ζ Phosphorylation Without ZAP-70 Activation Induced by TCR Antagonists or Partial Agonists

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Small changes in the peptide–major histocompatibility complex (MHC) molecule ligands recognized by antigen-specific T cell receptors (TCRs) can convert fully activating complexes into partially activating or even inhibitory ones. This study examined early TCR-dependent signals induced by such partial agonists or antagonists. In contrast to typical agonist ligands, both an antagonist and several partial agonists stimulated a distinct pattern of ζ chain phosphorylation and failed to activate associated ZAP-70 kinase. These results identify a specific step in the early tyrosine phosphorylation cascade that is altered after TCR engagement with modified peptide–MHC molecule complexes. This finding may explain the different biological responses to TCR occupancy by these variant ligands.

Both the development of immature thymocytes and the effector activities of mature $CD8^+$ and $CD4^+$ T cells are controlled by signals arising from the interaction of clonally distributed receptors (TCRs) with their natural ligands, short peptides bound to MHC class I or class II molecules (*1*). It has generally been assumed that such peptide–MHC molecule complexes were either activating TCR ligands (agonists) of varying potency or were recognized with such low affinity that no signaling occurred in their presence. On the basis of this model, TCR-transduced activating signals have most often been examined with the use of high-affinity antibodies to the TCR as model agonists (*2*). Recent evidence, however, indicates that the introduction of minor structural variations into the original peptide–MHC molecule pair used to prime a T cell may result in the formation not only of weak agonists, but of either selectively activating (partial agonist) or even inhibitory (antagonist) TCR ligands (*3–5*). The properties of these variant ligands appear to play critical roles in such fundamental processes as the positive (*6*) and negative selection (*7*) of developing thymocytes, the induction of peripheral T cell tolerance (anergy) (*8*), the regulation of the type of cytokines produced by activated T lymphocytes (*3, 5*), and viral escape from effector T cell activity (*9*). TCR antagonists also are being examined for their potential use in therapy for T cell–mediated autoimmune diseases (*10*). Therefore, determining the molecular basis for the unique biological effects of variant TCR ligands is critical for fully understanding and manipulating a wide range of T cell–related immune processes.

In our study here, we used nontransformed T cell clones to examine some of the intracellular biochemical events induced by structurally related, but functionally distinct TCR ligands (*11*). 3C6 is a murine T helper cell 1 (T_H1), $CD4^+$ T cell clone that responds with proliferation and interleukin-2 (IL-2) cytokine production ("agonist" response) to a ligand composed of pigeon cytochrome c fragment 81–104 [PCC(81–104)] bound to the syngeneic MHC molecule $E^k_\alpha E^k_\beta$ (Fig. 1A). In the absence of any added PCC(81–104), 3C6 cells also proliferate and produce a small amount of IL-2 in response to a mutant $E^k_\alpha E^k_\beta$ class II molecule in which the residues at positions 75 and 79 in the E_β chain have been changed to those present in the allelic variant E^s_β (*5*) (Fig. 1A). As shown here (Fig. 1A) and as previously reported (*5*), a complex consisting of PCC(81–104) bound to these mutant $E_\alpha E_\beta$ molecules constitutes a TCR antagonist that inhibits the IL-2 response stimulated by the mutant $E_\alpha E_\beta$ molecule itself.

Studies in this as well as other models have suggested that a parallel decline in all signaling events as a result of antagonist competition for TCR binding sites could not explain selective antagonism of specific effector functions. Rather, they raised the possibility that TCR engagement by antagonists may result in an atypical pattern of intracellular biochemical changes (*5, 12*). Such changes would likely involve steps preceding agonist-induced generation of inositol triphosphate and changes in intracellular calcium concentration ($[Ca^{2+}]_i$), as these events are inhibited by TCR antagonists (*4, 13*). Because one of the earliest events of TCR-mediated signaling is tyrosine phosphorylation of TCR subunits (*2*), we carried out phosphotyrosine antibody immunoblotting of CD3ε antibody immunoprecipitates from lysates of 3C6 cells exposed to agonist or antagonist ligands (*14*). Exposure of T cells to PCC(81–104) associated with wild-type $E^k_\alpha E^k_\beta$ class II molecules (agonist) resulted in a dose-dependent appearance of tyrosine-phosphorylated receptor chains of 21, 23, and 27 kD (Fig. 1B). These bands represent two phosphorylated isoforms of the TCRζ chain

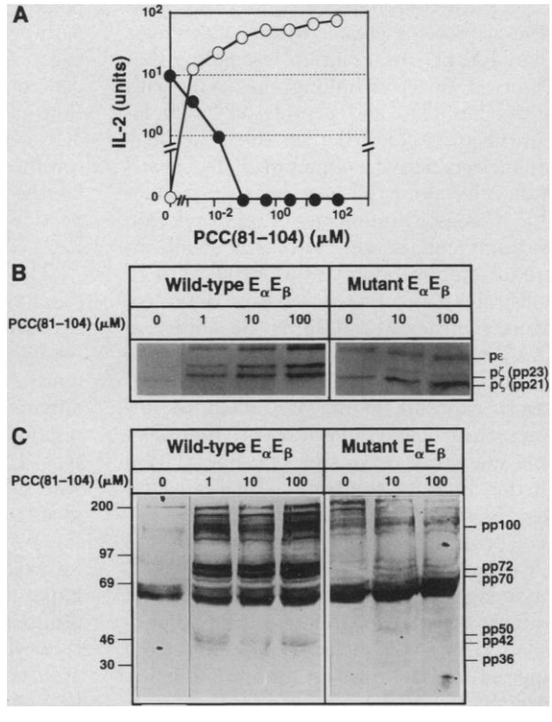

Fig. 1. Differential effects of exposure to agonist or antagonist peptide–MHC molecule ligands on cytokine production and protein tyrosine phosphorylation by the 3C6 $CD4^+$ T_H1 clone. (**A**) Interleukin-2 production by 3C6 cells in the presence of PCC(81–104) bound to wild-type $E^k_\alpha E^k_\beta$ (○) or mutant $E^k_\alpha E^k_\beta$ (●) MHC class II molecules. (**B**) Tyrosine phosphorylation of TCRζ and CD3ε chains from 3C6 cells stimulated for 10 min with increasing concentrations of PCC(81–104) with the use of transfected L cells expressing wild-type $E^k_\alpha E^k_\beta$ or mutant $E^k_\alpha E^k_\beta$, as seen after CD3ε antibody immunoprecipitation and phosphotyrosine immunoblotting. Similar results were obtained with TCRζ antibody immunoprecipitation (*16*). (**C**) Phosphotyrosine antibody–stained protein immunoblot of the total cell lysates used in (B). Labels on the right side of the panel indicate the approximate molecular sizes of the major species showing enhanced tyrosine phosphorylation upon antigen stimulation.

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(pp21 and pp23) and the CD3ε subunit (pε), respectively (*15, 16*). Unexpectedly, tyrosine phosphorylation of TCRζ increased in a dose-dependent manner in 3C6 cells exposed to the PCC(81–104)–mutant $E_\alpha E_\beta$ combination (antagonist) (Fig. 1B), despite the inhibition of IL-2 production mediated by TCR engagement of this ligand. This constitutes a qualitative change from the pattern seen in agonist-exposed cells, in which both ζ phosphorylation and IL-2 production increase together as more ligand is provided. This dichotomy between antagonist and agonist effects was also apparent in the specific pattern of tyrosine phosphorylation of TCRζ. Antagonist induced the formation of small amounts of pp23 in comparison to the amount of pp21 formed, whereas agonist induced the formation of relatively similar amounts of both (Fig. 1B). Finally, in contrast to 3C6 exposed to agonist PCC(81–104)–wild-type $E_\alpha E_\beta$ complexes, the antagonist-induced increase in pp21 ζ occurred without a strong increase in tyrosine phosphorylation of the CD3ε subunit, compared to the amount induced by the weakly stimulatory mutant class II molecule alone (Fig. 1B).

Tyrosine phosphorylation of TCR subunits precedes and is critical to tyrosine phosphorylation of many other intracellular substrates involved in downstream signaling. When analyzed with the use of cell lysates, exposure of 3C6 cells to increasing amounts of agonist ligand [PCC(81–104) bound to wild-type $E_\alpha E_\beta$ molecules] resulted in a clear concentration-dependent increase in tyrosine phosphorylation of several substrates (one or more proteins of 36 to 50 kD, three proteins of 70 to 75 kD, and several proteins greater in molecular mass than 100 kD) in a pattern resembling that observed by cross-linking the TCR with antibodies (*17*) and peptide–MHC molecule agonists (*15, 18*) (Fig. 1C). The weak stimulatory activity of mutant $E_\alpha E_\beta$ class II molecules alone did not lead to reproducible increases in substrate phosphorylation in total cell lysates, although small increases in phosphorylation of the 70 to 75 substrates were occasionally observed. Most significantly, despite the ability of PCC(81–104)–mutant $E_\alpha E_\beta$ complexes to stimulate increased ζ chain phosphorylation, exposure of the 3C6 TCR to this antagonist ligand did not lead to reproducible increases in tyrosine phosphorylation of the different substrates visualized after agonist stimulation (Fig. 1C).

These results are consistent with the idea that TCR antagonists alter the phosphorylation cascade by inducing a distinct pattern of TCR subunit tyrosine phosphorylation that fails to recruit or activate the appropriate downstream tyrosine kinases. Among these, ZAP-70, a 70-kD non-*src* family kinase, has been shown to play a critical role in effective $CD4^+$ T cell activation (*19*). The enzymatic function of ZAP-70 appears dependent on its binding to tyrosine-phosphorylated ζ and its own subsequent tyrosine phosphorylation (*20*). Because phosphorylation of ζ was induced by both agonist and antagonist, but only agonist and not antagonist stimulated the appearance of tyrosine-phosphorylated proteins in the size range corresponding to ZAP-70 (Fig. 1C), we examined ZAP-70 interactions with ζ as well as the phosphorylation state and enzymatic activity of ZAP-70 upon TCR engagement by these ligands. Phosphotyrosine immunoblotting of ZAP-70 antibody immunoprecipitates from lysates of 3C6 cells exposed to agonist complexes revealed a phosphorylated 70-kD triplet coprecipitating with phosphorylated ζ (pζ) and pε (Fig. 2, top). As before, both pp21 and pp23 ζ isoforms were present in relatively equal amounts. The identification of ZAP-70 as one of the 70-kD bands was confirmed by disruption of the material from the ZAP-70 immunoprecipitates with SDS sample buffer, renaturation, and reprecipitation with antibodies to ZAP-70 (*21*).

In contrast to agonist-stimulated T cells, direct immunoprecipitation of ZAP-70 from cells exposed to antagonist ligand resulted in the coprecipitation of pζ without evidence of detectable phosphorylated ZAP-70 (Fig. 2, top), although comparable amounts of total ZAP-70 were precipitated in each case (Fig. 2, middle) (*22*). Immunoprecipitation with antiserum to ZAP-70 appeared to favor coprecipitation of pp23 ζ in these antagonist-stimulated cells, although the ratio of pp23 to pp21 was still less than that seen with agonist under similar immunoprecipitation conditions. In agreement with these data on differential tyrosine phosphorylation of ZAP-70, only agonist-stimulated 3C6 cells showed a significant increase in specific ZAP-70 kinase activity as measured by the phosphorylation of an exogenous peptide substrate (*14*) (Fig. 2, bottom) or in ZAP-70 autophosphorylation activity (*16*).

The distinctive pattern of biochemical signaling seen with the 3C6 clone after exposure to antagonist TCR ligand was also observed with another T_H1 clone. A.E7 is a murine $CD4^+$ T cell clone that expresses a different TCR from that of 3C6 and that responds to PCC(88–104) bound to wild-type $E^k_\alpha E^k_\beta$. Some PCC(88–104) analogs with substitutions at the major epitopic residue (position 99) act as partial agonists for the A.E7 TCR, showing various capacities to evoke IL-2 or IL-3 responses (Fig. 3A). Exposure of A.E7 cells to agonist ligand resulted in a pattern of receptor subunit (Fig. 3B) and total cellular substrate (Fig. 3C) tyrosine phosphorylation similar to that of 3C6. In contrast, stimulation with

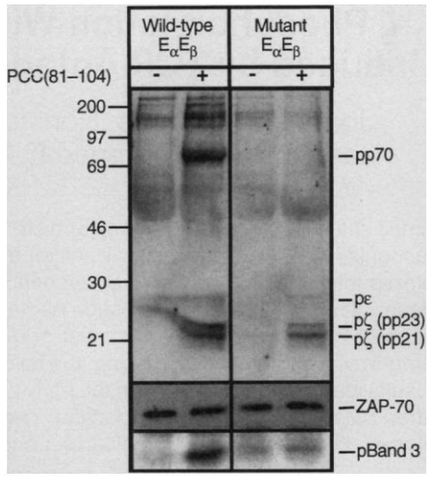

Fig. 2. ZAP-70 association with phosphorylated ζ, but not phosphorylation or activation in response to antagonist ligands. The top panel shows a phosphotyrosine antibody protein immunoblot of ZAP-70 immunoprecipitates from 3C6 cells cultured with transfected L cells bearing wild-type $E^k_\alpha E^k_\beta$ or mutant $E^k_\alpha E^k_\beta$ class II molecules in the absence (−) or presence (+) of 100 μM PCC(81–104). The estimated molecular sizes of the tyrosine-phosphorylated substrates are indicated on the right. A protein immunoblot developed with ZAP-70 antiserum showed constant amounts of total ZAP-70 in each lane (middle panel). The bottom panel shows an in vitro kinase assay performed with ZAP-70 antibody immunoprecipitates from lysates of the same groups of 3C6 cells, with the use of the cytosolic domain of erythrocyte band 3 as a substrate.

the partial agonists resulted in phosphorylation patterns resembling those of antagonist-exposed 3C6, with preferential formation of pp21 ζ (Fig. 3, B and C) and a lack of phosphorylation of multiple cellular substrates, especially those in the 70- to 75-kD range (Fig. 3C). Again, immunoprecipitation with antiserum to ZAP-70 showed association of pp21 ζ with ZAP-70, which was neither tyrosine-phosphorylated nor kinase-active (*16*). Not all substituted peptides evoked ζ phosphorylation, however, and the extent of ζ phosphorylation was not directly related to the amount of cytokine produced (Fig. 3, A and B).

Thus, these experiments provide evidence for distinct patterns of early biochemical events accompanying engagement of the TCR by structurally related peptide–MHC molecule ligands. In contrast to what is observed with typical agonists, antagonists can induce increasing receptor subunit phosphorylation in the face of decreasing downstream effector responses. Both antagonists and partial agonists stimulate the generation of tyrosine phosphoproteins in patterns that we have been unable to reproduce by titration of agonist ligand. A related phenomenon has also been noted after stimulation of $CD4^-$ cells with short peptides that fail to induce IL-2 secretion by

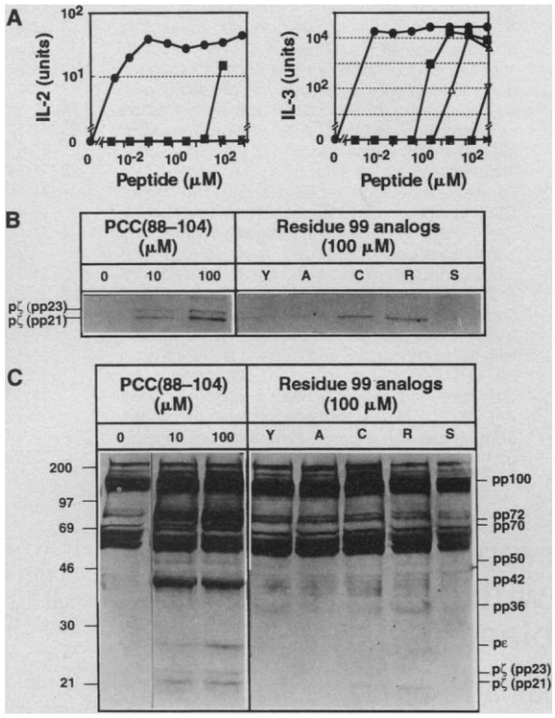

Fig. 3. Effects of variant peptide ligands with partial agonist properties on tyrosine phosphorylation in the A.E7 CD4$^+$ T_H1 clone. (**A**) IL-2 and IL-3 production of A.E7 cells in response to PCC(88–104) or its position 99 analogs [●, PCC(88–104); ■, Tyr^{99}; △, Ala^{99}; ▽, Cys^{99}; ▷, Arg^{99}; ◁, Ser^{99}], bound to wild-type $E^k_\alpha E^k_\beta$. (**B**) Tyrosine phosphorylation of ζ chains visualized after ζ antibody immunoprecipitation and phosphotyrosine immunoblotting of A.E7 cell lysates. Assays were performed after stimulation for 10 min in cultures containing transfected L cells expressing wild-type $E^k_\alpha E^k_\beta$ and 10 μM or 100 μM PCC(88–104) or 100 μM PCC(88–104) analogs substituted at position 99. (**C**) Phosphotyrosine antibody protein immunoblot of total cell lysates from (B). Labels on the right side of the panel indicate the approximate sizes of the major species showing enhanced tyrosine phosphorylation upon antigen stimulation.

such cells but that were complete agonists for CD4$^+$ cells with the same TCR (*23*). Finally, both antagonists and partial agonists lead to ZAP-70 association with pζ without subsequent phosphorylation or activation of this critical kinase, a finding that may account for some of the reported defects in functional T cell responses after exposure to variant TCR ligands (*24*).

Association of nonphosphorylated ZAP-70 with the pp21 form of ζ has recently been reported in freshly isolated thymocytes and lymph node cells and has been suggested to be the result of opposing constitutive tyrosine kinase and phosphatase activities not involving TCR engagement (*25*). Given our findings here that a similar pattern arises from TCR occupancy with partial agonists or antagonists, it is intriguing to speculate that this phenotype could in part arise from constant TCR interaction with self-peptide–MHC ligands possessing the properties of TCR partial agonists or antagonists. This may indicate that effective activation of T cells by foreign antigenic peptides requires overcoming TCR antagonism by endogenous ligands and that partial agonist stimulation is a significant mode of receptor signaling in developing thymocytes.

Our findings make unlikely a strictly competitive model of TCR antagonism in which the inhibitory ligand simply reduces the number of available TCR binding sites and thus decreases occupancy by available agonist (*5*, *24*). Instead, we favor a model in which TCR partial agonists or antagonists induce changes in either the stability or physical organization of activating TCR-coreceptor (CD4 or CD8) oligomers (*26*). This is in agreement with previously proposed models derived from studies of nonlymphoid cells, which postulate that effective intracellular signaling involves ligand-dependent and density-related receptor clustering, as well as a requirement for a minimal time of individual receptor occupancy (*27*). The description here of a common alternative pattern of intracellular signaling by partial agonists and antagonists should aid both in reaching a more refined understanding of TCR signal transduction pathways, as well as in relating studies on the affinities of TCR-ligand interactions and on TCR-dependent protein-protein interactions to the control of T cell function.

Note added in proof: Since submission of this report, Sloan-Lancaster *et al.* (*28*) have reported similar results regarding predominant induction of the TCRζ pp21 isoform and a lack of ZAP-70 activation after exposure of T cell clones to anergy-inducing variant peptide–MHC class II ligands.

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11. 3C6 and A.E7 are CD4$^+$ T_H1 cell clones specific for pigeon cytochrome c (PCC) bound to $E^k_\alpha E^k_\beta$ molecules and were grown as described [L. A. Matis *et al.*, *J. Immunol.* **130**, 1527 (1983); R. H. Schwartz, B. S. Fox, E. Fraga, C. Chen, B. Singh, *ibid.* **135**, 2598 (1985)]. Antigen-presenting cells (APCs) were DAP.3 L cells transfected with complementary DNA expression constructs encoding E_α, E^k_β, B7-1, and intercellular adhesion molecule 1 (P13.9) or B7-expressing DAP.3 L cells transfected with constructs encoding E_α and a mutant E^k_β with residues 75 and 79 corresponding to the allelic amino acids present in E_β (FT27.2). Use of these cells as APCs has been described [F. Ronchese, R. H. Schwartz, R. N. Germain, *Nature* **329**, 254 (1987); L. Ding, P. S. Linsley, L. Y. Huang, R. N. Germain, E. M. Shevach, *J. Immunol.* **151**, 1224 (1993)]. The synthetic peptides PCC(81–104) and PCC(88–104) and analogs of this latter peptide substituted at the major epitopic site (residue 99) were synthesized and purified by high-performance liquid chromatography in the National Institute of Allergy and Infectious Diseases Peptide Facility. The sequence of PCC(81–104) is IFAGIKK-KAERADLIAYLKQATAK. The single-letter amino acid code is as follows: 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; X, any amino acid; and Y, Tyr. Cytokine assays were performed as described [L. Racioppi, F. Ronchese, R. H. Schwartz, R. N. Germain, *J. Immunol.* **147**, 3718 (1991); R. König, L.-Y. Huang, R. N. Germain, *Nature* **356**, 796 (1992)].
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14. Stimulation and processing of T cell clones for protein immunoblot analysis was as follows. APCs (1×10^6 per well) were incubated overnight at 37°C in the indicated concentration of peptide in the wells of a 24-well tissue culture plate. T cells (10×10^6 per well) were added and, after a short spin at 1000 rpm for 30 s, incubated for 2, 10, or 20 min at 37°C. After incubation, T cells were harvested with phosphate-buffered saline containing EDTA (0.4 mM) and sodium orthovanadate (0.4 mM), transferred into Eppendorf tubes, spun at 8000 rpm for 5 s, and lysed in 1× lysis buffer [1% Triton X-100, 150 mM NaCl, 10 mM tris-HCl (pH 7.6), leupeptin (10 μg/ml), aprotinin (10 μg/ml), 1 mM sodium orthovanadate, and 25 μM *p*-nitrophenyl-*p'*-guanidino-benzoate] for 30 min on ice. After removal of nuclear debris by centrifugation, the resultant supernatants were analyzed directly or after immunoprecipitation by protein immunoblotting with phosphotyrosine or protein antibodies or by an in vitro kinase assay as described [R. L. Wange, S. N. Malek, S. Desiderio, L. E. Samelson, *J. Biol. Chem.* **268**, 19797 (1993)]. We used the following antibodies in these experiments: 4G10, a mouse immunoglobulin G2b (IgG2b) monoclonal antibody (mAb) to phopsphotyrosine [Upstate Biotechnology (UBI), Lake Placid, NY]; rabbit antiserum to human ZAP-70 (UBI) [A. L. Burkhardt *et al.*, *J. Biol. Chem.* **269**, 23642 (1994)]; 500A2, hamster IgG mAb to mouse CD3ε (Pharmingen, San Diego, CA); 387, rabbit antiserum to CD3ζ peptide [D. G. Orloff, S. J. Frank, F. A. Robey, A. M. Weissman, R. D. Klausner, *J. Biol. Chem.* **264**, 14812 (1989)]; and peroxidase-linked sheep antibodies to mouse Ig (Amersham, Buckinghamshire, England). Quantitative data were obtained from film exposures with a Molecular Dynamics Laser.
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21. The reprecipitation assay was as follows: Washed immunoprecipitates (50 μl of packed beads) were disrupted by incubation for 10 min at 95°C in 50 μl of 1% SDS–1% β-mercaptoethanol. Samples were then allowed to cool to room temperature and the supernatant transferred to a clean microfuge tube. Twenty microliters of 0.77 M iodoacetamide was added to give a twofold molar excess of iodoacetamide over β-mercaptoethanol, and incubation was continued for 30 min at room temperature. One milliliter of lysis buffer was then added to each sample and allowed to incubate for an additional 10 min at room temperature. Each sample was split into two equal aliquots, and 50 μl of a 50% slurry of protein A–Sepharose in phosphate-buffered saline was added to each aliquot. Either 0.4 μl of ZAP-70 antiserum or control antiserum was added to each aliquot, and immunoprecipitation was allowed to proceed overnight. Immunoprecipitates were washed two times with lysis buffer, eluted with reducing sample buffer at 95°C for 5 min, and analyzed by protein immunoblotting.
22. Parallel analyses of cell lysates with the use of CD3ε antibody immunoprecipitation followed by ZAP-70 antibody blotting, and ZAP-70 antibody immunoprecipitation followed by phosphotyrosine blotting, have shown, as expected, that the amount of ZAP-70 coprecipitated with the TCR is proportional to the amount of phosphorylated ζ and, further, that the stoichiometry of ZAP-70 association with these phosphorylated ζ chains is roughly similar in agonist- and antagonist-stimulated cells. Therefore, the failure to detect phosphorylated ZAP-70 in ZAP-70 antibody immunoprecipitates from lysates of antagonist-stimulated cells (Fig. 2, top) cannot be accounted for by the existence of only a very small amount of the total ZAP-70 associated with pζ in these cells, given the amounts of pζ in the two conditions.
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Cytostatic Gene Therapy for Vascular Proliferative Disorders with a Constitutively Active Form of the Retinoblastoma Gene Product

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Vascular smooth muscle cell (SMC) proliferation in response to injury is an important etiologic factor in vascular proliferative disorders such as atherosclerosis and restenosis after balloon angioplasty. The retinoblastoma gene product (Rb) is present in the unphosphorylated and active form in quiescent primary arterial SMCs, but is rapidly inactivated by phosphorylation in response to growth factor stimulation in vitro. A replication-defective adenovirus encoding a nonphosphorylatable, constitutively active form of Rb was constructed. Infection of cultured primary rat aortic SMCs with this virus inhibited growth factor–stimulated cell proliferation in vitro. Localized arterial infection with the virus at the time of balloon angioplasty significantly reduced SMC proliferation and neointima formation in both the rat carotid and porcine femoral artery models of restenosis. These results demonstrate the role of Rb in regulating vascular SMC proliferation and suggest a gene therapy approach for vascular proliferative disorders associated with arterial injury.

The arterial wall is a complex multicellular structure and is important in the regulation of inflammation, coagulation, and regional blood flow (*1*). Vascular SMCs are located predominantly in the arterial tunica media and are important regulators of vascular tone and blood pressure. These cells are normally maintained in a nonproliferative state in vivo (*1*, *2*). Arterial injury results in the migration of SMCs into the intimal layer of the arterial wall, where they proliferate and synthesize extracellular matrix components. This neointimal SMC proliferative response has been implicated in the pathogenesis of atherosclerosis (*2*). In addition, arterial injury after percutaneous balloon angioplasty of the coronary arteries results in neointimal SMC proliferation and clinically significant restenosis in 30 to 50% of patients (*3*). Many growth factors induce the proliferation of vascular SMCs in vitro and in vivo (*3*, *4*). This redundancy in growth factor signaling pathways has led to the suggestion that effective cytostatic therapies for vascular proliferative disorders should target nuclear cell cycle regulatory pathways rather than more proximal signal transduction molecules (*5*).

The Rb protein inhibits cell cycle progression in many mammalian cell types (*6*, *7*). For example, in resting (G_0) peripheral blood T cells, Rb is unphosphorylated and, in this state, binds to and inactivates a set of cellular transcription factors, including E2F and Elf-1, that are required for cell cycle progression (*8*). After T cell activation, Rb is rapidly phosphorylated and the Rb-E2F and Rb–Elf-1 complexes are thereby disrupted (*8*, *9*). The release of these transcription factors is associated with progression through the G_1/S checkpoint of the cell cycle and subsequent T cell proliferation. Given its important role in regulating cell cycle progression, we reasoned that Rb might also be an important regulator of vascular SMC proliferation.

To study the expression and regulation of Rb in vascular SMCs, we arrested cultured primary rat aortic SMCs in the G_0/G_1 phase of the cell cycle by incubating them in serum-free medium for 96 hours and then induced the cells to proliferate by exposing them to 10% fetal bovine serum (FBS). The expression and phosphorylation of Rb were assessed by immunoblot analysis of whole cell extracts. After serum withdrawal, >85% of the cultured SMCs were arrested in G_0 or G_1 of the cell cycle as assayed by propidium iodide staining and fluorescence-activated cell sorting analysis (*10*). These quiescent cells contained exclusively unphosphorylated Rb (*10*). Serum stimulation of the cells was associated with their progression into the S phase of the cell cycle, and the concomitant, progressive phosphorylation of Rb during the first 24 hours after stimulation (*10*).

To test directly the role of Rb in regulating cell cycle progression in SMCs, we constructed a replication-defective adenovirus vector, AdΔRb (*11*), that encodes a nonphosphorylatable, constitutively active form of human Rb (HAΔRb) containing a 10–amino acid, NH_2-terminal epitope tag from the influenza hemagglutinin molecule (*12*). In this vector, transcription of the Rb gene is controlled by the cellular elongation

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