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Loss of the retinoblastoma tumor suppressor protein (pRb) can contribute to breast tumor formation. However, many breast tumors retain expression of normal pRb, indicating that other genetic events may interfere with the function of this growth regulatory protein. Overexpression of cyclin D1 is such an event, since high levels of this protein may lead to constitutive inactivation of pRb through phosphorylation. We have demonstrated that cyclin D1 in oncogenic in cultured cells, and are using this assay to define regions and functions of cyclin D1 needed for transformation. Interestingly, a previously defined pRb-interaction domain in cyclin D1 is dispensable for phosphorylation and transformation by cyclin D1, but apparently not in the closely related cyclin D2. Instead, loss of a cyclin D1 domain just C-terminal the pRb-interaction domain may inactivate the protein. Using our knowledge of cyclin D1 function from these mutants and others, we hope to eventually create animal models of D1-dependent tumorigenesis useful for identifying suppressors of these tumors.

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INTRODUCTION

Breast cancer, like other cancers, results from the hyperactivity of growth-promoting oncoproteins and the loss of growth suppressing proteins (tumor suppressors). Many oncoproteins and several tumor suppressor proteins have recently been identified. Among the most commonly altered tumor suppressor proteins is the retinoblastoma protein, pRb. pRb function is lost in all retinoblastoma tumors, where it can lead to hereditary cancer, but is also involved in a variety of other tumors due to somatic inactivation. Reintroduction of the RB-1 cDNA into such cells inhibits their proliferation, supporting the role of pRb-inactivating mutations in formation of neoplastic cells (reviewed in Weinberg, 1991). This tumor-suppressive property of pRb is believed to result from pRb's ability to regulate progression through the cell cycle.

A central role for pRb in the control of cellular proliferation is also suggested by the observation that pRb is targeted by the oncoprotein products of several DNA tumor viruses (Whyte, et al., 1988; DeCaprio, et al., 1989; Egan, et al., 1989; Munger, et al., 1989; Dyson, et al., 1989). This interaction presumably serves to inactivate the growth-suppressive properties of pRb in infected or transformed cells. One mechanism by which oncoprotein-mediated inactivation may be achieved is through the dissociation of protein complexes between pRb and growth promoting molecules. For example, pRb has been reported to associate with the transcription factor E2F, which may be involved in the regulation of many genes required for DNA synthesis (Bagchi, et al., 1991; Bandara and LaThangue, 1991; Chellapan, et al., 1991; Chittenden, et al., 1991; Shirodkar, et al., 1992). The association of pRb with E2F may prevent the activation of these genes until the G1/S boundary, at which time the pRb/E2F complex dissociates, resulting in the release and activation of E2F. Because association of pRb with E2F seems to involve the same region of pRb (the "pocket") that is required for association with the viral oncoproteins, the binding of viral oncoproteins to pRb may release and activate E2F, resulting in the removal of a block to progression into S phase.

As is the case with a variety of human tumor types, some thirty percent of breast tumors show loss of pRb expression (Weinberg, 1991); however, other tumors have apparently wild-type pRb, and may have suffered alterations in one or another cellular proteins which interact with pRb. This may in turn lead to constitutive inactivation or circumvention of pRb function. A clue to the identity of such regulators of pRb is given by the fact that pRb is normally controlled by phosphorylation mediated by cyclin-dependent kinases (cdks; Lin, et al., 1991; Lees, et al., 1991). These cdks are controlled in turn by cyclins, regulatory subunits which lead to cyclic activity of their partner kinases. Work from many laboratories suggests that D-type cyclins in combination with cdk4 or cdk6 can initiate pRb phosphorylation in G1, cyclin E/cdk2 complexes may continue or expand on this phosphorylation just prior to S phase entry (Ewen, et al., 1993; Kato, et al., 1993). Indeed it has been suggested that both cdk4 and cdk2 activity may collaborate to fully inactivate pRb prior to S phase entry (Hatakeyama, et al., 1994). The activity of these cyclin/cdk complexes is further regulated by positive and negative phosphorylation of the cdk subunit. In addition, several proteins have recently been identified that serve to stoichiometrically inhibit the function of cyclin/cdk complexes (reviewed in Morgan, 1995). Thus, these cyclin-dependent kinase inhibitors, or CKIs, together with cdk-modifying

enzymes and cyclins represent potential targets for oncogenic mutations that may lead to deregulated cell cycle progression.

Importantly, cyclin D1 has been shown to be overexpressed in some thirty percent of breast tumors (Lammie, et al., 1991; Schuuring, et al., 1992; Buckley, et al., 1993; Keyomarsi and Pardee, 1993), as well as in cancers of the parathyroid, blood and squamous epithelium (Motokura, et al., 1991; Rosenberg, et al., 1991a,b; Withers, et al., 1991). Thus, deregulated D-type cyclin expression may be oncogenic, leading to aberrant cellular proliferation perhaps by interfering with the function of pRb. We have shown that cyclin D1 can indeed act as an oncogene, cooperating to transform cultured cells in cooperation with a mutant adenovirus E1A oncoprotein which has lost the wild-type capacity to bind and inactivate pRb (Hinds, et al., 1994).

In a conceptually similar manner, loss of CKI function may also lead to loss of pRb function in pRb-positive tumors. p16INK4a, an inhibitor tailored specifically to prevent the function of cdk4 and cdk6, is deleted or mutated in many cancers, presumably leading to hyperactivity of the cyclin D/cdk4(6) complex that initiates pRb phosphorylation (reviewed in Weinberg, 1995). p16 is thought to act as a direct competitor of D-type cyclins for cdk4/6 association, preventing the activation of these kinases when conditions are inappropriate for cellular proliferation. Thus, it is clear that tumor cells exploit at least three mechanisms (Figure 1) to abrogate pRb function: elimination of pRb itself, elimination of the negative regulator p16, and overexpression of cyclin D1 (Weinberg, 1995).

We have recently shown that deregulation of additional members of the p16/cyclin D1/pRb pathway can also promote cellular proliferation in the face of growth suppressive influences. Using rat cells expressing a conditional allele of p53, we have found that overexpression of cdk4 or cdk6 can restore the ability to phosphorylate pRb and progress through the cell cycle. Cells overexpressing these kinase subunits can proliferate continuously in the presence of functional p53, in part because the p21 CKI is prevented from association with other cdk/cyclin complexes required for cell cycle progression (Latham, et al., 1996). However, because p53 has growth suppressive influences separate from p21 induction (Brugarolas, et al., 1995; Deng, et al., 1995), overexpressed cdk4 and cdk6 may have pleiotropic effects on these cells that circumvent unidentified functions of p53. In support of this is the observation that rat cells proliferating in the presence of overexpressed cdk4/6 contain complexes between cdk4/6 and p16 CKI family members that are perhaps contributing to the deregulated proliferation of these cells. Indeed, our preliminary experiments suggest that even nonfunctional cdk4/6 can promote pRb phosphorylation upon overexpression in human tumor cells, perhaps by competing with endogenous, functional cdk4/6 for inhibitory subunits. Consistent with a role for cdk4 in human tumor formation, a mutant form of cdk4 that cannot bind p16INK4a has been found in melanoma cells (Wölfel, et al., 1995) . The molecular events outlined in Figure 1 may be oncogenic solely due to inactivation of pRb, and as such provide several alternatives to RB alteration in cancer. However, differences in the frequency of mutation of each pathway member occur in distinct tumor types, suggesting that alterations in cyclin D/cdk4 activity may be more profound than loss of pRb in some cell types. Our current work is focused on understanding the mechanims by which D-type cyclins and cdk4/6 can act as oncogenes.

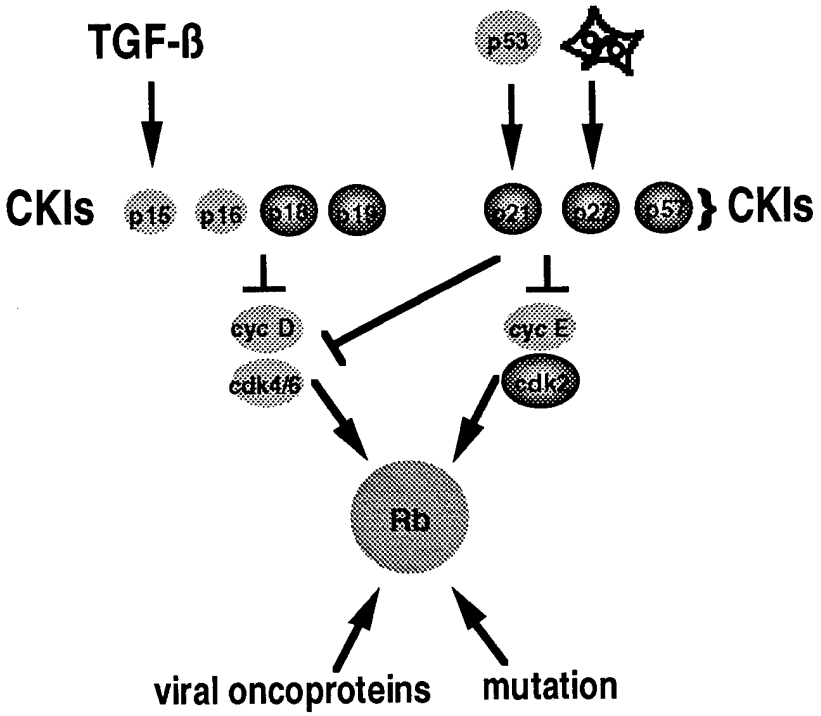

Figure 1. Interaction of cell cycle machinery, pRb and p53. Cyclin-dependent kinase (cdk) inhibitors (CKIs), cyclins and cdks involved in G1-S progression are shown. These proteins regulate the function of the Rb protein, a central target of oncogenic mutations. It has long been realized that such mutations inactivate pRb, and this can also be achieved by viral proteins, leading to uncontrolled proliferation. More recently, the cell cyclin components shown in gray have been demonstrated to be altered in tumors and/or function as oncogenes or suppressors in culture. Note that the CKIs are mediators of the growth suppressive influence of a variety of cell cycle inhibitors, such as TGF-ß, p53 and cell-cell contact. This pathway suggests that there are many ways to ultimately deregulate pRb, and most tumor cells may contain alterations in one of these components.

We and others have shown that D-type cyclins can physically associate with pRb in a manner analogous to the viral oncoproteins (Ewen, et al., 1993; Kato, et al., 1993; Dowdy, et al., 1993), a property of cyclin D1 that may explain its prevalence as a target of oncogenic activation. Paradoxically, however, this direct pRb binding is dispensable for transformation (Hinds, et al., 1994), and, as shown below, for phosphorylation of pRb. Intriguingly, we have recently found that a mutant cyclin D1 protein (KE) which is altered in the cdk-binding region not only fails to transform primary cells, but is dominant over the wild-type protein in this capacity. Thus, cointroduction of mutant and wild-type cyclin D1 genes leads to no increase in transformation frequency, suggesting a dominant-negative function of the mutant protein. This mutant protein can be expressed in conjunction with wild-type E1A, however, and is thus not itself lethal to cells in the absence of overexpressed cyclin D1. More detailed understanding of the mechanisms behind the

inhibition of cyclin function could lead to antiproliferative products using existing technologies and may be specific to those cells overexpressing certain cyclins, leaving normal proliferating cells relatively unaffected. The properties of D-type cyclins already uncovered in our prior research and those to be elucidated by the experiments described below provide an excellent opportunity for clinical intervention in aberrant cell cycle control, and could thus provide an important adjunct to the pharmaceutical treatment of human cancers. In addition, we seek to identify other upstream regulators or downstream targets of pRb which can inactivate pRb upon overexpression. Such candidate oncoproteins may be operative in the significant fraction of tumors which do not show direct inactivation of pRb. To address these issues, the following specific aims were originally proposed:

1) Use mutant pRb proteins that have been characterized for function as growth suppressors and substrates for the pRb kinase to determine if the association of D-type cyclins with pRb is required for pRb function or phosphorylation or both.

2) Identify a kinase activity precipitable with antibodies specific to a tagged D-type cyclin and use this functional assay to probe the effect of pRb binding on D-type cyclin function.

3) Introduce a dominant-negative D-type cyclin into cells transformed by wild-type D-type cyclins and control cells in effort to specifically prevent the proliferation of the transformed cells.

4) Identify other substances, e.g. antisense oligonucleotides or interfering peptides, which could cause cessation of proliferation of cyclin D-transformed cells in vitro and in vivo.

5) Use cell lines temperature-sensitive for growth due to the expression of temperature-sensitive pRb to identify upstream inactivators and downstream targets of pRb.

RESULTS

Specific aims 1 and 2: Mapping of pRb and cyclin D1 domains required for transformation.

Although it is clear that cyclin D1 can function as an oncogene and does so at least in part by interfering with the pRb protein, the exact mechanism by which this is achieved is unclear. For example, cyclin D1 may simply activate its cognate kinase partners, cdk4 and cdk6, to directly phosphorylate pRb. Alternatively, excess cyclin D1/cdk complexes may compete with or lead to inactivation of any of a variety of CKIs now known to regulate cdk activity. Finally, although the absence of pRb seems to negate the need for cyclin D1 in tumor cells (Lukas, et al., 1995; Parry, et al., 1995), cyclin D1/cdk complexes may have other cellular targets. Examples of such targets are the pRb homologues p107 and p130 (Beijersbergen, et al., 1995). These proteins are homologous to pRb in the region

used to associate with viral oncoproteins. Indeed, these proteins were first identified due to their association with E1A. It is now clear that p107 and p130 associate with transcription factors capable of binding to the E2F DNA site (Shirodkar, et al., 1992; Devoto, et al., 1992; Cao, et al., 1992; Cobrinik, et al., 1993), thus the homology to pRb is functional as well as physical. However, the E2F proteins that associate with pRb do not appear to interact with p107 or p130 in cells. It is therefore likely that pRb, p107 and p130 are members of a family of proteins which regulate the function of a family of E2F transcription factors whose roles may or may not overlap in cell cycle control. Deregulated phosphorylation of these targets and pRb together may be more profoundly oncogenic than deletion of pRb alone, consistent with the high incidence of cyclin D1 overexpression in certain tumor subsets, such as is the case in breast cancer. We are attempting to identify the functional regions of cyclin D1 operative in transformation and correlate these with an induction of pRb phosphorylation. In addition, we will identify the minimal elements required to produce a dominant-negative version of cyclin D1 that may be useful in interfering with cyclin D1-mediated tumorigenesis.

Phosphorylation and transformation properties of mutant cyclin D1 proteins.

To define the regions of cyclin D1 needed for transformation, we have initiated a series of experiments using mutant cyclin D1 proteins. First, we are examining the ability of these cyclin D1 mutants to transform primary BRK cells in cooperation with the pm928 allele of E1A. We have focused in particular on the N-terminus of cyclin D1 at the beginning of these experiments, since this region contains a known pRb-interaction domain. Previous work had shown that a point mutation (GH) in this domain does not alter cyclin D1's ability to transform cells or inactivate pRb in human tumor cells (Hinds, et al., 1994). Interestingly, this differs from the reported inability of a cyclin D2 mutant lacking the pRb binding domain to phosphorylate and inactivate pRb (Ewen, et al., 1993). However, the cyclin D2 mutant used had a different point mutation in this domain. We are thus attempting to elucidate any potential differences between these D-type cyclins. We constructed precise deletions of the pRb-binding, LxCxE domain in both cyclin D1 and cyclin D2 (Δ5 and Δ3, respectively) and tested both these mutants and the parent, wild-type alleles in transformation assays. As previously found, cyclin D1 can cooperate with 928 in BRK transformation. The Δ5 mutant of cyclin D1 was found to have equal or greater activity in these assays, consistent with previous results using point-mutated cyclin D1. Cyclin D2 cooperates at least as well as cyclin D1 in these assays, giving rise to foci with nearly fifty percent the frequency of wild-type E1A. Interestingly, preliminary results suggest that Δ3 cannot transform BRK cells efficiently, suggesting that cyclin D2, but not cyclin D1, depends on an interaction with the pRb pocket for transformation. Several additional transformation experiments are required for statistical significance and are in progress.

These mutant D cyclins and others have been used in parallel assays to determine their ability to promote pRb phosphorylation in transfected SAOS-2 cells. In this assay, cyclin D1 and cdk4 cotransfected with pRb leads to phosphorylation of pRb as judged by immunoblot of SDS-PAGE-treated cell extracts. The cyclin D1Δ5 mutant is wholly able to induce pRb phosphorylation in this assay, indicating that this interaction domain is dispensable for this activity in overexpression systems. Interestingly, the cyclin D2Δ5 mutant appears to only weakly induce pRb phosphorylation, again suggesting a

dependence of cyclin D2 on this domain for pRb inactivation. Further, these results suggest a positive correlation between the ability of D-cyclins to stimulate pRb phosphorylation and transformation. To test this correlation further, we have employed two additional cyclin D1 mutant proteins. These mutants, Δ15 and Δ35, lack five amino acids beginning at residue 15 and 35, respectively, and are unable to induce pRb phosphorylation in SAOS-2 cells, although they are produced in levels equal to the wild-type cyclin D1 protein. An example of one such experiment is shown in Figure 2 below.

Each of the D cyclins used in these studies so far has been found to retain an interaction with cdk4, and thus those mutants defective for pRb phosphorylation may be incapable of cdk4 activation, substrate recognition, or both. To test for activation of cdk4 directly, kinase assays using immunoprecipitated cyclin D1 and a C-terminal fragment of pRb fused to GST have been performed. The results of these assays correlate well with results from transfection experiments in that Δ15, Δ35 and KE each fail to precipitate pRb kinase activity. We are presently attempting to determine what the specific role of the 15-35 region of cyclin D1 is in activation of cdk4 or substrate recognition. As a first step in this analysis, we have examined the ability of cyclin D1 mutants to physically associate with pRb. As previously reported (Dowdy, et al., 1993), wild type cyclin D1 made in reticulocyte lysates binds pRb in vitro, and this association is abolished by point mutation in the LXCXE region (mutant GH). Mutants Δ15, Δ35 and KE retain their ability to associate with pRb. This may suggest that each of these mutants is unaltered in complex formation with cdk4 and pRb, and are defective in kinase activation. However, pRb binding results with mutant Δ5 surprisingly suggest that cyclin D1 may interact with pRb through a novel, undefined region. As shown in Figure 3, Δ5, although completely lacking the LXCXE domain, can bind to pRb in vitro. This result has been observed in several independent experiments, and thus suggests that cyclin D1 may have a "secondary" method of binding to pRb (and which may be compromised by the altered N-terminal structure of mutant GH). We are actively searching for this domain of cyclin D1 in effort to ascertain its role in pRb phosphorylation and transformation.

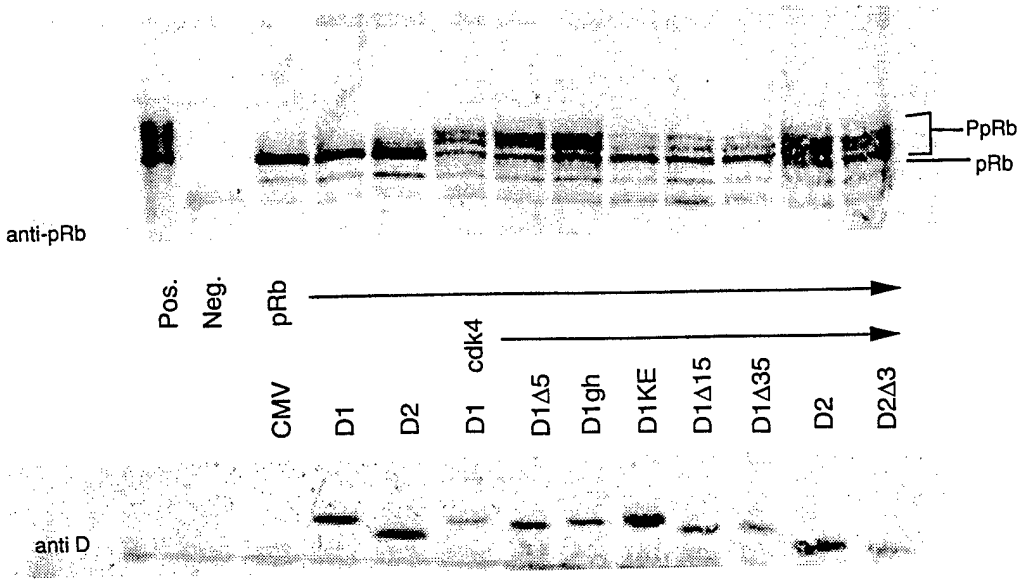

Figure 2. Induction of pRb phosphorylation by cyclin D1 mutants in transfected SAOS-2 cells. Saos-2 osteosarcoma cells were transfected with the indicated expression constructs and lysed two days later. Aliquots of lysate were run on SDS-PAGE, transferred to nitrocellulose and probed for pRb (top) or cyclin D1 and D2 (bottom). Hypo (pRb) and hyper (PpRb) phosphorylated pRb is indicated at right.

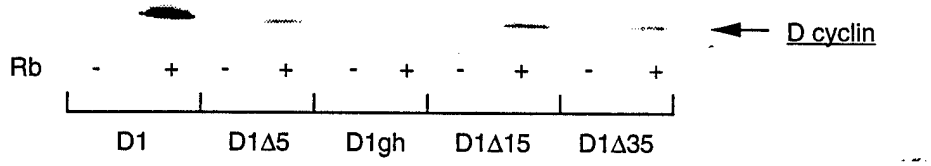

Figure 3. Binding of cyclin D1 mutants to pRb in vitro. Cyclin D1 and the indicated mutants were produced in reticulocyte lysate coupled transcription/translation reactions using labeled methionine. Aliquots of the translate were incubated with baculovirus-produced full length pRb and then immunoprecipitated with anti-pRb antibodies. The input level of wild-type cyclin D1 was elevated relative to the other lanes; subsequent experiments suggest the degree of binding is equivalent for Δ5, Δ15 and Δ35, but not detectable for GH.

The set of reagents described above is also of utility in analyzing a second mechanism by which cyclin D1 may antagonize pRb. As stated above, the introduction of pRb, cyclin D1 and cdk4 into SAOS-2 cells leads to phosphorylation of pRb. However, if cdk4 is omitted, pRb is not phosphorylated but the level of pRb is significantly lowered. By using co-introduced reporter constructs driven by promoters equivalent to those used for pRb, we have determined that this reduction of pRb level is post-transcriptional in nature. Intriguingly, we have also observed that this pRb reduction is not caused by cointroduction of cyclin D2, once again demonstrating a significant difference between the otherwise highly related cyclins D1 and D2. Using mutants of pRb, our preliminary data suggests that integrity of the pRb "pocket", or oncoprotein binding domain, is not required for this effect. Further, the N-terminal, pRb-pocket binding region of cyclin D1 is dispensable for pRb reduction, and there appears to be no requirement for kinase function, since Δ15 and Δ35 reduced pRb levels. However, in several experiments, the cyclin box mutant KE failed to reduce pRb levels. Thus, reduction of pRb levels by cyclin D1 appears to be a unique property of cyclin D1 mediated by elements of both pRb and cyclin D1 that differ from those conventionally thought to participate in phosphorylation. Analysis of other cyclin D1 and pRb mutants is ongoing in effort to define the biochemical nature of this interaction.

The apparent lack of a requirement for pRb binding by cyclin D1 in its role as an activator of the pRb kinase and as an oncogene in cultured cells may be due to the level of cyclin D1 achieved in these transfection experiments. Cyclin D1 produced at "normal" levels from its chromosomal site may require pRb interaction through the LXCXE domain for proper function. To test the role of the LXCXE domain of cyclin D1 more rigorously, we are constructing murine embryonic stem cells that contain the GH mutation in one allele. Production of mice from these cells followed by breeding to homozygosity will allow an assessment of the role of this domain in cyclin D1 function. To perform these experiments, we are collaborating with Piotr Sicinski, who has knocked out cyclin D1 in mice (Sicinski, et al., 1995). One of the striking features of these mice is a failure to develop lactating breast; thus the ability of GH-homozygous mice to develop lactating breast will provide a useful first indication of the function of this mutant cyclin D1 in vivo. Prior to constructing such animals, however, it is important to know that murine pRb and murine cyclin D1 produce from a genomic allele interact stably, and that the GH mutation will disrupt this. To test this, we have constructed a GST-murine pRb fusion protein containing the cyclin D1 interacting domain ("large pocket") of pRb. This reagent will be used to attempt pull-down experiments from extracts of cells transfected with a CMV-driven murine cyclin D1 genomic construct that we have recently produced. Should this control experiment yield positive results, the GH mutation will be introduce into the murine cyclin D1 construct and the encoded protein tested for loss of interaction with pRb. If the system passes these tests, the murine genomic GH mutant will be used to construct vectors for a "hit-and-run" approach to introducing this mutant into the ES cell genome. It is anticipated that mice homozygous for this mutation, and perhaps more importantly cells derived from them, will be most useful in ascertaining the role of the LXCXE domain in cyclin D1 function.

Specific Aims 3 and 4: Suppression of D1-dependent tumor cell growth

As mentioned above, cyclin D1 mutant KE acts as a dominant-negative protein in transformation experiments. This function was at first puzzling, since expression of KE in COS cells appeared to show a lack of interaction between KE and endogenous cdks. However, further experiments in several cell lines shows that KE retains an ability to interact with cdk4 in most cells. Thus, KE may act as a dominant-negative protein by binding to, but failing to activate, cdk4. This model is given further support by the recently-published structure of cyclin A/cdk2 (Jeffrey, et al., 1995). In this work, the analogous K residue in the cyclin box of cyclin A was found to interact directly with a residue in cdk2, probably resulting in conformational change of the cdk. We are currently testing this model of KE's dominant negative function in collaboration with Steve Dowdy of Washington University in St. Louis, and will extend these studies to mutants Δ15 and Δ35, which also bind but fail to activate cdk4 in our preliminary experiments described above. An understanding of the mechanism of cyclin D1 dominant negativity is anticipated to yield useful reagents for suppression of cellular proliferation, and may also be generally applied to other cyclins. After we gain a better understanding of this dominant-negative function, we will test potentially suppressive forms of cyclin D1 both in BRK transformants and breast tumor cell lines such as MDA-MB-134, 175, 330 and 453, all of which overexpress cyclin D1. As controls, we will use E1A-plus-ras transformed BRK cells and breast tumor lines such as MDA-MD-468, which has lost pRb expression and does not highly express cyclin D1.

An important goal of these aims is to move such growth suppression experiments in vivo. To do this, we are creating mice that should develop D1-dependent tumors. In collaboration with Dr. Terry van Dyke at UNC Chapel Hill we have created transgenic mice expressing cyclin D1 under the LPV enhancer. This construct allows cyclin D1 expression in the choroid plexus, a tissue in which Dr. van Dyke has already established a tumor model. Here, variants of SV40 Large T Antigen have been expressed that fail to bind to pRb and fail to cause tumors. We hope to complement this defect by mating the T mutant and cyclin D1 mice. At present, the LPV-D1 transgenics appear to express cyclin D1 in the choroid plexus, and at low penetrance develop hydrocephaly, indicating a potential choroid plexus defect. Most intriguingly, the weight of the brain of affected individuals is greatly increased, suggesting a general proliferation of cells in the brain in response to constitutive expression of cyclin D1 in the choroid plexus. The cause of this is under investigating. In any case, these mice appear suitable for establishing a D1-dependent proliferative phenotype in vivo, and should allow in vivo testing of potential anti-cyclin D1 reagents.

Specific Aim 5: Establishment of cell lines expressing temperature-sensitive pRb.

A linker-insertion mutation in pRb at codon 668, resulting in the insertion of four amino acids, produces a pRb protein (XX668) that fails to induce the G1 growth arrest in SAOS-2 cells that is characterized by the "flat cell" phenotype. However, when the construct encoding this mutant protein is introduced into SAOS-2 cells incubated at 32.5°C, the activity of the mutant pRb is indistinguishable from that of the wild-type protein. We have tested this protein for an ability to cause G1 growth arrest at both temperatures as well, and the results of the experiments are shown in Figure 4. Further,

preliminary experiments using an E2F-dependent reporter gene suggest that XX668 cannot repress transcription at 37°C, but is comparable to the wild-type protein in this capacity at 32.5°C. This temperature-sensitive effect should allow the establishment of cell lines that proliferate at 37°C, but uniformly undergo growth arrest in G1 at 32.5°C.

Preliminary experiments suggest that such cell lines can be created by transfection of XX668 into SAOS-2 cells. These cloned cell lines proliferate similar to parental cells at 37°C, but undergo growth arrest and phenotypic change at 32.5°C. Initial characterization of the cells at both temperatures indicates that the level of pRb doesn't change dramatically, but the protein is only able to bind substrates at 32.5°C. In addition, no significant change in the expression of G1 cyclins has been noted. Suitable cell lines now exist to allow a determination of transcriptional changes induced upon pRb activation and these experiments are in progress. Given this promising beginning, we are also in the process of introducing XX668 and derivatives into DU145 prostate adenocarcinoma and MDA-MB-468 breast carcinoma cell lines, both of which lack normal pRb expression.

Despite the significant differences between XX668 function and wild-type pRb function shown above, the mutant pRb does retain some growth suppressive effect (up to 20% flat cell induction), and may be poorly tolerated even at the nonpermissive temperature in some cells. We are exploiting our previous (unpublished) work using pRb mutants lacking the nuclear localization signal to produce a tspRb derivative that may be better tolerated at 37°C. These localization studies demonstrated that the loss of the nuclear targeting sequence produces a protein that localizes predominantly to the cytoplasm, yet maintains some nuclear localization. Further, the fraction of pRb that localizes to the nucleus appears functional, since it resists detergent treatment and causes growth arrest in the cells containing a significant nuclear fraction. This phenotype is consistent with the location of the NLS mutation at residue 860-876, a region that has not been implicated in any of the protein-protein associations required for pRb function. Curiously, when the NLS mutation is crossed with a tumor-derived inactivating mutation such as C to F at residue 706, the resultant doubly mutated protein is exclusively cytoplasmic and cannot induce growth arrest. We believe this indicates that the "pocket" of pRb is responsible for the "residual" nuclear localization of the protein, perhaps by association with nuclear structures upon their synthesis and transport to the nucleus.

We have introduced the NLS-minus mutation into the XX668 tsRb background in anticipation of creating a pRb protein that will be cytoplasmic and nonfunctional at 37°C, but will regain pocket function and thus partial nuclear localization at 32.5°C. Such a mutant may be better tolerated by SAOS-2 cells since very little of the protein will be nuclear at the nonpermissive temperature. Cell lines derived from the multiple mutant, or stable versions of breast tumor lines expressing the original tspRb, should be valuable in studying the molecular events that occur upon pRb activation as well as those that result in renewed proliferation after pRb is inactivated.

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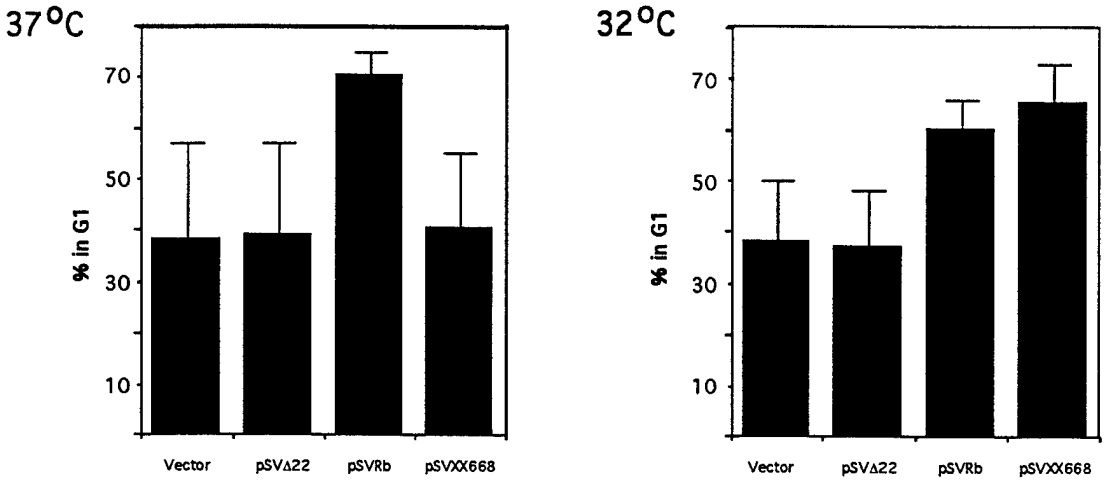

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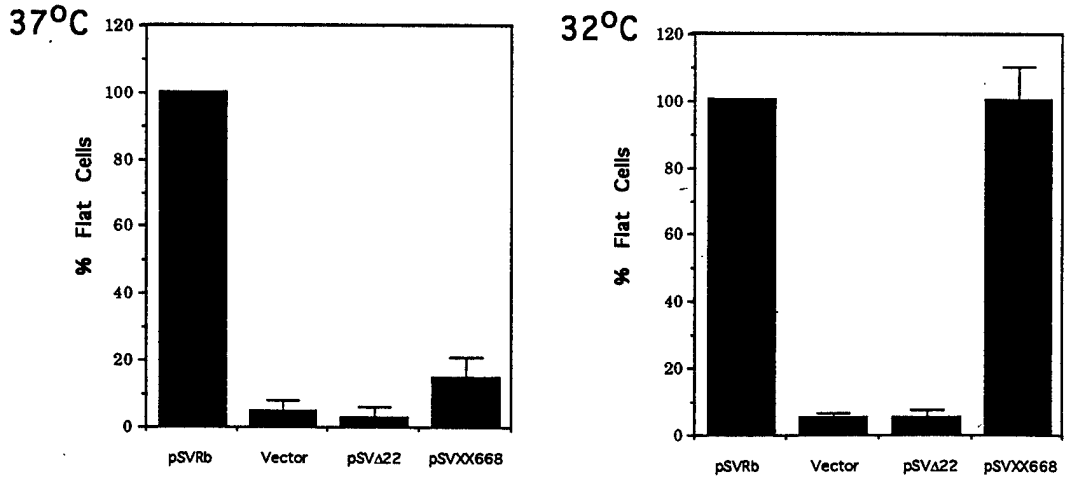

Figure 4. Temperature-dependent phenotypes of pRb mutant XX668. A. Control vector, Δ22 (nonfunctional, tumor-derived mutant Rb), SVRb encoding wild-type pRb, or XX668 plasmids were cotransfected with CMV-CD20 into SAOS-2 cells. Two days after transfection, cells were stained for CD20 (transfected cells) and treated with propidium iodide to determine position in the cell cycle. The percent of CD20-positive cells with a 2N DNA content is plotted. B. Plasmids as in A were transfected with pBabepuro and selected for resistance to puromycin. Drug-resistant "flat" cells were counted one week later.

CONCLUSIONS

Our ongoing work characterizing the method of action of cyclin D1 as an oncogene will be useful in identifying the functions of cyclin D1 that may serve as targets for anti-tumor approaches. Preliminarily, it seems that cyclin D1 differs from cyclin D2 in its biochemical effects on pRb in overexpression systems. These differences may explain the common observation of cyclin D1 as a human oncogene, in contrast to the rare incidence of cyclin D2 overexpression. Such differences are what we hope to exploit to reverse the aberrant proliferation resulting from cyclin D1 overexpression.

In a similar fashion, we believe that cyclin D1 overexpression may have consequences other than, or in addition to, direct inactivation of pRb. The different phenotypes associated with cyclin D1 overexpression as compared to pRb deletion support this notion. Attempts to address this issue in pRb-minus fibroblasts show some promise, but need to be extended in animal models. To address this, it may be possible to examine the ability of cyclin D1 to induce hyperproliferation or tumors in murine breast in the presence and absence of pRb. In collaboration with Tyler Jacks, we could employ embryonic stem cells lacking the pRb protein due to two independent gene targeting events as recipients of breast-specific cyclin D1 constructs. When compared to similar cells containing wild type pRb, the necessity for pRb function in observing a "cyclin D1 phenotype" can be assessed in chimaeric animals derived from the ES cells. The resultant cells should produce chimaeric mice with or without pRb and expressing cyclin D1 at high level in the breast. Based on published results, cyclin D1 overexpression should lead to hyperplasia and eventually tumor formation in the normal background. If this is also observed in pRb-minus breast tissue overexpressing cyclin D1, there are likely to be other targets of cyclin D1 relevant to breast tumor formation. A lack of hyperproliferation in this case would suggest that cyclin D1 cannot contribute to tumor formation in the absence of pRb. Because this experiment would require female ES cells for the desired result, new ES cell lines would need to be established from matings of Rb-minus heterozygotes, since all available lines are male. To circumvent this problem, expression of cyclin D1 in a different tissue may be desirable. An exciting alternative to expression of cyclin D1 in the breast in this case is to used the glial fibrillary acidic protein (GFAP) promoter. This will allow expression of cyclin D1 in glial cells, and the high incidence of cyclin D1 overexpression in human gliomas suggests this to be a compelling model system to test. If such animals show glial proliferation or tumors, the appearance of this phenotype in pRb-minus animals can be ascertained as described above.

Together, we hope that these experiments will extend our understanding of the role of cyclin D1 (and pRb) in breast cancer. As these experiments yield data on the functions of cyclin D1 regions in transformation, we will proceed with experiments designed to suppress D1-dependent tumor cell growth (specific aims 3 and 4). Precise knowledge of the functions of cyclin D1 in tumorigenesis may well demonstrate a specificity of overexpressed cyclin D1 that can be targeted in tumor cells, leaving normal cells unscathed.

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