We have examined the ability of the CD3-γδε and CD3-ζ signaling modules of the T cell receptor (TCR) to couple CD38 to intracellular signaling pathways. The results demonstrated that in TCR⁺ T cells that express the whole set of CD3 subunits CD38 ligation led to complete tyrosine phosphorylation of both CD3-ε and CD3-ζ polypeptide chains. In contrast, in TCR⁻ cells with a defective CD3-ζ association CD38 engagement caused tyrosine phosphorylation of CD3-ε but not of CD3-ζ. Despite these differences, in both cell types CD38 ligation resulted in protein-tyrosine kinase and mitogen-activated protein kinase activation. However, in cells expressing chimerical CD25-ζ or CD25-ε receptors or in a TCR-β⁺ Jurkat T cell line, CD38 ligation did not result in tyrosine phosphorylation of the chimeric receptors, or CD3 subunits, or protein-tyrosine kinase or mitogen-activated protein kinase activation. In summary, these results support a model in which CD38 transduces activating signals inside the cell by means of CD3-ε and CD3-ζ tyrosine phosphorylation. Moreover, these data identify the CD3-γδε signaling module as a necessary and sufficient component of the TCR/CD3 complex involved in T cell activation through CD38.

Human CD38 antigen is a 45-kDa type II transmembrane glycoprotein with a short N-terminal cytoplasmic domain and a long C-terminal extracellular domain (1, 2). It is widely expressed in different cell types including thymocytes, activated T cells, and terminally differentiated B cells (plasma cells) (3). Other reactive cells include natural killer cells, monocytes, macrophages, dendritic cells, and some epithelial cells. The CD38 antigen acts as a NAD(P)+ glycohydrolase (4) and plays a role in lymphocyte activation (3, 5). Recently it has been identified CD31, which is mainly expressed by endothelial cells, platelets, macrophages and a discrete subset of T cells, as a ligand for CD38 (6). CD31 and CD38 cognate interactions are found to modulate heterotypic adhesion as well as to induce increases in the concentration of intracellular free Ca²⁺ ([Ca²⁺]i) identical to those obtained by means of agonistic anti-CD38 mAbs (6). These results suggest that the interplay between CD38 and its ligand CD31 is an important step in the regulation of cell life and of the migration of leukocytes through the endothelial cell wall.

TCR/CD3-mediated signaling involves recruitment and activation of the PTKs of the Src-family Fyn or Lck to the proximity of the TCR/CD3 complex (7–14). As a result, CD3-ζ and CD3-ε are phosphorylated in the two tyrosines of the immunoreceptor tyrosine-based activation motif (ITAM), which is found three times in the CD3-ζ chain and once in each of the other CD3 subunits (γ, δ, and ε) (15). The most widely held structural model of the TCR/CD3 complex is one comprising a CD3-ζε dimer and two CD3 pairs (γε, δε). Therefore, 10 ITAMs may be present within a single TCR/CD3 complex. A view has emerged that in the TCR/CD3 complex there are at least two distinct functional units referred to as transduction modules and made of the CD3-ζδ dimers and the CD3 pairs (γε, δε), respectively (16, 17). Although the CD3-γε and CD3-δε pairs may have separate signaling capabilities (18), we will refer to them as the CD3-γδε transduction module (16). The ITAM tyrosine phosphorylation promotes a high affinity interaction of the CD3 chains with a second family of PTKs, the Syk/ZAP-70 family. The recruited Syk/ZAP-70 molecules are activated by phosphorylation and contribute to the recruitment and activation of other proteins such as LAT (linker for activation of T cells), Vav, and SLP-76. This leads to the formation of multimolecular complexes that activate several signaling cascades as the PLC-γ1-dependent pathway, and the ones emanating from activation of Ras such as the MAP kinase pathway and the phosphatidylinositol 3-kinase pathway (14, 19, 20). This signaling cascades ultimately converge on the nucleus, resulting in the changes of gene expression that characterize T cell activation.

In a previous paper, we have shown that CD38 ligation

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1 The abbreviations used are: mAb, monoclonal antibody; TCR, T cell antigen receptor; PLC-γ1, phospholipase C-γ1; ZAP-70, ϶-associated protein-70; MAP, mitogen-activated protein; Erk, extracellular signal-regulated protein kinase; MEK, MAP kinase or Erk kinase, also known as MAP kinase kinase; PTK, protein-tyrosine kinase; ITAM, immunoreceptor tyrosine-based activation motif, JK, Jurkat; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; PdBu, 4-phorbol 12,13-dibutyrate; FITC, fluorescein isothiocyanate-conjugated; Ab, antibody.
results in activation of at least two PTK-controlled signaling pathways, the CD3-ζ/ZAP-70/PLC-γ1-dependent cascade and the Raf-1/MAP kinase pathway, suggesting a functional relationship between signals delivered through CD38 and the TCR (21). This assumption was strengthened by our finding that, in Jurkat T cells, CD38-mediated signaling events as increases in [Ca^{2+}], or CD69 expression require the presence of the TCR/CD3 (22). These results parallel elegant studies done by Lund et al. (23, 24) in murine B cells, demonstrating that co-expression of the BCR is required for CD38-mediated signal transduction.

To further understand the molecular and functional relationship between the CD38 receptor and the TCR in human T cells, we investigated the participation of various components of the TCR/CD3 complex in CD38-mediated signaling. To address these questions, we compared the CD38- and CD3-mediated early signaling capabilities of a surface TCR /CD3+ Jurkat T cell variant with those signals delivered by surface TCR / CD3+ Jurkat cell transfectants that express either the complete TCR/CD3 complex or a TCR/CD3 complex with impaired CD3-ζ chain association. We have also studied the CD38-mediated increases in protein tyrosine phosphorylation in a TCR–Jurkat T cell variant expressing either CD25-ζ or CD25-e chimeric proteins on the cell surface. The data demonstrate that in T cells coexpression of the TCR/CD3 complex is absolutely required for coupling CD38 to downstream signaling events. Moreover, we show that the CD3-γδε signaling module is sufficient for CD38 to induce activation of PTK- and MAP kinase-mediated signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Lines—**Jurkat D8 cells were obtained from wild-type Jurkat cells (subclone E-6-1, American Tissue Culture Collection (ATCC), Rockville, MD) by the limiting dilution technique (26). JK-31-13 is a CD38–CD3+ TCR variant of Jurkat T cells lacking a functional TCR-β chain (26). This variant was transfected by electroporation with the cDNA encoding wild-type TCR-β chain (27), or a mutant TCR-β chain in which a transmembrane tyrosine to leucine mutation was created (27). These cell lines were made available by Dr. A. Alcover (Institut Pasteur, Paris, France), Dr. B. Alarcón (Centro de Biología Molecular, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain), and Dr. R. Bragado (Fundación Jiménez-Díaz, Madrid, Spain). CD25 chimeric receptor-expressing cell lines FEV20.3 (JK-CD25-ζ) and FEV19.5.4 (JK CD25-e) were generated by stable transfection of a TCR-β variant of the Jurkat cell line (4RT3-T3.5) with the cDNA encoding the complete human CD25 ecto- and transmembrane domains fused to either mouse CD3-ζ or D3-ζ cytoplasmic domains (28). These cells were kindly provided by Dr. Eric Vivier (Center d’Immunologie INSERM-CNRS de Marseille-Luminy, Marseille, France). Cells were cultured as described (21, 28).

**Transient Transfections—**The full-length TCR-β cDNA (pJ6) subcloned in the pRsv.5 vector (29) was transfected into JK-31-13 cells (30 μg of cDNA into 30 × 10^6 cells) by electroporation (using the BTX cell porator system (Genetronics, Inc., San Diego, CA) with a capacitance set at 975 microfarads). Twenty-four hours after transfection, dead cells were removed by Ficoll-Paque centrifugation, and live cells were cultured for another 24 h before performing the functional assays. Cells were always analyzed by fluorescence-activated cell sorting for surface expression of the transfected protein.

**Antibodies and Reagents—**Purified CD3 mAb OKT3 (IgG2a) was a gift from Dr. Goldein (Ortho Pharmaceutical, Raritan, NJ). Anti-CD3 mAb IB4 (IgG2a) was prepared and purified by affinity chromatography on Protein A-Sepharose and high performance liquid chromatography on hydroxyapatite, as described (30). Affinity-purified, fluorescein isothiocyanate-conjugated (FITC) Fab’2 fraction of goat antibody to mouse immunoglobulins (Fab’, FITC-Rm IgG) was purchased from Dako (Denmark, Affinity-purified Fab’2 fraction of goat antibody to mouse IgG (whole molecule) (Fab’, Gam IgG) was purchased from Cappel (Organon Teknika, Durham, NC). Anti-phosphotyrosine (anti-Tyr(P)) mAb 1G2 coupled to agarose beads (1G2-agarose) was obtained from Oncogene Research (Calbiochem, Cambridge, MA). Recombinant anti-Tyr(P) antibody coupled to horseradish peroxidase (RC20-HRP), anti-Grb2 mAb antibody, and anti-SLP76 mAb were obtained from Transduction Laboratories (Lexington, KY). The following affinity-purified rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-Erk-2 and anti-e-Cbl. An affinity-purified rabbit immunoglobulins to human anti-CD3-ε (Dako). Anti-ZAP-70 (Zap-7) rabbit antisera was a kind gift from Dr. S. C. Ley (Medical Research Council, London, United Kingdom) (31). Anti-CD3-ζ antisera 448 was a gift from Dr. B. Alarcon (Centro de Biologia Molecular, CSIC, Madrid, Spain). The anti-CD3-ζ mAb 1D4.1 and the anti-CD3-ε mAb 2F4.1 have been previously described (32, 33). The anti-PLC-γ1 polyclonal antibody (C-37) was made by immunizing New Zealand White rabbits with the synthetic peptide ADHFDSRERRAPRRTRVNGD conjugated to soluble keyhole limpet hemocyanin (Sigma-Aldrich Quimica, S.A., Madrid, Spain) as described previously (8). The anti-human CD25 mAb B1.49.9 (IgG2a, mouse) was purchased from Immunotech (Marseille, France). Affinity-purified goat anti-rabbit IgG (Fc) horseradish peroxidase (HRP) conjugate, and goat anti-mouse IgG (H+L) HRP conjugate were from Promega (Madison, WI). Prestained SDS-PAGE standards (broad range) were from Bio-Rad. Recombinant protein A-Sepharose was from Amersham Pharmacia Biotech. 4e-Phorbol 12,13-dibutyrate (PdBu) was purchased from Sigma-Aldrich Quimica, S.A.

**Fluorescence-activated Cell Sorting Analysis—**Cells were analyzed for surface expression of CD3, CD38, and CD25 by flow cytometry as described previously (21). Samples were analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

**Cell Stimulation, Immunoprecipitation, and Western Blotting—**Cells were grown up to a density of 10^6/ml, centrifuged, and serum-starved in RPMI + 0.5% FBS for 4 h, washed in RPMI without serum, and resuspended at 1–2 × 10^6 cells/sample or otherwise indicated, in serum-free RPMI-Hepes medium, at 4 °C. Stimulation, immunoprecipitation, and Western blotting were performed as described in detail elsewhere (21).

**RESULTS**

**In Jurkat T Cells, Cross-linking of CD38 Induces Tyrosine Phosphorylation of Both CD3-ε and CD3-ζ Subunits of the TCR/CD3 Complex—**In a previous study, we showed that, upon CD38 ligation, ZAP-70 is tyrosine-phosphorylated and recruited by phosphorylated CD3-ζ (21). Since tyrosine phosphorylation of both CD3-ε and CD3-ζ have been identified as the biochemical hallmarks of agonistic stimulation of the TCR (34, 35), in this work we examined whether CD3-ε became tyrosine-phosphorylated following CD38 engagement. To this end, Jurkat T cells were stimulated with the anti-CD38 mAb IB4 for various periods of time. Cells were then lysed in 1% Nonidet P-40 lysis buffer to dissociate the CD3-ζ chain from the other CD3 subunits (36), and the CD3-ε and CD3-ζ subunits were sequentially immunoprecipitated with specific antibodies. Note that CD3-ζ was immunoprecipitated with an anti-human CD3-ζ mAb, 1D4.1, that recognizes both the 32-kDa unphosphorylated and 42–44-kDa phosphorylated CD3-ζ dimers (10, 21, 33). The immunoprecipitates were resolved on SDS-PAGE under non-reducing conditions and subsequently immunoblotted with an anti-phosphotyrosine mAb. As shown in Fig. 1A, CD38 ligation resulted in the tyrosine phosphorylation of CD3-ζ (lanes 8–10) with similar kinetics as of CD3-ε (Fig. 1B, lanes 8–10). As compared with an anti-CD3 stimulation, which was used as a positive control, the extent and duration of anti-CD38-induced CD3-ε and CD3-ζ tyrosine phosphorylation was significantly lower (Fig. 1A and B, compare lanes 5–7 with lanes 8–10). Immunoblots with anti-CD3-ε antibodies confirmed that the anti-CD3 immunoprecipitations were specific and that equal amounts of CD3-ε were loaded in all lanes (Fig. 1C, lanes 4–10). Therefore, these results suggest that CD38 engagement with anti-CD38 Abs fully reproduces the distinct patterns of CD3 tyrosine phosphorylation seen after engagement of the TCR/CD3 complex with agonistic ligands or antibodies. Moreover, the data strongly suggest that in Jurkat T cells both CD3-γδε and CD3-ζ signaling modules are involved in CD38-mediated early signaling transduction events.

**CD38-induced PTK Activation Requires the Expression of a**
Fig. 1. CD38 induces tyrosine phosphorylation of both CD3-ε and CD3-ζ subunits of the TCR/CD3 complex. 1.5 × 10⁶ Jurkat cells, clone D8 (CD3-ε, CD3-ζ), were incubated for 10 min on ice with 5 μg/10⁷ cells of anti-CD3 mAb (OKT3) (lanes 2 and 5–7), or anti-CD38 mAb (IB4) (lanes 3 and 8–10), or RPMI-HEPES (nonstimulated, lanes 1 and 4). This was followed by cross-linking with 20 μg/10⁷ cells of the secondary antibody Fab'1, Goat IgG (lanes 3 and 8–10) for 10 min on ice. Then the time course was conducted at 37°C for the indicated times. Control unstimulated cells were incubated at 37°C for 1 min (panel B, lanes 1 and 4), or 5 min (panel A, lanes 1 and 4). A, cell lysates or anti-CD3-ε immunoprecipitates (OKT3) were separated on a 12.5% SDS-PAGE gel under non-reducing conditions and subjected to Western immunoblotting (WB) with an anti-Tyr(P) mAb RC20-HRP. Positions of the different tyrosine-phosphorylated forms of CD3-ε are indicated. C, the filter shown in A was stripped and reprobed with an affinity-purified anti-CD3-ε Ab. In all panels, the molecular mass markers are indicated to the left. All blots were developed by chemiluminescence using the ECL detection system and then exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech). D, surface expression levels of CD3-ε and CD3-ζ in Jurkat D8 cells. Cells were stained with the anti-CD3-ε mAb OKT3 (left panel) or with the anti-CD38 mAb IB4 (right panel), followed by F(ab')2 FITC-Goat IgG secondary Ab. Representative flow cytometric profiles are shown (filled histograms). Negative controls (open histograms) were obtained after staining with the secondary Ab alone. Flow cytometric data are presented as the logarithm of fluorescence intensity.

Functional TCR/CD3 Complex on the Cell Surface—The results on CD38-induced CD3-ε and CD3-ζ tyrosine phosphorylation suggested a functional relationship between signals derived through CD38 and the TCR/CD3. To address the question whether CD38 requires some of the CD3 subunits associated with the TCR to access the intracellular signal transduction machinery, we have used JK-31-13 cells, a CD38⁺ TCR/CD3 variant of the human T cell line Jurkat (26). This cell line does not express any of the TCR/CD3 chains on the cell surface due to a defective expression of the TCR-β gene (see phenotype in Fig. 2A). In these cells, CD38 ligation with the anti-CD38 mAb IB4 did not induce any significant increase in protein tyrosine phosphorylation (Fig. 2B, lane 4). Likewise and as expected, CD3 ligation by the anti-CD3 mAb OKT3 was unable to induce increased tyrosine phosphorylation of any substrate (Fig. 2B, lane 2). In contrast, treatment of JK-31-13 cells with the tyrosine phosphatase inhibitor sodium pervanadate for 5 min induced a marked increase in substrate tyrosine phosphorylation (Fig. 2C, lane 1). Overall, these results demonstrate that the lack of responsiveness of the CD3⁺ TCR-JK-31-13 cells to CD38 stimulation was not due to a general defect in PTK-mediated signals and strongly suggest that expression of a functional TCR/CD3 complex on the cell surface is required for CD38-mediated increases in protein tyrosine phosphorylation.

To further prove that indeed the surface expression of a functional TCR/CD3 is necessary for CD38-mediated early signaling in T cells, the TCR-⁡JK-31-13 variant of Jurkat was transfected with the cDNA coding for the human TCR-β chain. After drug selection, cells lines expressing the TCR/CD3 complex on their cell surface were isolated (27), and the signal transducing capability of CD38 was assessed. As shown in Fig. 2 (D and E), in cells transfected with the TCR-β cDNA as JK-B7 or JK-G6 cells increased tyrosine phosphorylation of a similar number of cellular proteins was induced in response to either CD38 or CD3 stimulation with specific antibodies. As expected
from previous experiments in untransfected CD38<sup>b</sup> TCR<sup>b</sup> Jurkat D8 cells (21), the extent of increased tyrosine phosphorylation induced by both stimuli differed, and was always significantly lower for anti-CD38 than for anti-CD3 stimulation (Fig. 2, D and E). Similar results were obtained with another three independent TCR<sup>b</sup> transfectants (data not shown). To avoid potential artifacts resulting from clonal variation, we also analyzed JK-31-13 cells transiently transfected with the TCR<sup>b</sup> cDNA. Transient TCR<sup>b</sup>-transfected JK-31-13 cells responded to anti-CD38 IB4 mAb or anti-CD3 OKT3 mAb stimulation, as measured by increases in protein tyrosine phosphorylation (lanes 3 and 2, respectively). B, A representative fluorescence histogram (left panel) shows the CD3 surface expression levels in JK-31-13 cells transiently transfected with the TCR<sup>b</sup> chain cDNA (filled histogram) versus CD3 surface expression levels in wild-type JK-31-13 cells (open histogram). In this particular experiment, about 20% of cells were CD3<sup>b</sup>. In the right panel (filled histogram), CD38 surface expression levels are shown. The negative control was obtained after staining with the secondary Ab alone (open histogram).

We have previously demonstrated in Jurkat T cells that Cbl, the product of the protooncogene c-cbl, is a prominent PTK substrate, becomes tyrosine-phosphorylated upon CD38 or CD3 stimulation (21). In addition, in T cells Cbl interacts with a number of molecules known to be critical in signal transduction. These include PTKs such as Fyn and ZAP-70, the adaptor molecule Grb2, and the lipid/protein kinase phosphatidylinositol 3’-kinase (38–41). We thus investigated in the TCR<sup>b</sup>-transfected cells whether Cbl would become tyrosine-phosphorylated in response to CD38 ligation. To this end, stimulated and nonstimulated TCR<sup>b</sup> JK-B7 cells were lysed and subjected to immunoprecipitation with antibodies directed at Cbl (Fig. 4A, lanes 5–8), or mock-stimulated (lane 6) were immunoprecipitated with the anti-Tyr(P) mAb 1G2-agarose beads (lanes 5–8). Lanes (lanes 1–4), and immunoprecipitates were resolved by SDS-PAGE (10% gel, reducing conditions), transferred to PVDF membrane and subjected to immunoblotting with anti-Tyr(P) mAb RC20-HRP. Position of tyrosine-phosphorylated c-Cbl is indicated by an arrow. B, the upper part of the panel shown in A was stripped and reprobed with an affinity-purified anti-c-Cbl Ab. Position of c-Cbl is indicated by an arrow. C–E, cells (4 × 10<sup>7</sup>) were prepared and stimulated as described in Fig. 2B. Lysates either from unstimulated (lane 5) or stimulated (lanes 7 and 8), or mock-stimulated (lane 6) were immunoprecipitated with the anti-Tyr(P) mAb 1G2-agarose beads (lanes 5–8). Lanes (lanes 1–4), and immunoprecipitates were resolved by SDS-PAGE (10% gel, reducing conditions), transferred to PVDF, and the filter was cut in parts and subjected to immunoblotting with polyclonal antibodies anti-PLCγ-1 (panel C), anti-SLP76 (panel D), and anti-Zap70 (panel E). Location of immunoprecipitated proteins is indicated by arrows. All blots were developed by ECL.
Role of CD3-γδε Transducing Module in CD38 Signaling

CD38-mediated MAP Kinase Activation Requires Surface Expression of the TCR/CD3 Complex—Activation of p21^{ras} leads to recruitment and activation of Raf-1, which then activates MEK-1 and MEK-2, which subsequently activate the MAP kinases Erk-1 and Erk-2 (43). Activation of MAP kinases occurs through phosphorylation of threonine 202 and tyrosine 204 of human MAP kinase (Erk-1) at the sequence TETY by MEK (44). We have previously demonstrated that, in Jurkat T cells, CD38-mediated MAP kinase activation is both PTK- and protein kinase C-dependent (21). To examine whether CD38-mediated Erk-2 activation required surface expression of the TCR/CD3 complex, we assessed in both the TCR^-^-JK-31-13 and the TCR^-^ JK-B7 cells the levels of Erk-2 tyrosine phosphorylation and its mobility shift following CD38 engagement with the agonistic antibody IB4. Tyrosine phosphorylation and reduced mobility on SDS-PAGE of Erk-2 (attributable to threonine phosphorylation) have been associated with activation of this enzyme by MEK (21, 45–47).

In untransfected JK-31-13 cells, which did not express the TCR/CD3 complex on the cell surface, Erk-2 became tyrosine-phosphorylated upon CD38 engagement. As expected, CD38 engagement with anti-CD38 mAb for 5 min resulted in tyrosine phosphorylation of PLC-γ1 and ZAP-70, and SLPI-76 became tyrosine-phosphorylated upon CD38 engagement. As expected, CD38 engagement with anti-CD38 mAb for 5 min resulted in tyrosine phosphorylation of PLC-γ1 (Fig. 4C) and ZAP-70 (Fig. 4E). Notably, we have also found for the first time that SLPI-76 became tyrosine-phosphorylated following CD38 stimulation (Fig. 4D, lane 8). In contrast, secondary antibody alone did not result in SLPI-76 tyrosine phosphorylation (Fig. 4D, lane 6).

In wild-type CD38^-^-Jurkat D8 cells, an immediate consequence of triggering CD38 is the tyrosine phosphorylation of PLC-γ1 and ZAP-70 (21), and elevation of intracellular calcium (22). It has been shown that the adaptor protein SLP-76 mediated Erk-2 activation required surface expression of the protein kinase C-dependent (21). To examine whether CD38- 

Overall, these results demonstrate that CD38-mediated activation of the Ras/MAP kinase signaling pathway strongly depends on the surface expression of the TCR/CD3 complex.

Association of the CD3-γδε Signaling Module with the TCR Is Sufficient to Mediate Anti-CD38 Induction of Protein Tyrosine Phosphorylation and MAP Kinase Activation—To ascertain which component of the TCR/CD3 complex is required for CD38-mediated signaling, we used JK-31-13 cells transfected with the cDNA coding for the TCR-β, but harboring a point mutation of a tyrosine residue to leucine in the transmembrane domain (named Y11L). This point mutation impairs the association of the CD3-ζ signaling module with the TCR (27). Despite this defect, cell lines as JK-C2 TCR-β^-mut expressing the TCR on the cell surface were isolated (Fig. 6A). In these cells, this TCR is tightly associated with the CD3-γδε signaling module (27). Moreover, these cells were able to transduce CD38-mediated signals as assessed by the ability of anti-CD3 mAbs to induce increases in protein tyrosine phosphorylation (Fig. 6B, lanes 3 and 4), demonstrating that the TCR devoid of CD3-ζ was functionally active. Thus, with these mutants, we can address the question whether the association of CD3-ζ to the TCR is required for CD38-mediated signaling events and whether the TCR-associated signaling module, CD3-γδε, can compensate for CD3-ζ function.

First, to examine whether CD38 engagement induced PTK activation, the JK-C2 TCR-β^-mut cells were stimulated with the anti-CD38 mAb IB4 and whole lysates were subjected to SDS-PAGE followed by anti-Tyr(P) immunoblotting. As shown in Fig. 6B (lanes 5 and 6), in these cells CD38 ligation resulted in tyrosine phosphorylation of a variety of endogeneous cellular proteins. Since the mutation in the transmembrane domain of
Fig. 6. The CD3-γε signaling module of the TCR is sufficient to allow CD38-mediated PTK activation. A, CD3 and CD38 surface expression in JKC2 TCR-βmut cells. Cells were stained with the same mAbs used in Fig. 1. B, patterns of CD38- and CD3-induced increases in protein tyrosine phosphorylation. Cells were prepared and stimulated for the indicated times as described in Fig. 1. C, Cell lysates (250,000 cell equivalents) from unstimulated (lane 1) or stimulated (lanes 3 and 4) with IB4+ F(ab')2 Goat IgG secondary Ab (lanes 5 and 6), or mock-stimulated with F(ab')2 Goat IgG secondary Ab (lane 2) were separated on 10% SDS-PAGE gel under reducing conditions and subjected to immunoblottinwith anti-Tyr(P) mAb RC20-HRP. Position of tyrosine-phosphorylated CD3-ε is indicated. D, the filter shown in C was stripped and reprobed with an affinity-purified antiserum 448 (Fig. 6E). E, Western blotting with anti-CD3-ε mAb, 1D4.1, followed by nonreducing SDS-PAGE and immunoblotting using either an anti-Tyr(P) mAb (Fig. 6E) or the anti-CD3-ζ antisemur, 448 (Fig. 6F). In contrast to CD3-ε, CD3-ζ became tyrosine-phosphorylated following CD38 ligation (Fig. 6E, lanes 4–6). F, with little appearance of the high molecular weight forms of phosphorylated CD3-ζ (Fig. 6E, lanes 7 and 8). The failure of the anti-CD3 mAb to induce an increase in CD3-ζ tyrosine phosphorylation was not due to the presence in unstimulated cells of a low molecular weight form of CD3-ζ (Fig. 6L, lane 4), because CD3 ligation with the anti-CD3-ε mAb OKT3 induced a significant increase in CD3-ζ tyrosine phosphorylation (Fig. 6E, lanes 5 and 6), with the appearance of 42–44-kDa CD3-ζ dimers (Fig. 6F, lanes 5 and 6). Note that these 42–44-kDa phosphorylated CD3-ζ dimers could also be seen in whole lysates blotted with the anti-CD3-ζ antisemur 448 (Fig. 6F, lane 2 versus lane 3). Since ITAM tyrosine phosphorylation is critical for ITAM-mediated signaling functions, these data indicate that association of CD3-ζ to the TCR is not necessary for CD38 to induce PTK activation. Moreover, the predominant CD3-ε tyrosine phosphorylation with little phosphorylation of CD3-ζ strongly suggests that, in these cells, the signals originated from CD38 converge on CD3-ε. Assessment of MAP kinase activation in mutant cells upon CD38 ligation was performed as described above (Fig. 5). In JKC2 TCR-βmut cells, increased tyrosine phosphorylation and mobility shift of Erk-2 was clearly detectable after 5 min of CD38, or CD3 stimulation with specific antibodies (Fig. 7A). These results prove that CD38-mediated MAP kinase activation does not require the association of the CD3-ζ chain with the TCR. Collectively, these results imply that, in these cells, the CD3-γε module couples CD38 to the signal transduction machinery.

Deficient Coupling of CD38 to Signaling in CD25-ζ and
tyrosine phosphorylation in JK-CD25-CD38 cross-linking resulted in very weak induction of protein expressed CD38 on the cell surface (Fig. 8). Both cell lines associate with endogenous CD3 subunits and therefore act as demonstrated earlier, these cells do not express the TCR/CD3 complex. Furthermore, we used two CD25 chimeric receptor-expressing cell lines (28) (Fig. 8). These cells were generated by stable transfection of a variant of the Jurkat cell line (J.RT3-T3.5) with the cDNA encoding the complete human CD25 ecto- and transmembrane domains fused to either the complete mouse CD3- or CD3-ε cytoplasmic domains (28) (Fig. 8A, left panels). As demonstrated earlier, these cells do not express the TCR/CD3 complex on the cell surface, and the chimeric molecules do not associate with endogenous CD3 subunits and therefore act as physically independent signaling molecules (28). Both cell lines expressed CD38 on the cell surface (Fig. 8A, right panels). CD38 cross-linking resulted in very weak induction of protein tyrosine phosphorylation in JK-CD25-ε cells and non-detectable induction in JK-CD25-ε cells (Fig. 8B, lanes 4 and 9, respectively). By contrast, in these cells direct cross-linking of either the CD25-ε or the CD3-ε chimeric molecules with an anti-CD25 mAb resulted in significant increases in protein tyrosine phosphorylation (Fig. 8B, lanes 3 and 8, respectively), which was still detectable at a 10-fold lower dose of anti-CD25 mAb (Fig. 8B, lanes 5 and 10, respectively).

We next examined whether CD25-ε or CD25-ε themselves, both of which contain cytoplasmic ITAMs, were tyrosine-phosphorylated when CD38 was cross-linked with the anti-CD38 mAb IB4. This was determined by immunoprecipitation of the chimeric proteins from control and stimulated cells, followed by immunoblotting with anti-Tyr(P) mAb. Whereas in JK-CD25-ε cells CD25-ε was weakly phosphorylated after CD38 ligation (Fig. 9A, lane 3), in JK-CD25-ε cells no signal could be detected for CD25-ε (Fig. 9B, lane 3). In contrast, in JK-CD25-ε cells CD25-ε was strongly tyrosine-phosphorylated when directly cross-linked with an anti-CD25 mAb (Fig. 9A, lanes 2 and 4). As reported earlier in TCR-β BW 5147 cells stably transfected with CD25-ε chimeras (52), in JK-CD25-ε-
cells CD25-ε resulted in very weak (if any) tyrosine phosphorylation after CD25 ligation (Fig. 9B, lanes 2 and 4). This could be due to the presence of a single ITAM in its cytoplasmic domain.

To examine whether the MAP kinase Erk-2 was activated upon CD38 or chimeric cross-linking, we monitored receptor-induced increases in Erk-2 tyrosine phosphorylation and changes in its electrophoretic mobility. To assess Erk-2 tyrosine phosphorylation, Erk-2 was immunoprecipitated from lysates of unstimulated or stimulated cells by using an anti-Erk-2 antibody and then immunoblotted with an anti-Tyr(P) mAb (Fig. 9, C and D, right panels). Whereas CD38 ligation only induced a faint increase in Erk-2 tyrosine phosphorylation in CD25-ε cells (Fig. 9C, lane 7), the same stimulus did not induce detectable Erk-2 tyrosine phosphorylation in JK-CD25-ε cells (Fig. 9D, lane 7). In contrast, cross-linking of the respective chimeric proteins with an anti-CD25 mAb led to a significant increase in Erk-2 tyrosine phosphorylation (Fig. 9, C and D, lane 6), even when a 10-fold lower dose of anti-CD25 mAb were used (Fig. 9, C and D, lane 8). To monitor changes in Erk-2 electrophoretic mobility, we performed in whole cell lysates from unstimulated or stimulated cells Western blot analysis with an anti-Erk-2-specific antibody (Fig. 9, C and D, left panels). No mobility shift of Erk2 was observed in cell lysates from anti-CD38-treated JK-CD25-ε and JK-CD25-ε’ cells (Fig. 9C, lane 3; Fig. 9D, lane 3), whereas a reduced electrophoretic mobility of Erk2 was readily detected in cell lysates from anti-CD25-treated cells (Fig. 9, C and D, lanes 2 and 4). These results provided evidence that the cytoplasmic domains of either CD3-ζ or CD3-ε alone are not sufficient to mediate CD38-induced signaling events, despite the fact that they are independently capable of signal transduction, leading to PTK and MAP kinase activation.

**DISCUSSION**

The results show that CD38 ligation leads to both PTK and MAP kinase activation, in TCR+T cells that express the whole set of CD3 subunits (e.g. the CD3-ζ-ε homodimer and the CD3-γ,ε and CD3-δ,ε heterodimers), or in TCR+ cells with a defective CD3-ζ association. Since the CD3-ζ-ε homodimer contributes with 6 out of 10 ITAMs within a given TCR/CD3 complex, these data highlight the importance of the so-called CD3-γε signaling module, identifying it as a necessary and sufficient component of the TCR/CD3 complex involved in T cell activation through CD38. The lack of PTK and MAP kinase activation in cells expressing chimerical CD25-ζ or CD25-ε receptors in response to CD38 stimulation strongly suggest that either the extracellular, or the transmembrane domain, of one, or more CD3 subunits are required for CD38-mediated signaling.

How does CD38 cross-linking lead to activation of Src family PTKs and tyrosine phosphorylation of CD3-ζ and CD3-ε ITAMs? That CD3-ε and CD3-ζ subunits become tyrosine-phosphorylated upon CD38 engagement could be explained by a model in which CD38 ligation by agonistic antibodies would induce CD38 association with the TCR/CD3 complex. In this model, a fraction of Lck could directly associate with CD38. The recruitment of CD38 to the proximity of the TCR/CD3 complex would allow the putative CD38-associated PTK (e.g. Lck) to reach a relative high concentration for the effective tyrosine phosphorylation of the CD3 chains. So far, there is little evidence to suggest that Lck is constitutively associated with CD38, but overall the results suggest that signals delivered by CD38 must be integrated at or near the T cell surface membrane that is in contact with both Lck and the TCR/CD3 complex. If so, CD38 could act as an authentic co-receptor bringing the associated PTK in the proximity of the CD3 chains to promote an augmented T cell response.

It is important to note that resident PTKs are associated with the TCR/CD3 complex in nonactivated T cells. These include Fyn that was found associated with the TCR/CD3 complex at a low stoichiometry (53), and Lck, demonstrated to be present in a complex that also includes CD4 or CD8 and CD5 at the cell surface of nonactivated T cells (54–56). In a variation of the model for CD38 signaling described above, we envision that the CD3 chains may function as docking molecules to link CD38 to the TCR/CD3-associated PTKs. In this scenario CD38 would be unable by itself to deliver PTK-dependent activating signals and CD38 signaling capabilities would be regulated exclusively by the ability of CD38 ligands to bring CD38 in...
close proximity to the TCR/CD3 complex.

In its simplest interpretation, therefore, CD38-mediated signaling capacity is determined by the efficiency of ligand-induced CD38/TCR/CD3 co-aggregation. However, we cannot rule out that conformational changes in either CD38, the TCR/CD3 complex, or both are required for optimal signaling. It is interesting that the two epitopic sites (the amino acid sequences of 220–241 and 273–285) recognized by all known agonistic anti-human CD38 mAbs, including IB4, are distinct to the site recognized by the nonagonistic anti-CD38 mAb OKT10 (amino acid sequence of 280–298) at the C-terminal portion of human CD38 (57). Despite the closeness of the sites recognized by IB4 and OKT10, the biological effects exerted by these antibodies are quite different. Thus, both IB4 and OKT10 mAbs induce the TCR/CD3 complex to co-cap with CD38 (30), suggesting that both antibodies are able to promote an association between CD38 and the TCR/CD3 complex. However, IB4, unlike OKT10, induces increases in PLC-γ1 tyrosine phosphorylation and Erk-2 activation (Figs. 8 and 9), which is likely, because in those cells ligation of the CD25-ε- and CD25-ζ-mediated signals may occur at certain critical points downstream of CD3 tyrosine phosphorylation and ZAP-70 activation. In this sense, SLP-76, which becomes tyrosine-phosphorylated upon CD38 cross-linking (Fig. 4D), may play such a role as it is suggested to operate in CD3-mediated signals (59).

We speculate that a specific pairing of CD38 with a particular CD3 subunit may endow CD38 with distinct signaling capabilities. This could allow different signaling responses depending on how a particular ligand (e.g. CD31, agonistic versus nonagonistic anti-CD38 mAbs, etc.) would affect the CD38/CD3 interaction. This may have some relevance to the observation that different CD3 antibodies and ligands can induce qualitatively different signals (6, 22, 57). Alternatively, the modular architecture of the TCR/CD3 complex permits the occurrence of multiple TCR “isofoms” made of distinct polypeptide combinations (60–62). These isofoms may coexist and be responsible for coupling antigen recognition to distinct signaling pathways (18, 63). Therefore, the capacity for CD38 to elicit different functional responses in T cells would rely, ultimately, on the plasticity in the CD3 composition of the TCR/CD3 complex (64).
