T Cell Receptor-mediated Signal Transduction Controlled by the β Chain Transmembrane Domain

APOTOPSIS-DEFICIENT CELLS DISPLAY UNBALANCED MITOGEN-ACTIVATED PROTEIN KINASES ACTIVITIES UPON T CELL RECEPTOR ENGAGEMENT*

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The bases that support the versatility of the T cell receptor (TCR) to generate distinct T cell responses remain unclear. We have previously shown that mutant cells in the transmembrane domain of TCR/β chain are impaired in TCR-induced apoptosis but are not affected in other functions. Here we describe the biochemical mechanisms by which this mutant receptor supports some T cell responses but fails to induce apoptosis. Extracellular signal-regulated kinase (ERK) is activated at higher and more sustained levels in TCR/β-mutated than in wild type cells. Conversely, activation of both c-Jun N-terminal kinase and p38 mitogen-activated protein kinase is severely reduced in mutant cells. By attempting to link this unbalanced induction to altered upstream events, we found that ZAP-70 is normally activated. However, although SLP-76 phosphorylation is normally induced, TCR engagement of mutant cells results in lower tyrosine phosphorylation of LAT but in higher tyrosine phosphorylation of Vav than in wild type cells. The results suggest that an altered signaling cascade leading to an imbalance in mitogen-activated protein kinase activities is involved in the selective impairment of apoptosis in these mutant cells. Furthermore, they also provide new insights in the contribution of TCR to decipher the signals that mediate apoptosis distinctly from proliferation.

The TCR1 expressed in the majority of peripheral T lymphocytes is a complex composed of the clonotypic TCR αβ heterodimer, responsible for antigen and superantigen recognition, linked to the monomorphic CD3-γ, -δ, -ε and -ζ chains, which are involved in signal transduction. This structural complexity, in comparison with other families of cytokines and growth receptors, might be responsible for the different outcomes of TCR engagement. The fact that TCR engagement triggers either cytokine-driven proliferation or cell death is particularly intriguing and raises the question of how these outcomes are differentially regulated. It has promoted the concept that the TCR does not function as a simple on-off switch upon activation. In fact, several approaches have provided evidence that TCR could signal to some activation while keeping other pathways functionally inactive (reviewed in Ref. 1). Because of the short length of the cytoplasmic tails and the apparent lack of inherent activity of both clonotypic chains, it is assumed that the heterodimer transmits the signal through the CD3ζ chains. In this context, questions that remain to be answered are the following: 1) How do engaged TCR transfer signals to initiate signal transduction? 2) Which domains of the TCR conserved regions are involved in such transmission? 3) What effector functions are modulated through the integrity of such domains?

Only a few reports have stressed the contribution of individual structural domains of the αβ clonotypic module to specific signal transduction. TCRα chain tail seems to be required for down-regulation of the TCR-CD3 complex (2), and chimeric TCRs containing clonotypic α instead of α residues in the connecting peptide of the TCRα chain fail to trigger IL-2 production upon cell activation (3). In addition, double positive thymocytes bearing this chimeric TCR undergo negative, but not positive, selection because of a failure in the activation of the extracellular signal-regulated kinase (ERK) (4, 5). A single mutation in the β chain connecting peptide results in a TCR that is specifically deficient in activating both the calcium pathway and IL-2 secretion (6). Furthermore, individual mutations at either of the two conserved Tyr residues in β chain transmembrane domain allow TCR-CD3 expression and are compatible with normal signaling. On the contrary, dual mutations affecting signaling efficiency to a greater extent than predicted by surface expression alone and resulted in severe reductions in IL-2 production and apoptosis (7, 8). Notwithstanding, although different mouse T cell hybridomas dramatically differed in their functional response to the same mutations, these results suggest that membrane-spanning domains in TCRβ are relevant for signal transduction (8). In all works, however, information on how mutations might affect biochemical events involved in the activation of upstream intermediates and signaling pathways is basically lacking.

The ability of the TCR to transduce quantitatively and qual-
itatively different signals is strongly supported by the existence of partial agonists, peptide analogues that can selectively stimulate only some T cell effector functions (9). Partial agonists, usually related to low affinity ligands, have been proved to be a useful tool to understand mechanisms regulating TCR differential signaling. In fact, a broad variety of such peptides has been described that trigger TCR to usually result in anergy, cytokine production, cytolytic activity, or Fas-mediated cytotoxicity as opposed to perf-in-dependent cytotoxicity or proliferation (10–12). Analogues that selectively impair triggering to apoptosis have not been reported, but, singularly, TCR ligands that uniquely trigger apoptosis in CD4⁺ lymphocytes have been described (13). No similar selective peptides have been reported for CD8⁺ cells. However, some partial agonists, being inducers of T cell cytotoxicity, can dissociate the induction of Fas-L-dependent CTL death from CTL activation and perforin-dependent (14) or Fas-L-dependent target cell death (10, 11). In some experimental systems, the ability to divorce killing of the targets and Fas-L-mediated CTL apoptosis has been demonstrated to occur by blocking CD8/MHC class I interaction (15). Two major biochemical events are characteristics of triggering with partial agonist: 1) the phosphorylation of CD3-ζ chains, which results in the predominant phosphorylation of the higher mobility phospho-ζ isofrom with the almost absent phosphorylation of the lower mobility isoform, was incomplete, and 2) although ZAP-70 was seen to bind to CD3-ζ following signaling, the ZAP-70 molecule itself was not phosphorylated, and its kinase activity was not induced (16, 17). In this regard, the fact that a consistent pattern of early signaling is not elicited by ligands capable of inducing similar functional responses (9, 13) suggests that differential signaling would take place after CD3-ζ and ZAP-70 phosphorylation.

Signal transduction through the TCR is initiated by Src kinase-mediated phosphorylation of the ITAMs of the CD3 and ζ chains, followed by the recruitment, phosphorylation, and activation of ZAP-70. The coordinated action of Src kinases and ZAP-70 results in the phosphorylation of multiple substrates. Among these substrates, LAT becomes heavily tyrosine-phosphorylated upon TCR activation, a fact that endows LAT with the capacity for recruiting multiple signaling molecules. It has been hypothesized that signaling through Ras is exquisitely dependent on tyrosine-phosphorylated LAT (18). However, several lines of evidence suggest that Ras activation involves additional interactions and that the Ras-MAPK activation can occur through alternative pathways independent of LAT phosphorylation (19). These alternative interactions might lead to distinct patterns of ERK activation, which could be associated with the regulation of T cell death or survival (20, 21). In addition to Ras, other small GTPases belonging to the Rho family (Rac-1, Cdc42, and RhoA) are activated upon stimulation of the TCR. These GTPases can activate other MAPK cascades, such as the c-Jun N-terminal kinase (JNK) and p38MAPK activation pathways that have also been related to apoptosis (22). Vav is a guanosine nucleotide exchange factor for Rho family GTPases whose exchange activity can be modulated by TCR signaling. Such a mechanism, on operating over Rac-1, has been reported to link the TCR to the activation of JNK and p38MAPK (23, 24). LAT phosphorylation appears to play a critical role on the assembly of ZAP-70-LAT-Vav complexes in lipids rafts and on its translocation in the vicinity of Vav downstream effectors (25). Additionally, it has been reported that Vav plays TCR-mediated roles other than acting as the GDP/GTP exchanger (26–28), but the mechanisms involved are poorly understood.

We have previously reported that Jurkat-derived mutant T cell clones, bearing a mutation (Tyr to Leu) in the C-terminal tyrosine of the conserved ITAM-like motif of the transmembrane domain of TCRβ chain, show normal ability: to secrete cytokines (IL-2 and interferon-γ); to express activation markers; to down-modulate their TCR-CD3 complex; and to mobilize intracellular Ca²⁺ upon TCR activation. However, these mutant cells show a resistance to TCR-induced apoptosis, a defect that is not observed upon direct Fas stimulation or after stimulating with reagents that bypass TCR engagement (i.e. phorbol esters plus calcium ionophore) (29–31). Altogether, these findings prompted us to further investigate the effects of the mutation on TCR signaling, in an attempt to clarify the mechanisms by which the integrity of the transmembrane domain of the TCRβ chain contributes to the early biochemical events and MAPK activation pathways associated with TCR-mediated signaling efficiency.

EXPERIMENTAL PROCEDURES

Cell Lines

Wild type and mutant clones have been previously described (29, 30). Briefly, they were obtained by reconstitution of the TCRβ-negative Jurkat variant 31.13 with either a wild type Vβ5 TCR cDNA or with a mutant cDNA that contains a tyrosine to leucine mutation in the C-terminal tyrosine of the transmembrane domain. No clonal variation was observed between different clones derived from independent wild type or mutant cDNA transfections (29, 30). The cells were maintained in RPMI supplemented with 10% fetal calf serum and antibiotics. Expression of TCR-CD3 complex was routinely tested by flow cytometry before each assay.

Antibodies and Reagents

The following antibodies were used: 1) for cytometry, anti-CD3e (UCHT-1-fluorescein isothiocyanate (Caltag, Burlingame, CA); anti-TCRβ3 (JOVI-3, a gift from Dr. M. Owen); and anti-CD69-fluorescein isothiocyanate (Becton Dickinson, Madrid, Spain); 2) for stimulation, anti-CD95 (UCHT-1, Immunokontact, Switzerland; used for stimulation at 10 μg/ml); anti-CD28 (Pharmingen, San Diego, CA; used for stimulation at 1 μg/ml); and the cross-linking antibody goat anti-mouse (Pierce; used for stimulation at 10 μg/ml); 3) for immunoprecipitation assays, anti-ZAP-70 (ZAP-4, a gift from Dr. S. Ley, NIMR, London); anti-LAT and anti-Vav (Upstate Biotechnology, Inc., Lake Placid, NY); anti-SLP-76 (kindly provided by Dr. G. Koretzky, University of Pennsylvania School of Medicine); 4) for Western blot, anti-PLC-γ1 and anti-Vav (BD Transduction Laboratories, Lexington, KY); anti-c-Bl and anti-Raf-1 kinase (Santa Cruz, Santa Cruz, CA); anti-SLP-76 (kindly provided by Dr. G. Koretzky); anti-ERK (Zymed Laboratories Inc., San Francisco, CA); anti-phospho-p44/42MAPK, anti-phospho-p38MAPK, and anti-JNK (New England Biolabs, Beverly, CA); anti-α-tubulin (Sigma-Aldrich); and anti-LAT, anti-Ras, and anti-phospho-tyrosine 4G10 (Upstate Biotechnology, Inc.). Staphylococcal enterotoxin B was purchased from Toxin Technology (Sarasota, FL; used for stimulation at 10 μg/ml). Biotinylated annexin-V was from Roche Molecular Biochemicals, and phycoerythrin-labeled streptavidin was from Caltag (Burlingame, CA). The construct for the glutathione S-transferase (GST) fusion protein containing the Ras-binding domain (RBD) of Raf was from Upstate Biotechnology, Inc.

TCR Stimulation

0.5–2.5 × 10⁵ cells/ml were resuspended in RPMI containing 15 mM HEPES. Unless otherwise detailed, stimulation was performed by incubation with anti-CD3 (10 μg/ml) together with anti-CD28 (1 μg/ml) antibodies for 10 min on ice followed by cross-linking with goat anti-mouse antibodies at 37 °C for the indicated times.

Flow Cytometry

10⁶ cells were incubated on ice for 1 h with a fluorescein isothiocyanate-conjugated specific antibody, washed, and analyzed in a flow cytometer (EPICS-XL MCL, Coulter).

Immunoprecipitations

2.5 × 10⁷ cells were unstimulated or stimulated as described and lysed for 30 min at 4 °C in a buffer containing 1% Brij-96 (or 1% Nonidet P-40), 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, leupeptin, pepstatin, and aprotinin (1 μg/ml each), 1 mM phenylmethyisulfonyl fluoride, 20 mM NaF, and 1 mM NaVO₃. After centrifugation, the lysates were pre-cleared sequentially with Sepharose beads and with protein A- or G-Sepharose beads coated with the appropriate control antibody and
after subjected to immunoprecipitation for 4 h at 4 °C with protein A- or G-Sepharose beads coated with specific antibodies. Immunoprecipitates were then washed four times with lysis buffer and subjected to SDS-PAGE followed by standard immunoblot analysis with the indicated antibodies.

Subcellular Fractionation

Upon stimulation, the cells were resuspended in ice-cold hypotonic buffer (42 mM KCl, 10 mM HEPES, pH 7.4, 5 mM MgCl₂, 10 µg/ml each aprotinin and leupeptin) and incubated on ice for 15 min. The cells were transferred to a 1-ml syringe and sheared by being passed five times through a 30-gauge needle. Whole cell lysates were centrifuged at 200 × g for 10 min at 4 °C. The supernatant was collected and centrifuged at 13,000 × g for 60 min at 4 °C. The supernatant (cytosol) was collected, and the pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, and 10 µg/ml each aprotinin and leupeptin), vortexed for 5 min at 4 °C, and centrifuged again at 13,000 × g for 60 min at 4 °C. The supernatant representing the particulate (membrane) fraction was then saved. Each fraction containing the protein of interest was diluted to a final concentration of 1/1000 representing the particulate (membrane) fraction was then saved. Each fraction and resuspended in 30 µl of kinase buffer, and proteins were eluted in Laemmli buffer and separated by SDS-PAGE, and the Western blots were analyzed with anti-PKC antibodies.

Kinase Activity Assays

**Raf Kinase, ERK, and p38MAPK Activities**—10⁶ cells/time point were not stimulated or stimulated for the indicated times and lysed for 30 min at 4 °C in 50 µl of a buffer containing 1% Nonidet P-40, 20 mM HEPES, 10 mM EDTA, 2.5 mM MgCl₂, 40 mM β-glycerophosphate, 1 mM dithiothreitol, and the indicated protease and phosphatase inhibitors (1 µg/ml each): leupeptin, pepstatin, and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, and 1 mM NaVO₄. Lysates were then centrifuged, and the supernatants were subjected to standard 10% SDS-PAGE for ERK and p38MAPK, or 8% (acrylamide/bisacrylamide 24/0.6) for Raf kinase to improve resolution of phosphorylated band shifts. Electrophoresed proteins were transferred to nitrocellulose membrane for immunoblot analysis with specific antibodies. In the cases of ERK and p38MAPK, their phosphorylation status, as determined by immunoblotting with antibodies that specifically recognize the phosphorylated forms of these kinases, is assumed to be an indication of their activation state. Raf kinase activation was evaluated by the band shifts corresponding to the protein phosphorylated form, which can be detected by immunoblotting with specific anti-Raf-1 kinase antibodies.

**Ras Activity**—5 × 10⁶ cells/time point were not stimulated or stimulated for the indicated times and lysed for 30 min at 4 °C in 300 µl of a buffer containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 10 mM MgCl₂, 1% glycerol, leupeptin, pepstatin, and aprotinin (1 µg/ml each), 1 mM phenylmethylsulfonyl fluoride, and 1 mM NaVO₄. The lysates were then centrifuged, and the supernatants were mixed with 20 µl of a freshly prepared GST fusion protein containing the Ras-binding domain of Raf-1 (GST-RBD), immobilized in glutathione-Sepharose beads (Amersham Biosciences, Inc.). The samples were incubated by rotating for 90 min at 4 °C, and the beads were then washed three times with lysis buffer. The bound Ras-GTP protein was eluted in Laemmli sample buffer and subjected to 12.5% SDS-PAGE. The levels of Ras in mutant cells that links the proximal and distal events of the TCR signaling pathway. Immunoprecipitation with specific anti-LAT antibodies followed by immunoblotting with anti-phosphotyrosine showed that in comparison with wild type cells, TCR-induced tyrosine phosphorylation of LAT was clearly diminished in mutant cells (Fig. 1A). A longer exposure of the LAT immunoprecipitation membrane revealed the presence of previously described tyrosine-phosphorylated proteins that co-precipitate with LAT upon TCR triggering (33) identified as PLC-γ, c-Cbl, Vav, and SLP76. Interestingly, although the levels of LAT-associated tyrosine-phosphorylated SLP76, Vav, and PLC-γ1 were reduced in mutant cells, the levels of LAT-associated phospho-c-Cbl were higher. Furthermore, blotting with specific antibodies indicated that mutant cells are defective in the recruitment of PLC-γ1, Vav, and SLP-76 to LAT and in a lesser extent in the recruitment of c-Cbl (Fig. 1B).

Because Zap70 was not detectable in LAT immunoprecipitates (33) and we had reported that Zap-70 was scarcely recruited to the plasma membrane in TCR-activated mutant cells (30), we performed reverse immunoprecipitation with specific anti-ZAP-70 antibodies to determine whether the low level of induced phospho-LAT in mutant cells was the result of poor activity of Zap-70 on its membrane substrate. The immunoprecipitation revealed lower levels of coprecipitated phospho-LAT in mutant cells than in wild type cells (Fig. 1C). Furthermore and as expected, the immunoprecipitation revealed similar levels of tyrosine-phosphorylated Zap-70 in both stimulated cells (Fig. 1C). Thus, despite the fact that both the tyrosine phosphorylation level of Zap-70 and in vitro Zap-70 activity were similar in both cell types, our results show that the in vivo phosphorylation of LAT and the recruitment to LAT of other signaling molecules are impaired in TCR-stimulated mutant cells.

**TCR Triggering in Mutant Cells Results in a Higher Activation of the Ras-ERK Pathway Than That in Wild Type Cells**—Most of the current evidence suggests that the activation of the Ras-Raf-ERK pathway, after TCR signaling, requires tyrosine-phosphorylated LAT (18). If this dependence were absolute, in light of the aforementioned results, we would expect that the impaired LAT phosphorylation would lead downstream to a deficient activation of the ERK pathway in mutant cells. We therefore studied whether the mutation could influence the activation of the Ras-ERK signaling pathway.

ERK is the last member in the MAPK cascade that is initiated when Raf is activated after its recruitment by the activated form of Ras. The analysis of the phosphorylation status of ERK, an indication of its activation state (34), upon TCR engagement revealed not only that the induced ERK activity was higher in mutant cells than in wild type cells but also that this activity lasted longer. Thus, although both activities peaked at 5 min, a high phosphorylation in both p42 and p44...
bands was still observed at 60 min in mutant cells, whereas in wild type cells, ERK phosphorylation was almost undetectable after 20 min of stimulation (Fig. 2A). Expression of ERK, assessed by immunoblotting with anti-ERK antibodies, was unaffected by TCR stimulation. As controls, phorbol 12-myristate 13-acetate alone or in combination with ionomycin induced similar kinetics and ERK activities in both wild type and mutant cells (not shown).

It was then reasonable to test whether this altered activity of ERK in mutant cells could be the consequence of a similar activation pattern of the upstream intermediates Raf and Ras. Interestingly, the activation of Raf in mutant cells was higher and was sustained longer compared with that in wild type cells, as evidenced by the band shift corresponding to the phospho-activated forms of Raf (Fig. 2B). Finally, we evaluated the levels of active (GTP-bound) Ras by pull-down experiments using an immobilized GST fusion protein containing the RBD of Raf. As shown in Fig. 2C (upper panels), the levels of Ras-GTP significantly increased as early as 1 min in mutant cells, were similarly maintained for 2 min, and were detectable after at least 15 min post-stimulation. In contrast, TCR triggering in wild type cells resulted in a modest increase in the level of Ras-GTP at 1 min, which peaked at 2 min and was undetectable at 15 min of stimulation. As an additional control that Ras activation follows an opposite pattern to that of LAT phosphorylation in mutant cells, a phosphotyrosine Western blotting performed on the same lysates showed that LAT phosphorylation was more weakly induced in mutant cells than in wild type cells (Fig. 2C, bottom strip). Similar results were obtained with different wild type and mutant clones that were derived from independent transfections (not shown). These data suggest a role for the TCRβ chain transmembrane region in modulating the Ras-ERK cascade and also support the notion that ERK activation can be modulated by LAT-independent pathways.

CD69 Expression Is Highly Induced in Mutant Cells—To further assess whether the differences in the activation of the Ras-Raf-ERK signaling pathway between wild type and mutant cells might have a functional relevance, we used CD69 expression as a readout that is clearly dependent on the activation of the Ras-ERK pathway (26). We previously showed that TCR-mediated induction of CD69 expression measured after 18 h of stimulation was similar in both cell types (30). However, CD69 is an early activation marker that can be detected by flow cytometry a few hours after TCR engagement. The time course of CD69 expression revealed that CD69 was more strongly induced in mutant cells than in wild type cells after 3–7 h post-stimulation (Fig. 3), consistent with the increased ERK activity in mutant cells (Fig. 2). As expected, the levels of CD69 expression in both cell types equalized at longer stimulation times (Fig. 3).
A

| TIME (min) | 0 | 5 | 15 | 30 | 60 |
|-----------|---|---|----|----|----|
| WT        | - | + | + | + | + |
| MUT       | - | + | + | + | + |

B

| TIME (min) | 0 | 1 | 2 | 5 | 15 |
|-----------|---|---|---|---|----|
| WT        | + | + | + | + |    |
| MUT       | + | + | + | + | + |

C

| TIME (min) | 0 | 1 | 2 | 5 | 15 |
|-----------|---|---|---|---|----|
| WT        | + | + | + | + |    |
| MUT       | + | + | + | + | + |

FIG. 2. TCR triggering of mutant cells results in overactivation of the Ras/Raf/ERK pathway. A, wild type (WT) and mutant (MUT) cells were unstimulated or stimulated for the indicated times and lysed in 1% Nonidet P-40-containing buffer. The cell lysates were subjected to 10% SDS-PAGE, and the proteins were transferred to a membrane. The blot was probed with anti-pERK to determine ERK activation. Both short (SE) and long (LE) exposures are shown to illustrate more adequately the normal activation kinetics in wild type cells. After developing, the blot was stripped and reprobed with anti-ERK antibodies to verify that ERK levels were not affected by stimulation. An additional loading control is shown as determined by immunoblotting of the same membrane with anti-tubulin antibodies. B, upon stimulation for the indicated times, lysates from wild type and mutant cells were subjected to 8% (acylamide:bisacrylamide 24:0.6) SDS-PAGE. Immunoblotting was then performed with antibodies to Raf kinase to detect kinase activity. The shift in the mobility of this protein to a slower migrating form of higher apparent molecular weight is caused by phosphorylation, and it is indicative of its activation. The shift is readily seen upon extended electrophoresis. After stripping, the blot was reprobed with anti-tubulin antibodies to control for loading. C, wild type and mutant cells were unstimulated or stimulated for the indicated times and lysed in 1% Nonidet P-40-containing buffer. Cell lysates were then subjected to pull-down assays with GST-RBD-Sepharose followed by SDS-PAGE and immunoblotting with an anti-Ras antibody to detect active (GTP-bound) Ras. Aliquots of the same total lysates were independently analyzed by immunoblotting with anti-tubulin antibodies to control for loading and with an antibody to phosphotyrosine (pTyr) to evaluate LAT phosphorylation. Representative results of three independent experiments of each type performed with different wild type and mutant clones are shown.

FIG. 3. CD69 is more strongly induced in mutant than in wild type cells. After stimulation for the indicated times, wild type (WT) and mutant (MUT) clones were stained with an anti-CD69 fluorescein isothiocyanate-labeled antibody. CD69 expression was measured by flow cytometry. The results are expressed as arbitrary units (A.U.), corresponding to the product of the mean value of CD69 fluorescence and the percentage of CD69 positive cells. This experiment is representative of five independent experiments performed in triplicate with different wild type and mutant clones.

The induction of the p38MAPK activity was studied by immunoblotting with antibodies that recognize the phosphorylated form, an indication of its activation state. Similarly to JNK, the activity of p38MAPK was severely reduced in TCR-stimulated mutant cells (Fig. 4B). Clonal variation was not observed when using different wild type or mutant clones in these assays (not shown). These results, together with those described for ERK activation (Fig. 2), show that the mutation in TCRβ differentially affects the activation of different MAPKs induced through the TCR, increasing ERK but decreasing both JNK and p38MAPK activities.

**TCRβ Mutation Results in Higher Than Normal Tyrosine Phosphorylation of Vav**—The results reported above suggest that other ZAP-70 substrates than LAT might contribute to the unbalanced MAPKs activation in mutant cells. Moreover, we did not know whether the TCRβ mutation was also affecting the in vivo ZAP-70 activity on other substrates than LAT. SLP-76 is also a major ZAP-70 substrate and an important linker of the TCR to cellular activation. Furthermore, both SLP76 and Vav are also considered to play critical roles in ERK activation. In fact, ERK activation is blocked in Vav-deficient mice as well as in SLP76-deficient cells (35, 36). In addition, it has been demonstrated that in T cells Vav can regulate not only Ras-dependent responses, such as ERK activation and CD69 expression (26, 27), but also the activation of JNK and p38MAPK through its GDP-GTP exchange activity (23, 24). Therefore, we performed immunoprecipitation with anti-SLP-76-specific antibodies. The immunoprecipitation from TCR+CD28 costimulated cells revealed similar levels of tyrosine-phosphorylated SLP-76 in both mutant and wild type cells (Fig. 5A). Furthermore, although in LAT immunoprecipitation we observed a slight decrease in the recruitment of phospho-SLP-76 to LAT in mutant cells with respect to wild type cells (Fig. 1A), we have found no substantial differences in the levels of phospho-LAT associated to SLP-76 between wild type and mutant cells (Fig.
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5A). Remarkably, however, the levels of tyrosine-phosphorylated Vav coprecipitated with SLP-76 were found to be notably higher in mutant than in wild type cells (Fig. 5A). Western blot analysis with specific anti-Vav antibodies revealed that the recruitment of Vav to phospho-SLP-76 was similar in both cell types (not shown), in agreement with the similar levels of induced phospho-SLP-76 in wild type and mutant cells. These data, together with those shown in Fig. 1, demonstrate that TCRβ mutation does not lead to a poor in vivo ZAP70 activity resulting in a generalized deficient phosphorylation of its substrates, consistent with our previous in vitro kinase experiments (30). More plausibly, our data suggest that TCRβ mutation seems to differentially affect the accessibility of ZAP70 to specific substrates (e.g. LAT versus SLP-76).

To examine whether the high phosphorylation of Vav associated with SLP-76 in mutant cells was a feature of such association or was reflecting an alteration of the total phosphorylation status of Vav upon TCR triggering, we performed immunoprecipitation with anti-Vav antibodies. In stimulated mutant cells, we found that Vav becomes tyrosine-phosphorylated at higher levels compared with wild type cells. Furthermore, the tyrosine-phosphorylated Vav signal was sustained at higher levels compared with wild type cells. Further-

Fig. 4. JNK and p38MAPK activities are substantially reduced in TCR-stimulated mutant cells. Wild type (WT) and mutant (MUT) clones were unstimulated or stimulated for the indicated times. A, cell lysates were subjected to pull-down assays with GST-Jun-Sepharose followed by solid phase kinase assays. The autoradiograph (upper strip) corresponds to a blot showing phosphorylation of the JNK substrate, c-Jun. Simultaneously, aliquots removed from the same pull-down precipitates were analyzed by immunoblotting (IB) with an anti-JNK as control for loading (bottom strip). B, cell lysates in 1% Nonidet P-40-containing buffer were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with anti-pp38MAPK antibodies to determine p38MAPK activity and with anti-α-tubulin to control for loading. P/I, phorbol 12-myristate 13-acetate plus ionomycin. The right panels show the induced JNK (A) and p38MAPK (B) activities after normalization to the densitometric values of their respective loading controls and to the relative activation level in unstimulated cells. The results shown are representative of three similar experiments performed with different wild type and mutant clones.

Furthermore, it has been reported that PKCθ is selectively required in the Vav signaling pathway that mediates up-regulation of CD69 expression (37). Additionally, it has been suggested that PKCζ acts as an intermediate between Vav and Ras (26). Given that the activation of the Ras/ERK pathway and the up-regulation of CD69 are both increased in stimulated mutant cells (Figs. 3 and 5), we decided to test whether PKCζ might also contribute as intermediate to these altered activation readouts observed in mutant cells. To do that, wild type and mutant cells were stimulated and subjected to subcellular fractionation to compare the translocation of PKCζ to cell membrane, a phospho-Vav dependent event that is assumed to be directly associated with the kinase activation and with its localization in the supramolecular activation complex together with the TCR (38, 39).

Analyzing the PKCζ immunoblots of membrane and cytosolic fractions, we found significant differences between the patterns in wild type and those of mutant cells. They revealed that PKCζ remained translocated in the cell membrane for longer time in mutant cells than in wild type cells (Fig. 6). Both cell types showed a similar and pronounced loss of PKCζ in the cytosolic fraction after 5 min of stimulation, correlating with a increase in the amount of PKCζ translocated to the membrane. However, a consistent level of the kinase was only observed in mutant cells after 20 min of stimulation. In fact, the return of PKCζ to the cytosol was quicker in wild type cells, and consequently PKCζ was almost undetectable on the membrane of these cells after 20 min of stimulation. Expression of PKCζ, assessed by immunoblotting on whole cell lysates, was unaffected by TCR stimulation. In light of the above reports (26, 37), these data not only support the notion that PKCζ is an intermediate in both Vav-dependent Ras/ERK pathway activation and CD69 up-regulation, but also they suggest that the long
normal Vav phosphorylation.

Wild type (WT) and mutant (MUT) clones were unstimulated or stimulated for the indicated times and lysed in 1% Nonidet P-40-containing buffer. A, cell lysates were immunoprecipitated (IP) with antibodies to SLP-76 and analyzed by immunoblotting (IB) with the antibody 4G10 to phosphotyrosine (pTyr). Individual phosphotyrosine-containing proteins were identified by reprobing the blot with antibodies to LAT and Vav (not shown) and to SLP-76. NGS, preimmune normal goat serum used as control for immunoprecipitation. B, cell lysates were immunoprecipitated with antibodies to Vav and analyzed by immunoblotting with the antibody 4G10 to phosphotyrosine. The identity of phospho-Vav (p-Vav) was confirmed by reprobing the blot with an anti-Vav antibody. The numbers below the phosphotyrosine blot represent the relative induction of tyrosine phosphorylation of Vav normalized to Vav loading in each lane and to the relative amount of p-Vav in the second lane (unstimulated wild type cells). NRS, preimmune normal rabbit serum used as control for immunoprecipitation.

The following question arises: If LAT is not involved, what other molecules can be invoked to explain the activation pattern of the Ras-MAPK pathway in TCRβ mutant cells? c-Cbl should be discarded, because the phosphorylation of this adapter has been correlated with the inhibition of the Ras-MAPK pathway (48). On the other hand, SLP-76, which has been shown to be required for ERK activation (36), is similarly phosphorylated in wild type and mutant cells (Fig. 5A). We also dismiss the putative contribution of Ras-GRB to Ras activation, because this event is phospho-PLCγ1-dependent, and we found a low level of phospho-PLCγ1 associated with LAT in mutant cells (Fig. 1A). The most probable candidate is Vav, based on two facts: 1) Vav is more highly phosphorylated in TCRβ mutant cells than in wild type cells and 2) Vav recruited to the relevant adapter SLP-76 is more phosphorylated in mutant than in wild type cells (Fig. 5). Two additional observations support this hypothesis: 1) the time course for Vav phosphorylation and Ras activation correlate much better in mutant than in wild type cells (Figs. 2 and 5) and 2) the intense induction of phospho-Vav in mutant cells matches the strong induction of CD69 expression in these cells (Figs. 3 and 5). Interestingly, Vav has been shown to play a relevant role in both ERK activation and CD69 up-regulation by acting upstream of Ras, through a Rac-1-independent pathway (26, 27).

The role played by constant domains of clonotypic TCR chains in TCR-mediated signaling is poorly understood. We describe here that the transmembrane domain of β chain is crucial in controlling signaling efficiency by coupling properly TCR stimulation to downstream effectors. Thus, engagement of a TCR bearing a single replacement in such a domain results in higher and more sustained ERK activation but in lower JNK and p38MAPK activation than in wild type cells. We have traced these defects as being due to a compendium of altered upstream events including low phosphorylation of LAT and high and long lasting phosphorylation of Vav. Because mutant cells are apoptosis-defective but IL-2-producing cells, our results provide new evidence for TCR-mediated differential signaling and shows that a combined effect of altered phosphorylation events is likely responsible for the impaired apoptosis in mutant cells but seems to be not relevant for IL-2 secretion. Further, the model emphasizes the role of phosphorylated linkers in dissociating signaling pathways leading to the development of effector T cell responses.

The most unexpected result has been the high and sustained activation of ERK in mutant cells compared with that in wild type cells. This sustained activation correlates with the activation time course of the upstream intermediates Ras and Raf (Fig. 2). Furthermore, the overactivation of this pathway in mutant cells was consistent with a higher up-regulation of CD69 (Fig. 3), a downstream response that is dependent on the activation of Ras (40), Raf (41), and ERK. Current evidence suggests that TCR-induced Raf activation is likely determined by the recruitment of adapter molecules to phosphorylated LAT (18, 42, 43). However, TCR-stimulated mutant cells show a significant defect on TCR-induced LAT phosphorylation, which consequently results in a defective recruitment of SLP-76 and PLCγ1 (Fig. 1A), which are both important for Raf activation (44, 45). Therefore, alternative pathways must contribute to elicit Ras-MAPK activation in mutant cells. In this regard, recent studies have shown that TCR-induced Ras-MAPK activation can occur through phospho-LAT-independent pathways. Thus, TCR triggering of SLP-76 deficient cells results in a blockade of ERK activation, although these cells show normal phospho-LAT (36). Furthermore, in ZAP-70-deficient cells, the Ras-MAPK pathway can be almost normally activated upon TCR engagement (46). Moreover, normal ERK activity can be induced in ZAP-70 mutant cells, Y/F 319, although they show a defective LAT phosphorylation (47). Additionally, TCR partial agonist ligands use a phospho-LAT-independent pathway to selectively activate the Ras-MAPK pathway (19).

The following question arises: If LAT is not involved, what other molecules can be invoked to explain the activation pattern of the Ras-MAPK pathway in TCRβ mutant cells? c-Cbl should be discarded, because the phosphorylation of this adapter has been correlated with the inhibition of the Ras-MAPK pathway (48). On the other hand, SLP-76, which has been shown to be required for ERK activation (36), is similarly phosphorylated in wild type and mutant cells (Fig. 5A). We also dismiss the putative contribution of Ras-GRB to Ras activation, because this event is phospho-PLCγ1-dependent, and we found a low level of phospho-PLCγ1 associated with LAT in mutant cells (Fig. 1A). The most probable candidate is Vav, based on two facts: 1) Vav is more highly phosphorylated in TCRβ mutant cells than in wild type cells and 2) Vav recruited to the relevant adapter SLP-76 is more phosphorylated in mutant than in wild type cells (Fig. 5). Two additional observations support this hypothesis: 1) the time course for Vav phosphorylation and Ras activation correlate much better in mutant than in wild type cells (Figs. 2 and 5) and 2) the intense induction of phospho-Vav in mutant cells matches the strong induction of CD69 expression in these cells (Figs. 3 and 5). Interestingly, Vav has been shown to play a relevant role in both ERK activation and CD69 up-regulation by acting upstream of Ras, through a Rac-1-independent pathway (26, 27).

The intermediates and the mechanisms relating Vav and Ras are presently unknown. However, in light of our results, one putative intermediate would be SLP-76, provided that it could perform a ZAP-70-dependent, but LAT-independent, function not previously reported. An alternative intermediate is PKCθ. Upon TCR engagement, this protein is activated by a phospho-Vav-dependent mechanism and induces CD69 up-regulation (37). In this regard, our cell fractionation experiments (Fig. 6) show that in TCR-stimulated mutant cells, PKCθ remains located in the membrane for a longer period than in stimulated wild type cells, indicating that PKCθ contributes to the enhanced activation of the Ras/ERK pathway and to the high up-regulation of CD69 in mutant cells.

Therefore, according to our data, it is tempting to speculate

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about two putative roles of Vav. Its recruitment to LAT-including complexes would be closely related to its role as a guanosine nucleotide exchange factor for Rho family GTPases and, consequently, to the JNK and p38MAPK activation (23, 24). This would explain the low level of activation of these kinases in mutant cells where Vav is poorly recruited to LAT. This role of Vav could be consistent with the previously described Rac-1-independent modulating effect of Vav on the Ras/Raf/ERK signaling pathway and on CD69 up-regulation (26). The latter function could be mediated by an inducible interaction with SLP-76 not necessarily in association with LAT. How the Vav-SLP-76 complex elicits Ras-ERK activation and how the two putative roles of Vav are balanced to contribute to a physiological response in normal cells are unknown.

In mutant cells, despite the fact that the induced association between ZAP-70 and ζ is very poor, ZAP-70 is inducibly tyrosine-phosphorylated at levels similar to those in wild type cells. Moreover, this protein shows comparable kinase activity in both cell types upon TCR activation. In addition, in TCR-activated mutant cells, ZAP-70 is incompletely concentrated near the plasma membrane, where the ZAP-70/ζ association is impaired (30). Thus, it can be hypothesized that subsequent defects following TCR triggering in mutant cells might be accounted for by the partial mislocation of this kinase. Further, TCR stimulation of mutant cells could resemble the interaction of altered ligands with normal TCRs. In this regard, it has been suggested that an altered signaling may derive from the different orientations adopted by membrane-associated ZAP-70 because this kinase somehow senses the nature of the TCR-ligand interaction and assumes a configuration that is differentially effective on its substrates (49). Alternatively, the phosphorylation of ZAP-70 could not be critical for Fas-L induction.

All of our studies have been performed without overexpressing constitutively active or dominant negative signaling proteins. Because the cause/effector relationships of these modified proteins is commonly examined many hours following their introduction, whereas most signaling molecules are normally only active for minutes, compensation and indirect effects are sometimes impossible to distinguish from direct effects. Therefore, in the absence of these studies, our results more than tentatively provide evidence that the serial biochemical alterations observed in mutant clones is responsible for the specific TCR-mediated apoptosis-defective phenotype of these cells. Two facts support this notion: 1) TCR-mediated apoptosis is the only activation outcome that is defective in mutant cells and 2) we have not observed significant differences between mutant clones derived from independent transfections in both functional and biochemical assays. Furthermore, our data would suggest that TCRβ transmembrane region imparts a specific signaling function to T cells and/or serves to assure a proper balance in the activation of the various MAPK involved in TCR signaling. Indeed, a function where this balance is more strongly regulated is the TCR-mediated cell death (activation-induced cell death). In this regard, several studies have reported relationships between ERK, JNK, and p38MAPK activities and the up-regulation of Fas-L and apoptosis (20, 22, 50).

Elucidating whether the functional and biochemical events we described here are relevant in the context of a physiological TCR is a hard task. In the absence of similar biological and biochemical studies, the more comparable situation is that provided by the stimulation of normal TCR with altered peptide ligands. Indeed, in both cases it is possible to activate some, but not all, functional features of the response elicited on wild type cells with cognate ligands. Although it is not clear why a consistent pattern of early signaling is not elicited by ligands capable of inducing similar functional responses (9, 13), the functional evidence that a TCR can interpret subtle changes in its ligand unequivocally highlights the exquisite sensitivity that the TCR-CD3 complex has in its recognition unit. In this sense, our results and previous works addressing the role of the TCR itself in controlling signaling efficiency (2–8, 29, 30) provide complementary evidence that the different domains of the TCR constitutes a support to differential signaling, at least from the point of view of its structural integrity.

Despite the functional similarities, two major biochemical differences distinguish the mutant cells from the altered peptide ligand system. First, stimulation with altered peptide ligands usually results in the absence of phosphorylation of the lower mobility phospho-ζ isoform, a fact that is not observed in stimulated mutant cells (30). It has been reported the ability of ζ chain to promote apoptosis (51); however, the comparison between the similar phosphorylation of the two phospho-ζ isoforms in stimulated mutant cells (30) with the partial phosphorylation in T cells selectively induced to apoptosis (13) would indicate that functional ζ ITAMs could be dispensable for antigen-mediated up-regulation of Fas-L and apoptosis. Indeed, it has been suggested that apoptosis does not depend on cooperative interaction between CD3-ζ and CD3-γδ6 modules, being the last one sufficient for qualitatively normal signaling (52, 53). Further support for this notion is the fact that although blocking of CD8 substantially reduces phosphorylation of the CTL TCRζ chain, CTLs are still fully competent to up-regulate Fas-L and undergo activation-induced cell death (15). Second, and similarly, the normal phosphorylation of ZAP-70 in stimulated mutant cells and the absence of such event in T cells selectively induced to apoptosis with a partial agonist ligand (9) would also suggest that the phosphorylation of the kinase is not critical in the apparent specificity engaged in TCR-stimulated cells to prevent or induce Fas-L induction and apoptosis. If the early phosphorylation of both ζ chains and ZAP-70 does not explain differential signaling, what other events are responsible for such kind of cell activation? Although the possibility of engagement of specific signaling pathways could exist, our results and those obtained with partial agonist could also be interpreted in the light of a new kinetic model of TCR signaling (54). By using partial agonists with similar occupancy to agonist ligands, it has been shown that all T cell
responses can be triggered by a cumulative signal that is reached at different time points for different TCR ligands, even in the absence of full phosphorylation of early intermediates. In fact, TCR-stimulated mutant cells show significant apoptosis but at a much more delayed time than do wild type cells (54). In this regard, the transmembrane region could help the overall TCR to sense the ligand affinity.

Notwithstanding, a question remains on how to conceal the fact that an inefficient signal emanating from the mutant TCR simultaneously results in an inefficient activity of some signaling intermediates, such as JNK and p38 activities, but in overactivation of others such as ERK. We do not know whether the functional phenotype and signaling events displayed by the agonist stimulation of mutant cells could be triggered in normal cells by stimulating with partial agonists. By assuming the existence of such ligands, our results would support a model of dissociated activation, such as previously described (19, 55), in which activation of Ras/ERK cascade could occur through the phospho-LAT-independent pathway we describe here, emphasizing the importance of ZAP-70 recruitment in determining the location of specific signaling complexes. In this case, the alternative pathway for ERK activation would be also triggered upon agonist TCR stimulation as a transient mechanism to control the risky decision to cell death commitment. However, it would not be detectable because the full signal provided by the agonist would interfere with that mechanism to induce the balanced MAPK activation related to a full spectrum of T cell effector functions.

The role played by altered peptide ligands has been enigmatic. Recently, it has been shown that these analogues could take part in mature T cell apoptosis as a mechanism of tolerance to self-peptides in vivo (56). Our data, however, preclude any conclusion regarding the putative role in vivo of T cells displaying a mutant cell-like phenotype induced by a self-peptide. Whether this phenotype mimics the “competence to die” signal delivered by some partial agonist self-peptides to promote the specific and benign deletion by apoptosis of the responding cells (56) deserves further investigation.

The present study was carried out in a Jurkat cell model, and therefore we cannot draw definite conclusions on how the TCRβ mutation could affect thymic selection. Furthermore, it remains unclear whether the signals mediated by the TCR during the selection of thymocytes and the activation of mature T cells are quantitatively or qualitatively different. This notwithstanding and in light of recent studies that report the direct contribution of the α chain connecting peptide domain (4, 5) and the conserved CD3β chain (57) in selective coupling the TCR to activation of the ERK signaling pathway, our results support the notion that different constant/conserved structural domains of the TCR complex might contribute to the fine balance of MAPK activation involved in thymic selection.

In conclusion, the results herein describe the signaling pathways affected by a mutation in the transmembrane domain of TCRβ. The data demonstrate how the mutation promotes an unbalanced phosphorylation of different adapters that leads to an unbalanced activations of the different MAPKs. Further, the data provide biochemical evidence to explain the ability for differential signaling of the mutant TCR to support some T cell responses but to impede the induction to apoptosis.

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