translocations and other chromosome aberrations in lymphocytes and fibroblasts, micronuclei formation in epithelial cells, and loss of heterozygosity in erythrocytes (*7, 21*). Hyperrecombination is a specific feature of the A-T phenotype rather than a generic consequence of defective DNA repair in that an XP cell line (which is defective in excision repair) exhibited normal spontaneous recombination rates [Table 1 and (*13*)]. Elevated spontaneous rates of mitotic recombination between homologous chromosomes in vivo could help contribute to the high incidence of cancer in A-T patients by causing loss of heterozygosity at recessive oncogene loci, a key genetic event in tumorigenesis.

Several hypotheses have been offered to explain the pleiotropic A-T phenotype, including defects in DNA recombination and repair (*5, 7, 8, 22*), unusual chromatin or cytoskeletal structure (*23, 24*), and cell cycle abnormalities (*23, 25*). Our findings of high spontaneous recombination rates are not consistent with an inability to productively rearrange DNA but may result from defects in damage-sensitive cell cycle checkpoints (*23, 26*) that allow A-T cells to replicate DNA or enter mitosis before repair of spontaneous DNA damage is complete. These same checkpoint defects could cause aberrant immune gene rearrangements by allowing DNA replication or mitosis to disrupt switch recombination and TCR gene rearrangement, leading to selective Ig deficiencies and low frequencies of T cells expressing α/β TCRs as well as a high frequency of T cells with interlocus TCRs and chromosome translocations.

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29. I thank E. Brainerd for technical assistance, L. B. K. Herzing for construction and transfection of the pLrec constructs, and R. M. Liskay for discussions. Supported in part by grants from the National Institutes of Health (GM38588) and the American Cancer Society (MG-15).

4 January 1993; accepted 9 March 1993

Regulation of the Ets-Related Transcription Factor Elf-1 by Binding to the Retinoblastoma Protein

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The retinoblastoma gene product (Rb) is a nuclear phosphoprotein that regulates cell cycle progression. Elf-1 is a lymphoid-specific Ets transcription factor that regulates inducible gene expression during T cell activation. In this report, it is demonstrated that Elf-1 contains a sequence motif that is highly related to the Rb binding sites of several viral oncoproteins and binds to the pocket region of Rb both in vitro and in vivo. Elf-1 binds exclusively to the underphosphorylated form of Rb and fails to bind to Rb mutants derived from patients with retinoblastoma. Co-immunoprecipitation experiments demonstrated an association between Elf-1 and Rb in resting normal human T cells. After T cell activation, the phosphorylation of Rb results in the release of Elf-1, which is correlated temporally with the activation of Elf-1–mediated transcription. Overexpression of a phosphorylation-defective form of Rb inhibited Elf-1–dependent transcription during T cell activation. These results demonstrate that Rb interacts specifically with a lineage-restricted Ets transcription factor. This regulated interaction may be important for the coordination of lineage-specific effector functions such as lymphokine production with cell cycle progression in activated T cells.

T lymphocyte activation and proliferation in response to signaling through the cell surface T cell antigen receptor (TCR) is a model system for studies of cell cycle–specific transcriptional regulation (*1*). Resting peripheral blood T cells remain in G0 until they encounter an antigen-presenting cell that displays a specific antigenic peptide in conjunction with the appropriate major histocompatibility complex molecule. Antigenic stimulation results in the transcriptional induction of more than 100 new genes, many of which are specific to T cells (*1*). Antigen-stimulated T cells progress through the cell cycle and proliferate concomitantly with these changes in gene expression.

Members of the Ets family of transcrip-

tion factors (2) regulate inducible gene expression in T cells (3–8). Purine-rich motifs corresponding to the consensus Ets binding site (2) are required for the transcriptional induction of multiple activation-specific T cell genes, including the *interleukin-2* (*IL-2*) and *granulocyte-macrophage colony-stimulating factor* (GM-CSF) genes (5, 8), and several inducible T cell trophic viruses including human immunodeficiency virus–type 2 (HIV-2) (6), murine sarcoma virus (3), and human T cell leukemia virus I (4). We have cloned a member of the Ets family, Elf-1 (5), that binds specifically to purine-rich sites in the *IL-2*, *GM-CSF*, and *HIV-2* enhancers that are required for the transcriptional induction of these genes after T cell activation (5, 8). These studies strongly suggest that Elf-1 regulates inducible gene expression during T cell activation. However, the mechanisms that underlie Elf-1–dependent transcriptional activation in T cells remained unclear because Northern (RNA) and protein immunoblot analyses showed equal amounts of Elf-1 mRNA (5) and protein (9) in resting and activated normal human T cells.

Rb negatively regulates the G1 to S transition of the cell cycle (*10*). Unphosphorylated Rb accumulates during G0 and G1 and binds to at least eight cellular nuclear proteins (*11, 12*), including RBP-1 and RBP-2 [whose functions remain unknown (*13*)], and c-Myc (*14*) and E2F (*11, 15*), transcription factors that are known to regulate cell cycle–specific gene expression. Rb is phosphorylated during the G1 to S transition (*16*). This phosphorylation event results in the dissociation of Rb from its cellular binding proteins (*11, 12*). The pocket region of unphosphorylated Rb (amino acids 379 to 792) also binds at least three viral oncoproteins, SV40 large T antigen (TAg) (*17*), adenovirus E1A (*18*), and human papillomavirus (HPV) E7 (*19*). Part of the oncogenic potential of these viral proteins may result from their ability to competitively dissociate Rb from its cellular binding proteins (*11, 12*). An examination of the predicted amino acid sequence of Elf-1 (5) revealed the presence of sequence motifs similar to the Rb binding domains of TAg, E1A, and HPV E7 (*17, 19*). The putative Rb binding site of Elf-1 is located at the NH_2-terminus of the protein (amino acids 21 to 72) (Fig. 1) and partially overlaps a highly acidic region (amino acids 55 to 100) that can function as a potent transcriptional activator domain when fused to the DNA binding domain of GAL-4 (9). The presence of a putative Rb binding site in Elf-1 suggested that regulated interactions of Elf-1 with Rb might represent an important mechanism for controlling transcription and cell cycle progression during T cell activation.

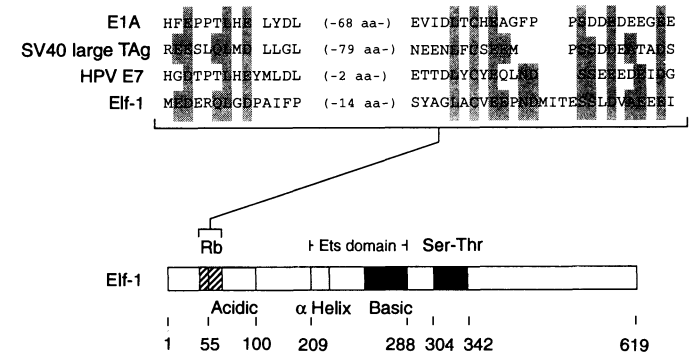

Fig. 1. Amino acid sequence (*32*) of the Rb binding site of human Elf-1. The bottom panel is a schematic illustration of the 619–amino acid human Elf-1 protein (*5*). Amino acid (aa) numbers are shown below the map. The Ser-Thr–rich domain (Ser-Thr) is shown as a shaded box, and the DNA binding domain (Ets domain) (*2, 8*) with adjacent α-helical (open box) and basic (solid box) regions are marked. An acidic transcriptional transactivation domain is located between amino acids 55 and 100. The putative Rb binding site is shown by the hatched box centered around amino acid 55. The upper panel shows a comparison of the amino acid sequences of the putative Rb binding site of human Elf-1 with the known Rb binding sites of adenovirus E1A, SV40 large TAg, and the HPV E7 protein (*17–19*).

To test whether Elf-1 can interact specifically with Rb in vitro, we bound a recombinant glutathione-S-transferase–Rb (GST-Rb) fusion protein containing the pocket region of Rb (amino acids 379 to 928) to glutathione-Sepharose and used the resulting beads as an affinity reagent for [^{35}S]Elf-1 protein translated in vitro. The pocket region of Rb used in this fusion protein binds to SV40 TAg, E1A, HPV E7, and several cellular proteins (*17–21*). The GST-Rb-Sepharose beads bound a 97-kD protein corresponding in size to the full-length Elf-1 protein translated in vitro (Fig. 2A). In control experiments performed to demonstrate the specificity of the Elf-1–Rb interaction, in vitro–translated Elf-1 failed to bind to GST-Sepharose (Fig. 2A). In addition, the Elf-1–related transcription factor Ets-1 (*22*), which contains a highly related DNA binding domain but lacks the putative Rb binding site present in Elf-1, also failed to bind to GST-Rb-Sepharose (9).

Several naturally occurring, loss-of-function alleles of Rb have been isolated from patients with retinoblastoma. Most stable variants contain mutations or deletions in the pocket region of Rb and fail to bind SV40 TAg, E1A, and HPV E7 (*20, 23*). Three of these Rb variants, containing a deletion of exon 22 (GST-RbdlEx22), a deletion of exon 21 (GST-RbdlEx21), or a point mutation resulting in a single amino acid substitution (Cys^{706} to Phe) (GST-RbΔ706), failed to bind in vitro–translated Elf-1 (Fig. 2A).

To determine whether the Rb binding site in Elf-1 corresponded to the NH_2-terminal sequence that is similar to the Rb binding sites of the viral oncoproteins, we produced a mutant form of recombinant Elf-1 in which the highly conserved amino acids Leu^{51}, Cys^{53}, and Glu^{55} were changed to Arg, Arg, and His, respectively. This mutant Elf-1 protein failed to bind to GST-Rb-Sepharose (Fig. 2B). A truncated version of Elf-1 lacking amino acids 1 to 108 also failed to bind to GST-Rb-Sepharose (9). The observed differences in Rb binding were not a result of differences in translational efficiencies because equal amounts of in vitro–translated wild-type and mutant Elf-1 proteins, as determined by SDS–polyacrylamide gel electrophoresis (PAGE), were used in each binding reaction.

In vitro binding experiments were also performed with recombinant GST–Elf-1 fusion protein bound to Sepharose and in vitro–translated [^{35}S]Rb. In vitro–translated Rb bound efficiently to GST–Elf-1–Sepharose but failed to bind to GST-Sepharose (Fig. 2C). In contrast, in vitro–translated RbΔ706 containing a point mutation that inactivates viral oncoprotein binding failed to bind to the GST–Elf-1–Sepharose. To determine directly if the HPV E7–related region of Elf-1 contained the Rb binding site, we attempted to compete away the binding of in vitro–translated Elf-1 to GST-Rb-Sepharose with a peptide corresponding to the Rb binding domain of the HPV E7 protein (*19*) (Fig. 2D). For a negative control, we used a mutant E7 peptide containing a single amino acid substitution (Glu to Gln). The wild-type E7 peptide completely inhibited the binding of in vitro–translated [^{35}S]Elf-1 to Rb (Fig. 2D). In contrast, the mutant peptide had little if any effect on the binding. Taken together, these experiments demonstrate that Elf-1 binds specifically to Rb in vitro and that this interaction is mediated by the

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pocket domain of Rb and an NH_2-terminal sequence of Elf-1 that is highly related to the Rb binding sites of SV40 TAg, E1A, and HPV E7.

The Rb protein is differentially phosphorylated during cell cycle progression (*16*). In G0 and G1, an unphosphorylated or underphosphorylated form of Rb predominates. Rb becomes highly phosphorylated at the G1 to S transition and is subsequently converted to the underphosphorylated form after mitosis. The underphosphorylated form of Rb binds preferentially to the viral oncoproteins (*17*). To determine whether Elf-1 could bind to Rb from cell extracts, COS cell (*24*) extracts were incubated with GST–Elf-1–Sepharose, the beads were washed, and the bound proteins were eluted and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and subjected to protein immunoblot analyses with the Rb monoclonal antibody (mAb) G3-245 (Fig. 3A). As described previously, COS cell extracts contained several forms of Rb with molecular sizes between 110 and 116 kD (*16, 17*). The band with the fastest mobility (pRb) represents underphosphorylated Rb, whereas the slower mobility bands (pRb^{phos}) represent multiple, hyperphosphorylated forms of the protein (*16, 17, 25*). Incubation of COS cell extracts with immobilized GST–Elf-1 resulted in the preferential retention of pRb (Fig. 3A). No binding of pRb^{phos} to Elf-1 was observed despite the fact that (as would be expected in extracts from exponentially growing COS cells) the majority of the Rb protein was hyperphosphorylated. In addition, no Rb protein bound to GST-Sepharose alone, again demonstrating the specificity of the Elf-1–Rb interaction. These results show that Elf-1 binds preferentially to the underphosphorylated form of Rb.

Two types of experiments were performed to determine if Elf-1 and Rb interact in vivo. First, we overexpressed the human Elf-1 protein in COS cells by transient transfection and then used a polyclonal

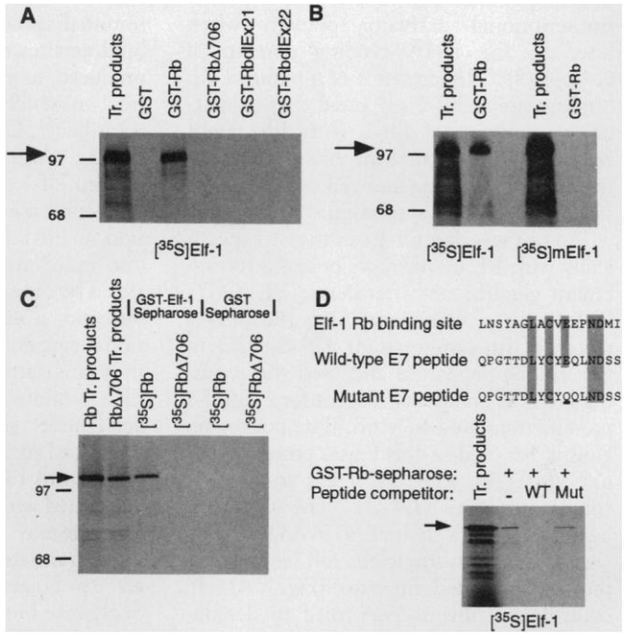

Fig. 2. Binding of Elf-1 to the pocket region of Rb in vitro. (**A**) In vitro–translated [^{35}S]Elf-1 was incubated with the following GST fusion proteins bound to glutathione-Sepharose: (i) wild-type GST-Rb fusion protein (GST-Rb), (ii) GST-Rb containing a point mutation at amino acid 706 (Cys to Phe) (GST-RbΔ706), (iii) GST-Rb containing a deletion of exon 22–encoded sequences (GST-RbdlEx22), (iv) GST-Rb containing a deletion of exon 21–encoded sequences (GST-RbdlEx21); and (v) GST lacking Rb sequences (GST) (*33*). The beads were washed, and bound proteins were eluted and resolved by SDS-PAGE. Gels were dried and subjected to autoradiography to visualize ^{35}S-labeled proteins. (**B**) Wild-type [^{35}S]Elf-1 or mutant Elf-1 ([^{35}S]mElf-1) protein containing three amino acid substitutions in the putative Rb binding site (Leu^{51} to Arg, Cys^{53} to Arg, and Glu^{55} to His) was translated in vitro in the presence of [^{35}S]methionine and incubated with GST-Rb-Sepharose. The eluted proteins were resolved by SDS-PAGE as described in (A) and detected by autoradiography. The first lane of each gel in (A) and (B) shows the in vitro translation products used for the binding reactions (Tr. products). Arrows denote full-length Elf-1 and mElf-1 in vitro–translated products. (**C**) In vitro–translated ^{35}S-labeled wild-type Rb ([^{35}S]Rb) or mutant Rb containing a point mutation at amino acid 706 (Cys to Phe) ([^{35}S]RbΔ706) was incubated with GST–Elf-1–Sepharose. Bound proteins were eluted from the beads and resolved by SDS-PAGE (*33*). Gels were dried and subjected to autoradiography. In control experiments, the same in vitro–translated Rb proteins were bound to GST-Sepharose. The first two lanes show the products of the wild-type and mutant Rb in vitro translation reactions used for binding (Rb Tr. products and RbΔ706 Tr. products, respectively). The arrow denotes full-length, in vitro–translated Rb. (**D**) ^{35}S-labeled, in vitro–translated Elf-1 protein was bound to GST-Rb-Sepharose beads in the absence or presence of wild-type (WT) or mutant (Mut) peptides from the Rb binding site of the HPV E7 protein. The top panel compares the amino acid sequences (*32*) of the Elf-1 Rb binding site with those of the wild-type and mutant E7 peptides. Conserved amino acids are shaded. The single amino acid substitution in the mutant E7 peptide is underlined. The bottom panel shows an autoradiogram of the proteins bound to GST-Rb-Sepharose after resolution by SDS-PAGE. The first lane shows the products of the Elf-1 in vitro translation reaction used for binding (Tr. products). The arrow denotes the position of full-length, in vitro–translated Elf-1. Size markers in kilodaltons are shown to the left of the autoradiograms.

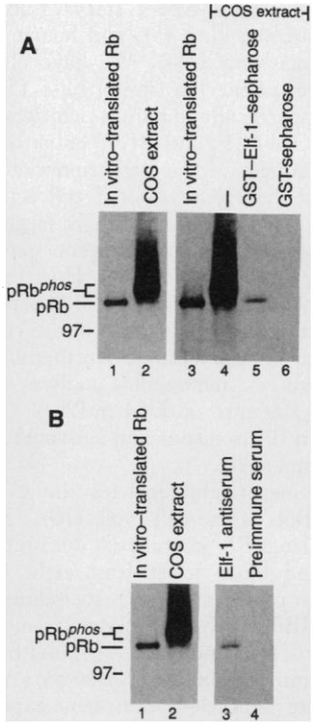

Fig. 3. Elf-1 binds preferentially to underphosphorylated Rb in COS cell extracts. (**A**) COS cell extracts were incubated with GST–Elf-1–Sepharose or GST-Sepharose. Bound proteins were eluted from the beads and resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and subjected to protein immunoblot analysis with a Rb mAb (*34*). Lanes 1 and 3, in vitro–translated Rb. Lanes 2 and 4, whole COS cell extracts. Lane 5, COS cell proteins eluted from GST–Elf-1–Sepharose. Lane 6, the COS cell proteins eluted from GST-Sepharose. The position of the 97-kD marker and that of underphosphorylated (pRb) and hyperphosphorylated (pRb^{phos}) are denoted to the left. (**B**) Immunoprecipitation of Elf-1–Rb complexes from Elf-1–transfected COS cell extracts. COS cells were transfected with a human Elf-1 expression vector (*34*), and extracts from these transfected COS cells were immunoprecipitated with an Elf-1 antiserum (lane 3) or preimmune serum from the same rabbit (lane 4). Products of the immunoprecipitations were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to protein immunoblot analysis with a Rb mAb (*34*). Lanes 1 and 2, in vitro–translated Rb and whole COS cell extract, respectively. The position of the 97-kD marker as well as that of underphosphorylated (pRb) and hyperphosphorylated (pRb^{phos}) Rb are shown to the left.

rabbit antiserum to human Elf-1 (Elf-1 antiserum) to immunoprecipitate Elf-1 from the transfected cell extracts. The resulting immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to protein immunoblot analysis with the Rb mAb G3-245 (Fig. 3B). The Elf-1 antiserum co-immunoprecipitated the underphosphorylated form of Rb (Fig. 3B). This coprecipitation reflected a specific interaction between Elf-1 and Rb because it was not observed with preimmune serum from the same rabbit.

We also attempted to demonstrate a regulated interaction between Rb and Elf-1 in normal human peripheral blood T cells. Protein immunoblot analyses demonstrated that the majority of Rb in resting human T cells was unphosphorylated (Fig. 4A). As expected, this Rb became progressively phosphorylated during the first 48 hours of T cell activation, the time period in which activated T cells progress through G1 and into the S phase of the cell cycle. Extracts from resting or activated normal human T cells were immunoprecipitated with the rabbit Elf-1 antiserum, and the immunoprecipitates were denatured and subjected to protein immunoblot analyses with a Rb mAb (Fig. 4). The unphosphorylated Rb in resting T cells was co-immunoprecipitated with Elf-1. In contrast, after T cell activation, most of the Rb became hyperphosphorylated and was no longer associated with Elf-1 (Fig. 4). Thus, the hyperphosphorylation of Rb during normal human T cell activation is associated with the disruption of the Elf-1–Rb complexes present in resting T cells.

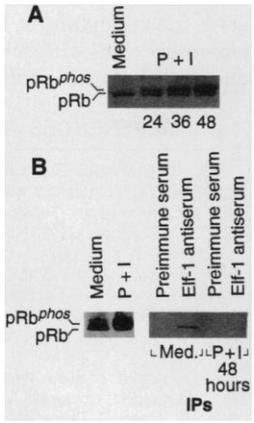

Fig. 4. Regulated association between Rb and Elf-1 during T cell activation (**A**) Time course of Rb phosphorylation during normal human T cell activation. Normal human T cells were cultured in medium alone or were activated by treatment with PMA + ionomycin (P + I) for 24 to 48 hours (indicated at the bottom of the gel) (*35*). Extracts prepared from 10^7 cells per time point were fractionated by SDS-PAGE and analyzed by protein immunoblotting with the Rb mAb G3-245 (*34*). (**B**) Co-immunoprecipitation of Elf-1 and Rb from resting or activated normal human T cell extracts. Normal human T cells prepared by elutriation were cultured in medium alone or in PMA + ionomycin (P + I) for 48 hours (*35*). Extracts prepared from 3.2×10^8 cells were immunoprecipitated with an Elf-1 antiserum or preimmune serum. Immunoprecipitates (IPs) were fractionated by SDS-PAGE and analyzed by protein immunoblot analysis with G3-245 (right panel) (*34*). As a control, unpurified lysates from the resting (Medium on left; Med. on right) and activated (P + I) T cells were run on the same gel (left panel). The underphosphorylated (pRb) and hyperphosphorylated (pRb^{phos}) forms of Rb are denoted to the left.

Elf-1 binds to a site in the GM-CSF promoter that is required for the inducible expression of the GM-CSF gene during T cell activation (Fig. 5) (*26*). A chloramphenicol acetyltransferase (CAT) reporter construct, which contained three copies of the wild-type GM-CSF Elf-1 binding site (Fig. 5A) that were cloned 5′ of a minimal SV40 promoter, was induced 35-fold after phorbol 12-myristate 13-acetate (PMA) plus ionomycin stimulation of Jurkat human T cells. Mutations of the Elf-1 binding site in this oligonucleotide (mGM-CSF; Fig. 5A) that abolished Elf-1 binding (Fig. 5B) also abolished the inducibility of CAT expression after T cell activation (Fig. 5C).

Thus, the GM-CSFCAT reporter plasmid can be used as an assay system for Elf-1–dependent transcription during T cell activation. To determine the effect of overexpression of Rb on Elf-1–dependent transcriptional activation, we cotransfected Jurkat T cells with the GM-CSFCAT reporter plasmid and an expression vector encoding a mutant form of Rb (Δp34-HA) that cannot be phosphorylated during cell cycle progression but retains the ability to bind viral oncoproteins such as TAg and E1A (*27*). These transfected cells were then activated by treatment with PMA and ionomycin. We used the phosphorylation-defective form of Rb because it should bind constitutively to Elf-1 even after T cell activation with PMA and ionomycin and because previous studies have shown that this mutant Rb is a potent inhibitor of adenovirus EIIaE promoter–dependent transcription (*27*). Overexpression of the phosphorylation-defective Rb protein reproducibly inhibited Elf-1–dependent transcriptional activation by more than 85% (Fig. 5C). In contrast, like overexpression of a wild-type Rb expression vector (9), overexpression of a control Rb expression vector encoding the ΔF706 mutant form of Rb that fails to bind to Elf-1 had little effect on the inducibility of the GM-CSF reporter

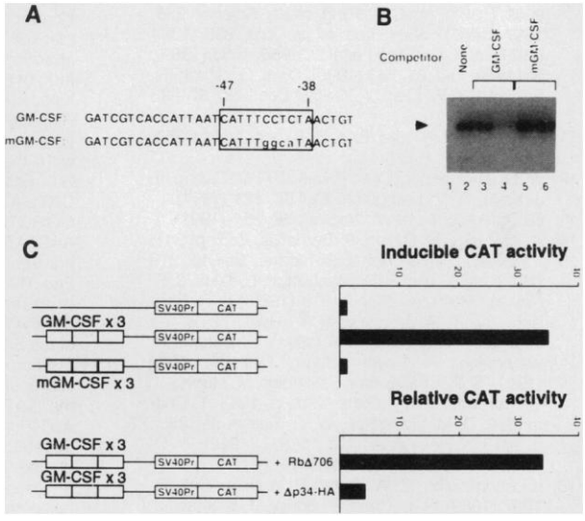

Fig. 5. Effect of overexpression of unphosphorylated Rb on Elf-1–dependent transcription in T cells. (**A**) Nucleotide sequences of the wild-type and mutant Elf-1 binding sites from the murine GM-CSF promoter (bp −33 to −63). Mutations are shown as lowercase letters. The Elf-1 binding site is boxed; mGM-CSF, mutant GM-CSF promoter. (**B**) EMSA with in vitro–translated Elf-1 (lanes 2 to 6) and a radiolabeled probe corresponding to the wild-type Elf-1 binding site from the murine GM-CSF promoter (*36*). Lane 1, radiolabeled probe incubated with an in vitro translation reaction lacking Elf-1 mRNA. Where indicated, 5 ng (lanes 3 and 5) or 50 ng (lanes 4 and 6) of unlabeled wild-type or mutant competitor oligonucleotide was added to the binding reactions. The arrow denotes the position of the specific complex corresponding to Elf-1 binding. (**C**) Effects of overexpression of unphosphorylated Rb on Elf-1–dependent transcription from a GM-CSF reporter plasmid. The structures of the reporter plasmids are shown schematically in the left panel. The reporter constructs contain the minimal SV40 promoter (SV40 Pr) alone or three copies of the wild-type (GM-CSF) or mutant (mGM-CSF) Elf-1 binding sites from the murine GM-CSF promoter cloned 5′ of the minimal SV40 promoter–CAT cassette. In the top panel, human Jurkat T cells were transfected with 10 μg of the indicated reporter plasmids alone. Thirty-six hours after transfection, half of the culture was left untreated and the other half was activated by treatment with PMA + ionomycin for 10 hours (*37*). Results are shown as the ratio of CAT activity in the activated cultures to CAT activity in the unactivated cultures after correction for differences in transfection efficiencies. In the bottom panel, Jurkat T cells were cotransfected with 4 μg of the wild-type GM-CSF reporter plasmid and 10 μg of either the RbΔ706 expression vector, which fails to bind Elf-1, or the Δp34-HA Rb expression vector (*27*), which binds Elf-1 and cannot be phosphorylated (*37*). Thirty-six hours after transfection, the cultures were activated by treatment with PMA + ionomycin. The results are shown as percent acetylation after correction for differences in transfection efficiencies. All transfections were repeated at least three times.

plasmid (Fig. 5C). The inhibitory effect of the phosphorylation-defective Rb protein was specific for Elf-1–dependent transcription because it had no effect on transcription from the pRSVβgal reference plasmid that was included in each transfection (9).

Previous studies have suggested that Elf-1 functions to regulate inducible gene expression in T cells (5, 8). However, the finding of equal amounts of Elf-1 mRNA and protein in resting and activated T cells raised important questions about how Elf-1 could function in an activation-specific context (5). To determine the basis of this apparent discrepancy, we examined the association of Elf-1 with other transcriptional regulatory proteins. Here, we have shown that Elf-1 forms complexes with the underphosphorylated form of Rb both in vitro and in vivo and that overexpression of unphosphorylated Rb inhibits Elf-1–dependent transcriptional activation in T cells. These results are consistent with a model in which underphosphorylated Rb in resting T cells sequesters or inactivates Elf-1, thereby rendering it unable to activate gene expression in these cells. After T cell activation, the phosphorylation of Rb results in the dissociation of the Elf-1–Rb complex, allowing Elf-1 to bind to and activate the expression of genes such as *GM-CSF* and *HIV-2*. In this model, Rb could inhibit Elf-1 function by preventing its DNA binding, inhibiting the transcriptional activity of the DNA-bound Elf-1, or both. The finding that the Rb binding domain is directly adjacent to an acidic transcriptional transactivator domain of Elf-1 is consistent with the hypothesis that Rb binding inhibits the activity of this transactivating domain, possibly by preventing important protein-protein interactions mediated by this region of Elf-1.

Although the data presented in this report are consistent with a model in which the binding of Rb to Elf-1 in resting T cells inhibits its transcriptional activation function, the mechanism by which Rb regulates Elf-1–dependent transcription in T cells may be more complex. Rb is capable of binding to DNA in concert with the cellular transcription factor E2F, and cyclin A is able to bind specifically to the E2F-Rb complex (*11, 15*). Similarly, Rb can form stable complexes with cyclins D2 and D3 and cdk4 (*28*). Moreover, there are additional proteins that bind to the Rb binding sites of TAg, E1A, and E7 (*17, 19, 29*). These include the Rb-related protein p107, which has been cloned (*30*), as well as p300. Finally, we have recently reported that Elf-1 can bind to DNA in conjunction with specific members of the AP-1 family of transcription factors (*31*). Thus, a complete understanding of the regulation of Elf-1–dependent transcription during T cell activation awaits a more detailed description of the regulated interactions between Elf-1, Rb, related pocket proteins such as p107 and p300, AP-1, and associated cyclins and kinases in normal T cells. The results presented here suggest that regulated interactions between Rb and the lineage-restricted transcription factor Elf-1 modulate inducible gene expression during T cell activation. Thus, Rb appears to integrate the regulation of lineage-specific effector functions with cell cycle progression during T cell activation.

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32. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
33. The pGST-Rb plasmid encoding a glutathione-S-transferase–Rb (amino acids 379 to 928) fusion protein has been described previously, as have variants of this plasmid encoding GST-Rb fusion proteins containing deletions of exon 21 or exon 22 (*11, 12*). GST-RbΔ706 contains a point mutation of Rb at amino acid 706 (Cys to Phe) (*11, 12*). pGST–Elf-1 contains the full-length human Elf-1 complementary DNA (cDNA) (*5*) cloned into the Sma I site of pGEX-3X (Pharmacia, Piscataway, NJ). pcDNA1/NEO–Elf-1 contains the full-length human Elf-1 cDNA cloned into the Eco RV and Xho I sites of pcDNA1/NEO (Invitrogen, San Diego, CA). The pSG5-Rb plasmid contains the full-length human Rb cDNA cloned into the Bam HI site of pSG5 (Stratagene, LaJolla, CA). pSG5-RbΔ706 is identical to pSG5-Rb except for a point mutation at amino acid 706 of Rb (Cys to Phe). A mutant form of Elf-1 containing three amino acid substitutions (Leu^{51} to Arg, Cys^{53} to Arg, and Glu^{55} to His) was produced with polymerase chain reaction–mediated mutagenesis as described (*8*) with the synthetic oligonucleotides GCCGGTCGGGCCCGTGTGCACGAGCCCAATGACATGATTACTGAGAGTTC (5′ primer) and GGGCTCGTGCACACGGGCCCGACCGGCATAACTATTGAGAATATCAGCACCAGG (3′ primer). The mutant Elf-1 cDNA was cloned into the Eco RV and Xho I sites of pcDNA1/NEO to produce pcDNA1/NEO–mElf-1. The pGM-CSFCAT reporter plasmid contains three copies of the Elf-1 binding site from the human GM-CSF promoter (bp −33 to −63) cloned into the Sma I site upstream of the minimal SV40 promoter and the CAT cassette in pSPCAT (*5*). The pcDNA-Δp34-HA plasmid contains the murine Rb cDNA mutated at eight possible phosphorylation sites cloned into the Eco RV site of pcDNA1/NEO (Invitrogen) (*27*). In vitro transcription and translation reactions were carried out with commercially available kits (Promega) as described (*8*). Before the binding reactions, 10 μl of in vitro translation mixture was diluted in 700 μl of NETN [20 mM tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40]. For GST fusion protein production, overnight cultures of *Escherichia coli* DH5α containing the pGEX-3X, pGST-Rb, or pGST–Elf-1 plasmids were diluted 1:10 in Luria broth and grown for 1 hour with shaking at 37°C. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and cultures were incubated at 37°C for 4 hours with shaking. Bacteria were pelleted and resuspended in NETN. After incubation at 4°C for 30 min, the cells were sonicated three times for 15 s on ice. For the in vitro binding assays, glutathione-Sepharose beads (Pharmacia) were washed three times in NETN plus 0.5% powdered milk. We bound GST fusion proteins to the glutathione-Sepharose by incubating 1 ml of crude bacterial extract with 30 μl of Sepharose beads

for 30 min at 4°C. The beads were washed three times with NETN and incubated with 700 μl of radiolabeled, in vitro–translated protein in NETN at 4°C for 1 hour. The beads were washed five times in NETN, and the proteins were eluted by boiling for 5 min in SDS-PAGE loading buffer [2% SDS, 10% glycerol, and 60 mM tris (pH 6.8)]. For peptide competition experiments, 2 μg of wild-type or mutant E7 peptide was included in each binding assay.

34. COS cells were grown on 100 mM tissue culture plates in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (FCS). We transfected cells with 10 μg of the pcDNA1/NEO–Elf-1 plasmid containing the full-length human Elf-1 cDNA under the control of the cytomegalovirus promoter using lipofectin (BRL, Gaithersburg, MD) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were washed with phosphate-buffered saline and lysed by incubation for 15 min at 4°C in EBC buffer [50 mM tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 0.1 mM aprotinin, 1 mM leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride]. Before their use in binding reactions and immunoprecipitations, the cell extracts were cleared by centrifugation in a microfuge at 14,000 rpm for 15 min at 4°C. For immunoprecipitations, 300 μl of COS cell extract or 400 μl of normal T cell extract was mixed with 700 μl of NETN and 10 μl of Elf-1 antiserum or preimmune serum and incubated for 1 hour at 4°C. Protein A–Sepharose (Pharmacia) was washed three times with 4% bovine serum albumin in NETN. Protein A–Sepharose (30 μl) was added to each immunoprecipitation reaction and incubated for 45 min at 4°C. The beads were washed five times with NETN, and the proteins were eluted by boiling in SDS-PAGE loading buffer. For the protein immunoblot experiments, Rb and Elf-1 proteins were resolved by electrophoresis in 7.5% SDS gels (*11, 12*). Protein immunoblots were probed with the Rb mAb G3-245 (Pharmingen, San Diego, CA) or Elf-1 antiserum produced by five sequential immunizations of a rabbit with purified recombinant Elf-1 protein. This antiserum immunoprecipitated Elf-1 but failed to immunoprecipitate other Ets family members, including Ets-1, Ets-2, GA binding protein α, and PU.1. It immunoprecipitates a single major protein of 97 kD from normal human T cells and from Jurkat cells, which corresponds in size to that of in vitro–translated Elf-1. It does not directly immunoprecipitate Rb. The second antibody was commercially available horseradish peroxidase–coupled goat antibody to mouse immunoglobulin (Ig) or goat antibody to rabbit Ig (BRL).
35. Normal human T cells were isolated by density gradient centrifugation from buffy coats obtained from normal volunteers as described (*31*). All preparations used in these studies contained more than 95% lymphocytes as assessed by staining with Wright's stain. Human T cells were cultured at a density of 2×10^6 cells/ml in either RPMI + 10% FCS or activated by culturing in RPMI + 10% FCS + PMA (10 ng/ml) + ionomycin (0.5 μg/ml) for 24 to 48 hours before harvesting. T cell extracts were prepared in EBC buffer as described above (400×10^6 cells per 0.5 ml of EBC buffer).
36. Electrophoretic mobility-shift assays (EMSAs) were carried out as described (*5*) with ^{32}P-labeled double-stranded synthetic oligonucleotides corresponding to the wild-type (GATCGTCACCATTAATCATTTCCTCTAACTGT) or mutant (GATCGTCACCATTAATCATTTGGCATAACTGT) Elf-1 binding sites from the GM-CSF promoter.
37. Human Jurkat T cell tumor cells were cultured in RPMI + 10% FCS and transfected with DEAE-dextran as described previously (*5*). All transfections contained 4 μg of reporter plasmid and 1 μg of the pRSVβgal reference plasmid. Cells were cultured for 36 hours after transfection and then, when indicated, activated by treatment with PMA (40 ng/ml) + ionomycin (1.4 μg/ml) for 8 to 10 hours at 37°C. Cells were harvested and extracts assayed for CAT and β-galactosidase activity as described (*5*). All transfections were repeated at least three times.
38. We thank D. Livingston, J. Lowe, M. Parmacek, and G. Nabel for helpful discussions and P. A. Hamel for generously providing the pECE-Δp34-HA plasmid. C. McNicholas-Serwecinski provided expert secretarial assistance and B. Burck provided help with the preparation of illustrations. Supported in part by NIH grant RO1 AI29673-01 (J.M.L.).

5 February 1993; accepted 7 April 1993

Inactivation of the Type II Receptor Reveals Two Receptor Pathways for the Diverse TGF-β Activities

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Transforming growth factor–β (TGF-β) is a multifunctional protein that regulates cell proliferation and differentiation and extracellular matrix production. Although two receptor types, the type I and type II receptors, have been implicated in TGF-β–induced signaling, it is unclear how the many activities of TGF-β are mediated through these receptors. With the use of cells overexpressing truncated type II receptors as dominant negative mutants to selectively block type II receptor signaling, the existence of two receptor pathways was shown. The type II receptors, possibly in conjunction with type I receptors, mediate the induction of growth inhibition and hypophosphorylation of the retinoblastoma gene product pRB. The type I receptors are responsible for effects on extracellular matrix, such as the induction of fibronectin and plasminogen activator inhibitor I, and for increased JunB expression. Selective inactivation of the type II receptors alters the TGF-β response in a similar manner to the functional inactivation of pRB, suggesting a role for pRB in the type II, but not the type I, receptor pathway.

TGF-β is a secreted protein that inhibits proliferation of many cell types, regulates the expression of extracellular matrix proteins and their integrin receptors, and is considered an important physiological regulator of cell proliferation and differentiation (*1, 2*). Cross-linking experiments have revealed that most cells have three types of TGF-β receptors at the cell surface. The type III receptor (or betaglycan) is a proteoglycan with a short cytoplasmic domain (*3, 4*) and is not likely to mediate any of the known biological activities of TGF-β (*5–7*). The type II receptor has recently been cloned, and its sequence indicates that it is a serine-threonine kinase (*8*). Both type I and type II receptors have been implicated in mediating the biological activities of TGF-β (*5–7, 9*). Complementation studies with mutant mink lung epithelial cell lines (Mv1Lu) resistant to growth suppression by TGF-β indicated that the type I receptors or both type I and type II receptors are necessary for all tested biological responses to TGF-β (*5–7*). In complementation analyses using different cell hybrids, increased type II receptor expression was associated with growth suppression but not induction of several matrix proteins (*9*). However, the concomitant increase in type I receptor levels in this study (*9*) and the possible interaction between these two receptor types (*7, 10*) complicates the interpretation of these data. In addition, the growth inhibitory effect of TGF-β can be abolished by inactivation of pRB without affecting the ability of TGF-β to induce an increased gene expression, suggesting that pRB is not involved in a defined set of responses (*11*). So far, it has remained unknown whether these different activities of TGF-β are mediated through a single receptor complex with divergent signaling pathways or by separate receptor-associated pathways.

To determine the specific roles of the type II and type I receptors, one could overexpress the type II receptors or abolish the function of the type II receptors and then evaluate the resulting changes in the spectrum of biological activities of TGF-β. Our first approach was to overexpress the type II receptor in transfected cell lines. Because very few cell lines lack endogenous TGF-β receptors (*1, 9, 12*), and other components of the TGF-β receptor signaling pathways could possibly be defective in these cells, we overexpressed type II receptors in cells that contain a low level of TGF-β receptors and are sensitive to TGF-β, such as the Mv1Lu cells (*5*). However, transient transfection assays with expression vectors for the human type II receptor (*8*) in QT6 and 293 cells resulted not only in increased cell surface expression of type II receptors but also in increased availability of type I receptors (Fig. 1A). This increase of type I receptors may be the result of the suggested interaction between both TGF-β receptor types (*6, 7, 10*). Thus, even

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