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AWARD NUMBER DAMD17-96-1-6092

TITLE: Biochemical Characterization of Complexes with p21, a CDK Inhibitor

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REPORT DATE: August 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1997	Annual (1 Aug 96 - 31 Jul 97)

4. TITLE AND SUBTITLE Biochemical Characterization of Complexes with p21, CDK Inhibitor

5. FUNDING NUMBERS
DAMD17-96-1-6092

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

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13. ABSTRACT *(Maximum 200*

The cyclin-dependent kinases (CDKs) are of pivotal importance for driving cell proliferation. The p21/WAF1 protein, by contrast, belongs to a family of growth regulators that are thought to restrain cell growth primarily as a consequence of their ability to inhibit CDKs. In vivo, p21 is known to form stable, higher order complexes with cyclin-dependent kinases and the DNA polymerase processivity factor, PCNA. As an important downstream target of the p53 checkpoint gene, p21 is thought to execute a critical growth arrest function in response to DNA damage and other cellular stress and differentiation signals. In addition, loss of p21 function may be associated with cellular transformation in some settings. Despite this information, it is not yet clear how p21 functions in vivo to regulate the multiple complexes with which it associates. Nor is it clear how p21 specifically regulates CDKs in vivo or whether all components of p21 complexes have been identified. Given the role of p21 and related proteins in kinase inhibition and growth control, a full understanding of the mechanisms used by this protein to restrain proliferation may yield clues toward the therapeutic intervention in breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, cell cycle, cyclin-dependent kinases (cdks), p21, growth control, cdk inhibitor, transformed cell	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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5. INTRODUCTION

Nature of problem/Background of previous work

The mammalian cell cycle is controlled in part by the periodic activation of specific cyclin-dependent kinases (cdks) at appropriate times. Cdk activation requires an association with a regulatory, or cyclin, subunit. At least five of these cyclins identified in mammalian cells (cyclins C, D1, D2, D3, and E) have been shown to function during G1 phase in specific combinations with cdk2, cdk4, and cdk6. In certain settings, the activities of the D- and E-type cyclins are essential for entry into S phase (reviewed in (Sherr and Roberts, 1995). The activity of cyclin A- and cyclin B-dependent kinases appears later in the cell cycle, during and subsequent to S phase. Each of the cell cycle kinases is proposed to function by phosphorylating critical substrates required for transitions through the cycle. One critical physiologic target of the G1 kinases cyclin D/cdk4 and cyclin D/cdk6 is believed to be the retinoblastoma (pRB) tumor suppressor protein, which has been shown to negatively regulate the E2F transcription factor. E2F itself is believed to play a critical role in cell proliferation by activating transcription of genes required for S phase entry (reviewed in (Hinds and Weinberg, 1994). Phosphorylation of pRB by these kinases, and possibly also kinases associated with cdk2, is believed to abolish its growth suppressive properties by preventing its interaction with E2F and other transcription factors that promote entrance into S phase.

Several factors, in addition to cyclin binding and post-translational modification, tightly control the activity of these cyclin-dependent kinases during the cell cycle. Association with cdk inhibitors is the most recently described level of cyclin/kinase regulation.

At least seven cdk inhibitors (CKIs), termed according to their molecular weights (p15, p16, p18, p19, p21, p27, and p57) have now been identified. They have been classified into two groups based on sequence homology and inhibitory specificity. One group, consisting of p21 (also known as Cip1 or WAF1; for simplicity, it will be called p21), p27, and p57, appears to inhibit a wide range of kinases, while p15, p16, p18, and p19 (collectively referred to as inhibitors of cdk4, or INK4 proteins) appear to inhibit the activity of cyclin D/cdk4 and cyclin D/cdk6 kinases specifically. CKIs appear to be components of multi-protein complexes in normal cycling cells. For example, in normal diploid human cells, p21 has been found in quaternary complexes that contain proliferating cell nuclear antigen (PCNA; a processivity factor associated with the DNA replication/repair enzyme DNA polymerase δ) and cyclin/cdk pairs. Interestingly, transformation by SV40 promoted profound rearrangement of these complexes (Xiong et al., 1993). In transformed cells, cdk4 no longer associated with cyclin D/PCNA/p21 complexes and instead associated exclusively with the p16 protein. Likewise, cyclin B/cdc2 and cyclin A/cdk2 complexes with PCNA and p21 were also disrupted. This pattern of rearrangement also occurred in cells transformed by other viral oncoproteins (encoded by adenovirus and papillomavirus), suggesting that cellular transformation might occur as a consequence of incorrect formation of cyclin/kinase/PCNA/CKI complexes. These data suggested that one pathway toward oncogenesis may involve alteration of the cell cycle machinery, although the exact biochemical consequences of this rearrangement are not known. Thus, detailed biochemical studies of the causes of these alterations may shed light on the mechanisms of transformation.

Several additional findings indicate that CKIs could potentially play a pivotal role in cell cycle progression and oncogenic transformation. First, each of these inhibitors can promote G1 phase arrest when overexpressed in certain tissue culture cell lines (reviewed in (Sherr and Roberts, 1995). Second, G1 arrest of certain human epithelial cells by the cytokine transforming growth factor-β (TGF-β) may be explained by the induced synthesis of p15, implicating this CKI as an effector of cell cycle arrest (Hannon, 1994). Third, in some settings, induction of p21 expression by certain agents correlates with the differentiation of human cell lines (Steinman et al., 1994). Finally, p16 expression can suppress neoplastic transformation of fibroblasts by H-ras and c-myc (Serrano, 1995). Although the CKIs do show some binding and inhibitory preferences in vitro, it has not yet been possible to distinguish any biochemical differences within the INK4 and p21/p27/p57 families of proteins. Thus, it is not clear why there are so many inhibitors and whether these proteins serve redundant functions. Such redundancy may help explain why mice lacking one CKI, p21, are viable and are not prone to tumorigenesis at an early age or to developmental defects, although the ability of p21-deficient cells to execute the p53-mediated G1 checkpoint is clearly compromised (Deng et al., 1995).

p21 inhibits with high affinity all kinases known to have a direct role in the G1 to S phase transition, including cdk2, cdk3, cdk4, and cdk6 (Harper et al., 1995). However, numerous studies suggest that p21 may carry out additional functions in growth control other than by acting as a kinase inhibitor. First, using an SV40-based DNA replication system, it was shown that p21 could block the ability of PCNA to stimulate the processivity of DNA polymerase δ ((Flores-Rozas et al., 1994); (Waga et al., 1994). Second, the cdk-inhibitory domain of p21, at concentrations similar to that of cyclin E/cdk2, could also effectively block DNA replication in *Xenopus* extracts (Chen et al., 1995). Third, previous studies suggested that p21 can exist in active kinase complexes and that low concentrations of p21 could reproducibly promote cyclin/kinase complex formation; the kinase activity of these complexes could, however, be abolished by the further addition of p21, suggesting that kinase inhibition required the binding of two p21 molecules (Zhang et al., 1994); (Harper et al., 1995). However, these studies did not rigorously address the specific activity of cellular p21/cyclin/CDK complexes, and definitive proof that such complexes are active is lacking. Fourth, in non-transformed cells, p21 is found in several different complexes that contain combinations of PCNA and cyclins A, B, D, and E with cdc2, cdk2, and cdk4. The function of each of these individual complexes is currently unknown.

p21 has also recently been studied in structural detail. Site-directed mutagenesis coupled with protein binding and activity assays suggests that p21 has two separate domains (Goubin and Ducommun, 1995); (Chen et al., 1995); (Chen et al., 1996); (Gulbis et al., 1996). An amino-terminal domain (residues 1-80) binds to and inhibits cyclin/CDK complexes, and a carboxy-terminal domain (residues 139-160) binds to PCNA and may suppress DNA replication. The N-terminal domain of p21 contains both cyclin-binding (residues 10-40) and CDK-binding (residues 40-60) regions and is highly homologous to the N-terminal region of p27, a closely related Kip/Cip family member. The structure of a ternary complex containing cyclin A, cdk2, and a p27 fragment has been solved (Russo et al., 1996). This crystallographic study suggests that a region of p21 between residues 74-79 (homologous to p27 residues 85-90) may be deeply buried in the catalytic cleft of CDK, occupying the ATP-binding site and playing a critical role in inhibition of CDK activity. Interestingly, this study also confirmed the structural importance of a cyclin-binding motif

in p27 ("LFG" amino acid motif) that is conserved in p21 and p57, as well as the pRB-related proteins p107 and p130 (Zhu et al., 1995). This domain in p107 is required for binding cdk2 complexes with cyclin A and cyclin E, inhibition of these associated kinases, and in some settings, growth suppression (Zhu et al., 1995).

By contrast, free p21 lacks stable secondary or tertiary structure in solution and undergoes a sharp transition to adopt an ordered, stable conformation upon associating with CDKs (Kriwacki et al., 1996). These observations would explain how a highly flexible p21 molecule is capable of binding to and inhibiting several G1/S phase cyclin/CDKs, including cyclin D/Cdk4, cyclin E/Cdk2, and cyclin A/Cdk2.

The ability of p21 to associate with a repair/replication factor and with multiple, different cell cycle kinases that play an important role in growth control, as well as a possible connection between p21 and complex assembly (LaBaer et al., 1997), suggest the importance of a rigorous examination of p21 function. Once we fully understand the ways in which CKIs such as p21 function, we can envision potential therapeutic strategies for the treatment of mammary and other cancers.

Purpose of present work/Experimental approach

It is clear that many questions regarding p21 function have not been resolved. Indeed, despite our knowledge of the existence of numerous complexes with p21, a complete understanding of the function of these different complexes is lacking. For example, it is not known whether all proteins associated with p21 have been identified, nor is it clear whether different p21/PCNA/cyclin/kinase complexes possess distinct functions. To begin addressing these questions and others, we have undertaken a biochemical examination of the proteins associated with p21.

In summary, we proposed four specific goals:

1) To identify and characterize p21-containing complexes in normal and transformed human cells, including multiple breast carcinoma cell lines, during various stages of the cell cycle. The subcellular distribution of such complexes in different cell lines will be determined.

2) To identify novel cell cycle-regulated proteins associated with p21. Interesting candidates would be isolated by molecular cloning.

3) To identify transcription factors of the E2F family that associate with p21 and study the regulatory properties of such complexes. We proposed to simultaneously study regulation of cdk and E2F activity by the pRB-related protein p107 which bears some structural and functional homology to p21.

4) To examine a role for p21 in the assembly of cyclin-dependent kinase complexes.

6. BODY

In the past year, we have made progress in several of the specific areas listed above, and some of these novel findings, described below, have suggested that we concentrate on certain areas and put less effort into others. Recent rapid progress in the cell cycle field in general, and on the p21 CKI specifically, has provoked a slight adjustment to our original Statement of Work as described below.

1) Characterization of p21-containing complexes in normal and transformed human breast cells and sub-cellular localization

Our studies in this area have brought to light several interesting and unexpected findings that we are currently pursuing. First and foremost, our biochemical studies have begun to resolve an ongoing dispute in the cell cycle field, namely, do active kinase complexes containing p21 exist in the cell? More generally, how do the relative levels of p21, cyclins, CDKs, and PCNA in multimeric complexes correspond to total cyclin-dependent kinase activity, and how do differences in such levels relate to cell cycle progression in the normal and cancer cell?

We have addressed this question as follows. Using several of our now well-characterized anti-p21 monoclonals, first described in our Preliminary Results section of the Research Proposal (and in (Dynlacht et al., 1997), we have immunoprecipitated p21-associated cyclin-dependent kinases from the breast carcinoma cell line MCF-7 and the normal fibroblast line WI38. We performed parallel immunoprecipitations (IPs) with anti-cyclin A, anti-cdk2, and anti-cyclin D antibodies in order to directly compare total kinase activity associated with a specific amount of cdk2 or cyclin D/cdk4. Amounts of immunoprecipitated protein and the resulting activity were judged by western blotting and kinase assays, respectively (Figure 1). The results were quite striking and cast doubt on the notion that cellular cyclin A/cdk2- and cyclin D-associated p21 complexes have kinase activity. Although either equivalent (anti-cdk2 IPs) or reduced (anti-cyclin A IP) amounts of cdk2 protein were obtained relative to anti-p21 IPs, the kinase activity associated with either cyclin A or cdk2 was much greater. Furthermore, immunoprecipitation of cyclin A and cdk2 complexes subsequent to depletion of p21 complexes did not result in reduced kinase activity relative to the non-depeleted control. Upon extended autoradiographic exposure of similar gels, a weak kinase activity associated with p21 is apparent. However, the activity is not significantly higher than that obtained with an unrelated control antibody. These experiments suggest that cellular p21-associated kinases are inhibited.

We have preliminary data suggesting how p21 can associate with cyclin/Cdk complexes in the kinase active and kinase inactive states. Briefly, this study has utilized our anti-p21 monoclonal antibodies to study conformationally important epitopes on the p21 protein. In our previous studies (Dynlacht et al., 1997), we demonstrated that certain antibodies (CP2, CP13, CP49, CP59, and CP68) immunoprecipitated all known cellular complexes containing cyclins, CDKs, PCNA, and p21, while others (CP23, CP36) immunoprecipitated only cyclin A/cdk2/p21, and a third group (CP55) immunoprecipitated only free p21. Interestingly, anti-p21 immunoprecipitations with this latter antibody fail to pull down any p21 from MCF-7 cells, althought the antibody is capable of recognizing recombinant, bacterial p21 and there is an abundant amount of p21 in these cells (Figure 2A). We conclude that the epitope recognized by CP55 must be masked in vivo, since

release of p21-associated proteins from in vivo complexes with urea restored antibody recognition (Figure 2B).

More importantly, we have performed simultaneous functional assays to discriminate whether CP55 recognizes in vitro assembled complexes containing cyclin A/cdk2 and p21. In these preliminary experiments, CP55 recognized p21 associated with active cyclin A/cdk2 complexes (assembled in vitro using baculovirus-produced proteins), but the antibody failed to recognize inactive kinase complexes assembled with a slightly higher molar ratio of p21 to cyclin/cdk2 (data not shown). These data, although preliminary, suggest a model in which p21 can bind to cyclin A/cdk2 via the amino-terminal cyclin-binding domain. In this conformation, the kinase is active, and CP55 recognizes p21 associated with it. At higher concentrations of p21, a second p21 molecule could bind the complex. This might favor a conformational rearrangement (reminiscent of similar concentration-dependent conformational changes in certain enzymes; see (Cai and Schirch, 1996), leading to insertion of the CP55 epitope into cdk2, causing its inactivation. This model could account for the apparent lack of kinase activity in anti-p21 immunoprecipitations and the complete inability of CP55 to recognize complexed p21 in cell extracts. Previous studies suggested that the epitope recognized by CP55 maps between residues 55 and 80, and crystallographic studies of p27 suggest that an analogous region may be buried in the cdk2 protein (Russso et al., 1996). We are currently fine-mapping the epitope recognized by the CP55 antibody to further test this model.

Because we believe that the above results are of substantial significance in terms of understanding the function of p21 in normal mammary cells, we intend to expand this line of research and are currently investigating p21-associated kinase activity in a large number of breast carcinoma cell lines, using normal breast cell lines for comparision. To this end, we first screened a panel of normal and transformed human cell lines for the presence of p21 and associated polypeptides (Figure 3). This panel included normal fibroblasts (WI38; as well as the same cell line transformed with SV40, WI38-VA13), cervical carcinoma line C33A, leukemia HL-60, osteosarcoma Saos-2, and several lines derived from breast cancers (MDA-MB-231, MDA-MB-468, T47D, MCF-7, and Hs578T). Two normal mammary cell lines, MCF-10A and Hs578Bst (derived from normal mammary cells but from the same patient used to obtain the carcinoma line Hs578T) will be used in parallel studies. These experiments suggested that two breast carcinoma cell lines, T47D and MCF-7, had abundant amounts of p21, cdk4, and PCNA, while others (both MDA lines and Hs578T) had considerably less p21 (apparent on longer autoradiographic exposure than the ones shown in Figure 3A). It should be most interesting to compare these cell lines for the total kinase activities associated with cyclin A, cyclin D, and cyclin E (normalizing again for the amount of each cyclin and CDK) given the large differences in the amount of p21 present in each. In addition, one breast carcinoma cell line (T47D) has abundant amounts of p21, PCNA, cyclin D, and cdk4, yet these do not appear to associate with p21 in anti-p21 immunoprecipitations of metabolically labeled extracts (Figure 4). This suggests the possibility that at least one breast carcinoma cell line may exhibit rearrangements in p21 complexes, and we are currently pursuing this idea more extensively.

Thus far, we have put only a limited amount of effort into studying the subcellular localization of p21 complexes. In these experiments, we have consistently reproduced our initial findings that the p21 protein can be found in both nuclear and cytosolic fractions in

both normal fibroblasts and breast carcinoma cell lines. We intend to pursue one potentially interesting finding from these studies. In anti-p21 immunoblots of some normal cell lines, we observed a band that migrated with slightly lower mobility than recombinant p21 and cellular p21 (Fig. 3). This species, which may represent a form of p21 that has undergone phosphorylation (or another post-translational modification), was found exclusively in the nuclear fraction of cell extracts. We are intrigued by this reproducible observation and intend to continue these studies by examining p21 localization at different stages of the cell cycle and in cells that have undergone treatment with DNA-damaging agents such as UV, γ-irradiation, or drugs. In addition, we will use phosphatase treatments to determine whether the reduced mobility band derives from phosphorylation of p21.

We intend to characterize the activity and sub-cellular localization of p21-containing complexes in a wide array of normal breast cells and breast carcinoma tissues once our initial characterization has been completed. We believe that this is the best sequence of events because prior to our studies described above, an extensive characterization of p21-containing complexes had not been attempted. Furthermore, we are now confident that our anti-p21 monoclonal antibodies represent the most effective reagents available to achieve this characterization, having now fully tested each of our monoclonal antibodies as well as a large number of p21 polyclonal and monoclonal antibodies available both commercially and through our colleagues in the field.

2) **Identifying novel p21-associated polypeptides**

In our Research Proposal we demonstrated that certain p21 Mabs immunoprecipitated several polypeptides (from metabolically labeled cells) in addition to the full complement of previously characterized polypeptides. All known p21-associated proteins had been eliminated from consideration by immunoblotting and parallel immunoprecipitations. In addition, two putative novel polypeptides of 28 and 40 kDa appeared to peak in abundance as cells progressed through the G1/S transition, and both proteins co-fractionated with p21 in large complexes on glycerol gradients and sizing chromatography columns (data not shown). Despite these data, we were concerned that these proteins could simply represent proteins the spuriously cross-react with our monoclonal antibodies. To rule out this scenario, we have further fractionated endogenous complexes by ion exchange chromatography, a method entirely different from the sizing methods we used previously. We observed that p28 completely co-eluted with cyclin A- and cyclin B-CDK complexes and was cleanly separated from cyclin D-containing complexes (Figure 5).

We have performed additional experiments to further characterize p28 and p40. First, we screened several normal and transformed cell lines to understand a correlation between transformed state and the presence of these polypeptides. In initial studies, we did not detect either putative novel protein in two leukemia cell lines (HeLa and ML-1). However, the protein was clearly present in a number of breast carcinoma cell lines, and the p28 protein was most abundant in the T47D line (Figure 4A). Interestingly, in this cell line, there appears to be a much reduced association between cyclin D, PCNA, and p21, although these proteins are present in these cells (Figure 4A, lane 5). We are currently testing this correlation between the presence of p28 and decreased association between p21 and the cyclin D/PCNA complex.

A second breast carcinoma cell line, MCF-7, also displays a potentially interesting profile of unidentified polypeptides precipitated with p21 MAbs (Figure 4B). This cell line appears to contain a polypeptide of slightly reduced mobility (relative to p28) that is immunoprecipitated by a second p21 MAb, CP2. This protein, p29, is not apparent in the normal fibroblast line WI-38.

Although we have made progess in the area of identifying novel p21-associated proteins, our studies have been hampered somewhat by the extremely low abundance of such complexes in the cell lines we tested previously, including WI-38 and MCF-7. Small-scale pilot experiments aimed at immuno-purifying putative novel p21-associated proteins have failed to yield quantities sufficient for visualization by non-isotopic methods (data not shown). Therefore, we are currently screening multiple cell lines for ones that have optimal amounts of p28 and p40. Since T47D appears to contain very large quantities of the p28 polypeptide (Figure 4A), this might be a suitable cell line for largescale purification. Should we be successful in purifying large enough amounts of p28, p29, or p40, we would attempt to get peptide sequence and ultimately clone the cDNAs encoding each.

3) **Regulation of E2F family members associated with p21 and p107/p130**

We have made progress in this area of the research proposal, and although certain areas of this specific aim were not a major focus of the proposal, recent results suggest that this could become an original and productive avenue in the future.

First, we have unambiguously identified the E2F component that consistently associates with the p21 protein in cells. Although at least five E2F genes have been isolated from human cells, only one of these family members, E2F-4, was found to interact with p21 in vivo (Figure 6 and data not shown). Interestingly, this E2F family member has been shown to associate with the p107 and p130 proteins as well (see below).

The pRB-related proteins p107 and p130 are thought to suppress growth in part through their associations with two important cell cycle kinases, cyclin A/cdk2 and cyclin E/cdk2, and the transcription factor E2F. Although each protein plays a critical role in cell proliferation, the functional consequences resulting from the association between growth suppressor, CDK, and transcription factor, have remained elusive. In an attempt to understand the biochemical properties of such complexes, we reconstituted each of the p130/cyclin/cdk2 and p107/cyclin/cdk2 complexes found in vivo using purified, recombinant proteins. Strikingly, stoichiometric association of p107 or p130 with either cyclin E/cdk2 or cyclin A/cdk2 negated the activity of these kinases. Kinase inhibitory activity was dependent upon an amino-terminal region of p107 that is highly conserved with p130. Further, a role for this amino-terminal region in growth suppression was uncovered using p107 mutants unable to bind E2F. To determine whether cellular complexes might display similar regulatory properties, we purified p130/cyclin A/cdk2 complexes from human cells and found that such complexes exist in two forms, one that contains E2F-4/DP-1 and one that lacks this heterodimer. These endogenous complexes behaved like the in vitro reconstituted complexes, exhibiting low levels of associated kinase activity that could be significantly augmented by dissociation of p130. These experiments suggest a mechanism whereby p130 and p107, like the p21 family of CKIs, might

suppress growth by inhibiting important cell cycle kinases. These studies culminated in a recent publication (Woo et al., 1997).

It is important to point out that having performed this initial characterization in normal human fibroblasts and other convenient tissue culture cell lines, we will begin to examine whether the p21-E2F, p107/p130-E2F, and p107/p130-CDK pathways are intact in mammary carcinoma cells as well. Although loss of p107 or p130 has not been associated with human cancers, a recent study detected the loss of the p130 gene in a small cell lung carcinoma (Helin et al., 1997), and it remains to be shown whether these proteins are lost in breast tumors.

These findings also have several broader implications. First, they suggest that in addition to the p21/p27/p57 and p15/INK4 family of proteins, certain members of the retinoblastoma protein family also function as CKIs. This potential redundancy may help explain why p21 nullizygous mice do not develop tumors, since both sets of proteins inhibit identical CDK complexes, cyclin A/cdk2 and cyclin E/cdk2. Second, the fact that both p21 and p107/p130 target the same E2F family member suggests that the p21 and p107/p130 growth restraining pathways may interconnect. Indeed, we had found previously that p21 and p107 bind the kinases in a mutually exclusive way and that p21 can displace p107 from such complexes (Zhu et al., 1995).

Experiments designed to examine p130/cyclin/cdk2 and p107/cyclin/cdk2 complexes in normal and cancerous mammary cell lines, in conjunction with those described in Aim 1 above, may eventually help to elucidate whether a "buffering" model for cell cycle progression is indeed correct: Is the state of the cell cycle predominantly determined by the overall cyclin-dependent kinases activity in a cell? That is, does the cell progress past G1 only in the instance where the inhibitory activity contributed by various CKIs is overcome by excess cyclin-dependent kinases?

4) **Examining a role for p21 in assembly of cdk complexes**

We have chosen not to pursue certain of these studies in the current funding period because recent observations (LaBaer et al., 1997) suggested that in some settings, over-expression of p21 could indeed facilitate association with specific cyclins and cdks (cyclin D and cdk4). However, the observation has also been made by these authors that in p21 null mouse fibroblasts, such complexes still form, suggesting that the p21-related proteins (p27 and p57) could compensate for the loss of p21. An alternate possibility is that assembly of CDK complexes in their system results only through the over-expression of p21 and that such a role for p21 does not occur under normal growth conditions.

We have chosen instead to focus our efforts on thoroughly understanding the biochemical activity of p21-containing complexes, identifying all components of these complexes, and understanding whether the loss of any specific components of cellular p21 complexes leads to changes found in breast cancer. In this sense, we will pursue the idea that transformation can lead to disassembly of certain p21 complexes as outlined in Aims 1 and 2 above. This approach may shed light on a role, if any, for p21 in complex assembly in vivo.

7. CONCLUSIONS

We believe that our experiments have begun to shed light on the topology and activity of native, cellular p21 complexes. We have systematically generated and characterized a set of anti-p21 monoclonal antibodies that have been invaluable for our studies. Using these reagents, we have initiated a thorough biochemical characterization of p21-containing complexes in normal breast and breast carcinoma cell lines. We believe these studies will help us pinpoint the function of p21 in vivo.

In this series of experiments, we found that p21-associated cyclin-dependent kinase complexes found in vivo display markedly reduced kinase activity relative to kinases that are not associated with this CKI. This observation appears to contradict previous observations (Zhang et al., 1994; Harper et al., 1995) of active kinases associated with p21 in vitro and in vivo. However, it is important to point out two differences between our studies and previous ones. First, we are strictly looking at native, endogenous complexes, not those assembled in vitro. We have not ascertained how many molecules of p21 are bound to these complexes. Second, unlike previous studies, we have compared p21-bound kinases to kinases lacking p21 and have rigorously compared the specific activity of similar amounts of each using immunoblotting. These studies may be considered preliminary because only two cell lines have been tested. However, if correct, our findings will certainly address a question in the field that had not been previously resolved. Our ultimate goal will be to understand how the relative amounts of cyclins, CDKs, and CKIs, particularly p21, determine the kinase activity and growth state of normal and transformed cells.

Our studies aimed at purifying and characterizing cellular p21 complexes have also demonstrated that certain polypeptides appear to associate with large cyclin/CDK/p21 complexes in some cell lines but not others. For example, one putative novel protein, p28, may associate with cyclin A/CDK/p21 complexes but not with cyclin D containing complexes, as judged by chromatographic fractionation. This protein also sediments in large complexes in glycerol gradients and appears to peak in abundance as cells approach S phase, making it a potentially interesting candidate to pursue in future studies. Further, the MCF-7 cell line has a protein of slightly higher molecular weight, p29, that is not apparent in other normal cell lines. We have identified a breast carcinoma cell line (T47D) that contains extremely high amounts of p28 and other cell lines that contain very low amounts of p21 and p28. It will be interesting to determine if these alterations in protein levels change the configuration of p21-containing complexes, the kinase activity of associated cyclin/CDKs, and the dynamics of cell cycle progression.

Since our intended studies on the role of p21 in CDK assembly might only duplicate a portion of those recently published by LaBaer and colleagues, we propose to focus our efforts in the next funding period on other aspects of the proposal that are likely to lead to more original findings. We may understand a role, if any, for p21 in assembling CDK complexes by virtue of our studies in Aims 1 and 2 in which we attempt to look for different p21-containing complexes in a wide array of breast cancer cell lines. We have already found differences between complexes in normal and breast cancer lines, and we will continue to focus on understanding whether (and how) these complexes are perturbed in the process of cellular transformation.

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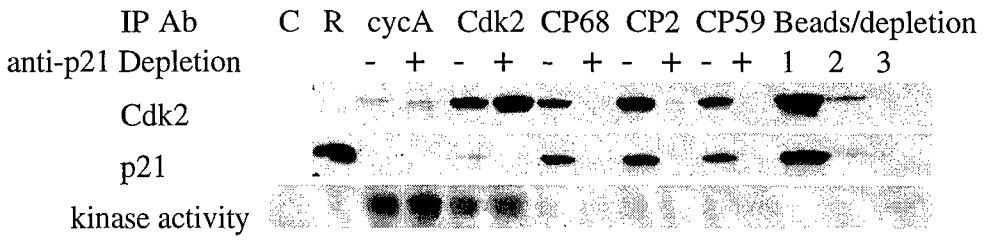


Figure 1. p21-bound cyclin/cdk2 is considerably less active than cyclin/CDK complexes lacking p21 *in vivo*. MCF7 lysate was split into two equivalent samples. One half was successively immunoprecipitated three times with CP68-protein A beads to deplete p21-containing complexes ("+" lanes and "beads/depletion, 1,2,3"). The last three lanes show the efficiency of p21 depletion. The second half was left undepleted. Immunoprecipitations were then performed on both depleted (+) and non-depleted (-) samples using different antibodies as labeled on the top panel. "C" is a control immunoprecipitation using a irrelevant MAb. "R" is a sample containing purified recombinant p21. Each immunoprecipitated sample was split and subjected to western blot analysis using anti-Cdk2 ("Cdk2") and anti-p21 ("p21", CP36) antibodies to normalize for the amounts of immunoprecipitated proteins and to kinase assays using histone H1 as a substrate ("kinase act."). From these data, it is clear that although cyclin A antibodies immunoprecipitate much less cdk2 than the anti-p21 antibodies, the associated kinase activity is greater than 10-fold higher, as judged by PhosphorImaging analysis. Similarly, cdk2 antibodies precipitate equivalent amounts of cdk2 protein compared to the anti-p21 samples, yet their associated kinase activity is also much greater than that associated with p21. Furthermore, if p21-associated kinase complexes did contribute kinase activity to cdk2 complexes in the cell, i.e., if they were still active kinases, the overall kinase activity of anti-cyclin A and anti-cdk2 immunoprecipitates would *decrease* after immunodepletion, and this is clearly not the case.

A

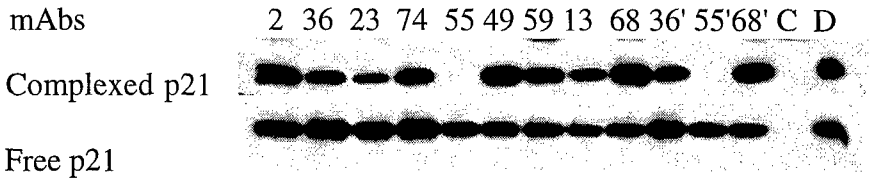


Figure 2A. An epitope recognized by Mab CP55 ("epitope 55") is buried in vivo. Immunoprecipitations were performed using MCF-7 or WI38-VA13 cell extracts ("complexed p21") or free, purified recombinant p21 ("free p21") with multiple MAbs as labeled above the top panel. Immunoprecipitations were performed in duplicate as denoted by " ' " next to each MAb. "C" is a control immunoprecipitation using an unrelated MAb. "D" is the direct western blot of the input samples. Immunoprecipitates were then electrophoresed and immunoblotted with anti-p21 MAb CP36. Note that CP55 immunoprecipitates recombinant p21 in a manner similar to other MAbs.

B

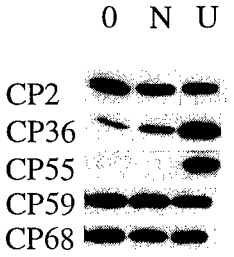


Figure 2B. After urea treatment (U), CP55 recognizes p21 from cell extracts. Whole cell extracts were either left untreated ("0"), treated with low concentrations of urea ("N'), or treated with denaturing concentrations of urea ("U'). Lysates were diluted in buffer after treatment and immunoprecipitated with each of the indicated anti-p21 MAbs.

A

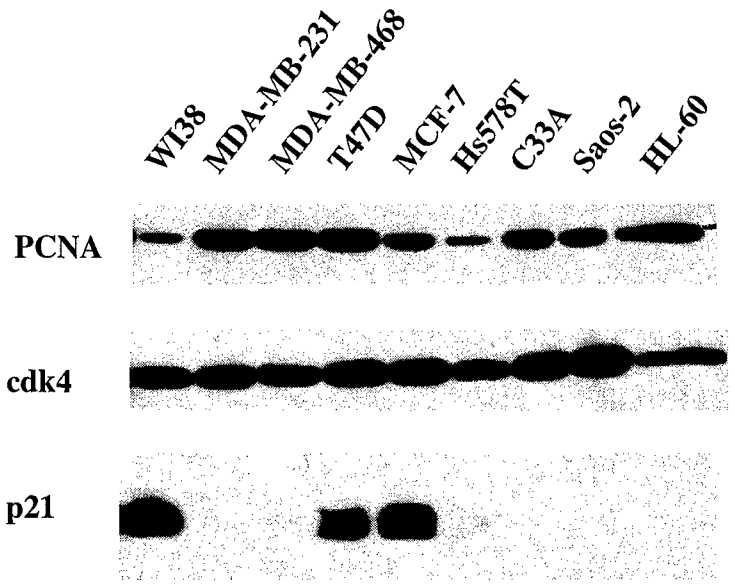
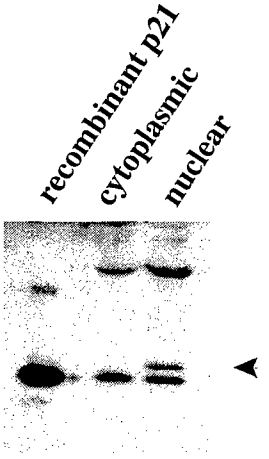


Figure 3A. Western blot analysis of several selected components of p21 complexes found in normal fibroblasts (WI38), breast carcinomas (MDA-MB-231, MDA-MB-468, T47D, MCF-7, and Hs578T), as well as cervical carcinoma (C33A), osteosarcoma (Saos-2), and leukemia (HL-60) cell lines. Most of the p53-deficient cell lines (MDA-MB-231, MDA-MB-468, C33A, Saos-2, and HL-60) lack substantial amounts of p21. T47D was the exception here, since it contained abundant p21. p21 protein is also visualized in the Hs578T lane after a longer exposure (not shown), while only a very weak signal can also be seen in the two MDA-MB lanes. Note that although T47D lysates have abundant p21, cdk4, and PCNA by direct immunoblotting, immunoprecipitations (Figure 4) show that these proteins are not associated with p21 in vivo.

B



B. WI38 extracts were fractionated into nuclear (N) and cytosolic (C) fractions and immunoblotted with p21 antibodies. A protein of slightly lower mobility (arrowhead) than p21 was seen in nuclear, but not cytosolic fractions.

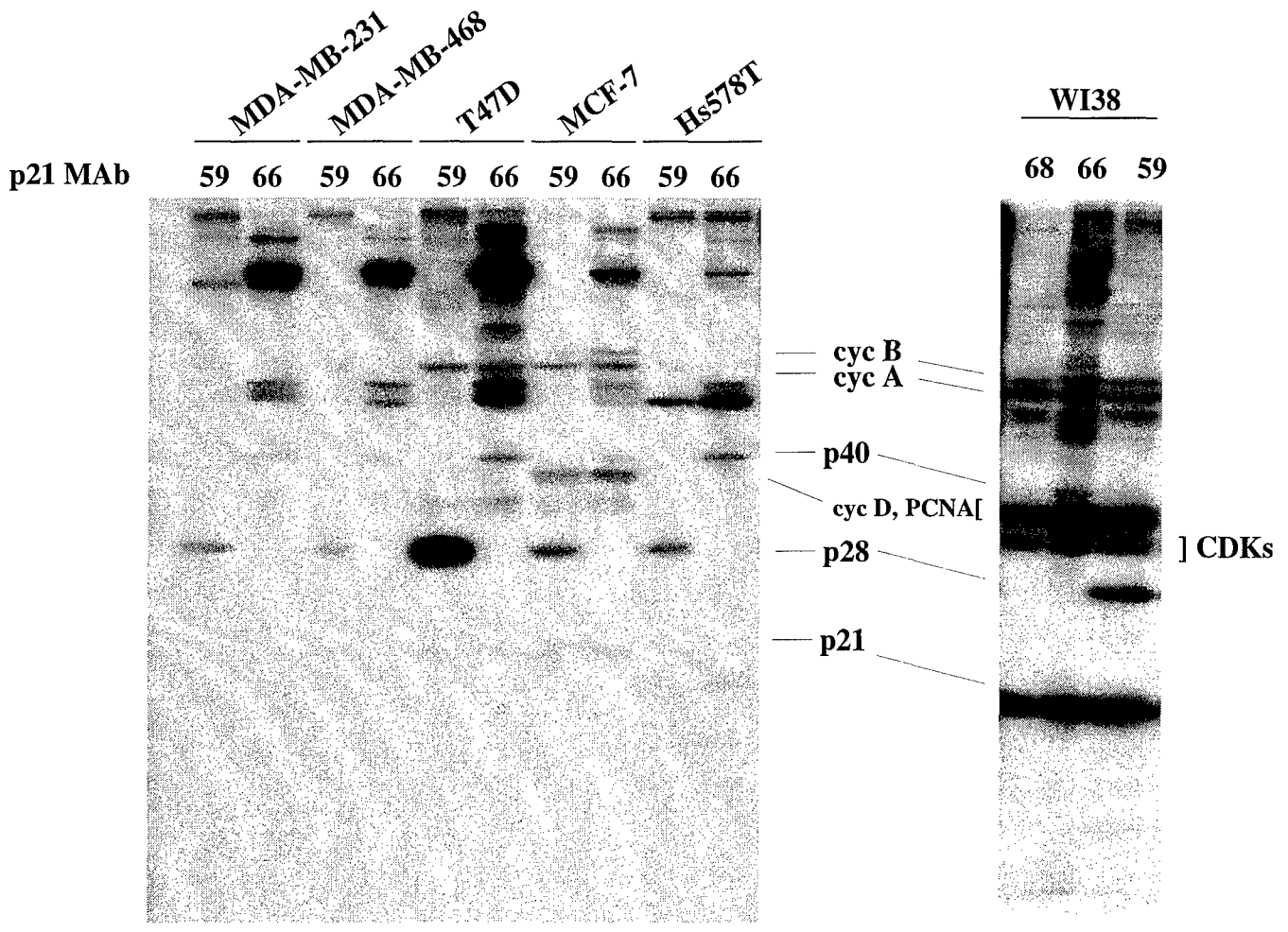
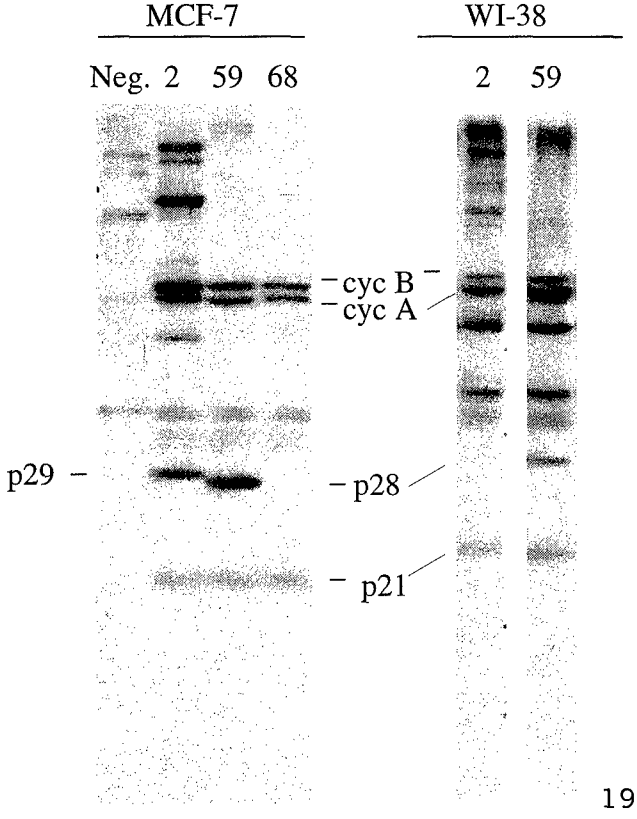


Figure 4A. Anti-p21 immunoprecipitations from different metabolically-labeled (35S) breast carcinoma cell lines and normal fibroblasts (WI38). The putative novel p28 protein is especially abundant in T47D cells, where a decreased association between p21, cyclin D, and PCNA is noted. T47D cells nevertheless contain each of these components by immunoblotting (see Figure 3).



4B. Comparison of immunoprecipitates of metabolically-labeled (35S) MCF-7 and WI38 cell extracts shows that MCF-7 cells contain another protein of mobility similar to p28. This putative novel protein, p29, was observed only in CP2 immunoprecipitates of MCF-7 cells and not in normal fibroblasts.

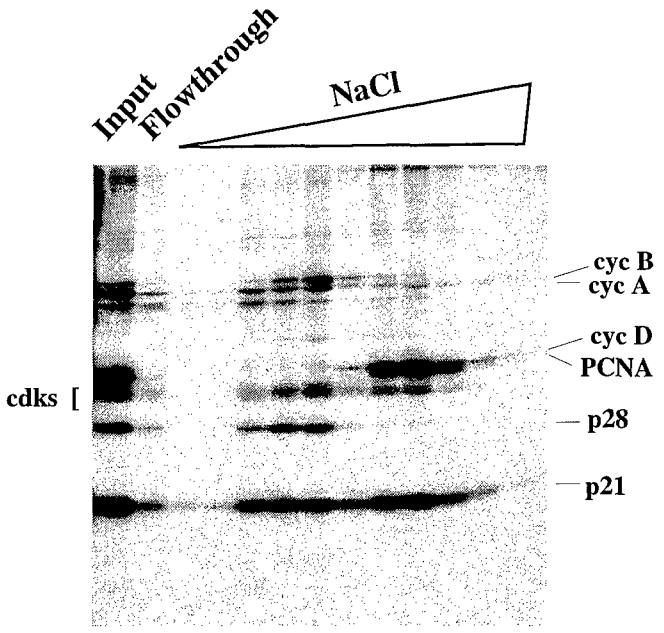


Figure 5. Co-fractionation of putative novel proteins with p21 complexes. Metabolically-labeled cell extracts were fractionated by ion exchange chromatography using an NaCl gradient and subsequently immunoprecipitated with anti-p21 antibody CP59. This method clearly separated cyclin A/cdk2/p21 and cyclin B/cdc2/p21 complexes from cyclin D/cdk4/PCNA/p21 complexes. The former complexes appear to lack PCNA and instead contain a putative, novel p21-associated protein, p28.

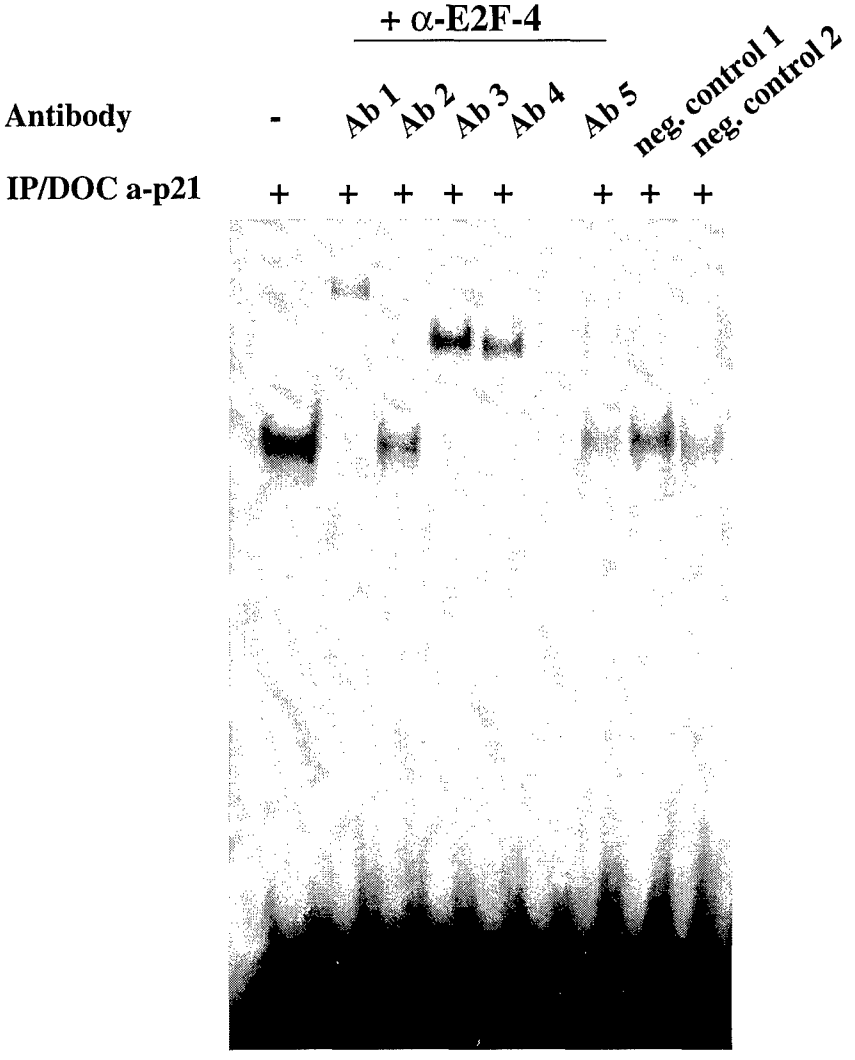


Figure 6. The E2F species associated with p21 in vivo is E2F-4. Extracts from normal human fibroblasts were immunoprecipitated with anti-p21 antibodies (lanes 1), and immunoprecipitates were treated with deoxycholate to release associated E2F activity. Proteins released in this way were incubated separately with a panel of anti-E2F-4 MAbs (gift of Dr. E. Harlow) or unrelated negative control antibodies. Several anti-E2F-4 antibodies "super-shifted" the released E2F-DNA complex, while other anti-E2F (data not shown) and the negative control antibodies did not.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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