ed DNA. If this is the case, then at the distances studied here, higher order chromosome structure is not required for activation either upstream or downstream of the promoter.

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9. The plasmid pΔ-38 contained the *E4* TATA box, start site, and 250 bp of coding sequence cloned into the Sma I site of pGEM3. The plasmid pG_5E4T was constructed by inserting five tandem GAL4 binding sites (G_5) into the Hinc II site of pΔ-38, 23 bp upstream from the *E4* TATA box (E4T). The plasmids $pG_5I54E4T$ and $pG_5I201E4T$ were constructed by inserting 54 (I54)– and 201 (I201)–bp Sau 3A fragments from the λ repressor and TFIIIA genes, respectively, into the Bam HI site of pG_5E4T. pΔ-63 contained the natural ATF site immediately adjacent to the *E4* TATA box, and all other sequences were identical to pΔ-38. The Hind III–Bam HI fragment of pG_5E4T, containing the GAL4 binding sites, was first transferred into pΔ-63 to produce pG_5AE4T, and then insertions of 54 and 201 bp were made as described above to generate the plasmids $pG_5I54AE4T$ and $pG_5I201AE4T$.
10. A 1139-bp Pvu II–Bgl I fragment from the adenovirus major late gene was inserted into the Bam HI site of $pG_5I160AE4T$ (*8*) to create $pG_5I1300AE4T$; alternatively, the Hind III–Bam HI fragment (containing the GAL4 binding sites) was excised and the Pvu II–Bgl I fragment was inserted to create pI1300AE4T. The Hind III–Bam HI fragment of pG5E4T was cloned into the Eco RI site of pΔ-63 to generate $pAE4T1320G_5$.
11. M. Carey and M. Ptashne, unpublished data.
12. Other workers have failed to observe chromatin formation at ratios of DNA to HeLa nuclear extract protein similar (with a factor of 2) to those used in our experiments [J. L. Workman, S. M. Abmayr, W. A. Cromlish, R. G. Roeder, *Cell* **55**, 211 (1988)].
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14. The 40-μl in vitro transcription mixtures contained 20 m*M* Hepes (*p*H 7.6), 60 m*M* KCl, 7.5 m*M* $MgCl_2$, 12.5% glycerol, 0.6 m*M* dithiothreitol, 0.5 m*M* nucleoside triphosphates, 25 μl (150 μg) of HeLa nuclear extract (*13*), 0.025 n*M* specific template, 200 ng of pGEM3 (Promega) carrier DNA, and either no protein or template-saturating amounts of GAL4(1–147)+AH or GAL4(1–147)+VP16 that had been purified as described previously (*8*) [D. Chasen, J. Leatherwood, M. Carey, M. Ptashne, R. Kornberg, *Mol. Cell. Biol.* **9**, 4746 (1989)]. After 1 hour at 30°C, the reactions were terminated, and the reaction products were purified and analyzed by primer extension [J. Lillie, M. Green, M. R. Green, *Cell* **46**, 1043 (1986)]. The hybridized primers were fractionated on 18-cm long, 1.5-mm thick, 10% polyacrylamide-urea gels. The gels were autoradiographed by exposure to Kodak XAR-5 film, and the autoradiographs were scanned with a Helena densitometer; dilutions of the primer were used to generate a standard curve. The transcription conditions differ from our previous experiments in an important way: the template DNA concentration is 100-fold lower than previously used. We found the amount of GAL4(1–147)+VP16 necessary to fill the binding sites at our standard DNA template concentration of 2.5 n*M* led to an inhibition of transcription, a phenomenon that may be related to "squelching" [G. Gill and M. Ptashne, *Nature* **334**, 721 (1988)]. We therefore lowered the DNA template concentration to 0.025 n*M* and found that the correspondingly lower amount of GAL4(1–147)+VP16 needed to fill the binding sites did not inhibit transcription. These new conditions more accurately mimic the effects observed in vivo with the two GAL4 derivatives.
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Suppression of Tumorigenicity of Human Prostate Carcinoma Cells by Replacing a Mutated *RB* Gene

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Introduction of a normal retinoblastoma gene (*RB*) into retinoblastoma cells was previously shown to suppress several aspects of their neoplastic phenotype, including tumorigenicity in nude mice, thereby directly demonstrating a cancer suppression function of *RB*. To explore the possibility of a similar activity in a common adult tumor, *RB* expression was examined in three human prostate carcinoma cell lines. One of these, DU145, contained an abnormally small protein translated from an *RB* messenger RNA transcript that lacked 105 nucleotides encoded by exon 21. To assess the functional consequences of this mutation, normal *RB* expression was restored in DU145 cells by retrovirus-mediated gene transfer. Cells that maintained stable exogenous *RB* expression lost their ability to form tumors in nude mice, although their growth rate in culture was apparently unaltered. These results suggest that *RB* inactivation can play a significant role in the genesis of a common adult neoplasm and that restoration of normal *RB*-encoded protein in tumors could have clinical utility.

THE RETINOBLASTOMA GENE (*RB*) encodes a nuclear phosphoprotein that is constitutively expressed in most cultured cells and normal tissues (*1, 2*). Retinoblastoma cells invariably lack normal expression of the *RB*-encoded protein (RB) because of mutation of both *RB* alleles, suggesting that loss of functional RB protein is an obligate event during retinoblastoma genesis (*3*). Furthermore, replacement of normal RB protein in retinoblastoma cells induces profound phenotypic alterations, including complete loss of their tumorigenic activity in nude mice (*4*). For this reason, *RB* is aptly termed a tumor suppressor gene (*5*). *RB* mutations are not restricted to retinoblastomas but are also found in some osteosarcomas, soft-tissue sarcomas, breast carcinomas, and small-cell carcinomas of lung (*6, 7*). These observations suggest a broad role for *RB* inactivation in the genesis of human tumors, even of those lacking a known association with retinoblastoma. However, direct biological evidence for this role has not been developed.

Prostate carcinoma is the most common cancer in men, and its incidence increases rapidly with age (*8*). Despite an abundance of clinical material, relatively few cell lines have been successfully established from human prostatic carcinomas (*9*). Three such lines, DU145 (*10*), LNCaP (*11*), and PC-3 (*12*), were readily available (*13*) and were screened for evidence of *RB* inactivation. Whereas LNCaP, PC-3, and control CV-1 cells expressed RB phosphoprotein isoforms in the expected size range (110 to 116 kD), the predominant RB form in DU145 cells migrated ahead of the least phosphorylated normal RB form (Fig. 1A). The same abnormal RB protein in DU145 was also detected by protein immunoblot analysis (*14*) with four polyclonal antisera (*7, 15*) and with monoclonal antibody PMG3-245 (*16*). These data suggested that this aberrant protein might result from a subtle *RB* mutation.

Northern (RNA) blot analysis of DU145 mRNA showed a single *RB* mRNA species

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of approximately normal size (4.7 kb) (*14*). In order to examine this *RB* mRNA for point mutations or small deletions, the reverse transcription–polymerase chain reaction (RT-PCR) method was used (*17*). Total RNA from DU145 cells was reverse-transcribed into cDNA, and then *RB*-specific sequences were amplified in vitro with several pairs of oligonucleotide primers made according to the *RB* cDNA sequence (*1*). Amplification with one pair of primers, located in exons 19 and 23, consistently yielded a shorter fragment from DU145 cDNA than from control *RB* cDNA (Fig. 1B); normal-sized fragments were obtained with other primer pairs (*14*). Sequence analysis of the aberrant fragment demonstrated the loss of 105 nucleotides corresponding precisely to the *RB* mRNA segment encoded by exon 21 (Fig. 1C). Because this exon contains exactly 35 triplet codons (*18*), its loss preserves the translational reading frame, and the shortened transcript would yield a protein product that is almost full-length.

A mutated *RB* mRNA and protein with exactly these features was reported by Horowitz *et al.* in human bladder carcinoma cell line J82 (*19*). We have shown that mutated RB proteins of both J82 and DU145 fail to form complexes with simian virus 40 (SV40) T antigen (*14*), consistent with the lack of E1A binding to RB of J82 (*19*). In J82 cells, a point mutation (ag → gg) in the intron 20-splice acceptor site was found to explain the loss of exon 21 during *RB* mRNA processing. By using similar techniques, we have confirmed the presence of this point mutation in J82 but have demonstrated a normal intron 20-splice acceptor site in DU145 (*20*). The DNA mutation giving rise to the abnormal *RB* transcript in DU145 cells is therefore different from that of J82 (*21*).

The significance of *RB* inactivation in the genesis of a particular tumor may be indicated by examining the biological effects of its replacement in tumor cells. Accordingly, DU145 cells were infected with either of two recombinant retroviruses: Rb, containing cloned normal *RB* cDNA, and Lux, containing the luciferase gene as a control (*4*). Both viruses also carried *neo* as a selectable marker. Mass cultures of G418-resistant DU145 cells were recovered 3 to 4 weeks after infection with both viruses. Rb- and Lux-infected cells (DU145/Rb and DU-145/Lux, respectively) were thereafter tested at 2-week intervals for expression of the exogenous *RB* (Fig. 2); such expression was demonstrated in DU145/Rb but not in DU-145/Lux cells as early as 3 weeks, and it persisted at least 13 weeks after infection. Metabolic labeling of cells with [^{32}P]orthophosphate was useful for detecting exogenous RB phosphoprotein because phosphorylated endogenous RB protein was only faintly seen (Fig. 2).

We suspected that bulk-infected cells such as DU145/Rb might be quite heterogeneous for expression from the viral *RB*, which could be an unfavorable property for further studies. Therefore, DU145/Rb cells were seeded at an average number of one cell per well in 96-well tissue culture plates. After 4 weeks of expansion, 16 clonal sublines were grown into mass cultures. Of these 16 lines, expression of the viral RB was readily detected in 12, whereas 4 had markedly decreased or absent exogenous RB expression (for example, B4 in Fig. 2). These data suggested that a subpopulation (25% or less) of the original DU145/Rb cells did not express exogenous RB. This heterogeneity may be due to the presence of defective viruses in Rb infectious stock, to instability of the integrated provirus, or to a combination of both (*22*).

The biological effects of *RB* replacement in DU145 cells were first examined in culture. Although some variation was apparent among individual cells, DU145/Rb, DU-

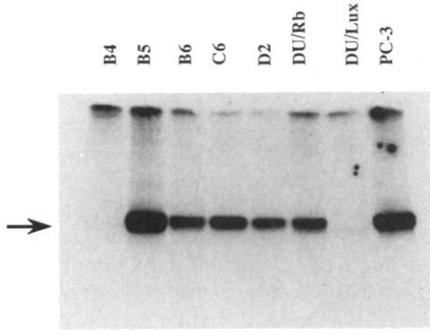

Fig. 2. Rb-infected DU145 cells expressed normal RB protein. Culture dishes (100 mm) of DU145 cells were infected five times in 48 hours by incubation in 10 ml of stock medium containing ~10^4 colony-forming units per milliliter of Rb or Lux recombinant retroviruses (*4*). Infected cells were then selected in Dulbecco's minimum essential medium containing G418 (800 μg/ml) for 4 weeks and passaged ad libitum. After 3 weeks, G418-resistant Rb-infected cells were seeded at approximately one cell per well in a 96-well culture plate to generate subcloned lines; 16 such lines (for example, B4, B5, B6, C6, and D2) were available 4 weeks later. Three weeks after infection and biweekly thereafter, 2 × 10^6 Rb- or Lux-infected DU145 cells (DU/Rb or DU/Lux, respectively) and control PC-3 prostate carcinoma cells were metabolically labeled with [^{32}P]orthophosphate, and cell lysates were immunoprecipitated with anti-fRB (7). Immunoprecipitates were separated by 7.5% SDS-PAGE and autoradiographed for 2 to 5 days. Expression of the viral RB was first detected at 3 weeks after infection, and it persisted at least 13 weeks (*14*). Five subcloned lines were first examined at 7 weeks (this figure), another 11 lines were subsequently examined with the use of protein immunoblotting, as in Fig. 4. In DU/Lux and B4 lanes, a phosphorylated form of endogenous RB (arrow) is faintly visible, migrating just ahead of exogenous phosphorylated RB.

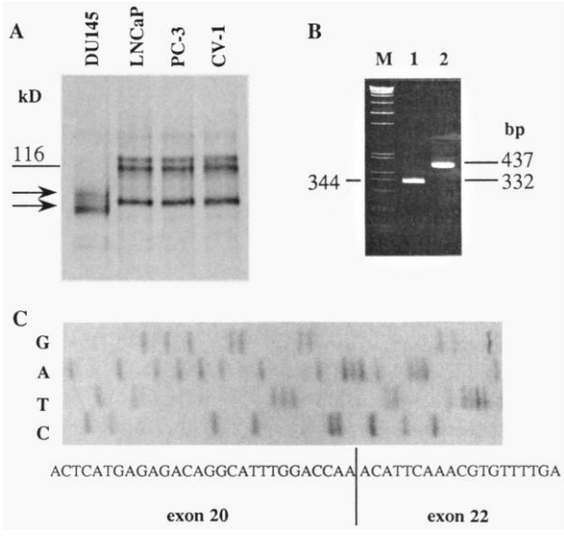

Fig. 1. (**A**) Abnormal RB protein in one of three human prostate carcinoma cell lines. About 2 × 10^6 DU145, LNCaP, and PC-3 human prostate carcinoma cells, and control CV-1 cells were metabolically labeled with [^{35}S]methionine and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 m*M* tris-HCl, *p*H 7.4, 150 m*M* NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% NP-40) with protease inhibitors [aprotinin (10 μg/ml), 50 μ*M* leupeptin, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)] and phosphatase inhibitors (30 m*M* tetrasodium pyrophosphate, 50 m*M* sodium fluoride, and 100 μ*M* sodium orthovanadate). Cell lysates were immunoprecipitated with antibody to a *Tryp* E-RB fusion protein (anti-fRB) (7). Immunoprecipitates were separated in 7.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiographed for 2 days. Abnormal RB protein forms in DU145 (arrows) are indicated. (**B**) Shortened *RB* mRNA in DU145 cells. Total RNA from DU145 cells was reverse-transcribed to cDNA by means of random primers (*17*). DU145 cDNA (lane 1) and normal *RB* cDNA (lane 2) were amplified with oligonucleotide primers having 3′ ends at nucleotide positions 2054 and 2490 (*1*). The resulting products were separated in a nondenaturing 8% polyacrylamide gel. Fragment sizes were inferred from results presented in (C). This experiment was replicated three times. M, size marker. (**C**) *RB* mRNA in DU145 cells lacked exon 21. Amplified DU145 *RB* cDNA was digested with Pst I and subcloned into M13. Two clones were analyzed by dideoxy chain termination sequencing (*28*). The reconstructed 332-bp sequence showed the absence of exon 21 (105 bp).

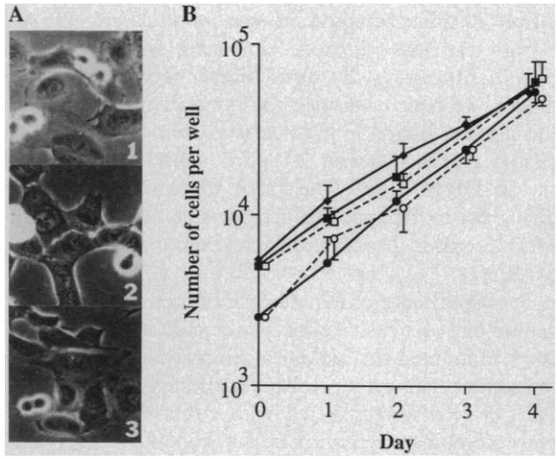

Fig. 3. Morphology and growth rate of cultured DU145/Rb cells. (**A**) Micrographs of representative uninfected DU145 (panel 1), DU145/Rb (panel 2), and DU-145/Lux (panel 3) cells (×100). No differences between these populations were distinguished. (**B**) Growth rate assay of bulk-infected cells and selected clonal lines. Equal numbers of each cell type were seeded into 12 replicate wells on 24-well plates. Cells in three wells were trypsinized and counted at daily intervals for 4 days thereafter. Plotted points are mean cell numbers per well, and error bars are 1 SD calculated over three wells. Symbols for cells are as follows: (■), DU145/Rb; (□), DU-145/Lux; (♦), D2; (●), B5; and (○),, B4.

145/Lux, and uninfected (parental) DU145 cells each demonstrated the same range of morphological appearance and could not be distinguished (Fig. 3A). Cells of clonal sublines had more uniform morphology (spindle-shaped or epithelioid); however, these phenotypes were uncorrelated with the presence or absence of exogenous RB (*14*) and were thought to merely reflect clonal expansion of morphological types in the original culture. Expression of exogenous RB did not appear to affect the growth rates of DU145 cells (Fig. 3B). These observations were in contrast to our previous results with bulk-infected retinoblastoma and osteosarcoma cells, for which morphology and growth rate in culture were significantly affected by *RB* replacement (*4*). This dissimilarity may have several causes, including different origins of the cells and the presence in DU145 cells of their particular endogenous RB. In another model system for cancer suppression, microcell transfer of a normal chromosome 11 into Wilms' tumor cells suppressed their tumorigenicity, but cells were unaltered in culture by the presence of the putative Wilms' tumor suppressor gene (*23*).

Suppression of the tumorigenic phenotype after *RB* replacement has been considered the most critical index of its cancer suppression function. Parental DU145 cells are tumorigenic in nude mice within 2 months after subcutaneous injection of 1×10^6 cells (*24*). Accordingly, 1×10^6 RB^+ cells (DU145/Rb, B5, or D2) were injected into the right flanks, and the same number of Rb^- cells (DU145/Lux or B4) were injected into the left flanks, of nude mice. After 2 months tumors had formed on both sides in most mice, but right-flank tumors were in every case smaller than those on the left (Table 1). Some right-flank tumors were exceedingly small (for example, 1 to 10 mm^3) and were confirmed as such only by histologic section. All tumors so examined had the same histologic appearance, that is, poorly differentiated adenocarcinoma, consistent with the tumor from which DU145 originated (*10*). Five of the larger right-side tumors were examined by protein immunoblot analysis for expression of both exogenous and endogenous RB proteins (Fig. 4). In every case, exogenous RB expression was undetectable, whereas the endogenous RB protein band was easily detectable, indicating that sufficient DU145 cellular protein was properly analyzed. Right-flank tumors were thus composed of DU145 cells that lacked expression of the exogenous *RB*; cells that retained exogenous *RB* expression were unable to participate in tumor formation. The smaller size of right-flank tumors is consistent with outgrowth from a minor subpopulation of RB^- cells intermixed in injected RB^+ cells.

These results indicated that *RB* inactivation had a significant role in determining the neoplastic phenotype of DU145 cells, and perhaps of the metastatic prostate tumor from which these cells were derived 14 years ago. In this report and in previous work (*4*), we have shown that *RB* replacement modifies the neoplastic properties of RB^- cells from three widely disparate types of tumors afflicting children and adults. It may be proposed that *RB* inactivation, when present, will usually be a significant mutation vis-à-vis the biological properties of tumor cells, and that replacement of this protein will usually be able to suppress tumorigenic properties of such cells. This proposal further motivates the development and clinical application of therapies aimed at restoring the normal *RB* gene product in tumor cells.

Table 1. Tumorigenicity of Rb-infected DU145 cells in nude mice. Bulk-infected DU145/Rb and DU145/Lux cells were harvested 4 to 5 weeks after viral infection, and 1×10^6 cells were injected into right and left flanks, respectively, of female homozygous *nu/nu* mice (*24*). The same number of cells from two RB^+ clonal sublines (B5 or D2) and one RB^- clonal subline (B4) were injected into right and left flanks, respectively, of additional mice, thereby controlling for host variation and nonspecific effects of viral infection and subcloning. Two months later, excised tumors were measured, and a relative volume ratio of RB^-:RB^+ sides was calculated.

Mouse number	Tumor size (mm)		Volume ratio
	Bulk-infected cells		
	DU145/Lux	DU145/Rb	
1	10 by 8 by 5	3 by 3 by 2	22
2	12 by 8 by 5	5 by 3 by 1.5	21
3	8 by 6 by 5	4 by 2 by 2	15
4	10 by 8 by 4	8 by 3 by 2	6.6
5	6 by 5 by 4	No tumor	
6	10 by 7 by 5	4 by 3 by 2	15
7	8 by 7 by 4	5 by 4 by 3	3.7
8	6 by 5 by 4	3 by 2 by 1.5	13
	Subcloned cells		
	B4 (RB^-)	D2 (RB^+)	
9	9 by 9 by 5	3 by 2 by 2	34
10	10 by 10 by 7	2 by 2 by 2	88
11	9 by 8 by 6	3 by 2 by 2	36
12	7 by 5 by 3	No tumor	
13	9 by 8 by 7	No tumor	
14	7 by 6 by 4	1 by 1 by 1	170
	B4 (RB^-)	B5 (RB^+)	
15	10 by 7 by 5	1 by 1 by 1	350
16	6 by 5 by 4	2 by 2 by 2	15
17	7 by 6 by 4	2 by 2 by 2	21
18	10 by 8 by 3	3 by 2 by 2	20
19	11 by 10 by 6	5 by 5 by 4	6.6
20	10 by 8 by 5	4 by 3 by 2	17

Although tumor suppression and growth suppression functions of *RB* are often taken to be equivalent, these activities have been dissociated in DU145 cells because their growth rate in culture was not significantly altered by *RB* replacement. Tumorigenicity is thus far the only consistent parameter for measuring the biological function of *RB*. Naturally occurring, mutated RB proteins shown to be inactive in tumor suppression include those of DU145 and J82, and a 95-kD protein in Saos-2 osteosarcoma cells that is truncated at its COOH-terminal region encoded by exons 21 to 27 (*25*). We have also characterized a mutated RB protein in a small-cell lung carcinoma that lacks the region encoded by exon 16 (*15*). These results suggest that the tumor suppression function of RB can be disturbed by deletion of any of several regions. Loss of exon 21 or 16 is also correlated with severely decreased or absent RB phosphorylation and with lack of binding to SV40 T antigen (*14, 15, 26*). Several studies have suggested that different phosphorylated forms of RB may have different functional properties (*27*). Analysis of other natural or experimental mutants may further elucidate mechanisms of tumor suppression by the *RB* gene product.

Note added in proof: We have recently identified an internal deletion of *RB* in a primary prostate carcinoma specimen.

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20. PCR amplification and direct sequencing of DU145 DNA has unexpectedly revealed a point mutation within exon 21 at nucleotide 2281. A single base substitution converts a lysine codon (AAG) to an in-frame stop codon (TAG), so this substitution cannot be a neutral sequence polymorphism. A coexisting normal base at this position was not observed. This preliminary result was duplicated in two separate amplification and sequencing experiments. This mutation does not explain the absence of exon 21 in *RB* mRNA by known splicing mechanisms.
21. J82 also differed from DU145 at a polymorphic Bam HI restriction site within *RB* [R. Bookstein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2210 (1988)], ruling out genetic identity of these two cell lines.
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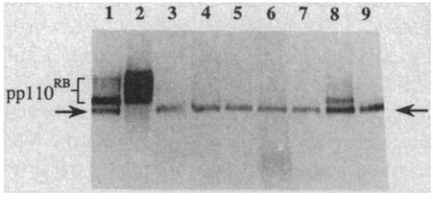

Fig. 4. Tumors formed from Rb-infected DU145 cells lacked expression of the exogenous *RB*. Five tumors (lanes 3 to 7) formed in right flanks of nude mice were individually pulverized in mortars on dry ice and lysed with RIPA buffer. In addition, 2.5×10^6 confluent cultured B5 cells (lane 1), DU145/Rb cells (lane 8), DU145/Lux cells (lane 9), and control Molt-4 cells (lane 2) were lysed with PLC buffer (50 m*M* tris, *p*H 7.4, 250 m*M* NaCl, 0.1% NP-40, 5 m*M* EDTA, 50 m*M* NaF, 2 μ*M* aprotinin, 50 μ*M* leupeptin, and 1 m*M* PMSF). Lysates were immunoprecipitated with anti-fRB, and immunoprecipitates were separated in 7.5% SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, Massachusetts) by standard techniques (*29*). The membrane was first incubated with blocking buffer, then incubated with monoclonal antibody PMG3-245 followed by alkaline phosphatase–conjugated goat antibody to mouse immunoglobulin G, as described (*16*). Endogenous RB protein (arrows) in lanes 1 and 3 to 9 served as a control for sufficient DU145 cellular protein and proper immunoprecipitation. Expression of exogenous RB is evident in cultured DU145/Rb or B5 cells and is undetectable in tumors; pp110^{RB}, normal RB protein.

Antigen Presentation Requires Transport of MHC Class I Molecules from the Endoplasmic Reticulum

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The role of exocytosis of major histocompatibility complex (MHC) class I molecules in the presentation of antigens to mouse cytotoxic T lymphocytes (CTLs) was examined by use of a recombinant vaccinia virus that expresses the E19 glycoprotein from adenovirus. E19 blocked the presentation of vaccinia and influenza virus proteins to CTLs in a MHC class I allele–specific manner identical to its inhibition of MHC class I transport from the endoplasmic reticulum. This finding indicates that (i) the relevant parameter for antigen presentation is the rate of MHC class I molecule exocytosis, not the level of class I cell surface expression, and (ii) association of class I molecules with antigen is likely to occur within the endoplasmic reticulum.

CYTOTOXIC T LYMPHOCYTES PLAY A critical role in host immunity to intracellular pathogens and cancer. CTLs recognize antigens only in association with class I molecules of the MHC (*1*). Class I molecules consist of an invariant β_2-microglobulin molecule light chain (molecular mass, 12 kD) noncovalently complexed to a 44-kD type I integral membrane glycoprotein heavy chain. Light and heavy chains associate in the endoplasmic reticulum (ER) within minutes of their assembly. Class I molecules are then transported to the plasma membrane via the cis, medial, and trans cisternae of the Golgi complex. An important question about the processing and presentation of antigens to CTLs is where and when class I molecules associate with foreign antigens.

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