Dissociation of Intracellular Signaling Pathways in Response to Partial Agonist Ligands of the T Cell Receptor

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Summary

The T cell receptor (TCR) is a versatile receptor able to generate different signals that result in distinct T cell responses. The pattern of early signals is determined by the TCR binding kinetics that control the ability of the ligand to coengage TCR and coreceptor. Coengagement of TCR and CD4 results in an agonist signaling pattern with complete tyrosine phosphorylation of TCR subunits, and recruitment and activation of ZAP-70. In contrast, TCR engagement without CD4 coengagement causes a partial agonist type of signaling, characterized by distinct phosphorylation of TCR subunits and recruitment but no activation of ZAP-70. The pathways triggered by partial agonist signaling are unknown. Here, we show that agonists cause association of active lck and active ZAP-70 with p120-GTPase–activating protein (p120-GAP). These associations follow engagement of CD4 or CD3, respectively. In contrast, partial agonists do not activate lck or ZAP-70, but induce association of p120-GAP with inactive ZAP-70. Despite these differences, both agonist and partial agonist signals activate the mitogen-activated protein kinase (MAPK) pathway. However, MAPK activation by partial agonists is transient, supporting a kinetic, CD4-dependent model for the mechanism of action of variant TCR ligands. Transient MAPK activation may explain some of the responses to TCR partial agonists and antagonists.

Key words: T cell receptor • partial agonist • signal transduction • mitogen-activated protein kinases • p120-GAP

The current paradigm of T cell activation proposes that signal transduction from the TCR is initiated after ligand binding by *src*-kinase–dependent phosphorylation of immune receptor, tyrosine- based activation motifs (ITAMs)[1] in the cytoplasmic domains of the CD3/TCR ζ chains (for review see reference 1). Phosphorylation of the ITAMs leads to recruitment and activation of ζ-associated protein (ZAP)-70 through its SH2 domain, and subsequent recruitment and activation of other proteins such as p36 (linker for activation of T cells) (LAT), vav, and SLP-76, through SH2–SH3 domain interactions. This leads to the formation of multimolecular complexes that activate several signaling cascades such as the phospholipase C-γ1 (PLC-γ1)–dependent cascade (2), and the ones emanating from activation of $p21^{ras}$ such as the mitogen-activated protein kinase (MAPK) pathway and the phosphatidyl-inositol 3-kinase (PI-3K) pathway (3–6). These signaling cascades ultimately converge on the nucleus resulting in the changes of gene expression that characterize T cell activation.

Implicit in this paradigm is the assumption that similar levels of TCR occupancy by different ligands will lead to identical signal transduction events. However, recent evidence indicates that the TCR is not an off/on signal transduction complex, but rather is able to transduce different patterns of signals that can cause dramatic effects on T cell function (7, 8). These distinct patterns of early TCR-mediated signaling are induced by variant TCR ligands that have conservative substitutions in the TCR contact points of the peptide or the MHC molecule (9, 10). As a result, they act as partial agonist or antagonist ligands of the TCR depending on whether they cause partial activation of the T cell or they inhibit agonist-induced activation (for review see references 11, 12). The differential T cell activation induced by these variant TCR ligands correlates with a distinct pattern of early TCR-mediated signaling that is characterized by predominant appearance of the p21 form of phospho-ζ with little phosphorylation of CD3-ε, and

[1]*Abbreviations used in this paper:* ERK, extracellular regulated kinase; MAPK, mitogen-activated protein kinase; p120-GAP, p120-GTPase–activating protein; PCC(81-104), pigeon cytochrome c fragment 81–104; ZAP, ζ-associated protein.

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with recruitment of ZAP-70 to the TCR-activating complex but no phosphorylation nor activation of this protein tyrosine kinase (13, 14). This distinct pattern of TCR-mediated signal results in an array of intracellular changes that include increase in cell size (10), induction of acidification of the extracellular environment (15, 16), transient or partial calcium signals (17, 18), modulation of TCR expression (19), and upregulation of some cell surface molecules such as IL-2 receptor and leukocyte function–associated antigen 1 (7, 8, 12, 20). These cellular changes translate functionally into inhibition of agonist-induced cell proliferation and/or cytokine production, split cytokine production profiles, and/or induction of T cell anergy. Together, this evidence strongly suggests that the distinct pattern of TCR-mediated signaling induced by partial agonist ligands can activate, at least, some signaling pathways emanating from the TCR.

It is not known how the partial agonist type of early TCR-mediated signaling is transmitted to downstream pathways. Two general possibilities can be considered. One is that this differential signaling may transduce distinct or diminished signals along each one of the pathways emanating from the TCR, resulting in less signal being delivered to the nucleus. Alternatively, the early TCR-mediated signaling in partial agonist mode could translate into dissociated delivery of signals, such as only some of the pathways emanating from the TCR are activated whereas others are not. Here, we explore this issue in relation to one of the pathways emanating from the TCR. Some of the functional responses induced by TCR partial agonist ligands, such as TCR downmodulation, can be attributed to serine phosphorylation (21–23). Therefore, we examined the effects of TCR partial agonists on the Ras-MAPK pathway of signal transduction, because it primarily involves serine/threonine kinases and regulates cell proliferation and differentiation (24). Here, we show that signaling from the TCR in a partial agonist mode causes recruitment of p120-GTPase–activating protein (p120-GAP), a potential Ras effector molecule, to the receptor, and is capable of activating the extracellular regulated kinase (ERK)-1 and ERK-2 MAPKs. However, it does so in a more transient fashion than full agonist ligands of the TCR do. Our results provide the first experimental evidence for activation of the MAPK after partial agonist signaling from the TCR. Activation of the Ras-MAPK pathway may explain some of the effects seen after TCR engagement with these variant ligands, such as downmodulation of TCR or split cytokine production without proliferation.

Materials and Methods

Cells. 3C6 is a Th1, $CD4^+$ murine T cell clone specific for pigeon cytochrome c fragment 81–104 [PCC(81-104)] bound to the $I\text{-}E^k$ class II MHC molecule (25). This clone was maintained by cycles of antigen stimulation with irradiated spleen cells, IL-2–induced cell expansion, and rest, as previously described (14). 3C6 T cells were used at least 10–14 d after last seeing antigen. L cells (P13.9), which had been transfected with cDNA constructs encoding Eα and Eβ chains of $I\text{-}E^k$, as well as the costimulatory molecules CD54 (ICAM-1) and CD80 (B7-1), were used as APCs (14, 26, 27).

Reagents. The following peptide was used for these experiments: PCC(81-104) (IFAGIKKKAERADLIAYLKQATAK). This peptide was commercially provided by Procyon Biopharma Inc. (London, Ontario, Canada) and purified to >85% by HPLC before use. Pervanadate was prepared by dissolving 100 μl of 0.1 M sodium orthovanadate (Sigma Chemical Co., St. Louis, MO) into 900 μl of double distilled water and 3.3 μl of 30% hydrogen peroxide, and kept at room temperature for 15 min before use.

Monoclonal Antibodies. 4G10 (provided by Dr. B. Druker, Oregon Health Sciences University, Portland, OR) is a mouse IgG2b monoclonal antibody against phosphotyrosine, B4F8 is a mouse IgG2a monoclonal antibody against p120-GTPase activating protein (p120-GAP; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit immune sera against lck and ZAP-70 were provided by L.E. Samelson (CBMB, NICHD, National Institutes of Health, Bethesda, MD), and H57-597 is a hamster IgG against a common epitope of the TCR β chain. Anti–ACTIVE MAPK (Promega, Madison, WI) is a rabbit antiserum against dual phosphorylated MAPK-derived peptide and recognizes MAPK2 enzyme bands at 42/44 kD after cell activation.

The anti-CD3-fos, anti-CD4-jun, and the bivalent anti-CD3-CD4 fos–jun antibody reagents were prepared by J. Tso (Protein Design Labs, Palo Alto, CA) and have been previously described (28–33). These chimeric antibodies combine a Fab fragment against CD3 or against CD4 linked to fos or jun, respectively. fos and jun, separately, will form homodimers and will lead to bivalent engagement of CD3 in the case of stimulation with anti-CD3-fos, or to bivalent engagement of CD4 for T cells incubated with anti-CD4-jun. However, when anti-CD3-fos and anti-CD4-jun chimeric antibodies are mixed, the equilibrium reaction is skewed towards fos–jun heterodimerization, and this will result in CD3-CD4 coengagement (34).

T Cell Stimulation. Ag stimulation of T cells was done as previously described (14, 33). In brief, P13.9 cells (10^6 cells/group) were plated in 24-well plates with or without PCC(81-104), and incubated at 37°C, overnight. The next day, T cells (10^7 cells/group) were added. Plates were then spun down (1,000 rpm for 30 s) and incubated at 37°C for 10 min. T cell stimulation with heterofunctional antibodies was performed in a final volume of 100 μl (10^7 cells/group) in Eppendorf tubes with the indicated heterofunctional antibodies (10 μg/ml) or different amounts of pervanadate for 10 min at 37°C. After stimulation, T cells were harvested in cold PBS containing sodium orthovanadate (400 μM) and EDTA (400 μM), and lysed in 1× lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.6, 5 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 25 μM *p*-nitrophenyl-*p*′-guanidino-benzoate).

Tyrosine Phosphorylation Analysis. Detection of tyrosine phosphorylation of proteins in ZAP-70 or p120-GAP immunoprecipitates was performed as described (14). 4G10 monoclonal antibody was used for immunoblotting. Rabbit antiserum to ZAP-70 was used to immunoprecipitate ZAP-70 and associated proteins, and mouse monoclonal antibody against p120-GAP was used for immunoprecipitation of p120-GAP. Immunoprecipitations were performed from 10^7 cells/group (14). Signal detection was performed by chemiluminescence (Boehringer Mannheim, Laval, Quebec, Canada) and intensity was quantitated using an imaging densitometer (model GS 700; BioRad Labs., Hercules, CA) and the Molecular Analyst® software (version 1.0, BioRad Labs.).

Detection of activated ERK-1 and ERK-2 was performed by

Western blotting of cell lysates with the anti–ACTIVE MAPK (Promega) rabbit antiserum. In brief, 10^7 cells/sample was stimulated with the appropriate antibodies at different concentrations or for different times at 37°C. Next, cells were washed once with PBS containing sodium orthovanadate, and subsequently lysed in 1× lysis buffer (500 μl) on ice for 30 min. Lysates cleared of nuclear debris by centrifugation at 14,000 rpm at 4°C for 10 min, and 300 μl of supernatant was mixed with 4× sample buffer. 30 μl of this solution (4.5×10^5cell equivalents/group) were run in 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted with anti–ACTIVE MAPK antiserum (Promega) using chemiluminescence (Boehringer Mannheim).

In Vitro Immune Complex Kinase Assays. T cells (10^7/sample) were stimulated with either the anti-CD3-CD4 fos–jun antibodies, the anti-CD3 fos antibodies, or the anti-CD4 jun antibodies for 10–12 min at 37°C, mixing gently on a thermomixer. Cells were lysed with 1× lysis buffer for 30 min on ice. In vitro immune complex kinase assays of lck, ZAP-70, and p120-GAP immunoprecipitates were performed as previously described (14). MAPK activity assay was performed according to manufacturer specifications (New England Biolabs, Boston, MA). In brief, collected cell lysates were immunoprecipitated with a phospho-specific p42/p44 MAPK monoclonal antibody on protein A–Sepharose beads with gentle rocking, overnight at 4°C. The next day, the bead pellets were washed twice with 1× lysis buffer and twice with 1× kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerol-phosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, and 10 mM magnesium chloride) and then incubated with the kinase reaction mix (1× kinase buffer containing 200 μM ATP) containing 2 μg of Elk-1 fusion protein for 30 min at 30°C. The reaction was terminated with 4× sample buffer and the samples were resolved on a 12% SDS-PAGE gel. The separated proteins were transferred onto PVDF membrane and the membrane was blocked for 1 h at room temperature. After blocking, the membrane was incubated in primary antibody solution (a monoclonal antibody against serine phosphorylated Elk-1) at 4°C overnight. The next morning, the membrane was washed three times in TBST and then incubated with a horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature. The membrane was again washed three times with TBST and then incubated for 1 min with chemiluminescence reagent before film exposure for 5–10 s.

Flow Cytometric Analysis of TCR Downmodulation. 3C6 T cells were incubated with different concentrations of heterofunctional antibodies for 1–3 h at 37°C. Subsequently, T cells were harvested and stained with FITC-labeled monoclonal antibody against mouse TCR-α/β (H57) or isotype-matched control antibody. Percentage of downmodulated receptors can be calculated as the ratio of mean fluorescence value of experimental samples over the mean fluorescence value of untreated control for the same time period (35).

Results

Agonist and Partial Agonist Signaling Patterns from the TCR Induced by CD3–CD4 Coengagement or by Bivalent CD3 Engagement, Respectively. As we have previously reported (33, 34), coengagement of CD3 and CD4 induces an agonist pattern of early signaling from the TCR. This pattern is characterized by the presence of two forms of phosphorylated TCR-ζ (p21 and p23), phosphorylation of CD3-ϵ, and recruitment and phosphorylation of ZAP-70 in phosphotyrosine blots of ZAP-70 immunoprecipitates (Fig. 1).

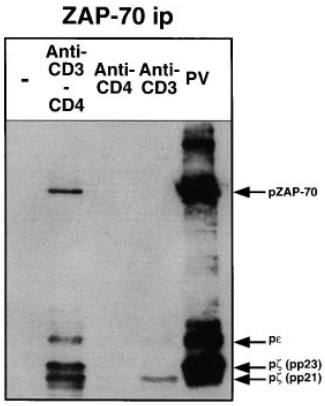

Figure 1. Induction of full agonist or partial agonist patterns of early TCR-mediated signaling by coengagement of CD3 and CD4 or bivalent engagement of CD3, respectively. 3C6 T cells (10^7 cells/lane) were stimulated with anti-CD3 fos antibodies, anti-CD4 jun antibodies, or anti-CD3-fos–anti-CD4-jun antibodies (10 μg/ml) for 10 min. Cells were lysed and the lysates underwent immunoprecipitation with an antiserum against ZAP-70. Immunoprecipitates were immunoblotted for phosphotyrosine. As positive control, T cells were stimulated with pervanadate, a phosphatase inhibitor.

In contrast, phosphotyrosine blots of ZAP-70 immunoprecipitates from T cells stimulated by bivalent engagement of CD3 show a partial agonist pattern of tyrosine phosphorylation. This is identified by predominant appearance of the p21 form of phospho-ζ, with little or no phospho-ϵ, and recruitment but no phosphorylation of ZAP-70 tyrosine kinase. In previous studies (14, 33), we have shown that the two patterns of early TCR-mediated signaling are not the result of quantitative differences in TCR engagement because they do not overlap after titration of antigenic peptide or stimulating antibody. Bivalent engagement of CD4 alone did not induce significant tyrosine phosphorylation of TCR subunits in ZAP-70 immunoprecipitates.

Further qualitative differences between the agonist pattern and the partial agonist pattern of early TCR-mediated signaling were seen when the kinase activity associated with the TCR complex under conditions of bivalent CD3 engagement or after CD3 and CD4 coengagement were examined. As genetic and biochemical approaches have previously demonstrated (36, 37), TCR-mediated signaling involves recruitment and activation of lck and ZAP-70 tyrosine kinases. Consistent with this, we found increased kinase activity associated with lck in T cells activated in an agonist pattern after CD3 and CD4 coengagement (Fig. 2). In these conditions, immune complex kinase assays after lck immunoprecipitation showed increased autophosphorylation of lck and phosphorylation of a 70-kD band as well as two bands of 23 and 32 kD, bands compatible with ZAP-70, TCR-ζ, and CD3-ϵ, respectively (Fig. 2). ZAP-70 immunoprecipitates showed significant autophosphorylation of ZAP-70 (Fig. 2). In contrast, no significant increase of lck phosphorylation or kinase activity was detected in lck immunoprecipitates or ZAP-70 immunoprecipitates from cell lysates of T cells activated in a partial agonist mode after bivalent CD3 engagement alone (Fig. 2). From these experiments, we concluded that bivalent engagement of CD3 and coengagement of CD3–CD4 fully reproduce the distinct patterns of early TCR signaling seen after engagement of TCR with ligands with partial agonist or agonist properties, respectively (13, 14).

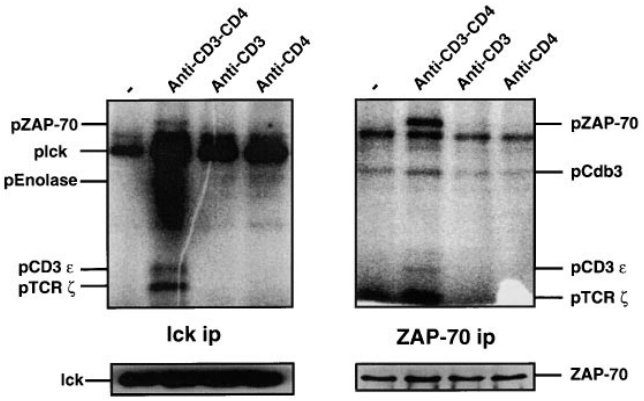

Figure 2. In vitro immune complex kinase assays of lck or ZAP-70 immunoprecipitates from T cells stimulated on a full agonist pattern or a partial agonist pattern of early TCR-mediated signaling. 3C6 T cells (10^7 cells/lane) were stimulated with the appropriate chimeric antibodies for 10 min. Cells were harvested and lysed, and the lysates subjected to immunoprecipitation of lck or ZAP-70. The immunoprecipitates were next used for in vitro kinase assay after addition of exogenous ^{32}P. Kinase reactions were run in SDS-PAGE and the gels exposed in a phosphorimager. Identity of the bands and equal loading were confirmed by parallel immunoblotting of these immunoprecipitates.

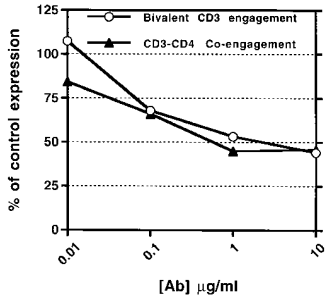

Figure 3. T cell receptor downmodulation induced by bivalent engagement of CD3 or by coengagement of CD3 and CD4. T cells were incubated with increasing concentrations of anti-CD3-fos–anti-CD4-jun antibodies or anti-CD3-fos antibodies for 3 h. T cells were then harvested and stained with an FITC-labeled monoclonal antibody against TCR-β constant region (H57-597) and examined by flow cytometry. Results are presented as a percentage of the mean fluorescence value of TCR expression for a given experimental group in relation to a time-matched, nonstimulated control group.

Despite these differences in early TCR-mediated signaling, we found that both agonist ligand and partial agonist ligand–induced similar loss of surface TCR expression as measured by flow cytometric analysis using a monoclonal antibody against mouse TCR (H57-597; Fig. 3). This finding implies that some signals from the TCR are equally induced by both types of ligands since TCR downmodulation is a reflection of T cell activation (19, 38) and protein kinase C–dependent serine phosphorylation of CD3 chains (23, 39–43).

Differential Effects of CD3 or CD3–CD4 Engagement on Recruitment and Phosphorylation of p120-GAP. To explore the downstream effects of these two qualitatively distinct patterns of early signaling, we initially looked at recruitment and activation of different molecules involved in the Ras-MAPK pathway of signal transduction. Among the many Ras effector molecules, we concentrated on p120-GAP for three reasons. First, Ag-induced T cell activation induces tyrosine phosphorylation of p120-GAP (44). Second, p120-GAP regulates Ras function by promoting GTPase activity (45–47). And third, p120-GAP may be itself a Ras effector molecule (48). We performed p120-GAP immunoprecipitates and examined phosphorylation and association of p120-GAP after agonist and partial agonist conditions of stimulation. T cell activation in an agonist mode (either by peptide–MHC molecule complexes on the surface of an APC or by heterofunctional antibodies against CD3 and CD4) induced a rapid, dose-dependent increase in phosphotyrosine content of p120-GAP (Fig. 4). In contrast, partial agonist signaling from the TCR did not induce tyrosine phosphorylation of p120-GAP. In addition, bivalent CD4 engagement alone did not induce tyrosine phosphorylation of p120-GAP either. Despite the differences in p120-GAP tyrosine phosphorylation, p120-GAP immunoprecipitation showed a tyrosine-phosphorylated 70-kD band that was immunoreactive with an antiserum against ZAP-70 in T cells stimulated in full agonist mode (Fig. 5, *a* and *b*). Surprisingly, this 70-kD protein was also associated to p120-GAP in T cells stimulated in partial agonist mode (Fig. 5 *b*), although it was not phosphorylated (Fig. 5 *a*), indicating that either pattern of early TCR-mediated signaling can induce p120-GAP recruitment to ZAP-70, but only agonists can induce its phosphorylation.

To further support that this 70-kD band was ZAP-70, the protein was depleted from the lysate by sequential rounds of immunoprecipitation with an antiserum against ZAP-70 (Fig. 6). Interestingly, the data suggest that the proportion of ZAP-70 that is associated with p120-GAP is relatively low compared with the total amount of ZAP-70 in the cells. Reblotting of the same membrane with an antiphosphotyrosine antibody showed a similar result for phospho–ZAP-70. In addition, in vitro immune complex kinase assays on p120-GAP immunoprecipitates showed phosphorylated lck and ZAP-70 bands as well as phosphorylation of the cdb3 exogenous substrate for ZAP-70 after

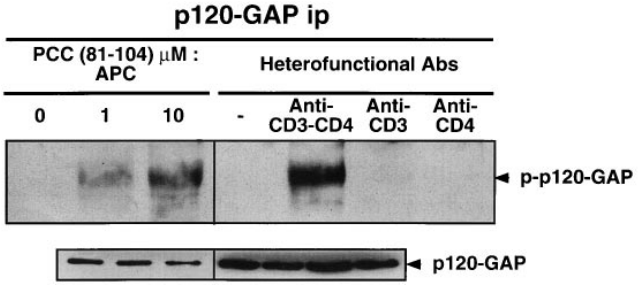

Figure 4. Induction of tyrosine phosphorylation of p120-GAP upon TCR engagement with agonist ligands. 3C6 T cells (10^7 cells/lane) were stimulated with antigenic peptide (1 or 10 μM) and APCs, or with chimeric anti-CD3-fos–anti-CD4-jun, anti-CD3-fos, or anti-CD4-jun antibodies at 10 μg/ml for 10 min. T cells were then harvested and lysed in Triton-X 1%. Cell lysates underwent immunoprecipitation with an antibody against p120-GAP and subsequently immunoblotted for phosphotyrosine. Equal p120-GAP immunoprecipitation in the different groups was confirmed by immunoblotting of the same membrane with an antibody against p120-GAP.

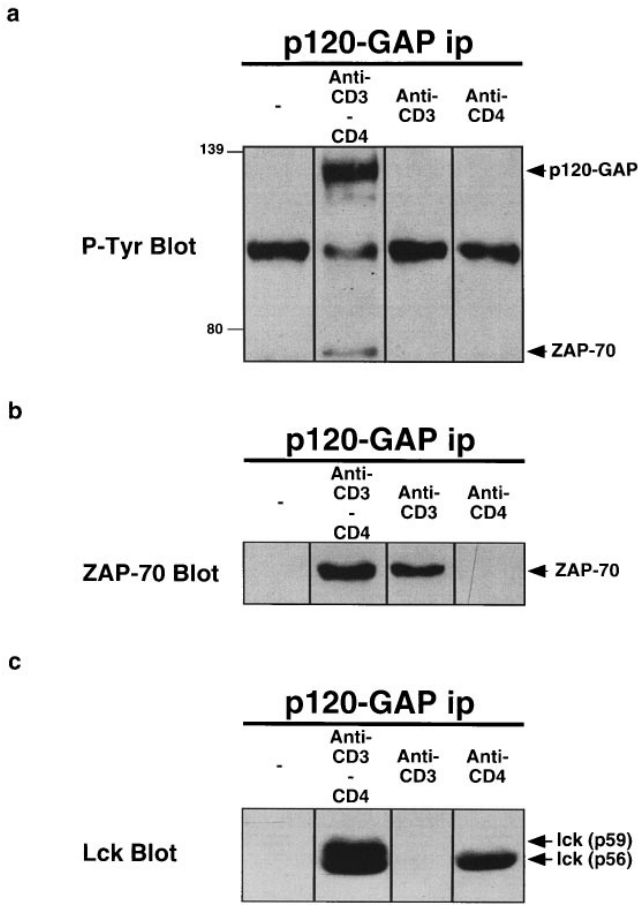

Figure 5. Association of p120-GAP with ZAP-70 and lck upon distinct patterns of early TCR-mediated signaling. 3C6 T cells (10^7 cells/lane) were stimulated with increasing concentrations of chimeric anti-CD3-fos–anti-CD4-jun, anti-CD3-fos, or anti-CD4-jun antibodies for 10 min. T cells were then harvested and lysed in Triton-X 1%. Cell lysates underwent immunoprecipitation with antibodies against p120-GAP and subsequently immunoblotted for phosphotyrosine (*a*). Similar experiments were performed and immunoblotted for ZAP-70 (*b*) or for lck (*c*).

CD3–CD4 coengagement (Fig. 7). In contrast, and consistent with our previous findings on kinase assays of ZAP and lck immunoprecipitates from partial agonist-stimulated T cells, no kinase activity was detected in p120-GAP immunoprecipitates from these cells. The association between ZAP-70 and p120-GAP is unlikely due to coassociation of these molecules through TCR subunits because these proteins were not detected in p120-GAP immunoprecipitates. In these kinase assays, p120-GAP was tyrosine phosphorylated only in the immunoprecipitates from T cells stimulated by agonist ligands.

Previous reports have demonstrated that p120-GAP can associate with lck and is a substrate of this *src*-kinase (49, 50). Given that lck is noncovalently associated with CD4 (51), the lack of coengagement of CD3 and CD4 might translate into lack of association between p120-GAP and lck and could explain the lack of tyrosine phosphorylation of p120-GAP. To test this hypothesis, we examined whether the two patterns of early TCR-mediated signaling differed in the ability of p120-GAP to associate with lck. As shown in Fig. 5 *c*, there is no association between p120-GAP and lck under basal conditions. However, after TCR-mediated signaling by the agonist, there was significant association of these two molecules. Both the p56 and the activation-dependent p59 form of lck were present in p120-GAP immunoprecipitates of T cells stimulated by coengagement of CD3 and CD4 confirming that T cell activation had occurred. Partial agonist type of TCR-mediated signaling resulted in no association of these two molecules. Of note was the observation that bivalent engagement of CD4 alone did induce the association of p120-GAP with lck as well as tyrosine phosphorylation of p120-GAP in immune complex kinase assays (Fig. 7); however, under these conditions only the resting p56 form of lck was detected.

Thus, our results indicate that p120-GAP is recruited and associated to ZAP-70 after engagement of the TCR in vivo. Early TCR signaling in agonist mode induces kinase activity of lck and ZAP and that correlates with tyrosine phosphorylation of the recruited p120-GAP. In contrast, early TCR-mediated signaling in partial agonist mode fails to induce kinase activity of lck and ZAP-70, and therefore it is not able to induce phosphorylation of the recruited p120-GAP.

Activation of ERK-1 and ERK-2 upon Early TCR-mediated Signaling in Agonist and Partial Agonist Modes. Activation of $p21^{ras}$ leads to recruitment and activation of Raf-1, which

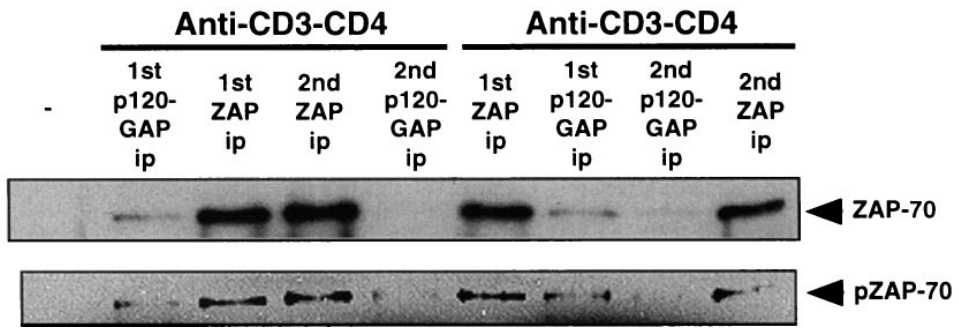

Figure 6. Depletion of ZAP-70 upon sequential immunoprecipitations with anti–ZAP-70 or with anti-p120-GAP monoclonal antibody. Two groups of 3C6 T cells (10^7 cells/group) were stimulated with anti-CD3-fos–anti-CD4-jun chimeric antibodies (10 μg/ml), and subsequently underwent immunoprecipitation with antibodies against p120-GAP or with antiserum against ZAP-70 followed by two rounds of immunoprecipitation with anti–ZAP-70 antiserum or anti-p120-GAP antibodies, respectively, and an additional immunoprecipitation with anti-p120-GAP or anti–ZAP-70 antibodies. The immunoprecipitates were immunoblotted with an antiserum against ZAP-70. Subsequently, the same membrane was stripped, reexposed to film to confirm complete stripping, and reblotted with a monoclonal antibody against phosphotyrosine.

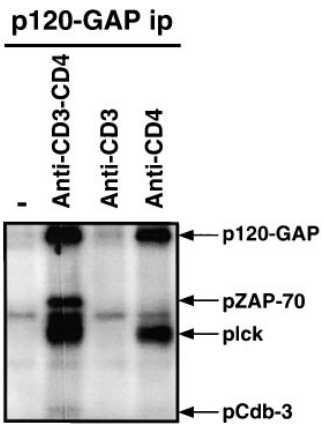

Figure 7. In vitro immune complex kinase assay of p120-GAP immunoprecipitates from T cells stimulated in agonist or partial agonist pattern of early TCR-mediated signaling. p120-GAP immunoprecipitates from 3C6 T cells (10^7 cells/lane) stimulated with the appropriate chimeric antibodies were used for in vitro kinase assay after addition of exogenous ^{32}P. Identity of the bands were confirmed by parallel immunoblotting of similar immunoprecipitates.

then activates MEK-1 and MEK-2, which subsequently activate ERK-1 and ERK-2 (24). Thus, we assessed the activation of these stages of the Ras-MAPK pathway by looking at the levels of double phosphorylated ERK-1 and ERK-2, as these are the active forms of ERK (24), and at the kinase activity of ERK-1 and ERK-2 measured as serine phosphorylation of exogenous Elk-1 as substrate. Immunoblotting with a monoclonal antibody against dual phosphorylated ERK-1 (p44) and ERK-2 (p42) showed a significant increase in these forms upon short stimulation with either anti-CD3-fos or with anti-CD3-fos–anti-CD4-jun (Fig. 8 *a*). ERK-1 and ERK-2 activation remained high for at least 10 min in T cells stimulated by the agonist ligand as well as in those stimulated by the partial agonist ligand. However, upon CD3 and CD4 coengagement, ERK-1 and ERK-2 activation did not start to significantly decrease until after 30 min and was still detectable after 60 min of stimulation. In contrast, ERKs activation decreased more rapidly after partial agonist signaling, with significantly decreased levels by 10 min after activation with bivalent CD3 engagement and disappearance after 30 min. The possibility of differential activation of p42 or p44 at the initial time points is unlikely given that the signal ratio for p44/p42 is similar for both groups. As ERK activation decreases, the loss of p44 phosphorylation occurs faster than the loss of p42 phosphorylation in both experimental groups.

These results were confirmed using in vitro kinase assays for the ERKs (Fig. 8 *b*). Coengagement of CD3 and CD4

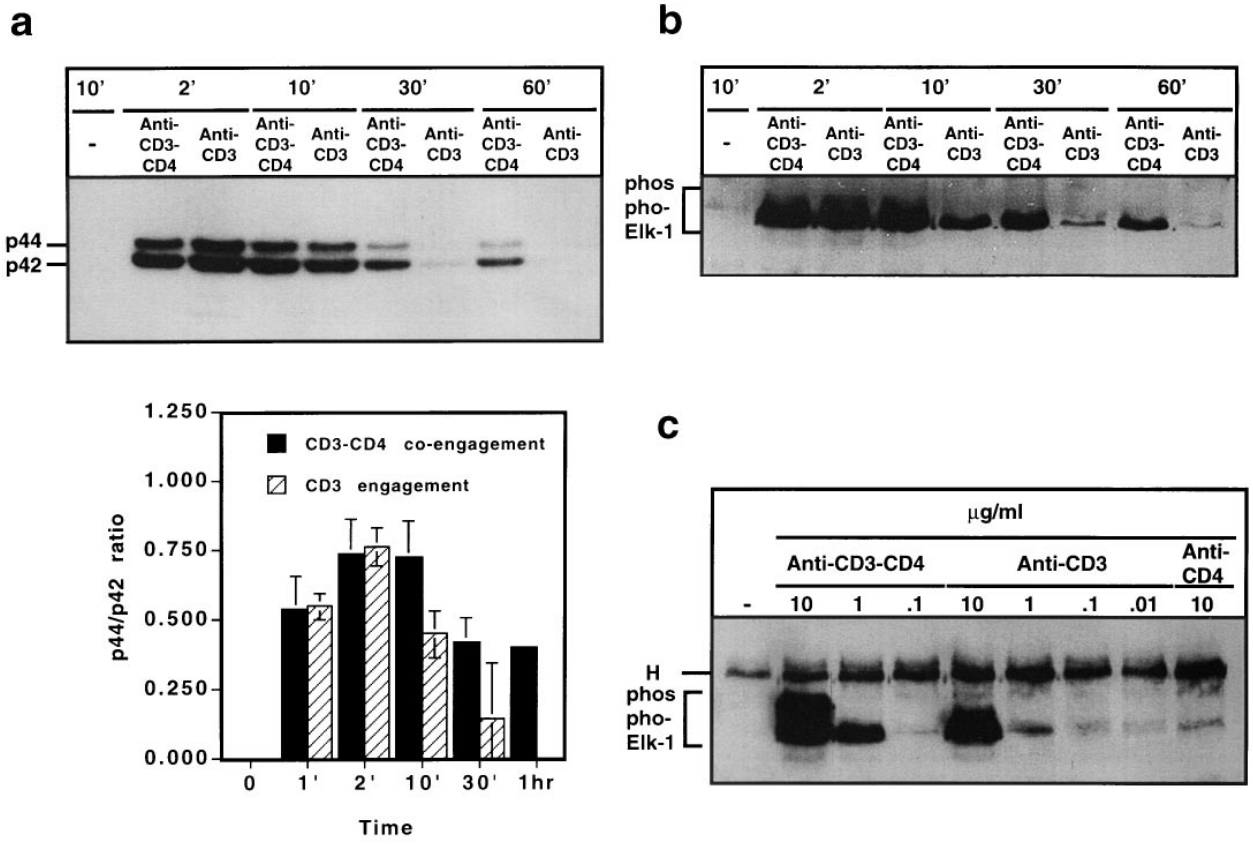

Figure 8. Activation of ERK-1 and ERK-2 after early TCR-mediated signaling in agonist or partial agonist pattern. (*a*) 3C6 T cells were stimulated with the indicated heterofunctional antibodies (at a final concentration of 10 μg/ml) for increasing times, after which T cells were harvested and lysed. Cell lysates (450,000 cell equivalents) were then immunoblotted with an antibody against dual phosphorylated ERKs. The bar diagram under *a* represents the p44/p42 ratio of signal intensity of these bands for at least three different experiments. (*b*) Similarly, T cells (10^7 cells/group) were stimulated for increasing times, after which T cells were harvested and lysed. Lysates underwent immunoprecipitation with phospho-specific anti-MAPK antibodies for 18 h. Immunoprecipitates were then incubated with exogenous Elk-1 substrate in kinase buffer containing ATP, and subsequently immunoblotted with a phospho-specific anti–Elk-1 antibody. (*c*) Similar experiment to *b*, but using increasing concentrations of the different antibodies.

for 2 min induced a significant increase in kinase activity of ERKs as indicated by increasing serine phosphorylation of exogenous Elk-1 (Fig. 8 *b*). Under these conditions, ERK kinase activity remained high for up to 60 min. Bivalent engagement of CD3 alone also induced a substantial increase in phosphorylation at 2 min, which was equal to that observed after full agonist TCR-mediated signaling. Therefore, the initiation of ERK activation is similar after either agonist or partial agonist engagement of the TCR. However, the kinetics of phosphorylation and phosphoprotein decay of this pathway are distinctly different, with significant loss of kinase activity after 10 min and almost complete loss of activity after 30 min under conditions of partial agonist signaling from the TCR. The increase in ERK kinase activity seen after TCR-mediated signaling in agonist mode or partial agonist mode were both dose dependent (Fig. 8 *c*).

Discussion

Variant TCR ligands with partial agonist properties induce a pattern of early TCR-mediated signaling that is distinct from the signals induced by agonist ligands (for reviews see references 12, 20). Although agonist ligands induce appearance of two forms of phospho–TCR-ζ (p21 and p23), phospho–CD3-ϵ, and subsequent recruitment and activation of ZAP-70, partial agonists of the TCR only induce the p21 form of phospho–TCR-ζ and fail to activate recruited ZAP-70. This distinct signaling pattern correlates with restricted T cell responses such as limited cytokine production or induction of T cell anergy (7, 8). Characterization of the steps linking the initial events after receptor engagement with the downstream effector responses is important to understand T cell differentiation after TCR ligation. The results presented in this paper demonstrate that stimulation of T cells in a partial agonist mode after bivalent engagement of CD3 causes recruitment and association of p120-GAP with ZAP-70, and activation of the Ras-MAPK pathway. However, in contrast to full agonist pattern of TCR-mediated signaling, partial agonist TCR-mediated signaling activates ERK-1 and ERK-2 only transiently, either because of lack of sustained stimulation and normal decay or because of faster inactivation of these serine/threonine kinases.

We have shown that p120-GAP can associate with ZAP-70 in vivo after early TCR-mediated signaling in agonist mode and in partial agonist mode. However, only full agonist type of signaling induced phosphorylation of p120-GAP. This is expected since only agonist ligands induce activation of lck and of ZAP-70 (13, 14), and lck can act as a kinase on ZAP-70 (36) and on p120-GAP (50). On the other hand, the partial agonist pattern of TCR-mediated signaling failed to activate the kinase activity of lck and did not induce association of p120-GAP to this *src*-kinase. This could explain the lack of tyrosine phosphorylation of ZAP-70 and of p120-GAP (34). The dissociated recruitment and activation of signaling molecules after partial agonist signaling from the TCR may have profound functional consequences. This is well established for ZAP-70, for which recruitment and activation are two separate, discrete events, and for which tyrosine phosphorylation correlates with kinase activity (14, 36, 52, 53). The biological implications of tyrosine phosphorylation of p120-GAP are still unknown.

What is the molecular basis for the association between p120-GAP and these kinases? In the agonist type of signaling, both lck and ZAP-70 are tyrosine phosphorylated and active. Given that p120-GAP has two SH2 domains (48), the association of p120-GAP to these molecules can be explained on the basis of SH2-mediated interactions with phospho-tyrosine-based motifs, as previously reported (54). However, the molecular mechanism by which the ZAP-70–p120-GAP association occurs after early TCR signaling in partial agonist mode is not clear because, in these conditions, ZAP-70 is not phosphorylated, excluding SH2-mediated interactions between these two molecules. Two possible mechanisms can be proposed. One is that p120-GAP associates with ZAP-70 through an interaction involving the SH3 domain of p120-GAP and a proline-rich region in ZAP-70. Close examination of the ZAP-70 sequence shows that it contains a PTLPAHP stretch between residues 267 and 274, downstream of the SH2(C) domain (52). This sequence conforms to the consensus sequence for SH3 domain binding (55). This claim is further supported by the similarity between this sequence and the proline-rich sequence in G3BP (PQRPQR) that likely mediates the high affinity binding between this protein and the SH3 domain of p120-GAP (56). The PTLPAHP sequence only contains relatively minor substitutions between the proline residues. This mechanism would be similar to that reported for MAPK recruitment to the TCR via SH3 interactions with lck (57). Alternatively, the association between ZAP-70 and p120-GAP may occur through an intermediate molecule to which both bind through their SH2 domains. One potential candidate could be a TCR subunit such as TCR-ζ or a CD3 chain because these are phosphorylated in tyrosines after partial agonist type of signaling from the TCR, and we know that this is sufficient for ZAP-70 recruitment (14). However, we have failed to detect the presence of TCR subunits by direct blotting of p120-GAP immunoprecipitates as well as by phosphotyrosine blotting of these samples. Another candidate could be p62-Sam68 (58, 59), although we do not have any indication of a tyrosine phosphorylated protein of this range in p120-GAP immunoprecipitates.

How does activation of ERK-1 and ERK-2 occur after TCR-mediated signaling in partial agonist mode? Our results indicate that coengagement of CD4 with CD3 is not necessary for activation of the Ras-MAPK pathway, but it is required for optimal and sustained activation of this pathway (60). Coengagement of CD3 and CD4 may be especially important when the affinity of the TCR for its ligand is low, such as in the case of weak agonist ligands (61) or partial agonists and antagonists of the TCR (62, 63). Under these conditions, CD4 coengagement may increase the stability of TCR–peptide–MHC molecule complex engagement and provide sustained TCR-induced signaling (64).

Our finding of transient activation of MAPKs in the absence of CD3–CD4 coengagement is therefore compatible with our previous model in which the efficiency of TCR–CD4 coengagement determines the quality of TCR-mediated signaling and partial agonism (33, 34). The transient kinetics of MAPK activation after partial agonist stimulation supports a CD4-dependent kinetic basis for differential TCR-mediated signaling by partial agonist ligands. TCR antagonism may represent conditions of a much lower range of TCR affinity that correlate with very transient or undetectable ERK activation (65).

It is important to note that bivalent engagement of CD3 may be a critical feature in the induction of partial agonist signaling in our experimental model, in line with recent claims that oligomerization of the TCR may be needed for initiation of signaling (66). The specific CD3 dimerization induced by our chimeric antibodies, in contrast to antibody-induced cross-linking (59), could explain the differences between our data showing lck-independent activation of ERKs and previous reports claiming that ERK activation is primarily lck dependent (67, 68). Some ligands, including the chimeric molecules used here, may induce receptor dimerization with the proper architecture to cause PKC-dependent activation of Raf-1 and ERK (69–72) in the absence of lck activation (68, 73). In vivo data would support this model. For example, although lck-deficient mice have a drastic reduction in the total number of double positive thymocytes, they still have a significant proportion of these cells (74). This would support a lck-independent pathway for MAPK activation given that differentiation into $CD4^+CD8^+$ thymocytes is critically dependent on activation of the MAPK pathway (75–77).

Based on the above mentioned considerations, we propose that partial agonist-induced differential phosphorylation of TCR subunits causes activation of Ras. This could be the result of activation of a kinase other than lck (possibly fyn; reference 78), which would concomitantly recruit ZAP-70 and activate some other signaling molecules like p95vav that shares homology with some guanine nucleotide releasing factors involved in Ras activation (79, 80). Ras activation would then recruit different effector molecules to the cell membrane, including Raf (81) and p120-GAP. Activation of Ras and Raf-1 would then result in activation of ERK-1 and ERK-2. In addition, the presence of p120-GAP in proximity to engaged and differentially phosphorylated TCR would favor the association of p120-GAP with recruited, TCR-associated ZAP-70. Although we have not ruled out an indirect association between these two molecules, we suggest that this would occur through proline-rich–SH3 domain interactions. An effector function of p120-GAP other than acting as GTPase is still unknown (45).

The demonstration of the flexibility of TCR-mediated recognition and consequent T cell responses raises an interesting question related to downstream signaling by variant TCR ligands. That is, does differential early TCR-mediated signaling result in a similar decrease in activation of all downstream pathways? Or conversely, does this differential signaling correlate with preserved activation of some pathways but not others? Our data would support a model of dissociated activation of some signaling pathways, but not others. This could reflect different requirements of sustained signaling for each signaling pathway emanating from the TCR. Such a model is also supported by two recent reports indicating that partial agonists of the TCR can induce low and transient calcium signals that do not correlate with detectable generation of inositol trisphosphates (17, 18). The different thresholds of activation for downstream pathways would then correlate with different patterns of effector T cell responses as recently reported (82). The ability to dissociate signaling pathways may have fundamental biological implications in the design of immunomodulatory strategies that target specific signaling pathways whose activation results in split T cell responses. In this sense, selective activation of the Ras-MAPK pathway may reproduce some of the effects seen after TCR engagement with partial agonist ligands.

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