Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein

Liang Zhu,^{1,3} Sander van den Heuvel,¹ Kristian Helin,¹ Ali Fattaey,¹ Mark Ewen,² David Livingston,² Nicholas Dyson,¹ and Ed Harlow¹

¹Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts 02129 USA; ²Dana-Farber Cancer Institute, Boston, Massachusetts 02115 USA.

The cellular protein p107 shares many structural and biochemical features with the retinoblastoma gene product, pRB. We have isolated a full-length cDNA for human p107 and have used this clone to study the function of p107. We show that, like pRB, p107 is a potent inhibitor of E2F-mediated *trans*-activation, and overexpression of p107 can inhibit proliferation in certain cell types, arresting sensitive cells in G₁. Several experiments, however, showed that growth inhibition by pRB and p107 did not occur through the same mechanism. First, in the cervical carcinoma cell line C33A, p107 was able to block cell proliferation, whereas pRB could not, even though both proteins were potent inhibitors of E2F-mediated transcription in this cell line. Second, growth arrest by pRB and p107 was rescued differentially by various cell cycle regulators. Third, some mutants of p107 that cannot associate with adenovirus E1A were still able to inhibit cell proliferation, whereas analogous mutants in pRB are known to be unable to block cell growth. Together, these results suggest a biological role of p107 that is related, but not identical, to that of pRB.

[Key Words: p107; pRB; E1A; E2F; growth suppression]

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The cellular protein p107 was first identified through its association with adenovirus E1A and SV40 large T antigen (Yee and Branton 1985; Harlow et al. 1986; Dyson et al. 1989; Ewen et al. 1989; Whyte et al. 1989). The same regions of E1A or large T that form the binding sites for p107 are also needed for interaction with pRB, one of the best characterized tumor suppressor gene products (DeCaprio et al. 1989; Whyte et al. 1989; Kaelin et al. 1990; Dyson et al. 1992; Weinberg 1992). Although the affinity and exact contact points for these interactions appear to be somewhat different, the overlapping nature of the binding sites was the first suggestion that p107 and pRB might be related. The interactions of the viral proteins with pRB and p107 are thought to be functionally important because viral mutants that fail to bind are defective for transformation (Kalderon and Smith 1984; Cherington et al. 1988; DeCaprio et al. 1988; Whyte et al. 1988, 1989).

Recent studies have extended the similarities between pRB and p107. The amino acid sequence of human p107 has revealed homologies between these two proteins (Ewen et al. 1991). The regions of homology correspond primarily to the carboxy-terminal two-thirds of these proteins, a region needed for pRB to bind E1A or large T (Hu et al. 1990; Huang et al. 1990; Kaelin et al. 1990). This interaction domain for E1A and large T is often termed the pocket (Kaelin et al. 1991), and in pRB it is

composed of two essential segments named A and B (A is amino-terminal to B) separated by a spacer region with a specific sequence that is not essential for binding. In vitro binding assays have shown that the homologous sequences in p107 also constitute the E1A/large T-binding domain (Ewen et al. 1991). The sequences in the spacer regions of the two proteins are not homologous and are not essential for binding. Interestingly, p107 also has the ability to associate with cyclin A (Faha et al. 1992), and the p107 spacer is the site for this association (Ewen et al. 1992).

Following the prediction that pRB might use the E1A/large T-binding pocket to interact with cellular proteins for some aspects of its normal function, the pocket region of pRB was shown to bind specifically to a set of cellular proteins in vitro (Defeo-Jones et al. 1991; Huang et al. 1991; Kaelin et al. 1991). When the pocket region of p107 was tested in the same assay, it associated with the same set of proteins as pRB, except that two additional proteins were seen, one of which was cyclin A (Ewen et al. 1992). In vivo, pRB and p107 both associate with the transcription factor E2F, although in independent complexes (Bandara and La Thangue 1991; Chellappan et al. 1991; Mudryj et al. 1991; Cao et al. 1992; Devoto et al. 1992; Shirodkar et al. 1992). pRB associates with E2F mainly in G₁ and S phases (Mudryj et al. 1991; Shirodkar et al. 1992), whereas p107 forms two complexes with E2F; one with cdk2 and cyclin A that is found predominantly in S phase (Devoto et al. 1992; Shirodkar et al. 1992), and one with cdk2 and cyclin E found in G₁ phase

³Corresponding author.

(Lees et al. 1992). E1A or large T is able to bind to pRB and p107, dissociating these complexes and releasing free E2F (Bandara and La Thangue 1991; Chellappan et al. 1991, 1992; Mudryj et al. 1991).

Despite the common features outlined above, the biological function of p107 has remained a matter of speculation. A logical prediction would be that p107 is also a tumor suppressor gene product. Attempts to link p107 mutations with tumorigenesis, however, have not been successful. This might be attributable to two possibilities: Either p107 is not a tumor suppressor gene product or the conventional approaches that have been used cannot uncover the role of p107 as a negative growth regulator. Given these difficulties in assessing the role of p107, we have turned to a more direct approach. We have isolated a full-length cDNA for human p107 and have begun to investigate its biological functions. Here, we report that, like pRB, p107 inhibits cell proliferation and E2F-mediated transcription; however, p107 acts by mechanisms different from those of pRB.

Results

Cloning and expression of full-length human p107 cDNA

The first cDNA for human p107 was cloned by Ewen and colleagues and shown to encode a protein of ~100 kD (Ewen et al. 1991). Because p107 actually migrated with a relative molecular mass of ~120 kD in the same gel systems and the coding sequence of this p107 cDNA was still open at its 5'-end, it was assumed that this clone was not full length. In further screening of several human cDNA libraries, a clone that contained an additional 467 bp of 5' sequence was isolated. DNA sequencing suggested that this clone could code for an additional 189 amino acids upstream from the first methionine in the published sequence (Fig. 1). The extended sequence predicts an open reading frame of 1068 amino acid residues (calculated molecular mass of 120.875 kD), and the first methionine of the open reading frame can be used for translation initiation (Kozak 1986). Protein sequence comparisons of the predicted amino-terminal region did not reveal homologies with pRB or with any other proteins in GenBank. This amino-terminal region, however, like other regions of p107, is highly conserved with the mouse and *Xenopus* p107 (M.-H. Lee and E. Harlow, in prep.), indicating that the amino terminus may be functionally important.

Expression studies with the new p107 cDNA have indicated that this clone is full length. When used as a template for *in vitro* transcription-translation, the largest protein product comigrated with the cellular p107 on SDS-PAGE (Fig. 2A, cf. p107 in lanes 1 and 2). When the clone was inserted into an expression vector, pCMV-neoBam (Baker et al. 1990), and transfected into several human cell lines, high levels of expression of a protein with molecular masses identical to the endogenous p107 were seen after immunoprecipitation with an anti-p107 monoclonal antibody (Fig. 2A, cf. lanes 4 and 5). Both

immunoprecipitation and immunoblotting experiments showed that the levels of p107 expression were similar to that of pRB expressed from the same expression vector, pCMVpRB (Qin et al. 1992), (Fig. 2A, lanes 5,6; data not shown).

For experiments that required the separation of exogenous p107 from the endogenous p107, we attached a hemagglutinin epitope (HA) to the carboxyl terminus of p107. The exogenous p107 was distinguished from the endogenous p107 by use of the anti-HA monoclonal antibody 12CA5 (Field et al. 1988). The HA tag was also used to detect the expression of certain deletion mutants that had lost the epitopes for the available monoclonal antibodies. For example, the mutant p107DE contains a deletion from residue 409 to residue 826 (see Fig. 7, below) and was not recognized by anti-p107 monoclonal antibodies, but could still be detected with the anti-HA antibody (Fig. 2B).

Several other criteria were used to confirm that the protein expressed from the new p107 cDNA had the expected properties. Consistent with previous data from studies of adenovirus infections and transformations, the exogenously expressed p107 was able to associate with E1A when mixed with a lysate from 293 cells, an E1A-containing cell line (Fig. 2B). The full-length p107 was similarly coimmunoprecipitated with large T antigen when p107 was expressed in COS cells, which express large T (data not shown). p107 is known to bind E2F *in vivo*. Using the epitope-tagged constructs in transfection experiments, we were able to show that overexpressed wild-type p107 but not the mutant p107DE (which lacks most of the pocket and spacer sequences, see Fig. 7, below) associated with E2F (Fig. 2C). In this type of experiment, immune complexes are prepared by use of the anti-HA antibody 12CA5, associated proteins are released by treatment with deoxycholate (DOC), and the E2F DNA-binding activity is assayed in the supernatant. The HA-tagged p107 was also used in transfection for immunofluorescence staining to show that the exogenously expressed p107 was localized to the nuclei (Fig. 2D); a property of cellular p107 as determined by biochemical fractionation experiments (L. Zhu, unpubl.). Taken together, we conclude that the p107 protein expressed from this cDNA clone has the known properties of the cellular p107.

p107 is a cell-type and cell-cycle stage-specific growth suppressor

A common feature of p53 and pRB, two well-characterized tumor suppressor gene products (Marshall 1991), is their ability to inhibit cell proliferation when overexpressed in sensitive cell lines (Baker et al. 1990; Diller et al. 1990; Mercer et al. 1990; Templeton et al. 1991; Hinds et al. 1992; Qin et al. 1992). Such inhibition is both cell-type and cell-cycle stage dependent. To determine whether p107 can inhibit cell proliferation, we employed the colony formation assay used previously for p53 and pRB. The p107 expression construct pCMVp107

CGGGTAGCGCGCTGGGAGGAGAAAGTAGCGGGCGGTGGCGCGAGCCCGGGGCGCTGAAGGATGTTTCGAGGACAAAGCCACGCTAGGGGGC 100
 GCGGTGGTGCAGCGAGCGGGGAGCGCTACAGCCCCGTGCCAGGAGCTGAACCTGGACGAGGGAGCGCGCGGCGGAGCCCTGGACGACTTACTGCC 110
 AVVAAGAALQALCQELNLD EGSAAEALDDFT A 120
 ATCCGAGGCACTACAGGCTAGAGGAGAAATACACACTGGTGGCATGTTTATATGTTGATGCGCGGAGGAGGATTTCCCGAGGTTGGAAAGG 130
 IRGNYSLEGEVTHWLACSLYVACRKSIIPTVKGK 140
 GTATCATGGAAGGCACTGTGTTTACCTACAGAACTACGCTACGCTAAATTAAGTTTAAATACAAATTTTAAATGAAGAAATGGATGGACAT 150
 IMEGNCVSLTRLRLRSRKA LSLIQFP SKMKKWM DM 160
 GTCAAATCTACCAAGAAATTCGTGAACGTATAGAAAGGCTAGAGAGAAATTTGAGGTGCTACTGTAATATCAAAAAATAGGCAATTTTAA 170
 S N L P Q E F R E R I E R L E R N F E V S T V I F K K Y E P I F L 180
 GATATATTTCAAAATCCATATGAAGAACCAAAAGTTACACGAAGCGGAGGAGGAGGATTCCTTGCAGTGTAAAGATCTGTTAAATTTCTGTT 190
 D I F Q N P Y E E P P K L P R S R K Q R R I P C S V K D L F N F C W 200
 GGACACTTTTGTCTTAAAGGTAATTTCCGGATGATGGGATGACTTAGTAACTCTTATCATTTACTTCTATGCTGCTTGGATCTGATTTTGC 210
 T L F V Y T K G N F R I G D D L V N S Y H L L L C C L D L I F A 220
 CAATGCGATTATGCGCAATAGACAAGACTTGCTAAATCATCATTTAAAGGTTTACCATCTGATTTTCATCTGCTGACTTTACGGCTTCTGAAGAG 230
 N A I M C P N R Q D L L N P S F K G L P S D P H T A D F T A S E E 240
 CCACCTGCTCATGCTGCTGTAACCTGCTGATGAGGCTTCTGTAAGCAAGAAAGAAATGAAGGAGCACTTTAAAGCCATATTTCAAAAC 250
 P P C I I A V L C E L H D G L L V E A K G I K E H Y F K P Y I S K L 260
 TCTTTGACAGGAATATTAAGGAGAAATGCTTCCGACCTTTCAAGTTTACTGATAATAGCAAGCAGTGAATAAGGAGTATGAAGATGATGCTTCT 270
 F D R K I L K G E C L L D L S S F T D N S K A V N K E Y E E Y V L 280
 AACTGTTGGTATTTGATGAGAGGATCTTTTGGGAGCAGCAGCAAGAGGAAATGGAAACCTCGAAAGTTCACCTGACACCCCTTAGGGA 290
 T V G D F D E R I F L G A D A E E E I G T P R K F T R D T P L G K 300
 CTGACACGAGGCTAATGTGGATATAACCTTCAAGCACTTTGAAAAAAGGTCATTTGCACTTCTACCCCACTGACCGGAGGAGATTTAT 310
 L T A Q A N V E Y N L Q Q H F E K K R S F A P S T P L T G R R Y L R 320
 GAGAAAAAGCAGCTTACTCTGTTGCTATCAGCCACCCAAAGTGTGAGCGGTACAGAGTATGTGGCTGCTGAAAAATGCACCAAGTGACCA 330
 E K E A V I T P V A S A T Q S V S R L Q S I V A G L K N A P S D Q 340
 ACTTATAATATTTTGAATCTGTGTCGTAATCTCTGTGAAACATTATGAAAAATCTAAAAGGAATAGGAGAGCTTTCTGTCAACACTATATCAA 350
 L I N I F E S C V R N P V E N I M K I L K G I G E T F C Q H Y T Q 360
 TCACAGATGAACAGGAGGATCTCACATAGACTTTGCTGTAAACAGACTAAAGCTGGCAGAAATTTGTATTATAAAATAGAGACTGTAATGGTTC 370
 S T D E Q P G S H I D F A V N R L K L A E I L Y Y K I L E T V M V Q 380
 AGGAAACAGGAGCTTATGGAATGGACATGTCAGTCTTTTAGAGCAAGATATATTCATGTTCTTGTATGGCTTGTGTTTGGAAATTTGCTCTT 390
 E T R L H G M D M S V L L E Q D I F H R S L M A C C L E I V L F 400
 TGCTTATAGCTCAGCTGCTTCTTTGATTTGAGTTTCAAGTCTCAACTTGCAACCTTTTACTTTTAAAGGTTATGAGGTGGTGTGCGCTCAGAA 410
 A Y S S P R T P P W I I E V L N L Q P F Y F Y K V I E V I R S E 420
 GAGGGCTCTCAAGGAGATGTTGAACACCTAAACAGCATTGAAGAACAGATTTGGAGAGTTTACATGGAGTACAGATTTCTGCACTGTGGGAGGCTC 430
 E G L S R D M V K H L N S I E E Q I L E S L A W S H D S A L W E A L 440
 TCCAGGTTCTGCAAAACAAAGTTCTACCTGTGAAGAAATATATCCCAAAATCTTGAACAGGAAATGAGGAAATGTCAGGCACTTTCCCTT 450
 Q V S A N K V P T C E E V I F P N N F E T G N G G N V Q G H L P L 460
 GATGCGAAGTCTTCCCTTAAGTACCAAGAGTCAAGGAGTTTCAAGTACAGGAGTCTTGAAGAGATATGCAACCTGCTTCCCAATTTGCTC 470
 M P M S P L M H P R V K E V R T D S G S L R R D M Q P L S P I S V 480
 CATGAAGCTACAGTCTTCTTCCAGGAGGAGTGTGAAGAAAGACTCTTGGAGAGGAGCCCAAGGAAATGCTTATGAGCAAGATCATTAACAGAG 490
 H E R Y S S P T A G S A K R R L F G E D P P K E M L M D K I I T E G 500
 GAACAAATGAAATCGCTCTTCTTCAAGCATTACTGCTGAAATGTATCAATTTTACCTGGTCAAACTCTTCAACAATGGCCAGCCCGCACTA 510
 T K L K I A P S S S I T A E N V S I L P G Q T L L I M A T A P V T 520
 AGGAACAGAGGACATAAGATTACAATCCATTACATGGTGTGCAATGATGCTGGAGAGTACACTGATACCTTTTCCATGAATACAAATCAGGAG 530
 G T T D G H K V T I P L H G V A N D A G E I T L I P L S M N T N Q E 540
 TCCAAAGTCAAGAGTCTGTAATCTTACTGCTCATTCAATATTTGGTCTTCTTCAAAACAGACCAATCTGACTAAAGCACAGAGGTACATCACTG 550
 S K V K S P V S L T A H S L I G A S P K Q T N L T K A Q E V H S T G 560
 GAATACAGGCAAGAGAGTGGGCTTACGACTATTTACAGAAAGGCTATCATTTGGCAAGTGTAGCTTACGTGATCTATGCTTAAAGTGA 570
 I N R P R T G S L A L F Y R K V Y H L A S V R L R D L C L K L D 580
 TGTTTCAATGAGTTACGAAGAGATATGACGCTGTTTGAATTCATTTAGTTTCACTGCTGATCTAATGAAGACAGGCAATTTGATACGCTCTC 590
 V S N E L R R K I W T C F E F T L V H C P D L M K D R H L D Q L L 600
 CTTTGGCTTTTATATATGCGCAAGGTAACAAAAGAGAAAGAACTTTTCAAGAAATATAGGAATCAGCCCCAAGCTAATAGTACAG 610
 L C A F Y I M A K V T K E E R T F Q E I M K S Y R N Q P Q A N S H V 620
 TATATAGAAGTGTCTGCTGAAAAGTATCCAAGAGAAGTTGTGGCATATAATAAAATATAAATGATGACTTTGAAATGATAGATTGTGACTTAGAAGA 630
 Y R S V L L K S I P R E V V A Y N K N I N D D F E M I D C D L E D 640
 TGCTACAAAACCTGACTGTTCCAGTGGACAGTGAAGAGGAGAAAGTATCTTATAAAATTTTCAATACAATATATGAGGAAGAGTGAAGTCA 650
 A T K T P D C S S G P V K E E R S D L I K F Y N T I Y V G R V K S 660
 TTTGCACTGAAATAGACTTGCGCAATCAGGACCATATGATGATGCTCCACCACTCTCTCTTTTCCACATATTAACAAAGCCAGGCTCACCAGCC 670
 F A L K Y D L A N Q D H M M D A P P L S P F P H I K Q Q P G S P R R 680
 GCATTTCCAGCAGCACTCCATTTATATTTCCCGCAGAAAGTGGTGAAGGCTTACACCAAGAGGCTCTGCTGTACAAGTTCAATGGCAGCCCTTC 690
 I S Q Q H S I Y I S P H K N G S G L T P R S A L L Y K F N G S P 700
 TAAGAGTTTGAAGATATCAACAACTGATAGGCAAGGTGAGCAGAGAACCAAGAGGAGTAAATAGGCACTGATGATGATGAGAAATCCCTTGGCAAA 710
 K S L K D I N N M I R Q G E Q R T K K R V I A I D S D A E S P A 720
 CGGCTGTGTAAGAAATGATGAGTTTACTGAAGCACTACAGGATGTTGTGAGTGAAGAGCAATCAATATGTTGTTCTTGTCTATGATATAAA 730
 R V C Q E N D D V L L K R L Q D V V S E R A N H - 740
 GCACCTTCAGATGTTCTGCGAAGTTGGACCTCTGCTCTCAAACTTTTACGCTTATAGATGATAAATATCACTGGGTATAAGAAAAAATTCGACA 750
 AAAATATGTCGCTTTTAAAAATTTTATCAAAAATGATGTTGACAGAGATGATTTTGTAGTTGGATTTGGAAGGAATATTTTAAAGTCTTTTAAAAATA 760
 CTAATAGTCCGCGCAGGCGTGTGGCTACGCTCTAATCCAGGACTTTGGAGGCGCAAGCGCGGAGATCAGCGAGTTCAGAGACAGGCT 770
 GACCAACATCGAGAAACCCATCTCTACTAAAAATACAAAATAGCCGGGTGGTGTGGCGCATGCTTATAATCCAGCTACTTGGGAGGCTGAGCGAGAA 780
 TTGCTTGAACCCAGGAGCGGAGGTTGTGTGAGCCAGGTTGCGCCACGCTCCAGCTGGGCAACAGAGATAAACTCCATCTCAAAAAATATATA 790
 TATATATATAAATAGGGAATTTTAAATGTTTGTCTTGTAGTTTCAAGATGAAATAGGAGAAACCCCACTAATTTTGTGCTCTTTTAAAAATA 800
 AATGTCCTCTGCTGTTCTGTAATATAGGATAAATATCTGCTTTTGTAGCAAAAAA 810

Figure 1. Complete nucleotide sequence of human p107 cDNA. Nucleotide sequences of the first 467 bp and the 3'-untranslated region were determined in this study. The two initiating Met codons that were used in this study (full-length) and previous studies (Ewen et al. 1991) are boxed. Nucleotide positions are at right, with amino acid residue positions shown in parentheses under the nucleotide positions.

contains a neomycin resistance marker. Transfected cells were cultured in G418-containing media for ~3 weeks to allow resistant cells to form colonies. As shown in Figure 3, transfection of Saos-2 cells with the p107 expression construct significantly reduced colony numbers. The levels of reduction were comparable to that seen with the pRB expression construct pCMVpRB as reported previously (Qin et al. 1992). Interestingly, p107 expression appeared not to cause significant reduc-

tion of colony numbers in U2OS cells (Fig. 3). U2OS cells were also resistant to the suppression effect of pRB. To confirm that p107 overexpression inhibited Saos-2 cells but had no effect in U2OS cells, individual colonies from p107-transfected plates were isolated and examined for exogenous p107 expression. Colonies expressing exogenous p107 could not be found in Saos-2 transfections, whereas U2OS colonies overexpressing p107 were detected and U2OS cell lines expressing p107 at high levels

Figure 2. Expression and characterization of full-length p107. (A) p107 produced from the cDNA clone comigrates with the cellular p107. ML1 cells (lane 1) were labeled with [35 S]methionine, lysed, and cellular p107 was immunoprecipitated by an anti-p107 monoclonal antibody (SD4; N. Dyson and M. Dembski, unpubl.). p107 was synthesized by in vitro transcription (T3 RNA polymerase) of the cDNA clone and translation of the RNA in rabbit reticulocyte lysate in the presence of [35 S]methionine (lanes 2,3).

Labeled proteins were immunoprecipitated with antibodies to p107 (SD4; lane 2) or pRB (XZ91; lane 3). Saos-2 cells were transiently transfected with pCMVAS (lane 4; this negative control is indicated in the figure as vector), pCMVp107 (lane 5), or pCMVpRB (lane 6). Transfected cells were metabolically labeled with [35 S]methionine and the cell lysates were immunoprecipitated with anti-p107 antibody SD4 (lanes 4,5) or anti-pRB antibody XZ91 (lane 6). Immune complexes were separated on an 8% SDS-polyacrylamide gel. The positions of protein molecular mass markers are indicated in kilodaltons. (B) Exogenously expressed p107 binds to E1A. Saos-2 cells were transfected with pCMVAS (lanes 1,5,9), pCMVp107 (lanes 2,6,10), pCMVp107-HA (indicated as pCHA in lanes 3, 7, and 11), and pCMVp107DE (as pDE in lanes 4, 8, and 12) and labeled with [35 S]-methionine. Equal amounts of cell lysate were used for immunoprecipitation with the anti-p107 monoclonal antibody SD4 (lanes 1–4), anti-HA monoclonal antibody 12CA5 (lanes 5–8), or mixed with extracts prepared from unlabeled 293 cells and immunoprecipitated with anti-E1A antibody M73 (Harlow et al. 1985) as described previously (Dyson et al. 1989) (lanes 9,10,11,12). (C) Exogenously expressed p107 binds to E2F. Saos-2 cells were transfected as described above. After 24 hr, cell extracts were prepared and immunoprecipitated with either the anti-p107 antibody SD4 (lanes 1–4) or the anti-HA antibody 12CA5 (lanes 5–8). E2F associated with the immune complexes was released by DOC treatment and detected in a mobility retardation assay using [32 P]-labeled E2F-binding site-containing oligonucleotide. Note that because the DOC release experiment is very sensitive, it detects the endogenous p107 activity at relatively higher levels as compared with the low amount of endogenous p107 detected by immunoprecipitation in B. (D) Nuclear localization of p107 was observed 36 hr after transfection of pCMVp107-HA into Saos-2 cells. (Left) Nuclear staining with Hoechst 33258; (right) staining with anti-HA antibody 12CA5. Both photographs were taken from the same field at 40 $\times$ magnification.

could be readily established (data not shown). Thus, p107 can function as a growth suppressor in Saos-2 cells but not in U2OS cells.

Here, it is interesting to note that another phenotype of pRB expression in Saos-2 cells is the large, flat cell morphology that resembles senescent cells (Templeton et al. 1991; Hinds et al. 1992; Qin et al. 1992). This morphology change was not seen in p107-transfected Saos-2 cells, although a few somewhat enlarged cells were detected (data not shown). This observation sug-

gests that p107 arrests cell growth in a state that is different from pRB.

We then investigated whether the proliferation suppression by p107 was specific to a certain stage of the cell-cycle progression. The point of cell-cycle arrest induced by overexpression of pRB in Saos-2 cells has been reported to be in mid-to-late G₁ as determined by microinjection (Goodrich et al. 1991) and transfection studies (Hinds et al. 1992). We employed a modified flow cytometry technique to study the effect of p107 on cell cycle.

Figure 3. Colony formation assay with Saos-2 and U2OS cells. Osteosarcoma cell lines Saos-2 and U2OS were transfected with 20 μ g of the indicated plasmids. Twenty-four hours after transfection, cells were trypsinized, diluted 1 : 5 for Saos-2 cells or 1 : 20 for U2OS cells, and cultured in media containing 500 μ g/ml of G418 for ~3 weeks. The plates were fixed and stained with violet blue.

The p107, pRB, or control vectors were cotransfected with a plasmid expressing a cell-surface protein that could be identified easily by immunofluorescent cell staining. The cell-surface marker CD20 was chosen for this purpose because it is only expressed in B lymphocytes and, even when expressed at high levels, is not toxic to the cells (Stamenkovic and Seed 1988). After transfection, cells that contained transfected DNA were identified by staining with an anti-CD20 monoclonal antibody, and the DNA content of the transfected cells was determined by propidium iodide staining. In transfection studies with pRB, this method was compared with a method in which the anti-pRB monoclonal antibody XZ91 (Hu et al. 1991) was used to detect pRB-transfected cells. Results obtained from both types of methods were identical (data not shown); therefore, we have routinely used the CD20 marker for flow cytometry analysis.

In Figure 4, representative results obtained from U2OS and Saos-2 cells are shown. Consistent with the colony formation assay, cell-cycle profiles of U2OS cells transfected with vector, pCMVpRB, or pCMVp107 were virtually identical. In Saos-2 cells, however, transfection with pRB or p107 reproducibly resulted in significant decreases of S-phase cells and corresponding increases in

G₁-phase cells. This result confirmed the previous reports of similar experiments with pRB (Hinds et al. 1992) and demonstrated that p107 generated a similar cell-cycle arrest, at least within the resolution of this experiment. From the above results we conclude that p107 has growth suppression activity that is both cell-type and cell-cycle stage dependent.

Rescue of p107-mediated growth arrest

Several conceivable explanations exist for the growth suppression activity of p107. Because growth suppression by 107 is both cell-type and cell-cycle stage specific, it seemed unlikely that the arrest was attributable to nonspecific toxic effects. It did seem possible, however, that p107 might inhibit cell growth by mimicking the biochemical role of pRB because pRB and p107 have extensive sequence homology and Saos-2 cells are very sensitive to pRB arrest. To address this question we examined the abilities of several proteins that have been implicated in the functional roles of pRB and p107 to influence their growth suppression activities in this assay system. As shown in Table 1 we coexpressed the E1A12S product, a set of human cyclins, and the recently cloned E2F1 (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992) with pRB or p107 in Saos-2 cells and examined their effects on growth suppression. Our results demonstrated clear differences in the ability of these proteins to rescue the G₁ arrests caused by pRB or p107.

As expected, the G₁-to-S block caused by p107 was prevented by coexpression of the E1A12S product. Interestingly, in three separate experiments, E1A coexpression resulted in a more efficient rescue in p107-transfected cells than in pRB-transfected cells, indicating that

Figure 4. Flow cytometry analysis of transfected Saos-2 and U2OS cells. Saos-2 and U2OS cells were transfected with 20 μ g of the indicated plasmids plus 2 μ g of pCMVCD20. Two days after transfection, cells were harvested for flow cytometry analysis (see Materials and methods for details) to determine the cell-cycle profile of the transfected cells. Solid bars represent the percentages of G₁-phase cells; open bars represent S-phase cells; hatched bars represent G₂ + M-phase cells.

Table 1. Flow cytometry analysis of transfected Saos-2 cells

Rescue	Vector			pRB			p107		
	G ₁	S	G ₂ + M	G ₁	S	G ₂ + M	G ₁	S	G ₂ + M
E1A									
+ CMV vector	56.8	23.1	20.1	77.4	7.1	15.6	78.4	8.8	12.8
+ CMVE1A12S				65.4	16.8	17.9	31.2	37.9	30.9
Cyclin									
+ CMV vector	48.6	36.0	15.4	74.2	10.6	15.2	74.5	12.2	13.4
+ CMV cyclin A				29.6	53.7	16.6	74.6	18.8	6.6
+ CMV cyclin B1				80.9	7.4	11.7	81.0	7.0	12.0
+ CMV cyclin B2				82.5	5.2	12.3	81.0	4.9	14.1
+ CMV cyclin E				36.4	51.6	12.1	66.3	20.0	13.7
E2F1									
+ CMV vector	39.9	25.6	34.5	79.3	9.2	11.5	73.8	11.5	14.7
+ CMVE2F1				54.6	38.9	6.5	69.6	16.0	14.4

Cell lines were transfected with the plasmids indicated and subjected to flow cytometry analysis to determine the cell-cycle profiles of cells that expressed the cotransfected cell-surface marker CD20. In E1A rescue experiments pRB and p107 were expressed from CMV-based vectors, whereas in cyclin and E2F1 rescue experiments, p107 and pRB were expressed from SV40-based vectors (see Materials and methods for details).

E1A was more efficient in overcoming the arrest in p107-transfected cells. Although we do not know the biochemical differences in the ability of E1A to overcome the p107 and pRB inhibitions, these results were the first indication that the growth arrests induced by these proteins were different. This suggestion was reinforced by rescue experiments with other genes.

Hinds et al. (1992) have reported that the growth arrest of Saos-2 cells by pRB could be rescued completely by coexpression of cyclins A or E but not by cyclins B1 or B2. Examination of the phosphorylation status of coexpressed pRB indicated that the ectopic expression of cyclin A or cyclin E resulted in pRB hyperphosphorylation and, thus, presumably inactivated the function of pRB. Using the same cyclin expression constructs (the kind gift of P. Hinds, The Whitehead Institute, Cambridge, MA), we were able to confirm their pRB results (Table 1). Cell-cycle arrest caused by p107, however, was not efficiently rescued by cyclin A or cyclin E in the same experiments (Table 1). Thus, in a second example, pRB and p107 cell-cycle arrests were rescued differentially by known growth regulators.

Recently, Qin and colleagues have shown that Saos-2 cells arrested by pRB overexpression can be rescued by cotransfection of an E2F1 construct (X.-Q. Qin and D. Livingston, in prep.). We have extended their observations and tested whether p107-arrested cells could also be rescued by E2F1 expression. Although both pRB and p107 associate with E2F proteins, they exist in different, independent complexes (Bandara and La Thangue 1991; Chellappan et al. 1991; Mudryj et al. 1991; Cao et al. 1992; Devoto et al. 1992; Shirodkar et al. 1992). The recently cloned E2F1 associates with pRB both in vitro and in vivo. To date, however, we have been unable to detect E2F1 association with p107 in vivo, although there is clearly an E2F-related protein that binds to p107 (N. Dyson and M. Dembski, unpubl.). This observation has now been supported by the isolation of a family of

E2F factors (J. Lees, M. Vidal, K. Helin, and E. Harlow, unpubl.). As shown in Table 1, cotransfection of E2F1 completely rescued the arrest by pRB but had no significant effect on p107 suppression. This finding is most consistent with the suggestion that pRB and p107 interact with different E2F proteins and that there is a functional difference in the growth suppression by pRB and p107.

Taken together, these rescue experiments show that p107 is not merely acting as a pRB surrogate in these assays. The differential rescue indicates that either there are differences in the actual mechanism of inhibition or the method of rescue is different.

p107 and pRB do not function in the same manner in C33A cells

To study growth suppression by p107 further, we expanded the types of cell lines used for the flow cytometry analysis. As shown in Table 2, p107 has a cell-type specificity that closely resembles pRB. The osteosarcoma cell line MG63 (L. Zhu, unpubl.), the breast carcinoma cell line MCF7 (Horowitz et al. 1990), and the glioblastoma cell line T98G (A. Fattaey, unpubl.) all contain apparently normal pRB and are resistant to the suppression effect of pRB or p107 expression. Interestingly, the bladder carcinoma cell line J82 that contains deletion of exon 21 in pRB (Horowitz et al. 1989) was not sensitive to either pRB or p107 expression. Thus, the apparent loss of normal pRB function was not in itself sufficient to allow these cells to be growth suppressed by the reintroduction of wild-type pRB or p107.

Although p107 and pRB behaved similarly in most of the cell lines tested, they had different effects on the cervical carcinoma cell line C33A (Table 1). C33A cells express a mutated pRB with an in-frame deletion of four amino acid residues in exon 20 (Scheffner et al. 1991). Exogenous expression of pRB in this cell line did not

Table 2. Flow cytometry analysis of various transfected cell lines

Cell lines	Transfection plasmids								
	pCMVAS			pCMVpRB			pCMVp107		
	G ₁	S	G ₂ + M	G ₁	S	G ₂ + M	G ₁	S	G ₂ + M
Glioblastoma T98G	49.4	31.0	19.6	54.4	31.6	14.1	53.0	31.2	15.8
Breast carcinoma MCF7	59.2	11.7	29.1	55.6	13.0	31.4	66.8	6.9	26.3
Osteosarcoma MG63	52.1	21.9	26.0	53.2	30.6	16.2	53.7	22.1	24.3
Bladder carcinoma J82	54.9	33.9	11.2	59.4	28.4	12.1	54.1	36.6	9.4
Cervical carcinoma C33A	39.9	43.3	16.8	39.1	57.7	3.2	78.0	18.8	3.2
	36.8	36.0	27.1	49.4	42.7	7.9	86.9	8.8	4.3
	37.9	37.8	24.3	39.6	40.7	19.7	60.1	21.2	18.6

Cell lines were transfected with the indicated plasmids and subjected to flow cytometry analysis to determine the cell-cycle profiles of cells that expressed the cotransfected cell-surface marker CD20. For cell line C33A, data from three separate experiments are shown.

arrest cells in G₁, although it reduced the G₂ + M cell population reproducibly. In contrast, p107 expression reduced the population of S-phase cells significantly with corresponding increase in G₁-phase cells. The difference between pRB and p107 could not be attributed to the levels of expression of pRB and p107, as they were sim-

ilar in all three cell lines, U2OS, Saos-2, and C33A (Fig. 5A). Importantly, pRB expressed in C33A cells appeared to be phosphorylated to a degree that was between the phosphorylation status of pRB in Saos-2 cells and that in U2OS cells on the basis of the migration behavior of the pRB proteins (Fig. 5A, cf. lanes 2, 4, and 6). It is known

Figure 5. Expression of pRB and p107 in cell lines after transient transfection. (A) Three different cell lines (as indicated) were transfected with pCMVAS as a control (lanes 1,3,5,7,9,11), with pCMVpRB (lanes 2,4,6) or with pCMVp107 (lanes 8,10,12). Transfected cells were labeled with [³⁵S] methionine and subjected to immunoprecipitation with XZ91 for pRB-transfected cells or SD4 for p107-transfected cells. Samples were separated on a 7% SDS-polyacrylamide gel and fluorographed. (B) Cells were transfected with 20 μg of pCMVAS (lanes 3,4,6,8), 10 μg of pCMVpRB plus 10 μg of pCMVAS (lanes 2,9), 10 μg of pCMVpRB plus 10 μg of pCMVp107 (lanes 1,10), and 20 μg of pRB (lanes 5,7). Immunoprecipitation with anti-pRB antibody XZ91 was carried out as in A. pRB proteins in lanes 1–3 were detected by immunoblotting with XZ91. pRB in lanes 4–10 were detected by autoradiography (pRB proteins are indicated).

that the phosphorylation status of pRB oscillates during the cell cycle being hypophosphorylated in G₁ phase and gradually hyperphosphorylated when the cells advance through S phase into G₂ phase (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989, 1992; Mihara et al. 1989; Ludlow et al. 1990). Therefore, the phosphorylation status of exogenously introduced pRB in C33A cells is another indication that expression of pRB in this cell line did not arrest cells before S phase, as was the case in Saos-2 cells. On the basis of these observations, we predicted that if p107 expression arrested C33A cell growth at a point that was unaffected by pRB, this difference might be revealed by the phosphorylation status of pRB. Figure 5B shows that this was the case. When cotransfected with p107, pRB expressed in C33A cells was hypophosphorylated (Fig. 5B). From these results we conclude that p107 has a growth suppression effect on C33A cells at a point that is not apparently affected by pRB.

Growth suppression by p107 does not correlate with the ability to bind E1A

Previous studies (Ewen et al. 1991, 1992) have demonstrated that p107 contains a pocket region that is homologous to the pRB pocket. This is based both on sequence comparisons and the ability of p107 to bind to E1A, large T, and a set of cellular proteins first identified by their ability to associate with pRB. The ability to bind large T is required, although not sufficient, for the growth suppression activity of pRB (Qin et al. 1992). To study the functional properties of p107 further, we engineered several mutants to determine whether p107 has similar requirements for its growth suppression activity. One

commonly used mutant of pRB that disrupts binding to the viral proteins is a missense mutant at Cys706, first identified in a small cell lung carcinoma cell line (Kaye et al. 1990). p107 contains a Cys residue at position 846 that is in a similar position to Cys-706 of pRB. When the Cys → Phe mutation was introduced into this position of p107, the ability of p107 to associate with T was lost (Ewen et al. 1992). This mutant and a set of five others were engineered and shown to be expressed at levels comparable with the wild-type p107 protein (Fig. 6, lanes 1–7,12). Most of the mutants, with the exception of p107DE, were recognized by anti-p107 antibodies. HA-tagged versions of some of the mutants (p107N385, p107EC, and p107DE) were examined by the 12CA5 antibody, and again the expression levels of these mutants were very similar (Fig. 6, lanes 9,11,12). All of the mutant forms of p107 were also able to localize to the nuclei after transient transfection of Saos-2 cells (data not shown). These mutants were then used in the growth suppression assays (as outlined in Fig. 4 and Table 1) and E1A-binding assays (as outlined in Fig. 2). The results of these assays are shown in Figure 6 (lanes 13–20) and Figure 7. Although the pocket region was essential as indicated by p107DE and p107EC, the ability of p107 to bind E1A was surprisingly not required for growth suppression. Two mutants, the Cys-846 → Phe mutation and the deletion of the sequences from amino acid 768 to the carboxyl-terminus, were unable to bind to E1A but were still competent to inhibit cell proliferation in Saos-2 cells. We conclude that the ability to associate with E1A is not essential for p107-mediated growth suppression at least in this cell line. This observation indicated another difference between pRB and p107; they have different structural requirements for the growth suppression activity.

Figure 6. Expression and E1A binding of p107 mutants. C33A cells were transfected with equivalent amounts of expression constructs for different p107 mutants and labeled with [³⁵S] methionine. Equal amounts of cell extracts were used for either immunoprecipitation with a mixture of five monoclonal anti-p107 antibodies (N. Dyson and M. Dembski, unpubl.) (lanes 1–7), with an anti-HA antibody 12CA5 (lanes 8–12), or mixed with unlabeled 293 cell extract and immunoprecipitated with anti-E1A antibody M73 (lanes 13–20). The immune complexes were separated on a 10% SDS–polyacrylamide gel with positions of standard protein molecular mass markers indicated. Note that the p107 and pC949 proteins in lanes 8 and 10 were not HA-tagged and were used as controls for the anti-HA antibody.

Figure 7. E1A binding and growth suppression abilities of p107 mutants. The full-length p107 protein is shown schematically with the A and B segments numbered according to sequence homology with corresponding regions of pRB (Ewen et al. 1991). The boundaries of each deletion are drawn to scale, and the exact number of residues at the boundary are indicated in the names of some mutants (e.g., p107N385 indicates an amino-terminal truncation at residue 385). p107DE and p107EC were generated by deletions and in-frame fusions between two restriction sites (see Materials and methods). p107F846 is a C → F mutation at position 846. Growth suppression results were obtained from flow cytometry analysis of Saos-2 cells transfected with each mutant construct. The activity of each protein was determined as the net difference between G₁- and S-phase cell populations as compared with the vector control. The activity of the full-length protein was set as 100%, and the activities of each mutant were determined accordingly. The inability of p107EC to suppress cell growth might be attributable to an unfavorable and general conformation alteration of the mutant protein or to the masking of an active domain(s) present in p107C768.

p107 is an inhibitor of E2F transcriptional activity

pRB and p107 are found in different, independent E2F complexes. One consequence of the high-level expression of pRB is the repression of E2F transcriptional activity (Hamel et al. 1992; Hiebert et al. 1992; Zamanian and La Thangue 1992). Because E2F-binding sites are present in the promoter regions of many genes involved in cell proliferation (Nevins 1992), the ability of pRB to repress the E2F transcriptional activity has been assumed to be important for its role as a negative growth regulator. We were able to express the full-length p107 protein to study the effects of p107 on E2F transcriptional activity. Comparing the E2F repression activity of pRB and p107 in the C33A cell line became especially interesting with the demonstration that p107 and pRB have different effects on the growth of this cell line. A set of CAT reporter plasmids for endogenous E2F activity was employed in transient transfection studies with p107. Results in Figure 8A represent a typical experiment with reporter construct E2CAT and its mutant derivatives (Loeken and Brady 1989). Cotransfection with p107 consistently repressed the activity from the E2CAT ~10-fold. This level of repression was comparable with

the repression effect of pRB as reported previously (Hamel et al. 1992; Hiebert et al. 1992; Zamanian and La Thangue 1992). Repression of E2F activity was not seen with the mutant p107DE that contained a deletion in the pocket region. Like pRB, this repression was specific to E2F, because there was no significant repression on an E2CAT reporter with mutations in E2F-binding sites of the promoter. Analogous results were also obtained in Saos-2 cells (data not shown). These results indicate that p107, like pRB, is a potent inhibitor of E2F-mediated transcription.

Because no obvious differences in the repression activities were seen between pRB and p107 (Fig. 8A), we titrated the levels of expression constructs to compare the repression of both proteins in more detail. From the results in Figure 8B it is evident that pRB and p107 repressed the E2F activity with similar levels of input plasmids. Analogous titration results were also obtained in Saos-2 cells (data not shown). We also noted that whereas the repression by p107 could be reversed completely by the E1A12S product, the reversal of E2F activity in pRB-transfected cells was not complete (Fig. 8A). This difference was also apparent in Saos-2 cells (data not shown). Furthermore, we have also observed in both

Figure 8. Repression of E2F activity by p107 and pRB. (A) C33A cells were cotransfected with (1) pRB or p107 expression constructs (8 μ g) as indicated; (2) 5 μ g of E2F reporter construct with upstream wild-type E2F-binding sites (*left* and *right* panels) or mutated E2F-binding sites (*middle* panel); (3) 1 μ g of pRSV β -gal as internal control; (4) 5 μ g of E1A12S expression construct for the *right* panel; and (5) pBSK DNA for a total 25 μ g of DNA. CAT activities were normalized with the internal control β -galactosidase activity and are the averages of duplicate samples. (B) CAT assays were performed as described for E2CAT reporter (A, *left*), except decreasing amounts (as indicated) of p107 or pRB expression constructs were used.

B

C33A and Saos-2 cells that coexpression of p107 and E1A12S resulted in a higher CAT activity than that observed when E1A12S alone was expressed (Fig. 8A). The mechanism for these differences is not understood and is currently under investigation.

We conclude that p107 and pRB repress E2F activity in a similar manner, at least in these two cell lines, and the difference seen in the growth suppression of C33A cells does not correlate with the repression of E2F activity.

Discussion

p107 has many similarities to pRB. These similarities include stretches of extended amino acid homology and associations with the same viral oncoproteins and similar cellular proteins, including the transcription factor E2F. Previous work, however, has also demonstrated differences between pRB and p107. Most interestingly, pRB could be detected in a complex with E2F in late G₁ and S phases of the cell-cycle, whereas p107 could be detected in independent complexes with cyclin E, cdk2, and E2F in G₁ phase and with cyclin A, cdk2, and E2F in

S phase. Furthermore, the cloned E2F1 (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992) appears to bind exclusively to pRB but not to p107. This fits well with the recent cloning of a family of E2F proteins (J. Lees, M. Vidal, K. Helin and E. Harlow, unpubl.), and the demonstration that the E2F proteins bound to pRB are structurally distinct from those bound to p107 (N. Dyson and M. Dembski, unpubl.). A likely explanation for these findings would be to suggest that functions of pRB and p107 are related but distinct. Our functional studies of p107 establish this distinction.

We have identified several functional activities of p107 in this study. When overexpressed, p107 can inhibit the growth of tumor cells in tissue culture with a cell-type specificity that is similar but not identical to that of pRB. This inhibitory effect blocks cell-cycle progression of the sensitive cells before S phase. p107 is also an inhibitor of E2F-mediated *trans*-activation. Unlike pRB, however, the p107-mediated growth arrest cannot be rescued efficiently by cyclin A, cyclin E, or E2F1, and the ability to associate with E1A is not correlated with the growth suppression activity of p107. Taken together, our results demonstrate a role for p107 as a negative cell

growth regulator that functions through similar but not identical mechanisms to pRB.

The differences between the functions of pRB and p107 are best seen in the analysis of the cervical carcinoma cell line C33A. Exogenously expressed p107 inhibited C33A proliferation at a point apparently not affected by pRB. This difference can be explained in different ways. p107 and pRB could function through completely independent pathways so that a particular property of C33A cells, perhaps caused by a mutation in this cell line, makes them sensitive to p107 but resistant to pRB. Alternatively, p107 may have a function in addition to the one shared by both p107 and pRB. In this case, C33A cells would be insensitive to the shared activity but sensitive to the unique p107 function. On the other hand, it is also conceivable that C33A cells are resistant to the G₁ suppression effect of pRB because they contain a strong kinase for pRB phosphorylation. If this were the case, one would envision that pRB and p107 are regulated differently, either through different kinases or through different mechanisms. This speculation is in agreement with the ability of cyclins A and E to rescue pRB-mediated, but not p107-mediated, growth suppression in Saos-2 cells (Table 1).

Our functional analysis of pRB and p107 in Saos-2 and C33A cells raises an interesting question as to whether E2F is an essential target for this type of growth inhibition. The efficient rescue of pRB-mediated growth arrest of Saos-2 cells by E2F1 would seem to point to the E2F1 factor as a major target for growth suppression. This rescue, however, may be attributable to a simple sequestering effect that prevents the E2F1-bound pRB from interacting with its other targets. On the other hand, the fact that pRB overexpression in C33A cells repressed E2F activity but failed to arrest cell proliferation in G₁ makes it unlikely that growth suppression in G₁ is simply a result of E2F repression. This result suggests that the repression of E2F activity is not sufficient to lead to the inhibition of cell proliferation, at least in C33A cells. The repression of E2F activity, as assayed with the E2CAT construct, and the growth suppression activity, as assayed in transient transfection experiments, may well be independent activities. This possibility is favored by our preliminary results that mutant p107C768 is a much less potent repressor of E2F activity but is still active in growth suppression, both assayed in C33A cells. In any event, these studies have important implications for the functional significance of E2F activity in the G₁-to-S transition. If, in pRB-transfected cells, E2F activity is repressed but G₁-to-S transition is not, the activation of E2F—at least the E2F that is repressed by pRB—cannot be the only promoting factor for this transition.

The efficient repression of E2F activity by p107 (Fig. 8) provides a functional consequence for the E2F-p107 interactions, a consequence that can also result from pRB-E2F interactions. Given the observation that pRB and p107 interact with different E2F proteins, however, it becomes somewhat surprising that the repression of the total E2F activity in a cell (as measured by E2CAT) by each protein alone was near complete. Several possibi-

ties could be responsible for this. It is very likely that in the transient transfection experiments, pRB (or p107) proteins are expressed to such high levels that interaction with E2F factors that are not normally regulated by pRB (or p107) could take place. It is also possible that the high-level expression of pRB or p107 may remove an essential and common cofactor from the E2F-activation machinery. Also, the E2 promoter of adenovirus (E2CAT reporter construct) commonly used in the E2F studies may not be able to fully reflect the complex and delicate regulation of E2F activities in a cell. Further work is needed to understand the mechanism of regulation of E2F activity by pRB and p107.

The correlation between oncoprotein binding and growth suppression, and other properties such as nuclear tethering (Mittnacht and Weinberg 1991), has been taken to indicate that pRB normally associates with certain cellular proteins that have domains similar to these viral oncoproteins. Although, to date, there is an unbroken correlation between the ability of viral oncoprotein binding and growth suppression for pRB, two of the p107 mutants lost the ability to bind E1A but still retained their growth suppression ability. In vitro, the mutant p107F846 has lost the ability to bind E1A and is also unable to associate with a set of cellular proteins similar to those associated with pRB (Ewen et al. 1992). Because this mutant still retains most of the growth suppression activity, it would seem that the in vitro protein association studies are not an indication of the in vivo function of p107. The difference in the requirement to associate with viral oncoproteins is another indication that p107 operates, at least in part, through a different afferent or efferent pathway from pRB. It is important to note that the region of p107 that is sufficient for growth suppression includes most of the spacer (Fig. 7). Given its ability to associate with cyclin A, the p107 spacer may play a role in growth suppression. The ability of p107 to associate with cyclin E in the cdk2/cyclin E/p107/E2F complex could also be involved. Nevertheless, overexpression of cyclin A or E could not efficiently rescue the growth arrest by p107. On the other hand, cyclins A and E have not been shown to associate with pRB but they can efficiently rescue the growth arrest of Saos-2 cells by pRB, presumably by hyperphosphorylating pRB (Hinds et al. 1992).

Finally, the observation that p107 can inhibit cell proliferation in a manner that is reminiscent of pRB raises the question of whether p107 can act as a tumor suppressor gene product. Others have reported previously that growth suppression is not an absolute indicator of tumor suppression. For example, Bookstein et al. (1990) have reintroduced wild-type pRB into the prostate carcinoma cell line DU145, which contains the same mutant exon 21-deleted pRB as the J82 cell line used in this study, and the resultant cells did not show altered growth rate but did fail to form tumors in nude mice. Whether pRB or p107 overexpression can change the tumorigenicity of any of the cells that we have tested is an interesting issue yet to be determined. The most obvious difference between pRB and p107 is that no naturally

occurring mutations of p107 have been detected so far, whereas mutations of pRB are present in all retinoblastomas and many other tumors. Theoretically, several possibilities may exist to explain the lack of p107 mutations in tumor cells. It is possible that the p107 gene can tolerate only subtle mutations that are not easily detected. Such mutations might exist in cells that are sensitive to p107 overexpression such as Saos-2 or C33A cells, although the ability of p107 to bind E1A and E2F in these cell lines seems to be normal (L. Zhu, unpubl.). Alternatively, p107 may have an essential function so that cells cannot proliferate in its absence. This possibility has become attractive since the discovery of the associations between p107 and cyclins E and A, a characteristic that pRB apparently lacks. A third possibility is that deletion of p107 function would not give the cell a growth advantage. With the isolation of the full-length p107 cDNA and the demonstration of its growth suppression and E2F repression activities, we are now in a position to investigate further the mechanisms through which p107 regulates cell growth. Pathways through cyclins and cyclin-dependent kinases and/or E2F are obvious candidates for investigation.

Materials and methods

Cell culture and transfection

Human tumor cell lines Saos-2, U2OS, C33A, and J82 were purchased from the American Type Culture Collection. Osteosarcoma cell line MG63 was kindly provided by S. Friend (Massachusetts General Hospital). ML-1, 293, and T98G have been described previously (Helin et al. 1992). All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. Cell lines were transfected by the standard calcium-phosphate precipitation method (Ausubel et al. 1990). DNA precipitates were left on the cells for 12–15 hr. The cells were then washed twice with PBS and cultured in fresh media.

Cloning of the full-length p107 cDNA

A cDNA library prepared from the pre-B lymphocyte cell line Nalm 6, kindly provided by A. Bernards (Massachusetts General Hospital), was screened at high stringency with a DNA probe spanning the first 800 bp of the previously published p107 sequence. The probe was generated by restriction digestion with *EcoRI* in the polylinker of the vector and *DraIII* in the p107 sequence. One positive clone (clone 7) contained 467 bp 5' upstream of the previously published sequence and was used in this study. This clone, however, did not contain the complete carboxy-terminal half of the molecule and was therefore digested at the *NdeI* site (nucleotide 517 in Fig. 1) and ligated onto the same *NdeI* site of another 5'-truncated cDNA to generate the full-length clone.

Plasmid constructs

The full-length clone was inserted into vector pBluescript II SK+ (Stratagene) generating pBSKp107. A derivative plasmid was constructed by deleting a GC-rich sequence between two *SacII* sites (one in the polylinker of the vector and the other at nucleotide 52, just 5' of the ATG codon) and was used in vitro

transcription experiments to give better yield of the full-length product. For in vivo expression, the full-length clone was removed from pBSKp107 by *XbaI* and *KpnI* digestion and inserted into the same sites of the SV40-based expression vector pJ3 Ω resulting in pJ3p107. The p107 sequences were then excised from pJ3p107 by *BamHI* and *BglII* and inserted into the *BamHI* cloning site of pCMVneoBam (Baker et al. 1990) to generate pCMVp107. The antisense construct pCMVAS was used for controls. The HA epitope was added to the natural carboxyl terminus of the protein by PCR with a 5' primer from p107 nucleotide sequences (TAATAGCCATCGATAGTGATGC) and a 3' primer containing the epitope (CCGCTCGAGCTACAA-GCTAGCGTAATCTGGAACATCGTAT). The PCR product was digested with *Clal* and *XhoI* and ligated into the *Clal* site of the gene. The sequence was confirmed by DNA sequencing.

p107 mutations p107DE and p107EC were generated by in-frame deletions of sequences between two convenient restriction sites: *DraIII* at 1287 (nucleotide position as shown in Fig. 1) and *EcoRI* at 2541 were used to generate the DE deletion, and *EcoRI* at 2541 and *Clal* at 3170, to generate the EC deletion. Mutations p107N385 and p107C949 were generated by PCR as follows: For p107N385 the 5' primer was CCGGGATCCGC-CACCATGGTTACTCCTGTTGCATCAGCC, and the 3' primer was CTGTCTTTCATTAGATCAGG. The PCR product was cut with *BamHI* and *EcoRI* and ligated into the *EcoRI* site of the gene. The *DraIII*(1287)–*EcoRI*(2541) fragment of the resulting construct was then replaced by the same fragment from the original clone and the region 5' of the *DraIII* site was confirmed by sequencing. p107C949 was constructed similarly (the 5' primer was GATCGGTACCTTCGCTAGCACTATTTTACAGAAAG and the 3' primer was GAAGATCTGGTACCTAG-TATTTTCAGTGCAAATGACT). Mutation in p107C768 was generated by a random exonuclease III nuclease digestion followed by the insertion of a *XbaI* linker with stop codons in three frames (New England BioLabs). Mutation p107F846 was described previously (Ewen et al. 1992) and moved into the full-length clone with convenient restriction enzymes. All mutated versions of p107 were inserted into the pCMVneoBam vector at the *BamHI* site. pCMVpRB was described previously (Qin et al. 1992). pRB cDNA was excised from pCMVpRB as a *BamHI* fragment and inserted into pJ3 Ω to yield an SV40-based expression construct. The pCMVneoBam vector was also used to express CD20 (Stamenkovic and Seed 1988) and E1A12S (Moran and Zerkler 1988) cDNAs. Expression constructs for human cyclins A, B1, B2, and E were kindly provided by P. Hinds (Hinds et al. 1992). pCMVE2F1 contains the full-length open reading frame of the E2F-1 cDNA (Helin et al. 1992) and was constructed as described (K. Helin, E. Harlow, and A. Fattaey, in prep.).

Detection of p107 expression, cellular localization, E1A and E2F association

For p107 expression, 20 μ g of pCMVp107 constructs was used in transfection assays in 100-mm plates of subconfluent cells. Twenty-four hours after the removal of DNA precipitates, the transfected cells were incubated for 3 hr in 3 ml of DMEM minus methionine containing 0.5 mCi of [35 S] Protein Labeling Mix (NEN). Immunoprecipitations were carried out as described previously (Hu et al. 1991). A panel of anti-p107 monoclonal antibodies (N. Dyson and M. Dembski, unpubl.) were used to detect p107, monoclonal antibody 12CA5 was used to precipitate the HA-tagged p107, and XZ91 (Hu et al. 1991) was used to detect pRB. For Western blotting, proteins were transferred to Immobilon-P membranes (Millipore) according to manufacturer's instructions. pRB was detected by use of XZ91

followed by a color development system with an alkaline phosphatase-conjugated anti-mouse IgG antibody (GIBCO-BRL).

For immunofluorescence staining, the transfected cells were washed with PBS, fixed with 3% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.3% Triton X-100 for 10 min. Monoclonal antibody 12CA5 in the form of tissue culture supernatant was diluted 1 : 1 with PBS containing 1% normal goat serum and applied to the cells for 1 hr at room temperature. After washing with PBS for three times, the cells were incubated with rhodamine-conjugated goat anti-mouse IgG antibody (Cappel Laboratories, 1 : 100 dilution) for 1 hr. The cells were washed with PBS, stained with Hoechst 33258 for 5 min, and analyzed by immunofluorescence microscopy.

The ability of the in vivo-expressed p107 to bind E1A was determined by a mixing experiment as described previously (Dyson et al. 1989). Equivalent amounts of [³⁵S]methionine-labeled cell lysate were used both in anti-p107 immunoprecipitation and in mixing with the 293 cell extract (an E1A-transformed human embryonic kidney cell line expressing high levels of E1A).

To determine the ability of the in vivo-expressed p107 to associate with E2F, the DOC release experiment was performed after transient transfection of p107 expression constructs according to a modified protocol. Twenty-four hours after transfection cells were washed with cold PBS, pelleted, and resuspended in an extraction buffer [20 mM HEPES at pH 7.4, 1 mM MgCl₂, 40 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM NaF, and 0.5 mM sodium orthovanadate]. The cell suspension was sonicated briefly on ice with a Branson Sonifier 250 and subjected to one cycle of freezing-thawing. The resulting extract was cleared by centrifugation at 4°C in a microcentrifuge for 10 min. The supernatant was used for DOC release experiment as described previously (Cao et al. 1992).

Colony formation assay

The colony formation assay was performed as described (Qin et al. 1992), except that the transfected cells were split with certain dilutions before starting the G418 selection.

Flow cytometry analysis

For flow cytometry analysis, 2 µg of an expression vector for the cell-surface protein CD20 (pCMVCD20) was cotransfected with 20 µg of p107 or pRB constructs into the cells indicated as described above. In rescue experiments, 10 µg of pRB or p107 constructs and 10 µg of plasmid constructs expressing E1A12S, human cyclins, or E2F1 were used. Forty-eight hours after the removal of DNA precipitates, cells were washed once with PBS, once with PBS containing 0.1% EDTA, and incubated in PBS containing 0.1% EDTA for 5 min at 37°C to dislodge the cells from the plates. Five milliliters of media was added, and the cells were pelleted at 1000g for 5 min. The cell pellet was resuspended in a small volume (50–100 µl) of medium containing 20 µl of an FITC-conjugated anti-CD20 antibody (Becton-Dickinson). Cells were incubated with the antibody on ice for 30 min, followed by washing twice with PBS containing 1% calf serum. The cells were fixed with 90% ethanol on ice for several hours. Before flow cytometry analysis, the cells were pelleted, washed once in PBS, and stained with 20 µg/ml of propidium iodide containing 200 µg/ml of RNase. Flow cytometry analysis was performed on a Becton-Dickinson FACScan machine. The intensity of propidium iodide staining was analyzed on cell populations that were positive for FITC staining to determine the DNA content and, hence, cell-cycle profiles of these cells using

the CellFIT Cell Cycle Analysis software. The data presented are representatives of multiple experiments. The cell-cycle distributions of vector control samples vary to some extent from experiment to experiment; however, the effect (differences between vector control and testing samples) observed in one particular experiment is significantly consistent in separate experiments.

CAT assays

For CAT assays, 25 µg of transfection DNA included 5 µg of the CAT reporter construct, 1 µg of pRSVβ-gal plasmid, the indicated amount of p107 or pRB expression construct, and pBSK DNA for a total mass of 25 µg. Forty hours after transfection, CAT activities were determined as described previously (Helin et al. 1992). CAT activities (cpm) were normalized on the basis of the internal control β-galactosidase activity, which did not reproducibly vary more than twofold between samples.

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Note added in proof

The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned the accession number L14812.

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