We demonstrate that p107 and p130 immune complexes exhibit kinase activity. We have tested such immune complexes with four substrates commonly utilized to assay Cdk activity, including all three known members of the retinoblastoma family. Immunodepletion revealed this kinase activity could be abolished by removal of either cyclin A or Cdk2 but was unaffected by removal of Cdk4 or any D-type cyclin. The appearance of p107 associated activity followed the accumulation of p107 protein. In contrast, the kinase activity associated with p130 immune complexes became apparent only after mid-G1, coincident with p130 hyperphosphorylation. GST-Rb, GST-p107, and GST-p130 (where GST indicates glutathione S-transferase) were equally suitable substrates in p107 and p130 immune complex kinase assays, yielding activity equal to 25% of the cyclin A activity present. The p107 and p130 associated activity was unable to phosphorylate histone H1, suggesting the p107 and p130 associated cyclin A/Cdk2 may represent a distinct pool with a distinct substrate specificity. The p107 and p130 associated activity was released from the immune complexes upon incubation with ATP and Mg2+ and exhibited the same substrate preference observed with the untreated immune complex. Our data suggest that p107 and p130 recognize, or form by association, a distinct pool of cyclin A/Cdk2 that preferentially phosphorylates retinoblastoma family members.

The retinoblastoma family of proteins includes three family members, pRb, p107, and p130. The major similarity among these proteins is a core region, termed the "pocket," consisting of two homologous domains, A and B, separated by a variable spacer region. The pocket region dictates interactions of Rb family members with a variety of viral oncoproteins and cellular proteins, including members of the E2F transcription factor family (1), cyclin/Cdkks (2–5), and c-Myc (6).

pRb was the first protein identified in this group and, consequently, is characterized in more detail than p107 and p130. The importance of pRb in decisions concerning cell fate has been documented with biochemical and genetic evidence as a key element in a regulatory pathway that includes cyclin D1/Cdk4 (7) and E2F (8, 9). It is well established that Rb frequently mutated in a variety of human cancers (10–13); these loss of function alterations gave rise to the notion pRb functioned as a growth suppressor. Such a role has been substantiated through studies in which ectopic expression or microinjection of pRb resulted in growth arrest (14–16). Rb-mediated growth inhibition occurs, at least partly, through sequestration of the E2F family of transcription factors, preventing transcriptional activation of E2F target genes, such as cyclin E (reviewed in Ref. 17).

p107 and p130 may also function in a manner similar to pRb, and all three share the ability to inhibit the growth of Rb−/− human osteosarcoma cells (18–20). All of these proteins have been found to exist in multiple states of phosphorylation, which dictates their ability to interact with E2F targets and correlates with their ability to function as growth inhibitors (21–24). Although the Rb family members all seem to regulate E2F, each member appears to have a distinct binding pattern with respect to different E2Fs (21, 25–28), an idea that is consistent with the appearance of specific complexes during cell cycle traverse. p130 exhibits association with E2F-4 during quiescence, which is lost upon mitogenic stimulation as a result of p130 phosphorylation. In such stimulated cells, E2F-4 is predominantly associated with p107, as p107 is induced during late G1 and S phase (20, 21, 25, 27–29). Unlike E2F-4, which appears to remain constant during cell cycle traverse (30), E2F-1 and -3 are not induced until cells approach G1/S (27, 30). Although p107 and p130 associate with E2F-4 and -5 (20, 30, 31), pRB is found to predominantly associate with E2F-1–3 (20, 26, 31). The association of E2Fs with p107 and the ability of cyclin/Cdkks to phosphorylate one or both of these members has been postulated to serve as the basis for continuously monitoring and regulating E2F-dependent transcriptional activity (32).

In this study we have demonstrated that cyclin A, which has been found to be the major cyclin partner for p107 (30–33), is also the major cyclin partner for p130 in stimulated Balb/c 3T3 cells. Furthermore, we also demonstrated that although cyclin A immune complexes can phosphorylate both histones and GST-Rb very efficiently, the p107 and p130 associated kinase is unable to efficiently utilize histone H1 as a substrate. Therefore, we conclude that there may be distinct cyclin A complexes that exhibit substrate specificity that may be distinguished by their association with p107 or p130.

MATERIALS AND METHODS

Cell Culture—BALB/c 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. Density-arrested quiescent cultures were prepared as described previously (34). Primary cultures of mouse keratinocytes were established from newborn ICR mice as described (35). Keratinocytes were cultured in KGM (Clonetics, San Diego), containing 0.05 μM insulin (Clonetics), 0.05 μM hydrocortisone (Sigma), 1.5 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA, receptor grade), 7.5 μg/ml bovine pituitary extract (Clonetics), and

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against a C-terminal peptide), and anti-Kip (36). Western blotting.

Antibodies utilized in this study were anti-p107 (sc-318), anti-p130 (sc-317), anti-cyclin A, a kind gift from Ed Leof (Mayo Clinic), anti-Cdk2 (directed against a C-terminal GST-Cdk2 fusion protein), anti-Cdk4 (directed against a C-terminal peptide), and anti-ß-catenin (31). The demonstration that p107 can be found in complexes associated with Cyclin A and Cdk2 (29) led us to examine if either p107 or p130 complexes exhibited kinase activity. We tested for this possibility by conducting kinase assays on p107 or p130 complexes exhibiting kinase activity, demonstrating the activity was specific for p107 and p130 (Fig. 1A). Parallel immunoprecipitations with appropriate antibodies was performed on 200 µg of protein followed by p107 analysis with Western blotting as described above, except we utilized protein A-horseradish peroxidase (1:2000 dilution in PBS) as a secondary antibody. Cell lysates, various cyclins and Cdk's (Fig. 2) were prepared by conducting immunoprecipitation on naive cell extracts with 1–2 µg of the appropriate antibody. Resulting immune complexes were harvested on protein A agarose beads, and the supernatant fraction was removed and subjected to a second immunoprecipitation with p107 or p130 antibodies. We have previously demonstrated this protocol results in the removal of D1, D3, and Cdk4 (36).

**RESULTS**

The demonstration that p107 can be found in complexes containing cyclin A and E2F-4 (30–33) and p130 associates with cyclins A and E (22, 29, 37) led us to examine if either p107 or p130 complexes exhibited kinase activity. We tested for this possibility by conducting kinase assays on p107 or p130 immune complexes using GST-Rb as a substrate, and we found immune complexes of both proteins exhibited kinase activity (Fig. 1A). Parallel immunoprecipitations with p107/p130-specific serum blocked with immunizing peptide did not yield complexes exhibiting kinase activity, demonstrating the activity was specific for p107 and p130 (Fig. 1A). To determine whether the p107/p130 associated activities displayed cell cycle-specific regulation, we examined immune complexes prepared from density arrested cultures of Balb/c 3T3 fibroblasts were stored frozen. For analysis of suspension cultures, confluent 3-day-old primary mouse keratinocytes were refed 12–15 h before cells were induced to undergo differentiation using suspension in semi-solid medium in 50-ml flasks coated with 0.4% polyHEMA (35). Cells in semi-solid medium were diluted 10-fold with PBS (4 °C) and pelleted by centrifugation. Frozen pellets were thawed on ice, resuspended in immunoprecipitation buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 1 mM EGTA, 0.1 mM orthovanadate, 0.5 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 µg/ml leupeptin, and 1 mM DTT), and sonicated for 5 s. Following centrifugation to remove cellular debris, protein concentrations were determined by the Bradford assay. The crude cell extract (20 µg) was boiled 3 min in Laemmli buffer and incubated on 11% discontinuous SDS-polyacrylamide gels, and the separated proteins were transferred to nitrocellulose membrane by electroblotting. Nonspecific binding was prevented by blocking the membrane in BLOTTO (5% dry milk in 1× phosphate-buffered saline/Tween 20 (PBS-T; 1× PBS, 0.1% Tween 20) and incubated with the primary antibody (1:1000 dilution in PBS-T) for 1 h at room temperature. After washing in PBS-T, the membranes were incubated with anti-rabbit-horseradish peroxidase (1:10,000) for 1 h, washed, and visualized by enhanced chemiluminescence, as recommended by the supplier. To identify p107 in specific cyclin complexes, immunoprecipitation with appropriate antibodies was performed on 200 µg of protein followed by p107 analysis with Western blotting as described above, except we utilized protein A-horseradish peroxidase (1:2000 dilution in PBS) as a secondary antibody. Cell lysates, various cyclins and Cdk’s (Fig. 2) were prepared by conducting immunoprecipitation on naive cell extracts with 1–2 µg of the appropriate antibody. Resulting immune complexes were harvested on protein A agarose beads, and the supernatant fraction was removed and subjected to a second immunoprecipitation with p107 or p130 antibodies. We have previously demonstrated this protocol results in the removal of D1, D3, and Cdk4 (36).
Altered Specificity of Cyclin A/Cdk2

Figure 2. p107 and p130 are associated with active cyclin A in proliferating cells. Density-arrested Balb/c 3T3 cells were stimulated with 10% serum and 10 ng/ml PDGF-BB. After stimulation for 24 h, extracts were analyzed for in vitro Rb kinase activity. To perform depletions, lysates were first subjected to immunoprecipitation (“Materials and Methods”) with the indicated antibodies. After addition of protein A-agarose, immune complexes were harvested by centrifugation, and the supernatant fraction was removed to a new tube and subjected to a second immunoprecipitation with either anti-p107 or anti-p130, as indicated and then assayed for Rb kinase activity as described under “Materials and Methods.” A, p130 associated kinase activity was measured as described above. B, p107 associated kinase activity was measured as described above.

Since multiple cyclin/Cdk associations have been identified for both p107 and p130, we performed immunodepletions to identify those cyclins responsible for the immune complex associated activity we observed. It is clear that depletion of cyclin A or Cdk2 resulted in quantitative removal of p130 associated activity (Fig. 2A), whereas depletion of D-type cyclins, the Cdk inhibitors p27kip1 and p21Cip1, or Cdk4 had no discernible effect. This result is consistent with previous studies that demonstrated p130 is associated with cyclin A/Cdk2 (22, 29, 37).

We also identified the cyclin partner associated with p107 Rb kinase activity in a similar manner and found immunodepletion of cyclin A, but not D-type cyclins, resulted in the removal of p107 kinase activity (Fig. 2B). These results are consistent with several reports demonstrating the association of p107 and cyclin A (30–33). The activity profile for the induction of p107 associated kinase, observed after mitogenic stimulation of density arrested Balb/c 3T3 cells, is almost identical to the cyclin A activity profile (Fig. 3A), and both proteins are induced with similar kinetics (Fig. 1C). To determine the amount of p107 that was associated with cyclin A, we immunoprecipitated cyclins A, D1–3, and E, separated the proteins within the immune complex by SDS-polyacrylamide gel electrophoresis, and determined the amount of p107 associated with the cyclins by Western blotting. As shown in Fig. 3B, approximately 25% of the total p107 is bound to cyclin A in extracts from proliferating fibroblasts, and a very small amount is bound to cyclins E and D3.

Cyclin A kinase activity assays were performed using either GST-Rb or histones as substrates, and these substrates showed similar induction of activity (Fig. 3A). When the kinase activity found in immune complexes of cyclin A, p107, and p130 was compared, utilizing GST-Rb as a substrate, p107 and p130...
Altered Specificity of Cyclin A/Cdk2

FIG. 4. p107 and p130 are unable to efficiently phosphorylate histones. Density-arrested Balb/c 3T3 cells were stimulated with 10% serum and 10 ng/ml PDGF-BB. A, after stimulation for 24 h, cell extracts were assayed for the presence of cyclin A, p107, and p130 associated kinase activities utilizing histones or GST-Rb as substrates. Rb kinase reactions were conducted with Rb-K buffer containing 10 mM ATP (final concentration). B, after stimulation for 24 h, cell extracts were assayed for the presence of p107 associated kinase activities utilizing GST-Rb as a substrate. p107 immunoprecipitates from stimulated cell extracts were preincubated for 5 min at 30°C with buffer (50 mM Tris, pH 7.5, 10 mM DTT) containing 50 mM EDTA (2nd lane), 10 mM MgCl2 + 10 mM cold ATP (3rd lane), or 10 mM MgCl2 (4th lane), and then pellets were washed once with 750 µl of kinase buffer, and Rb kinase assays were conducted. Kinase assay on sample in the 1st lane was performed without preincubation and extra washing steps.

Our data demonstrate, for the first time, that the association between p107 or p130 and cyclin A/Cdk2 contains active cyclin-Cdk components. A report describing kinase activity in p130 immune complexes has been recently published; however, the component(s) responsible for this activity were not identified.
Immunodepletion of all G1 cyclins revealed that removal of D-type cyclins did not have an effect on the p107 or p130 kinase activity. It is somewhat surprising that depletion of D-type cyclins did not affect the kinase activity of p107 or p130, since both contain the pocket region, which is known to interact specifically with D cyclin-Cdk4 complexes. It is possible that this association may be less stable than that with Cdk2 complexes. Preincubation with ATP in the presence of Mg2+ releases or inactivates a component(s) required for phosphotransferase activity; the appearance of activity in the supernatant of such washes suggests the former. Since the activity released from p107 and p130 complexes can be recovered specifically with cyclin A antibodies, we believe the activity recovered in p107 and p130 complexes is due to the presence of cyclin A/Cdk2. However, we cannot rule out the possibility that an unidentified kinase controlled by cyclin A/Cdk2 may be involved in the Rb kinase activity we have measured. A more detailed study of the protein complex is therefore being conducted. Our observations are consistent with the idea that this association of the components in the p107 and p130 immune complex reflects a transient enzyme/substrate interaction. We have not mapped the regions of p107 or p130 that are involved in these interactions, but it has recently been demonstrated that cyclin-Cdk2 complexes interact with p107 in a region distinct from the pocket, between residues 655 and 666 (33). Interestingly, a similar sequence is found in p130 between residues 633 and 644.

The p107 protein and associated kinase activity is induced during the same time frame that cyclin A protein and its associated kinase activity is induced. We have found that approximately 25% of the total p107 can be recovered in cyclin A immune complexes. The remaining p107 that can be obtained in p107 immune complexes from cyclin A-depleted extracts has no detectable kinase activity, thus the remaining p107 must be free of at least cyclin A-Cdk2 complexes. We do not yet know the relative abundance of other proteins known to bind p107, such as E2F and p21Cip1; however, it is certainly possible to conclude from the data we have presented that p107 may exist in distinct pools, perhaps with distinct regulatory functions. The p130 associated kinase, on the other hand, is activated 6 h prior to the p107, which has been previously demonstrated. However, the kinase activity from cells during S phase is clearly associated with cyclin-Cdk2 complexes and not depleted by D-type cyclins. It is possible, therefore, that the kinase activity associated with p130 could come from multiple cyclin/Cdk pairs, which are determined in a cell cycle-dependent manner.

Cdk2 complexes formed with either cyclin E or cyclin A have been found to efficiently phosphorylate either histone H1 or Rb protein, in vitro (39–42). It is clear from our work, however, that these specificities may be the aggregate activity of distinct cyclin A-Cdk2 complexes. This conclusion is based on our demonstration that histones are unable to serve as credible substrates for either the p107 or p130 associated activity. Therefore, it is likely that either cyclin A specificity is altered when p107 or p130 are associated with the complex or that these two proteins specifically interact with a subpopulation of Cdk2 complexes that do not have histone H1 kinase activity. We do not yet understand how such a shift in substrate specificity occurs. If cyclin A exists in more than one distinct complex, it is possible that in a subset of the complexes, the p107- or p130-containing portion, one of the associated proteins may confer the ability to exclude certain substrates like histones, perhaps...
as a result of steric hindrance. The kinase activity released from p107 and p130 immune complexes by incubation in the presence of ATP and magnesium, however, exhibits a substrate preference that is identical to the untreated immune complex. Thus, if the differential substrate specificity is the result of steric hindrance, the excluding factor must also be released in the ATP/Mg²⁺ wash. Definitively ruling out this possibility awaits more defined studies with well characterized Cdk2 substrates, which are less likely to be subject to such a problem. At first glance, our findings would seem to contradict those of Mayol et al. (23), who demonstrated that p130 associated complexes could phosphorylate histones. We also found that p107 and p130 could phosphorylate histones, but the activity is 50-fold less when compared with cyclin A/Cdk2, whereas p107 and p130 phosphorylate GST-Rb almost as well as cyclin A/Cdk2.

We note two recently published studies that have revealed parallel observations made for Cdk activity (43, 44). Both studies demonstrated a marked substrate preference for Cdk7-cyclin H complexes that depended on whether it was assayed as a “free” complex or bound to TFIIH, a multi-subunit basal transcription factor containing Cdk7 as one of its subunits. The TFIIH bound Cdk exhibited a preference for an RNA polymerase II C-terminal domain-derived substrate, whereas free Cdk exhibited a preference for Cdk2. These reports and our study demonstrate that Cdk activity may be regulated by substrate binding and illustrate how Cdk substrates may participate in dynamically modulating Cdk activity.

According to current models, G₁ and G₂/S control is mediated through interactions between Rb family members and E2Fs, which in turn is regulated by Cdk activity. An important aspect of this circuit is a release of E2F-4 from p130 as cells leave quiescence and undergo G₁ traverse. We have demonstrated that the association of p130 with an active kinase begins at 6 h after stimulation and persists, a pattern that is consistent with the appearance of the hyperphosphorylated form. The p107 protein and its associated kinase activity, in contrast, does not appear until S phase, at which time it also associates with members of the E2F family (20, 21, 25, 27–29). It has been suggested that one function served by independent binding domains for E2F and cyclins may be to cause dissociation of the E2F from p107 under conditions that support continued proliferation (33). We have considered the possibility that E2F immune complexes may also bind cyclin Cdk complexes utilizing p107 as a bridging molecule; however, we found a very low level of associated activity.

Our data reveal that the interactions that have been found between cyclin/Cdks and at least one important physiological class of substrates, members of the Rb family, are sufficiently stable to allow isolation of active kinase activity from p107 and p130 complexes. Although all the data we have presented is consistent with the notion that the kinase activity is isolated as a “trapped” enzyme/substrate interaction, we cannot conclusively exclude the possibility that p130 or p107 are integral members of the active complex and, as we have shown, modify the cyclin A/Cdk2 activity. Nonetheless, further examination of the activity before and after release from the immune complex and characterization of the proteins that are involved in the activities we have observed, as compared with those present in the remaining cyclin/Cdk pool(s), should allow interesting

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3 P. J. Hauser and W. J. Pledger, unpublished observations.