osteoblasts. This inhibitory activity is readily recruited by the cyclin E complexes of differentiated osteoblasts but is not found in cyclin E immunoprecipitates of the proliferating cells themselves. Strong inhibitory activity on *diff.ECx* kinase activity is excerted by proliferating ROS 17/2.8 osteosarcoma cells. However, unlike the normal diploid cells, the *diff.ECx-i* activity of proliferating ROS 17/2.8 cells is recovered by cyclin E immunoprecipitation. The cyclin-dependent kinase inhibitor p21$^{CIP1/WAF1}$ inhibits *diff.ECx* kinase activity. Thus, our results suggest the existence of a unique regulatory system, possibly involving p21$^{CIP1/WAF1}$, in which inhibitory activity residing in proliferating cells is preferentially targeted towards differentiation-related cyclin E-associated kinase activity. J. Cell. Biochem. 66:141–152, 1997. © 1997 Wiley-Liss, Inc.

Key words: differentiation; osteoblasts; cyclin E-associated kinase; cyclin dependent kinase inhibitors; RB related proteins

Cyclins and cyclin dependent kinases (cdk's) play primary roles in control of cell cycle progression [reviewed by King et al., 1994; Nurse, 1994; Sherr, 1994; Hunter and Pines, 1994; MacLachlan et al., 1995; Sherr and Roberts, 1995; Morgan, 1995]. Activity of these cell cycle regulatory factors is determined by periodical association of cyclins with their kinase partners, resulting in both phosphorylation and dephosphorylation of specific sites on the kinases. These periodical activities promote cell cycle progression by phosphorylation of regulatory factors such as the retinoblastoma susceptibility protein (pRB) [reviewed by Sherr, 1994; Hunter and Pines, 1994; Sherr and Roberts, 1995]. While the levels of cdk's such as cdc2 and cdk2 are not significantly altered during the cell cycle, the formation of active cyclin-cdk complexes is regulated by the representation of the cyclins. Although each cdk is activated primarily by a specific cyclin, the partial structural similarity among different kinases and among different cyclins allows some promiscuous partnerships. For example, cdc2 which plays its major role during mitosis as a cyclin B/cdc2 complex [Draetta and Beach, 1988; Pines and Hunter, 1989; reviewed by King et al., 1994; Nurse, 1994; Hunter and Pines, 1994], can also be activated by cyclin A [Draetta et al., 1989; Giordano et al., 1989] and by cyclin E [Koff et al., 1991, 1992]; and, cdk2, which is activated primarily by cyclin E, can also form active com-

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plexes with cyclin A [Koff et al., 1992; Dulic' et al., 1992; Tsai et al., 1991; Pagano et al., 1992] or with cyclins D2 and D3 [Ewen et al., 1993]. Additional regulatory levels of cdk activities are contributed by cdk-kinases, cdk-phosphatases, and cdk-inhibitors (cdi's) [Sherr, 1994; Hunter and Pines, 1994; Sherr and Roberts, 1995].

Cyclin E is believed to play a major role in the G1/S transition of the cell cycle [reviewed by Sherr, 1994; Sherr and Roberts, 1995]. While cyclin D/cdk4 complexes appear to predominate during early G1, the level of cyclin E increases at late G1, resulting in binding to and activation of cdk2 [Koff et al., 1992; Dulic' et al., 1992; Lew et al., 1991; Matsushime et al., 1991, 1992; Kato et al., 1993; Quelle et al., 1993; Resnitzky et al., 1994]. Consequently, the underphosphorylated forms of pRB or the p107 RB-related protein, which bind to and restrain the activities of E2F family transcription factors, are phosphorylated to allow E2F-mediated cell cycle events [Hinds et al., 1992; Hatakeyama et al., 1994; Bremmer et al., 1995; Lees et al., 1992; Suzuki-Takahashi et al., 1995; Zhu et al., 1995; and reviewed by MacLachlan et al., 1995]. In addition to cyclin E levels, phosphorylation of pRB by cyclin E/cdk2 is also regulated by cdk inhibitors. For example, high levels of $p21^{CIP1/WAF1}$ in cyclin E/cdk2 complexes inhibit the phosphorylation of pRB even when cdk2 has already been activated [Zhu et al., 1995; Harper et al., 1993; Xiong et al., 1993; Gu et al., 1993; Zhang et al., 1994; El Deiry et al., 1993; Dulic' et al., 1994; and reviewed by Sherr, 1994; Hunter and Pines, 1994; Sherr and Roberts, 1995].

Unexpectedly, several studies with various differentiation systems have indicated postproliferative retention of significant levels of cyclins. Quiescent hepatocytes in vivo exhibit cyclin D1 and cyclin E levels similar to those observed during liver regeneration [Loyer et al., 1994]; differentiated neurons in vivo also retain significant cyclin E levels [Miyajima et al., 1995]. Differentiated cell lines derived from P19 embryonal carcinoma cells express cyclins D1 and D2 [Kranenburg et al., 1995] while C2C12 myoblasts differentiation is accompanied by increased cyclin D3 expression [Rao and Khotz, 1995]. Murine erythroleukemia (MEL) cells induced to differentiate by hexamethylene bisacetamide stop dividing but retain high levels of cyclins D2, D3, and E [Kiokawa et al., 1994]. Finally, nerve growth factor-treated pheochromocytoma PC12 cells undergoing differentiation exhibit increased levels of cyclins D1 and E [Yan and Ziff, 1995; Dobashi et al., 1995; van Grunsven et al., 1996]. The emphasis in most of these studies was to reveal which are the cell cycle regulatory factors that must be downregulated to allow differentiation. Accordingly, it was found that reduction of cdk4 but not cdk2 is critical for commitment of MEL cells to develop the erythroid phenotype [Kiokawa et al., 1994]. Suppression of cdk2 activity was found to be critical for PC12 cells differentiation [Dobashi et al., 1995]. Finally, limited overexpression of cyclin D1 inhibited C2C12 myoblast differentiation [Rao et al., 1995].

In studies of the spontaneous development of normal diploid rat calvarial osteoblasts in vitro, we have observed differentiation specific upregulation of nuclear cyclin E whereas the levels of the cell cycle protein kinases cdc2 and cdk2 were substantially reduced [Smith et al., 1995]. We initiated the current study to address whether in postproliferative osteoblasts cyclin E may exhibit differentiation related activities. Indeed, we report that the elevated levels of cyclin E are associated with kinase activity, designated *diff.ECx,*[1] distinct from that operative during the cell cycle. Further, we have revealed the existence of a unique regulatory system in which proliferating cell extracts exert inhibitory activity on the cyclin E associated kinase activity of differentiating cells. $p21^{CIP1/WAF1}$, which also inhibits *diff.ECx* kinase activity, may play a role in this system.

MATERIALS AND METHODS

Cell Culture

Fetal rat calvarial osteoblasts were cultured and subjected to a differentiation protocol as previously described [Owen et al., 1990; Smith et al., 1995]. Cells were collected at either the early proliferation period, at the matrix maturation stage, when nodules start to form, or at a late differentiation stage, when the extracellular matrix mineralizes. Essentially the same results were obtained with primary cultures and with cells that were trypsinized and replated before reaching confluency as described [Smith et al., 1995]. ROS 17/2.8 osteosarcoma cells [Majeska et al., 1980] were maintained as described [van den Ent et al., 1993] and col-

[1] *diff.ECx* = differentiation-related cyclin E complexes. *diff.ECx-i* = *diff.ECx* inhibitory activity.

lected at either an early proliferation time point or at a late time point of density-mediated growth inhibition. Both the normal and the tumor-derived cells were collected 18–20 h after medium change.

Whole Cell Extracts

Between 10–80 plates (100 mm in diameter) were thoroughly washed with ice-cold phosphate-buffered saline, and the cells were scraped, centrifuged, and lysed in a 50 mM Tris-HCl buffer (pH 7.4) containing 250 mM sodium chloride, 0.5% (v/v) Nonidet P40 (NP40), 20 μg/ml TPCK, 20 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, and 1 mM PMSF. The cells were then homogenized on ice in a tight-fitting dounce homogenizer (35 strokes), centrifuged at 13,000*g* for 10 min at 4°C and the supernatant stored at −70°C. Total protein concentration in the cell lysate was determined using the Micro BCA Protein Assay kit (Pierce, Rockford, IL).

Immunoprecipitation and Western Analysis

Immunoprecipitations from whole cell extracts were performed essentially as described [Koff et al., 1992]. During all steps, samples were kept at 0–4°C. For immunoprecipitations with mouse monoclonal antibodies, the extracts were first precleared with non-specific mouse immunoglobulins and protein G-Agarose beads. For immunoprecipitations with rabbit polyclonal antibodies, the extracts were first precleared with rabbit serum and protein A-Agarose beads. Following 1 h incubation on a rotator and removal of the beads by spinning at 300*g* for 10 s, the specific antibodies (or the respective non-specific control antibodies) were added to the supernatant and incubated for 1 h on a rotator, at which time the respective Agarose beads were added for additional 1 h. The immunocomplexes were pelleted by centrifugation at 300*g* for 10 s, then washed twice with NP40 lysis buffer containing the above protease inhibitors, and four times with kinase buffer (see below) containing 0.2 mg/ml bovine serum albumin. For Western analysis, the immunoprecipitates were washed 4 times with NP40 lysis buffer and resuspended in SDS loading buffer [60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 0.01% bromophenol blue] prior to boiling and SDSPAGE. Western analysis was performed as previously described [Smith et al., 1995].

Kinase Assays

Immunoprecipitates were subjected to kinase assay for 30 min at 37°C essentially as described [Koff et al., 1992]. The reaction mixture (30 μl) contained 20 mM Tris-HCl (pH 7.4), 10 mM magnesium chloride, 1 mM DTT, 0.3 mM ATP, and 5 μCi [^{32}P-γ]ATP (3,000 Ci/mmol). For kinase assays of immunoprecipitates obtained using rabbit polyclonal antibodies, protein kinase A inhibitor (10 μg/ml) was also included [Tam et al., 1995]. When indicated, the following proteins were added as exogenous substrates: histone H1 (300 μg/ml), GST-RB pocket (50 μg/ml; see below), or GST-p107 pocket (50 μg/ml; see below). The reaction was stopped by adding SDS loading buffer, and the mixture boiled for 5 min and subjected to SDSPAGE under non-reducing conditions. The gel was then fixed in 5% tetrachloroacetic acid for 10 min at 80–100°C, dried, and the phosphorylated products visualized by autoradiography.

GST-Fusion Recombinant Proteins

cDNA's encoding p21$^{CIP1/WAF1}$ and the pocket-containing domains of pRB (p105) and p107 were cloned in pGEX vectors (Pharmacia, Gaithersburg, MD). The recombinant proteins, as well as the control GST protein alone were prepared using Glutathione Sepharose 4B beads (Pharmacia) essentially as recommended by the manufacturer. BL21 *Escherichia coli* cells transformed with the respective plasmids were grown overnight in L. Broth at 37°C, diluted 1:100 into 400 ml, and grown at 30°C to ~1.3 OD_{600} U. Following additional 3 h at 30°C in the presence of 0.1 mM IPTG, cells were pelleted (4°C), resuspended in 20 ml of high salt lysis buffer [20 mM Tris HCl (pH 8.0), 0.2 mM EDTA, 1 M NaCl, 1 mM PMSF] containing 1 mg/ml lysosyme, incubated for 10 min, and DTT added to 1 mM. The cells were then gently sonicated, 1 ml of 20% Triton X-100 in PBS was added, and the mixture incubated for 30 min at 4°C on a rotator. Following 1 h centrifugation at 12,000*g* at 4°C, the supernatant was incubated with 400 μl PBS-washed Glutathione beads (50% slurry) for 30 min at room temperature. The beads were then washed 3 times with 2 ml of PBS and the GST recombinant proteins eluted in 0.5 ml of 50 mM Tris buffer (pH 8.0) containing 10 mM reduced Glutathione and separated from the beads using Spin-X filters (Costar, Cambridge, MA).

Materials

Anti cyclin E antibodies (sc247 for Western analysis and sc248x for immunoprecipitation) as well as anti cdc2 and cdk2 antibodies (sc54 and sc163) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cyclin B antiserum was a gift from R. Schlegel (Harvard School of Public Health, Boston, MA). Anti cdk5 antiserum was prepared by A. DeLuca and A. Giordano (Jefferson Cancer Institute, PA). Purified mouse IgG (Sigma, St. Louis, MO, no. M9269) and rabbit serum were used as controls for immunoprecipitations with mouse monoclonal and rabbit polyclonal antibodies, respectively. Histone H1, protein kinase A inhibitor, and the phosphotyrosine inhibitor Genistein were purchased from Sigma.

RESULTS

Cyclin E in Differentiated Osteoblasts Is Associated With Kinase Activity

Normal diploid fetal rat calvarial osteoblasts in culture undergo distinct developmental stages characterized by the timed expression of cell growth and bone phenotypic genes [reviewed by Stein et al., 1996]. Unexpectedly, during this process, nuclear cyclin E is distinctly upregulated as compared to proliferating osteoblasts or confluent osteoblasts maintained under a non-differentiation protocol [Smith et al., 1995]. To address the function of the upregulated cyclin E in differentiated osteoblasts, cyclin E immunocomplexes of proliferating and differentiated whole cell extracts were isolated and assayed for kinase activity. Consistent with our previous data, Western analysis of the cyclin E complexes obtained from differentiated osteoblasts show an elevated cyclin E level (Fig. 1A). Notably, this immunoprecipitate exhibits only one of three immunoreactive bands detected by direct Western analysis of these cells [Smith et al., 1995], probably due to restricted specificity of the antibody used for immunoprecipitation compared to that used for the Western analysis. Kinase activity of the cyclin E complexes from proliferating and differentiated osteoblasts was evaluated using histone H1 as exogenous substrate. As shown in Figure 1B, significant H1 kinase activity is evident not only in the proliferating cells (consistent with the role of cyclin E during cell cycle progression), but also in cyclin E complexes of differentiated osteoblasts. In multiple osteoblast preparations, the latter histone H1 kinase activity ranged from 80–130% of that observed in proliferating cells, as determined by densitometric scanning of the autoradiograms. Of particular interest, all these osteoblast preparations exhibited the phosphorylation of two high molecular weight endogenous substrates of 78 and 105 kD, co-immunoprecipitated with cyclin E (Fig. 1B). Unlike the histone H1 kinase activity, phosphorylation of the 105 kD endogenous substrate is preferential in differentiated compared to proliferating osteoblasts (Fig. 1B). In most of the osteoblast preparations assayed, we also observed preferential phosphorylation of the 78 kD endogenous substrate at the differentiation stage. This is demonstrated, for example, in Figure 1C, in which phosphorylation of both the 78 and the 105 kD co-immunoprecipitated endogenous substrates was assayed in the absence of added histone H1. Cells maintained under nondifferentiation culture conditions [Smith et al., 1995] did not exhibit the postproliferative cyclin E-associated kinase activity (data not shown). Thus, the postproliferative development of the osteoblast phenotype is accompanied by formation of *diff*erentiation-related cyclin *E* *c*omple*x*es (*diff.ECx*), which phosphorylate both histone H1 and two endogenous substrates.

Specificity of the differentiation related kinase activity to cyclin E complexes is further demonstrated in Figure 1D, showing that cyclin B immunocomplexes of differentiating cells do not exhibit significant kinase activity on either histone H1 or the high molecular weight endogenous substrates. Interestingly, we observe postproliferative cyclin B-associated histone H1 kinase activity in an earlier time point that precedes mineralization (differentiation stage) and is referred to as the matrix maturation period [Owen et al., 1990]. This cyclin B associated kinase activity, as well as the concomitant phosphorylation of an endogenous ~50 kD protein (Fig. 1D), is under investigation. Cyclin E-associated kinase activity (Fig. 1B,C) was also evident at this matrix maturation stage (data not shown).

Activity of the Differentiation-Related Cyclin E-Associated Complexes (*diff.ECx*) Is Not Recovered by cdc2 or cdk2 Immunoprecipitation

During the G1/S transition in cycling cells, cyclin E serves as the regulatory unit of the cyclin dependent kinase cdk2 [Koff et al., 1992;

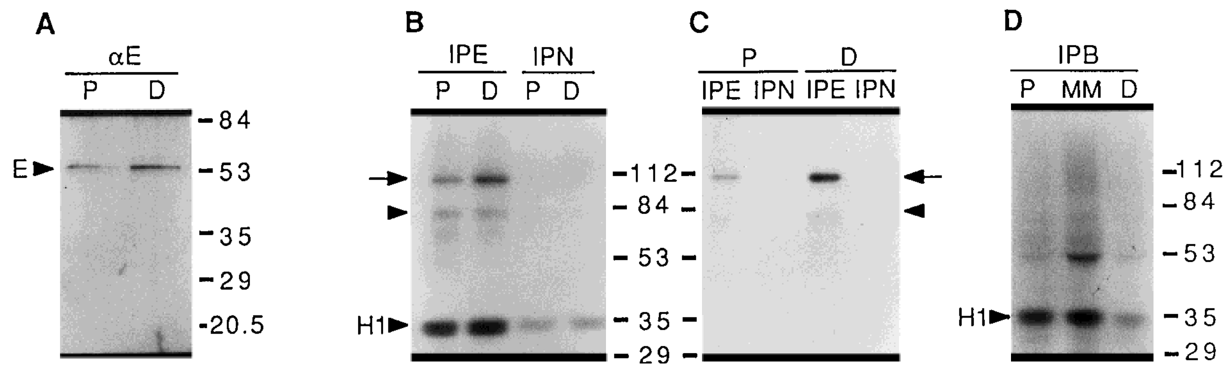

Fig. 1. Cyclin E complexes of differentiated osteoblasts (*diff.ECx*) exhibit kinase activity. Rat calvarial osteoblasts were harvested during exponential growth (P, proliferating) or at a late differentiation stage (D). **A:** Whole cell extracts (1 mg protein) were immunoprecipitated with anti cyclin E antibody as described in Materials and Methods and the immunoprecipitates subjected to 10% SDS-PAGE, followed by Western analysis with cyclin E antibody. **B:** Immunoprecipitations were performed as in A on 0.5 mg protein using either anti cyclin E antibody (IPE) or a non-specific antibody (IPN). The immunoprecipitates were subjected to kinase assay using [^{32}P-γ]ATP and histone H1 as substrate, and the phosphorylated proteins separated on 10% SDS-PAGE and visualized by autoradiography. **C:** Immunoprecipitation and kinase assays were performed exactly as in B, except that histone H1 was eliminated. **D:** Immunoprecipitations and kinase assays were performed as in B using anti-cyclin B antibody and 0.5 mg of whole cell extracts obtained from osteoblasts at the beginning (P), the end (D), and the middle (MM, matrix maturation) of the differentiation time course. E = cyclin E; H1 = histone H1; arrows and arrowheads indicate 105 and 78 kDa endogenous substrates, respectively. Positions of molecular weight markers are depicted in kD.

Dulic' et al., 1992; and reviewed by Sherr 1994; Sherr and Roberts, 1995]. In addition, cyclin E can form active complexes with cdc2 [Koff et al., 1992]. However, kinase assays of both cdc2 and cdk2 immunoprecipitates show a dramatic decrease during osteoblast development in culture (Fig. 2). At the late differentiation stage, the histone H1 kinase activity of cdc2 (Fig. 2A) and cdk2 (Fig. 2B) immunocomplexes approached background levels (measured with respective nonspecific antibodies) and there was no evidence for phosphorylation of the 78 or the 105 kD endogenous substrates, which were observed in kinase assays of cyclin E immunocomplexes (Fig. 1). Thus, it appears that the cell cycle related kinases cdc2 and cdk2 do not contribute to the kinase activity of *diff.ECx.* Alternatively, they may reside in *diff.ECx* in a configuration that renders them unaccessible to the cdc2 and cdk2 antibodies. Based on the recent identification of cdk5 in cyclin E complexes of differentiated neurons [Miyajima et al., 1995], we have also performed cdk5 immunoprecipitations and kinase assays. No phosphorylation was observed at the 105 kD range (data not shown).

Proliferating Normal Diploid Osteoblasts Exert an Inhibitory Effect on *diff.ECx* Kinase Activity

It has recently become evident that the cyclin-dependent kinases in proliferating cells are subject to negative regulation by cyclin dependent kinase inhibitors (cdi's), which have been suggested to play a role in induction of growth arrest and terminal differentiation [reviewed by Sherr, 1994; Hunter and Pines, 1994; Sherr and Roberts, 1995]. We speculated that the cyclin E associated kinase activity in differentiated osteoblasts may be reciprocally inhibited by factor(s) present in proliferating cells. In Figure 3A, we have mixed proliferating and differentiated cell extracts prior to cyclin E immunoprecipitation and kinase assay. The results clearly indicate inhibition of *diff.ECx* activity, suggesting the presence of a *diff.ECx*-inhibitory (*diff.ECx-i*) activity in proliferating osteoblasts (Fig. 3A, compare lane D+P to lane D). Of the three phosphorylated substrates, inhibition of *diff.ECx* kinase activity on the 105 kD endogenous substrate is the most significant. Phosphorylation of the histone H1 exogenous substrate is also inhibited, as lack of inhibition would have resulted in a signal representing the sum activities of both the proliferating and the differentiated cells, because the assay was performed with antibody excess. In contrast to the 105 kD and the histone H1 substrates, phosphorylation of the endogenous 78 kD substrate was approximately equal to the sum of activities observed in the two separate extracts (Fig. 3A), suggesting resistance to inhibition by the *diff.ECx-i* activity.

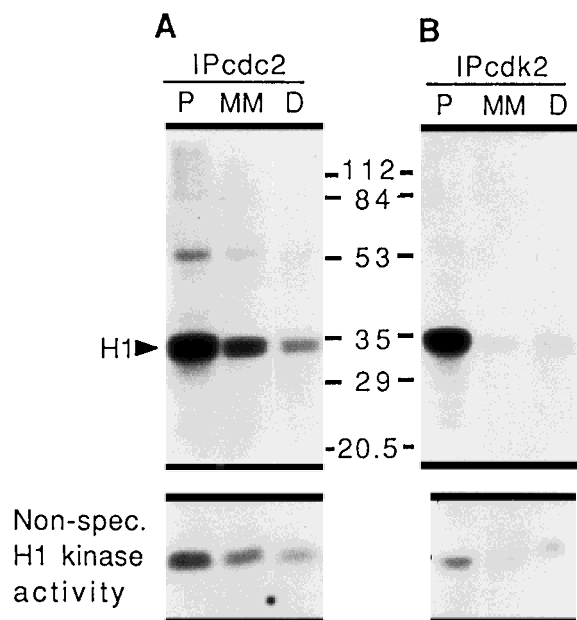

Fig. 2. Differentiated osteoblast cyclin E-associated kinase activity is not recovered in cdc2 or cdk2 immunoprecipitates. Immunoprecipitations and kinase assays were performed as described in Materials and Methods using 0.5 mg protein extracted from osteoblasts at the proliferation (P), matrix maturation (MM), or differentiation (D) stages. The kinase activities associated with cdc2 (**A**) and cdk2 (**B**) are presented in the top panels and the respective non-specific activities are shown below.

The *diff.ECx* Inhibitory (*diff.ECx-i*) Activity in Proliferating Osteoblasts Is Heat Resistant and Readily Associates With Cyclin E Complexes of Differentiated, Not Proliferating Cells

Because some cell cycle kinase inhibitors are heat resistant [Dulic' et al., 1994; Polyak et al., 1994; Slingerland et al., 1994; Hengst et al., 1994], we have assayed *diff.ECx-i* activity in proliferating cell extracts that have been subjected to boiling for 5 min. As shown in Figure 3B, while this treatment completely abolished the kinase activity in the cyclin E immunoprecipitate from proliferating cells (lane ΔP), the *diff.ECx-i* activity was retained (Fig. 3B, compare lane D+ΔP to lane D). To further validate that an inhibitory activity is present in the cyclin E immunocomplexes obtained from the mixture of proliferating and differentiated osteoblasts, we next attempted to extract this inhibitory activity by boiling. First, cyclin E immunocomplexes were obtained from a mixture exactly as in Figure 3A (D+P). Then, instead of subjecting the pellet to a kinase assay, it was boiled, centrifuged, and the supernatant assayed for its effect on active cyclin E complexes of differentiated cells. This supernatant significantly inhibited *diff.ECx*-activity on the 105 kD substrate, when compared to a supernatant obtained by the same procedure using a nonspecific antibody (Fig. 3C), which itself had no effect on *diff.ECx* activity (data not shown). Phosphorylation of histone H1 was not inhibited, possibly reflecting incomplete immunoprecipitation of the inhibitory activities present in the mixture of proliferating and differentiated cells. Inhibition at the 105 kD endogenous substrate by the supernatant obtained after boiling the cyclin E complexes was evident whether the supernatant was added to the differentiated cell extracts prior to cyclin E immunoprecipitation (Fig. 3C) or added directly to active cyclin E immunocomplexes, although it was less profound in the latter protocol (data not shown).

We next asked whether the *diff.ECx-i* activity residing in proliferating osteoblasts is present in the cyclin E complexes of these cells. Cyclin E immunoprecipitate obtained from proliferating osteoblasts alone was boiled and the material released to the supernatant was assayed for *diff.ECx-i* activity by addition to differentiated cell extracts. In several experiments, no inhibition of *diff.ECx* kinase activity was observed, regardless of whether the supernatant from the proliferation related cyclin E complexes was added to the differentiated cell extracts prior to cyclin E immunoprecipitation (data not shown) or added directly to active *diff.ECx* immunoprecipitates (Fig. 4A). These results indicate absence of *diff.ECx-i* activity in cyclin E immunocomplexes of proliferating cells. This was not the case, however, when cyclin E complexes were obtained from a mixture of proliferating and differentiated cell extracts (Fig. 3C). Taken together, our results suggest that the *diff.ECx-i* activity present in proliferating osteoblasts preferentially associates with the cyclin E complexes of differentiated cells when extracts from these two developmental stages are mixed.

Variations in *diff.ECx-i* Activity of ROS 17/2.8 Osteosarcoma Cells Compared to Normal Osteoblasts

ROS 17/2.8 is an osteosarcoma cell line in which, as opposed to normal diploid osteoblasts, proliferation and differentiation-related genes are co-expressed. Upon density induced growth inhibition in culture, ROS 17/2.8 cells

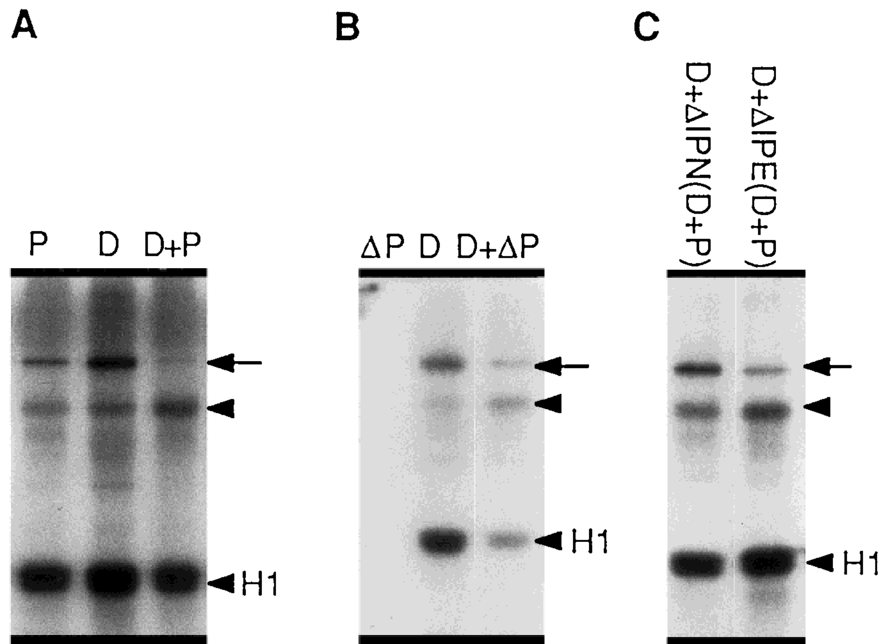

Fig. 3. Inhibition of *diff.ECx* kinase activity by proliferating osteoblasts. **A:** Immunoprecipitation and kinase assays were performed with anti-cyclin E antibody as in Figure 1B, using 0.5 mg protein from proliferating osteoblasts (P), 0.5 mg protein from differentiated cells (D), or a mixture of 0.5 mg protein of each (D+P). **B:** Immunoprecipitation and kinase assays were performed exactly as in A, except that the proliferating cell extracts were boiled for 5 min prior to the immunoprecipitation (ΔP = boiled proliferating cell extracts). **C:** Cyclin E associated kinase activity was assayed as in A, lane "D," except that ΔIPE(D+P) or ΔIPN(D+P) were added to the differentiated cell extracts prior to immunoprecipitation of cyclin E complexes. ΔIPE(D+P) represents the supernatant obtained after boiling the cyclin E immunocomplexes from a mixture of proliferating and differentiated cell extracts (0.5 mg of each). ΔIPN(D+P) represents a control supernatant obtained using non-specific IgG. The positions of histone H1 (H1), as well as the 105 kD (arrows) and the 78 kD (arrowheads) endogenous substrates, are shown.

exhibit upregulation of several differentiation related genes such as osteocalcin, but development of the complete bone phenotype is abrogated [van den Ent et al., 1993; Bortell et al., 1993]. Figure 5 presents a semi-quantitative analysis of *diff.ECx-i* activity in ROS 17/2.8 cells compared to normal diploid osteoblasts. *Diff.ECx-i* activity is stronger in the tumor-derived osteoblasts, but declines postproliferatively in both cell types. Interestingly, *diff.ECx-i* activity is completely lost in normal diploid differentiated osteoblasts, whereas substantial inhibitory activity is retained in the osteosarcoma cells when they are density mediated growth inhibited. In addition, Figure 5 indicates lack of *diff.ECx-i* activity in exponentially growing cervical carcinoma He La cells, serving as control.

We further addressed whether *diff.ECx-i* activity present in the proliferating osteosarcoma cells would be retrieved from cyclin E complexes of these cells. Cyclin E immunocomplexes obtained from proliferating ROS 17/2.8 cells were boiled and the supernatant subjected to *diff.ECx* inhibition assay, using the same protocol employed in Figure 4A for proliferating normal diploid cells. As shown in Figure 4B, the ROS 17/2.8 supernatant exhibits strong *diff.ECx-i* activity (right lane) when compared to the supernatant obtained with the same protocol using a non-specific antibody (left lane). This inhibitory activity is comparable to the *diff.ECx-i* activity exerted by boiled proliferating ROS 17/2.8 whole cell extracts (see Figs. 5 and 6B below). Thus, similar to normal proliferating osteoblasts, proliferating ROS 17/2.8 osteosarcoma cells contain *diff.ECx-i* activity; but, in contrast to the normal cells (Fig. 4A), this inhibitory activity is recoverable from the cyclin E immunocomplexes of the proliferating osteosarcoma cells (Fig. 4B).

Preferential Phosphorylation of RB Family Proteins by Cyclin E Complexes of Differentiated as Compared to Proliferating Osteoblasts

In our pursuit of candidates that may serve as physiological substrates for the *diff.ECx* kinase activity in differentiated osteoblasts, and

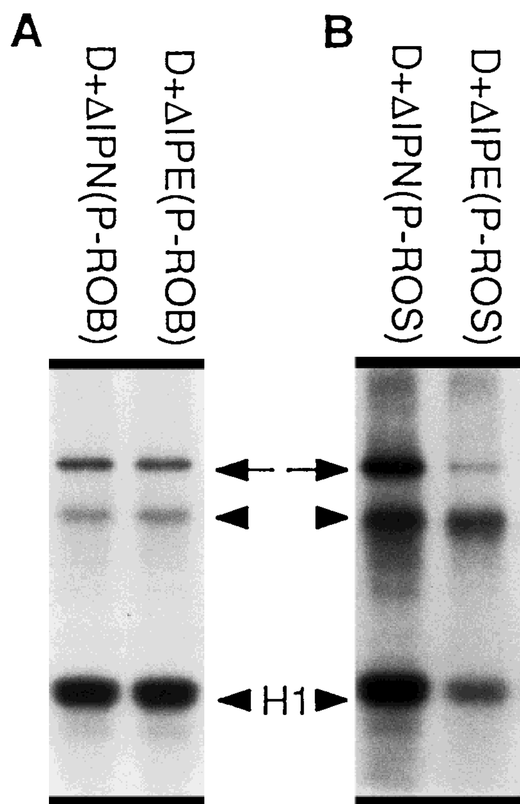

Fig. 4. Relationship between *diff.ECx-i* activity and cyclin E in proliferating cells: dissociation in normal osteoblasts vs. association in osteosarcoma cells. Inhibition of *diff.ECx* kinase activity was assayed as in Figure 3C, except that the source for the inhibitory activity (*diff.ECx-i*) was cyclin E complexes of proliferating cells, rather than a mixture of proliferating and differentiated cells. **A:** Cell extracts from normal proliferating osteoblasts (0.5 mg protein) were immunoprecipitated with either anti-cyclin E antibody (right lane) or a non-specific antibody (left lane), boiled, and added to differentiated cell extracts (0.5 mg) prior to immunoprecipitation of active cyclin E complexes. These active immunocomplexes were then subjected to kinase assay as in Figure 1B. **B:** Cyclin E complexes of proliferating ROS 17/2.8 cells were subjected to *diff.ECx* kinase inhibition assay exactly as in A. The positions of histone H1 (H1), as well as the 105 kD (arrows) and the 78 kD (arrowheads) endogenous substrates, are shown.

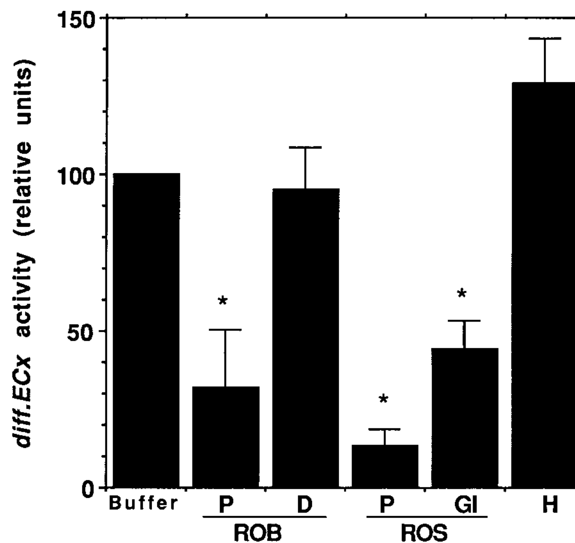

Fig. 5. Comparison between *diff.ECx-i* activity in normal and tumor-derived cells. Whole cell extracts (0.5 mg protein) from either proliferating (P) or differentiated (D) rat osteoblasts (ROB), proliferating or density-mediated growth inhibited (GI) ROS 17/2.8 cells (ROS), or exponentially growing HeLa cells (H) were boiled for 5 min and subjected to a kinase inhibition assay as in Figure 3A. In all cases, the source for kinase activity is differentiated ROB cell cyclin E immunocomplexes. Phosphorylation of the 105 kD substrate was quantitated by densitometric scanning of the autoradiograms (UVP, San Gabriel, CA), and is expressed relative to the phosphorylation level in the presence of buffer alone, set as 100 U. Results are mean ± SEM for 3–5 independent cell preparations of each type. *Statistically different from the activity obtained with buffer alone ($P < 0.05$, paired *t*-test).

since *diff.ECx* kinase activity on the endogenous 105 kD substrate was found insensitive to the tyrosine kinase inhibitor Genistein (data not shown), we assayed phosphorylation of the retinoblastoma susceptibility protein (pRB) and the p107 RB-related protein by cyclin E complexes derived from proliferating and differentiated osteoblasts. Recombinant human GST-RB and GST-p107, which include the pocket domains, were used as substrates. As shown in Figure 6A, these proteins are readily phosphorylated by the osteoblast derived cyclin E complexes. The signal obtained with the RB family members was severalfold higher than that obtained with equal amounts of histone H1 as substrate (data not shown). Moreover, in contrast to the activity on histone H1 (Fig. 1B), activity on the RB family members is higher in differentiated compared to proliferating osteoblasts (Fig. 6A), reminiscent of the activity on the endogenous 105 kD substrate (Fig. 1B,C). Sensitivity of pRB and p107 phosphorylation to physiological inhibitory activities was then examined by kinase inhibition assays, using boiled extracts from proliferating normal osteoblasts, osteosarcoma, and HeLa cells. As shown in Figure 6B, phosphorylation of pRB was only moderately inhibited by proliferating normal or transformed osteoblasts. However, p107 phosphorylation was strongly inhibited by the osteoblast-derived extracts especially those from osteosarcoma cells (Fig. 6C). Notably, the inhibition profile of p107 phosphorylation was very similar to that of the endogenous 105 kD

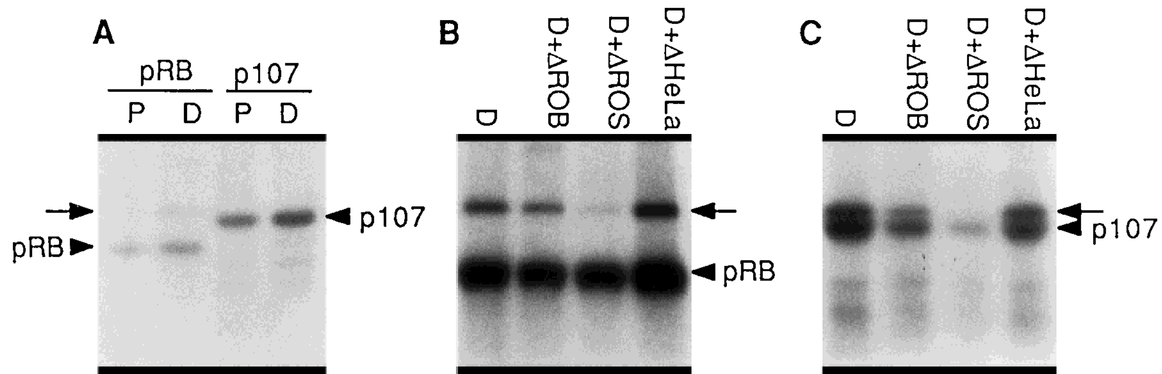

Fig. 6. *diff.ECx* kinase activity on RB family proteins and sensitivity to *diff.ECx*-inhibitory activity. **A:** Immunoprecipitation of cyclin E complexes from proliferating (P) or differentiated (D) osteoblasts as well as kinase assays were performed as in Figure 1B, except Histone H1 was substituted for by 1.5 μg of the GST-fusion pocket domain of either pRB (p105) or p107 (see Materials and Methods). Phosphorylation levels obtained with non-specific IgG were negligible (not shown). **B:** Immunoprecipitations and kinase assays on the GST-pRB pocket were as in A, lane 2, in the absence (D) or presence of the supernatant obtained after 5 min boiling of 0.5 mg extracts from normal proliferating osteoblasts (D+ΔROB), proliferating ROS 17/2.8 osteosarcoma cells (D+ΔROS), or proliferating HeLa cells (D+ΔHeLa). **C:** Same as B, except that the GST-p107 pocket was used as substrate. The positions of the GST-RB (pRB) and the GST-p107 (p107) recombinant proteins, as well as the endogenous 105 kD substrate (arrows), are indicated.

protein (Fig. 6C). Thus, both pRB and p107 are potential physiological substrates for the *diff.ECx kinase* activity, but only p107 phosphorylation is sensitive to *diff.ECx-i* activity from proliferating osteoblasts.

Differentiation Related Cyclin E Associated Kinase Activity Is Inhibited by $p21^{CIP1/WAF1}$

It has recently been suggested that the p107 RB-related protein and the cdi $p21^{CIP1/WAF1}$ bind to cyclin E/cdk2 complexes in a mutually exclusive manner [Zhu et al., 1995]. Because p107 mimicked the endogenous 105 kD substrate in the inhibition assay (Fig. 6C), we suspected that inhibition of phosphorylation of the 105 kD endogenous substrate by proliferating cell extracts (Fig. 3) may be attributable to $p21^{CIP1/WAF1}$, which is highly abundant in proliferating osteoblasts (data not shown). Indeed, Figure 7 demonstrates that recombinant $p21^{CIP1/WAF1}$ inhibits phosphorylation of the 105 kD endogenous substrate in cyclin E complexes of differentiated osteoblasts, suggesting that $p21^{CIP1/WAF1}$ in proliferating osteoblasts may contribute at least partially to the inhibition of the *diff.ECx* kinase activity of the differentiated cells.

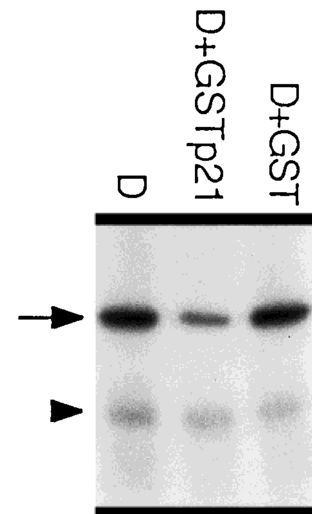

Fig. 7. Inhibition of *diff.ECx* kinase activity by $p21^{CIP1/WAF1}$. *Diff.ECx* kinase assay was performed in the absence of exogenous substrates as in Figure 1C. Following pre-clearing, the cell extracts were incubated for 30 min at 4°C without (left lane) or with either 2 μg of GST-p21 (middle lane) or GST as control (right lane). The arrow and the arrowhead indicate the 105 and the 78 kD endogenous substrates, respectively.

DISCUSSION

We propose the existence of a regulatory system in which inhibitory activity residing in proliferating osteoblasts is preferentially targeted towards a post-proliferative cyclin E associated kinase activity observed in differentiated cells.

Upon differentiation of normal diploid osteoblasts, upregulation of nuclear cyclin E is accompanied by increased associated kinase activity. The differentiation related cyclin E complexes, designated *diff.ECx,* are functionally distinct from cell cycle related cyclin E complexes. Cyc-

lin E immunocomplexes of differentiated osteoblasts exhibit increased phosphorylation of two endogenous substrates of 78 and 105 kD (Fig. 1B,C), as well as preferential phosphorylation of exogenous retinoblastoma susceptibility protein pRB and the p107 RB related protein (Fig. 6A). Unlike the RB family members, exogenous histone H1 was similarly phosphorylated by cyclin E complexes of proliferating and differentiated cells (Figs. 1B, 3A). Differentiated osteoblast cyclin E associated kinase activity was not recovered by cdk2 or cdc2 immunoprecipitations (Fig. 2), indicating either lack of or configurational alteration of these cell cycle kinases in the *diff.ECx.* Although postproliferative upregulation of D- and E-type cyclins has been reported in a number of differentiation systems [Miyajima et al., 1995; Kranenburg et al., 1995; Rao and Khotz, 1995; Kiyokawa et al., 1994; Yan and Ziff, 1995; Dobashi et al., 1995; van Grunsven et al., 1996] this report is, to our knowledge, the first describing differentiation related increase in cyclin E-associated kinase activity.

Proliferating normal diploid osteoblasts exert heat resistant inhibitory activity (*diff.ECx-i*) on the differentiation related cyclin E associated kinase activity. The inhibition is preferential on phosphorylation of the co-immunoprecipitated 105 kD endogenous substrate and on phosphorylation of exogenous p107 RB related protein. The inhibitory activity cannot be retrieved from cyclin E complexes of proliferating cells (Fig. 4A) but can be retrieved from cyclin E complexes immunoprecipitated from combined extracts of proliferating and differentiated cells (Fig. 3C). Interestingly, no increase in the heat resistant *diff.ECx-i* activity occurs upon boiling of the proliferating cells (Fig. 3A,B). This is unlike the activity of known cyclin dependent kinase inhibitors (cdi's), which is augmented by boiling indicating sequestration by heat labile factors [Dulic' et al., 1994; Polyak et al., 1994; Slingerland et al., 1994; Hengst et al., 1994]. Hence, the inhibitory activity residing in proliferating osteoblasts is readily available and preferentially recruited by the differentiation related cyclin E complexes.

Strong *diff.ECx-i* activity is exerted by proliferating ROS 17/2.8 osteosarcoma cells. Similar to *diff.ECx-i* activity of proliferating normal osteoblasts, this inhibition of *diff.ECx* kinase activity is preferential on the phosphorylated 105 kD endogenous substrate and the p107 RB related protein (Figs. 4, 6). However, unlike the inhibitory activity residing in proliferating normal diploid osteoblasts, inhibitory activity that resides in proliferating ROS 17/2.8 osteosarcoma cells can be retrieved from cyclin E immunoprecipitates (Fig. 4). This result may be related to co-expression of proliferation and differentiation related genes in ROS 17/2.8 osteosarcoma cells [van den Ent et al., 1993; Bortell et al., 1993]. Interestingly, in contrast to differentiated osteoblasts, substantial *diff.ECx-i* activity persists in density induced growth inhibited ROS 17/2.8 cells (Figure 5) in which several bone related genes are upregulated but development of bone cell phenotype is impaired [van den Ent et al., 1993; Bortell et al., 1993].

Whether the 105 kD endogenous substrate phosphorylated by *diff.ECx* is related to the RB family remains to be explored. Although both pRB and the p107 RB related protein, much like the endogenous 105 kD substrate, were preferentially phosphorylated by cyclin E complexes derived from differentiated as compared to proliferating cells (Fig. 6A), only p107 was strongly inhibited by *diff.ECx-i,* exhibiting the same inhibition profile as the endogenous substrate using a panel of *diff.ECx-i* sources (Fig. 6B,C).

The inhibition of *diff.ECx* kinase activity by p21$^{CIP1/WAF1}$ (Fig. 7), the higher p21$^{CIP1/WAF1}$ representation in proliferating compared to differentiated osteoblasts, and the partial loss of *diff.ECx-i* activity following immunodepletion of p21$^{CIP1/WAF1}$ (data not shown) suggest contribution of p21$^{CIP1/WAF1}$ to *diff.ECx-i* activity. This possible new role for p21$^{CIP1/WAF1}$ may contribute to an understanding of several reported observations, which are apparently inconsistent with the implicated function of this inhibitor in induction of growth arrest and terminal differentiation. These observations include: (1) presence of p21$^{CIP1/WAF1}$ in cycling cells, and its upregulation when quiescent cells are induced to proliferate [Sherr and Roberts, 1995; Firpo et al., 1994; Li et al., 1994; Noda et al., 1994; Nourse et al., 1994; Sheikh et al., 1994]; (2) downregulation during contact inhibition of normal human fibroblasts [figure 6C in Hengst and Reed, 1996]; (3) temporal upregulation of p21$^{CIP1/WAF1}$ in murine erythroleukemia cells induced to differentiate, followed by a decrease to uninduced levels when the cells undergo several last cell cycles and terminal differentiation [Macleod et al., 1995]; (4) in contrast to (3)

above, senescence was reported to be accompanied by upregulation of p21$^{CIP1/WAF1}$ [Dulic' et al., 1994; Noda et al., 1994]. These observations may now be interpreted in the light of the possibility that p21$^{CIP1/WAF1}$ may function not only in relation to periodical kinase activities during the cell cycle, but also to prevent premature differentiation-related cyclin associated kinase activities, such as *diff.ECx* activity of differentiated osteoblasts.

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