CD38 Is Associated with Lipid Rafts and upon Receptor Stimulation Leads to Akt/Protein Kinase B and Erk Activation in the Absence of the CD3-ζ Immune Receptor Tyrosine-based Activation Motifs*

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T lymphocytes can be activated via the T cell receptor (TCR) or by triggering through a number of other cell surface structures, including the CD38 co-receptor molecule. Here, we show that in TCR+ T cells that express a CD3-ζ lacking the cytoplasmic domain, cross-linking with CD38- or CD3-specific monoclonal antibodies induces tyrosine phosphorylation of CD3-ζ, ζ-associated protein-70, linker for activation of T cells, and She. Moreover, in these cells, anti-CD38 or anti-CD3 stimulation leads to protein kinase B/Akt and Erk activation, suggesting that the CD3-ζ-immunoreceptor tyrosine-based activation motifs are not required for CD38 signaling in T cells. Interestingly, in unstimulated T cells, lipid rafts are highly enriched in CD38, including the T cells lacking the cytoplasmic tail of CD3-ζ. Moreover, CD38 clustering by extensive cross-linking with an anti-CD38 monoclonal antibody and a secondary antibody leads to an increased resistance of CD38 to detergent solubilization, suggesting that CD38 is constitutively associated with membrane rafts. Consistent with this, cholesterol depletion with methyl-β-cyclodextrin substantially reduces CD38-mediated Akt activation while enhancing CD38-mediated Erk activation. CD38/raft association may improve the signaling capabilities of CD38 via formation of protein/lipid domains to which signaling competent molecules, such as immunoreceptor tyrosine-based activation motif-bearing CD3 molecules and protein-tyrosine kinases, are recruited.

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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) (3–6). Other reactive cells include natural killer cells, monocytes, macrophages, dendritic cells, and some epithelial cells. The CD38 antigen acts as a NAD(P)+ glycyrrhetinic acid phosphohydrolase (7) and plays a role in lymphocyte activation (3, 8). CD31, which is mainly expressed by endothelial cells, platelets, macrophages, and a discrete subset of T cells, has been identified as a ligand for CD38 (4, 9). The interplay between CD38 and its ligand CD31 may be an important step in the regulation of cell life and of the migration of leukocytes through endothelial cells.

Plasma membranes of many cell types, including T cells, contain specialized microdomains, or lipid rafts, enriched in sphingolipids, cholesterol, sphingomyelin, and glycosylphosphatidylinositol-anchored proteins. These membrane domains are characterized by detergent insolubility at low temperatures and low buoyant density. Based on these biochemical properties, they are often referred to as glycosphingolipid-enriched membranes, or detergent-insoluble glycolipid fractions (10, 11). Several signaling proteins are enriched in lipid rafts. Src family kinases and LAT, both of which require acylation for raft targeting, are constitutively present in rafts. The densely packed, liquid-ordered environment of rafts excludes most integral membrane proteins. However, antibody-mediated clustering can recruit receptors on several cell types to rafts. These include some components of the T cell antigen receptor (TCR)1. CD3 complex (12–15), BCR (16–18), FcεR1 (19), CD20 (20), and human CD2 (21).

The molecular basis for signaling by CD38 remains unclear. We have previously demonstrated that CD38 ligation leads to PTK, phospholipase C-γ1, and mitogen-activated protein kinase activation in TCR+ T cells that express the whole set of CD3 subunits, but not in TCR-negative T cells (22, 23). More recently, we have extended these observations to TCR+ cells

1 The abbreviations used are: TCR, T cell antigen receptor; PI, phosphatidylinositol; ZAP-70, ζ-associated protein-70; Erk, extracellular signal-regulated protein kinase; PKB/Akt, protein kinase B; PTK, protein tyrosine kinase; PTK/N, phosphatase and tensin homolog; LAT, linker for activation of T cells; ITAM, immunoreceptor tyrosine-based activation motif; Tyr(P), phosphotyrosine; mAb, monoclonal antibody; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; αHms, Fabα goat anti-hamster IgG; †mo, Fab† goat anti-mouse IgG; MβCD, methyl-β-cyclodextrin; WB, Western blot; IP, immunoprecipitation; GM1, ganglioside Galβ1-3GalNacβ1-4Gal(3-2NεNeuAc)β1-4GlcPβ1-1Cer.
with a defective CD3-ζ association (24). While the distinct signaling pattern induced by CD38 in these cells underscored the importance of the CD3-γδε module in mediating CD38 signaling, we could not exclude a functional role for CD3-ζ in this process. Thus, CD3-ζ has also been found to associate rather promiscuously with various non-TCR components, including the transferrin receptor (25) and the CD16 receptor expressed on natural killer cells (26). Hence, several possibilities exist. CD3-ζ could associate directly with CD38 independently of the other CD3 chains. Alternatively, CD3-ζ could associate with CD38 indirectly through other surface receptors or intracellular adapter proteins. Moreover, the site where the CD38/CD3 interaction could take place is unknown. One possibility is that lipid rafts may be involved in this process by grouping together CD38, TCR-CD3, and Src family tyrosine kinases.

The TCR-CD3 is a multimeric protein complex comprising ligand-binding (TCR) and signal-transducing subunits (CD3). The signal transduction processes are mediated by the immunoreceptor tyrosine-based activation motifs (ITAMs), and up to 10 ITAMs are present within a single TCR-CD3 complex (with a CD3-ζ-ζ homodimer and two CD3-γ-ε and CD3-δ-ε heterodimers). This multiplicity may allow for signal amplification and/or the formation of qualitatively distinct intracellular signaling events. Notably, the disulfide-linked CD3-ζ-ζ homodimer contains six ITAMs within a given TCR-CD3. Production and characterization of CD3-ζ-negative murine thymoma T cells that had been reconstituted with either full-length wild-type (DC262) or partially deleted CD3-ζ lacking all of its ITAMs (DC64) has been reported previously (27). Because DC262 and DC64 cells differ in their CD3-ζ subunits and both lack CD3-η and FceRIγ mRNAs, these cells could allow a direct examination of the role of the cytoplasmic tail of CD3-ζ in CD38-mediated signal transduction and in the hypothetical translocation of CD38 to lipid rafts. To this end, these cells were transfected with the cDNA encoding human CD38. Results demonstrate that in DC262 with wild-type CD3-ζ or in DC64 with a truncated CD3-ζ, CD38 ligation leads to tyrosine phosphorylation of similar sets of cellular substrates, including CD3-ε, ZAP-70, and Shc, and the activation of the PI 3-kinase/Akt and Ras/Erk signaling pathways. We also demonstrate the constitutive association of CD38 with lipid rafts. CD38/raft association, together with induction of PI 3,4,5-trisphosphate synthesis, may help to colocalize kinases such as Akt/PKB with their substrates. These results support a model for CD38 signaling where accumulation in lipid rafts of supramolecular signaling complexes containing ITAM-bearing CD3 molecules and protein-tyrosine kinases is a prerequisite for CD38-mediated signal transduction.

EXPERIMENTAL PROCEDURES

Cell Lines—Production and characterization of murine BW5147 cells expressing either the wild-type (DC262) or partially deleted CD3-ζ (DC264 series) molecule have been reported elsewhere (27). Briefly, CD3-ζ-negative mutants of BW5147 cells were transfected with either the wild-type (DC262) or partially deleted CD3-ζ molecule (DC264) expressing either the wild-type (DC262/wt) or a truncated CD3-ζ molecule (DC264ζ)

RESULTS

Cell Phenotyping—In this study, we have used murine T cell lines expressing TCR-CD3 complexes containing either the wild-type (DC262/wt) or a truncated CD3-ζ molecule (DC264ζ)
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with the anti-pS473 Akt antibody, is relatively high (36). To reduce the basal level of PI 3-kinase activity and Akt phosphorylation, cells were serum-starved for 20 h (0.5% fetal calf serum) before stimulation (37, 38). Thus, in serum-starved cells and in the absence of stimulation, weak basal phosphorylation at Ser\(^{733}\) was observed, which was substantially increased at 5 min following CD38 or CD3 stimulation with specific mAb (Fig. 2, upper panel). Phosphorylation of Akt/PKB was transient, with a peak at 5–15 min (lanes 4, 5, 9, and 10) and a slow decline to basal levels at 60 min (lanes 2 and 7). Subsequently, blots were stripped and reprobed with an anti-Akt-specific antibody that detects total Akt (phosphorylation state-independent) (Fig. 2, lower panel). Interestingly, there was a shift in Akt mobility on SDS-PAGE gels at 5 and 15 min following mAb stimulation (lanes 4, 5, 9, and 10), which has been linked to phosphorylation at Thr\(^{308}\) and Akt activation (39, 40).

Next, CD38-mediated Akt activation was assessed in the CD38-transfected murine T cells. To reduce the basal level of Akt phosphorylation at Ser\(^{733}\), DC262\(^{\text{wtCD38}}\) or DC264\(^{\text{mutCD38}}\) cells were serum-starved overnight. Then cells were mock-stimulated with secondary antibody alone or stimulated with anti-CD38 or anti-CD3 mAbs plus secondary antibody. Then, cell lysates were probed with the anti-pS473 Akt antibody (Fig. 3). In cells stimulated with the secondary antibody alone, weak basal phosphorylation was observed on Ser\(^{733}\) (Fig. 3, A and B, upper panels, lanes 1 and 4), which significantly increased at 5 min following anti-CD38 or anti-CD3 stimulation (Fig. 3, A and B, upper panels, lanes 6 and 3, respectively). Subsequent reprobing of the filters with anti-Akt-specific antibodies demonstrated that Akt levels were similar throughout the experiment (Fig. 3, A and B, lower panels), demonstrating equal protein loading. As in Jurkat human T cells, in the CD38-transfected murine T cells, we also observed a shift in Akt mobility on SDS-PAGE gels coincident with the maximum levels of Akt phosphorylation.

To investigate whether CD38-mediated Akt/PKB activation is under the control of PI 3-kinase activity, serum-starved cells were preincubated with the inhibitor wortmannin, which specifically prevents PI 3-kinase activation (41). Indeed, preincubation with 100 nM wortmannin for 30 min completely prevented CD38- or CD3-induced Akt/PKB activation (Fig. 3, C and D, upper panels). The total amount of Akt/PKB did not change during the wortmannin treatment. These results indicate that CD38-mediated Akt/PKB activation requires PI 3-kinase activation. Overall, the results demonstrate that CD38 triggering induced Akt activation in both human and murine T cell lines and that CD38- and CD38-mediated activation of the PI 3-kinase/Akt signaling pathway is not negatively regulated by the absence of a functional CD3-ζ.
CD3-ζ Cytoplasmic Domain Is Not Required for CD38- or CD3-mediated Erk Activation—Among the signaling components thought to be downstream of the TCR-CD3 complex and PI 3-kinase is Erk. We have previously demonstrated that in Jurkat T cells Lck is required for CD38-mediated Erk activation (22). Moreover, CD38-mediated Erk activation occurs in Jurkat T cells with a point mutation in the TCR-β chain that results in a defective CD3-ζ association with the TCR-CD3 complex (24). To assess the role of the cytoplasmic tail of CD3-ζ on CD38-mediated Erk activation, serum-starved DC262 wtCD38- and DC264 mutCD38- cells were left unstimulated or were stimulated with anti-CD3 or anti-CD3 mAb for 1 and 5 min. Then cell lysates were probed with a diphospho-Erk-specific mAb, which recognizes dually phosphorylated Erk at the TEY motif. Dual phosphorylation at these sites correlates with Erk activation. As shown in the upper panels of Fig. 4, A and B, in both DC262 wtCD38- and DC264 mutCD38- cells, anti-CD38 stimulation caused a significant activation of Erk-1 and Erk-2 at 5 min following mAb cross-linking, as compared with that in control unstimulated cells (Fig. 4, A and B, upper panels, lane 7 versus lane 1). This increase in Erk activation was somewhat weaker than that induced upon CD3 cross-linking (lane 7 versus lane 4), but there were no major differences when the comparison was carried out between cells (Fig. 4A, lane 7 versus Fig. 4B, lane 7). Likewise, CD3-mediated Erk activation was similar in both cell types (Fig. 4A, lane 4 versus Fig. 4B, lane 4). Subsequently, the blots were stripped and reprobed with total Erk-specific antibodies to verify that equivalent amounts of proteins were loaded per lane. Therefore, these results suggest that CD38- or CD3-mediated Erk activation is independent of the cytoplasmic domain of CD3-ζ.

Effect of Wortmannin Treatment on CD38-mediated Erk Activation—It has been previously shown that the PI 3-kinase inhibitor, wortmannin, inhibits CD3-mediated Erk2 activation in T cells (42). Moreover, active (but not mutant) PTEN also decreases TCR-induced activation of Erk2, as seen after inhibition of PI 3-kinase (43). We therefore decided to ask whether PI 3-kinase activity is required for CD38-mediated Erk activation. To this end, DC262 wtCD38- and DC264 mutCD38- cells were treated with 100 nM wortmannin for 30 min and then stimulated with cross-linked anti-CD3 mAb BI4 or anti-CD3 mAb 2C11. Activation of Erk was tested by immunoblotting of total cell lysates of anti-CD38-stimulated cells and compared with those stimulated with the cross-linked anti-CD3 mAb 2C11, control unstimulated cells, or cells mock-stimulated with secondary antibody alone. As shown in the upper panels of Fig. 4, C and D (lanes 6 and 7), CD38-mediated Erk activation was sensitive to wortmannin, with a lower increase in the amount of phospho-Erk detected as compared with that in untreated cells (upper panels of Fig. 4, A and B, lanes 6 and 7). Consistent with previous reports (44), wortmannin blocked CD3-mediated Erk activation (upper panels of Fig. 4, C and D, lanes 3 and 4). We therefore conclude that PI 3-kinase activity is required for Erk activation in response to CD38 engagement.

CD3- but Not CD38-mediated LAT Tyrosine Phosphorylation Is Positively Regulated by the Presence of a Functional CD3-ζ—Given that LAT seems to be essential for TCR-mediated Ras/Erk activation (44, 45), we addressed the question whether...
cross-linking of CD38 leads to LAT tyrosine phosphorylation. To this end, anti-LAT immunoprecipitates from unstimulated, anti-CD38-stimulated, or anti-CD3-stimulated cells were immunoblotted with an anti-Tyr(P) mAb. As shown in Fig. 5A, in DC262\(\text{mutCD38}^+\) cells, CD38 ligation induced a weak increase in LAT tyrosine phosphorylation (lane 6), whereas the same stimulus did not cause any increase in DC262\(\text{wtCD38}^+\) cells (lane 3). In contrast, the latter cells did respond to anti-CD3 stimulation with a relatively high increase in LAT tyrosine phosphorylation (lane 2), as compared with that in DC262\(\text{mutCD38}^+\) cells (lane 5), despite the fact that Sos was readily detected in LAT immunoprecipitates from both CD38- and CD3-stimulated DC262\(\text{mutCD38}^+\) cells (Fig. 5C, lanes 6 and 5, respectively), and not in those from CD38-stimulated DC262\(\text{wtCD38}^+\) cells (Fig. 5C, lane 3) nor from unstimulated cells (Fig. 5C, lanes 1 and 4, respectively). Since phosphorylated LAT binds Grb2 directly (46) and does not contain an Src homology 3 domain to interact with the prolinc-rich region of Sos, Grb2/Sos interaction might mediate the association of phosphorylated LAT–Sos described here. LAT tyrosine phosphorylation and recruitment of Grb2–Sos are important steps for Ras activation (44). Overall, these results suggest that CD3 but not CD38-mediated LAT tyrosine phosphorylation is positively regulated by the presence of an intact and functional CD3–ζ cytoplasmic domain.

**CD38-mediated Erk Activation in LAT- or ZAP-70-deficient Cells**—To further explore the role of LAT in CD38-mediated Erk activation, we performed the following experiment in the LAT-deficient Jurkat T cell line JCaM2.5 (44). Activation of Erk was analyzed by using the anti-diphospho-Erk antibody. As shown in Fig. 6, in both anti-CD3- and anti-CD38-stimulated cells there was a significant increase in Erk activation (lanes 6 and 7). Consistent with this, phospho-LAT-independent activation of Erk has been previously described for TCR stimulation with partial agonist peptides (47). Moreover, a small and transient activation of Erk has been observed in JCaM2.5 cells following TCR stimulation (44), suggesting additional mechanisms for Ras/Erk activation independent of inducible LAT tyrosine phosphorylation.

In a similar experiment, the requirement for ZAP-70 in mediating Erk activation in response to CD38 cross-linking was also assessed, using the ZAP-70-negative Jurkat T cell line P116 (48). As in LAT-negative cells, activated Erk could be detected in lysates from P116 cells stimulated with anti-CD38 mAbs or anti-CD3 mAbs (Fig. 6, lanes 9 and 10), which was somewhat weaker than that in Jurkat D8 (lanes 3 and 4) or in JCaM2.5 cells (lanes 6 and 7). This was not likely to be due to the basal level of Erk activation observed in unstimulated P116 cells (lane 8), because a similar basal level of Erk activation was observed in JCaM2.5 cells (lane 5). These results suggest that the presence of ZAP-70 exerts a positive role in CD38- or CD3-mediated Erk activation.

**CD38 Ligation Induces Shc Tyrosine Phosphorylation and Recruitment of Grb2 to Shc**—A primary candidate as an alternative recruitment site of Grb2-Sos complexes in the absence of phosphorylated LAT is the adapter protein Shc. To examine the tyrosine phosphorylation status of Shc in the CD38-transfected cells, the following experiments were performed. Anti-Shc immunoprecipitates from DC262\(\text{wtCD38}^+\) or DC262\(\text{mutCD38}^+\) cells stimulated for 5 min with anti-CD3 were blotted with anti-Tyr(P) mAb and compared with those from unstimulated or anti-CD3-stimulated cells. As shown in Fig. 7A, in both DC262\(\text{wtCD38}^+\) and DC262\(\text{mutCD38}^+\) cells, Shc tyrosine phosphorylation increased following anti-CD38 stimulation (lanes 3 and 6), as compared with control unstimulated cells (lanes 1 and 4). Moreover, both cells responded to anti-CD3 stimulation with a marked increase in Shc tyrosine phosphorylation, which was more pronounced in DC262\(\text{wtCD38}^+\) than in DC262\(\text{mutCD38}^+\) cells (Fig. 7A, lanes 2 and 5). To assess whether tyrosine-phosphorylated Shc recruits Grb2 upon CD38 cross-linking, the Shc immunoprecipitates were analyzed by immunoblotting with an anti-Grb2 antibody. Low amounts of Grb2 were detected in Shc immunoprecipitates from control unstimulated cells, which may reflect the basal level of Shc tyrosine phosphorylation (lanes 1 and 4). However, in CD38-stimulated DC262\(\text{mutCD38}^+\) cells, there was more Grb2 associated with Shc than in unstimulated cells (lane 6 versus lane 4). The relative low level of Grb2 associated with Shc found in CD38-stimulated DC262\(\text{wtCD38}^+\) cells may reflect the lower increase in Shc tyrosine phosphorylation observed in these cells as compared with those in DC262\(\text{mutCD38}^+\) cells. Overall, these results suggest that, at least in DC262\(\text{mutCD38}^+\) cells, Shc-Grb2 complexes may cooperate to fully activate Erk in response to CD38 ligation. Moreover, these results demonstrate that CD38-mediated Shc tyrosine phosphorylation and Grb2 recruitment are not positively regulated by a functional CD3–ζ chain.

**CD38 Ligation Induces CD3-ζ Tyrosine Phosphorylation in CD3-ζ Tailless DC262\(\text{mutCD38}^+\) Cells**—The CD3-ζ and CD3–ζ...
subunits are among the earliest substrates that become tyrosine-phosphorylated upon CD38 stimulation in Jurkat T cells (24). Therefore, we addressed the question of whether cross-linking of CD38 leads to tyrosine phosphorylation of CD3-ε and ZAP-70 in T cells with a CD3-ζ lacking most of the cytoplasmic tail. To this end, CD3-ε immunoprecipitates from unstimulated or anti-CD3-8- or anti-CD3-stimulated cells were examined for inducible CD3-ε tyrosine phosphorylation. Fig. 8A shows that in DC262/wtCD38 cells, CD3-ε was tyrosine-phosphorylated upon CD38 stimulation (lanes 2 and 3). Likewise, in DC264/mutCD38 cells, similar treatment caused CD3-ε tyrosine phosphorylation, although with less intensity (lanes 5 and 6). These results are similar to those obtained following anti-CD3 stimulation, where CD3-ε tyrosine phosphorylation was weaker in DC264/mutCD38 than in DC262/wtCD38 cells (Fig. 8C, lanes 5 and 6 versus lanes 2 and 3). However, the extent and duration of anti-CD3-mediated CD3-ε tyrosine phosphorylation was significantly lower, as compared with that upon anti-CD3 stimulation (Fig. 8, A versus C), which is consistent with our previous results in Jurkat T cells (24). These results also suggest that both CD3- and CD38-mediated CD3-ε tyrosine phosphorylation is positively regulated by the presence of a functional CD3-ζ chain.

CD38 Ligation Enhances the Association of p85 PI 3-Kinase Subunit with CD3-ε—To investigate whether p85 PI 3-kinase associated with CD3-ε following CD38 stimulation, anti-CD3-ε immunoprecipitates from DC262/wtCD38 or DC264/mutCD38 cells were analyzed for the presence of co-immunoprecipitated p85 by Western blotting with an anti-p85 PI 3-kinase-specific antibody. As shown in Fig. 8E, p85 PI 3-kinase was found to interact with CD3-ε in unstimulated cells, and this association was significantly enhanced after CD38 stimulation in both cell types. These data are consistent with our previous results in Jurkat T cells, where we found a 2-fold increase in p85 PI 3-kinase association with the intracellular domain of CD3-ε upon engagement of the TCR-CD3 with anti-CD3 mAbs (37). The relatively modest increase in p85 binding to CD3-ε resulted in a 5–10-fold increase in PI 3-kinase enzymatic activity associated with CD3-ε (37), which suggested that the binding of p85 to phosphorylated CD3-ε-ITAM could induce a conformational change in the p85/p110 PI 3-kinase structure, leading to PI 3-kinase activation. Our data also demonstrate that association of p85 PI 3-kinase with CD3-ε is not impaired by the absence of the cytoplasmic tail of CD3-ζ, which is consistent with the Akt/PRK results.

CD38-mediated ZAP-70 Tyrosine Phosphorylation—We have previously shown in Jurkat T cells that CD38 ligation leads to ZAP-70 tyrosine phosphorylation (22). Since the CD3-ζ ho-modimer contributes with 6 out of 10 ITAMs within a given TCR-CD3 complex, it was of interest to know whether CD38 ligation resulted in ZAP-70 tyrosine phosphorylation in the CD3-ζ tailless cells. To this end, anti-ZAP-70 immunoprecipitates from unstimulated and CD38- and CD3-stimulated cells were immunoblotted with anti-Tyr(P) mAb. In contrast to CD38-mediated ZAP-70 tyrosine phosphorylation, CD38 ligation in-
and DC264 cells were solubilized in octyl-β-D-glucoside to concentrate CD38, CD3-ζ, near the top of the discontinuous sucrose gradient. These fractions were precipitated at the 5%/30% interface. Three low density fractions were collected, corresponding to fractions 1–3, near the top of the discontinuous sucrose gradient. Likewise, in DC264 cells, there was a significant increase in the amount of CD38 detectable in low density fractions 1–3 and a concomitant decrease in the CD38 present in high density fractions 7 and 8, as compared with that in unstimulated cells. In contrast, the raft marker LAT remained unchanged, since in unstimulated cells most of it was already present in the raft fractions. These results demonstrate that CD38 becomes more resistant to detergent solubilization upon CD38 cross-linking, which is a hallmark of raft-associated proteins or lipids (49). Moreover, a significant proportion of CD38 is associated with lipid rafts independently of the activation status of the cell or the presence of the cytoplasmic tail of CD3-ζ. These data suggest that CD38 is constitutively associated with lipid rafts.

**Cholesterol Depletion Inhibits Akt Activation and Enhances Erk Activation Induced by CD38 or CD3 Cross-linking**—Because cholesterol is a critical component of rafts, we investigated whether reduction of cholesterol content of the DC262 wtCD38 or DC264 mutCD38 cells affects CD38- and CD3-mediated Akt activation. To this end, cells were incubated with methyl-β-cyclodextrin (MβCD), known to reversibly extract membrane cholesterol and therefore disrupt raft interactions (50, 51) and greatly affect TCR-CD3-mediated signaling (12, 52). Thus, cells were incubated with 10 mM MβCD for 20 min before stimulation with either anti-CD38 or anti-CD3 mAbs and cross-linking with a secondary antibody. Based on the work of others, these conditions are expected to extract 50–60% of cellular cholesterol content (53). As shown in Fig. 11, MβCD treatment caused a substantial inhibition of CD38-
Role of PI 3-Kinase in CD38-mediated Signaling—PI 3-kinase and at least one of its targets, Akt/PKB, plays an important role in cell growth control, cell survival, and protection from apoptosis (35, 54, 55). The participation of PI 3-kinase in CD38-mediated signaling in B cells is well documented (56, 57), but not in T cells. In this study, to determine whether CD38 is coupled to Akt activation in both Jurkat T cells and CD38-transfected murine T cells, we assessed the phosphorylation status of Akt upon CD38 cross-linking. The results demonstrate that CD38 ligation induces Akt/PKB activation not only in T cells bearing the complete set of TCR-CD3 subunits but also in T cells lacking all CD3-ζ-ITAMs. CD38-mediated Akt/PKB activation requires PI 3-kinase activation, since the PI 3-kinase inhibitor, wortmannin, completely blocks Akt/PKB activation. Likewise, CD38-mediated Akt/PKB activation is not restricted to CD3-ζ-ITAM-positive T cells. Therefore, these observations lead us to conclude that the ability of CD38 or CD3 to induce PI 3-kinase/Akt activation does not depend on the presence of a functionally competent CD3-ζ chain.

In LAT-deficient Jurkat T cells, CD38-mediated Erk activation occurs, which suggests the existence of alternative pathways leading to Erk activation not involving LAT tyrosine phosphorylation by Zap-70. Consistent with this, in both DC262wtCD38⁺ and DC264mutCD38⁺ cells, there is a relatively weak induction of LAT or Shc tyrosine phosphorylation upon CD38 ligation. Consequently, the amount of Grb2-Sos complexes recruited by these adapters is also low. In addition, we have shown that in Jurkat T cells, CD38-mediated Erk activation strongly depends on protein kinase C activation (22), which may up-regulate Ras or Raf activity in a tyrosine kinase-independent manner. Likewise, PI 3-kinase may play a pivotal role in CD38- or CD3-mediated Erk activation. This is suggested by the strong inhibition of Erk activation in response to CD38 or CD3 cross-linking in cells pretreated with the PI 3-kinase inhibitor wortmannin. Interestingly, TCR-CD3-induced activation of other upstream Ser/Thr kinases, such as c-Raf, B-Raf, Mek1, Mek2, and Mekk, is not affected by wortmannin (42). These results suggest that PI 3-kinase is involved in the activation of Erk but does not regulate the enzymes that are thought to be upstream of Erk. Notably, expression in Jurkat T cells of the phosphoinositide 3-phosphate phosphatase, PTEN, decreases TCR-induced activation of Erk2 (36, 43), which is consistent with the positive role of PI 3-kinase activity in Erk activation.

The molecular mechanism for recruitment of PI 3-kinase to the CD38 signaling cascade is not clear. In B cells, it has been proposed that the coupling intermediate is CD19, which binds directly to the p85 subunit of the PI 3-kinase (57). We believe that in T cells the CD3 chains are likely to fulfill this function, because both the p85 PI 3-kinase subunit and the PI 3-kinase enzymatic activity specifically associate with tyrosine-phosphorylated CD3-ζ-ITAMs, CD38, or CD3 ligation, delivers an intracellular signal that leads to PTK, Akt/PKB, and Erk activation. With the exception of inductive CD3-ζ, LAT, and Shc tyrosine phosphorylation, the CD38-mediated response is indistinguishable from that delivered in TCR⁺ cells expressing the wild-type CD3-ζ. In contrast, the CD38-mediated PTK activation, and to a lesser extent Akt/PKB and Erk, is clearly amplified in the latter cells. We therefore conclude that CD3-ζ ITAM-mediated signals are dispensable for CD38-mediated PI 3-kinase and Erk activation, although it may be quantitatively important to recruit signaling molecules to the rafts. Therefore, in the absence of a functional CD3-ζ, the remaining ITAM-bearing CD3 subunits (i.e. CD3-γ, -δ, and -ε) are sufficient to mediate signaling by cross-linked CD38.

DISCUSSION

Functional Dependence of CD38 on Signaling by TCR-CD3—Our previous work demonstrated that CD38 relies on the TCR-CD3 complex for signal transduction (22–24). The data of the present study show that in TCR⁺ T cells lacking most of the CD3-ζ cytoplasmic tail, and therefore without the six CD3-ζ-ITAMs, CD38, or CD3 ligation, delivers an intracellular signal that leads to PTK, Akt/PKB, and Erk activation. With the exception of inductive CD3-ζ, LAT, and Shc tyrosine phosphorylation, the CD38-mediated response is indistinguishable from that delivered in TCR⁺ cells expressing the wild-type CD3-ζ. In contrast, the CD38-mediated PTK activation, and to a lesser extent Akt/PKB and Erk, is clearly amplified in the latter cells. We therefore conclude that CD3-ζ ITAM-mediated signals are dispensable for CD38-mediated PI 3-kinase and Erk activation, although it may be quantitatively important to recruit signaling molecules to the rafts. Therefore, in the absence of a functional CD3-ζ, the remaining ITAM-bearing CD3 subunits (i.e. CD3-γ, -δ, and -ε) are sufficient to mediate signaling by cross-linked CD38.

FIG. 11. Effect of cholesterol depletion on CD38- or CD3-mediated Akt/PKB and Erk activation. A, DC262wtCD38⁺ cells were serum-starved for 20 h and incubated with 10 mM MβCD for 20 min at 37 °C (lanes 1–7) or left untreated (lanes 8–14). Then cells were washed and left unstimulated (lanes 1 and 14), mock-stimulated with secondary antibody alone (lanes 2, 5, 10, and 13), stimulated with anti-CD3 2C11 + αH11001 for 1 min (lanes 3 and 12) or 5 min (lanes 4 and 11), or stimulated with anti-CD38 IB4 + αH11001 for 1 min (lanes 6 and 9) or 5 min (lanes 7 and 8). Cell lysates were separated on 10% SDS-PAGE under reducing conditions and subjected to immunoblotting with anti-phospho-Akt (Ser473) (upper panel) or with anti-diphospho-Erk mAb (third panel). Then filters were stripped and reprobed with anti-total Akt (second panel) or with anti-pan-Erk polyclonal antibody (fourth panel). B, DC264mutCD38⁺ cells were serum-starved, treated with MβCD, stimulated, lysed, and immunoblotted as in A.
co-localize kinases such as Akt with their substrates. In this sense, it has been shown in mature T cells that the recruitment of lipid rafts to the site of TCR-CD3 co-stimulation is mediated by PI 3-kinase (60).

**Association of CD38 with Lipid Rafts**—It has been shown that upon TCR-CD3 stimulation, specific components of the signaling machinery are incorporated into glycolipid-enriched membrane microdomains known as lipid rafts (12, 14, 15, 31). In the present study, we show that human CD38 is constitutively associated with lipid rafts. Thus, the substantial expression of CD38 in the low density Nonidet P-40-insoluble fractions from unstimulated cells and its stabilization in these fractions upon CD38 cross-linking are in favor of this interpretation. These results are consistent with the work of Harder et al. (49), demonstrating that cross-linking of raft-resident proteins or lipids promotes coalescence of membrane rafts and increased resistance to nonionic detergent solubilization. The data on CD3-ζ and CD3-ε are consistent with other studies showing that the CD3-ζ chain seems more detectable in detergent-insoluble membrane complexes than the other CD3 chains (12, 15, 61, 62). It is important to note that Nonidet P-40 can solubilize a substantial amount of lipid raft components, especially those weakly associated with the rafts. Thus, CD3-ε is only detectable in rafts isolated by using a milder detergent as Brij-58 (15) or by lowering the concentration of Triton X-100 to 0.2% (21) or by confocal fluorescence microscopy (14). Therefore, the CD38/raft association observed here is different from the TCR-CD3 based on its stability under various extraction conditions. In particular, the CD38/raft interaction is more robust than CD3-ε, and similar to CD3-ζ, being stable even using 1% Nonidet P-40 extraction conditions.

Our results also demonstrate that pretreatment of DC262wtCD38- or DC264mutCD38- cells with MβCD causes a substantial inhibition of CD38- or CD3-mediated Akt activation, whereas the same treatment enhances receptor-mediated Erk activation. Since cholesterol depletion is associated with the loss of compartmentalization of the PI 3-kinase substrate, PI 4,5-bisphosphate, in the low density membrane domains (63), our data are consistent with the hypothesis that localization of signaling proteins and lipids to cholesterol-enriched domains is required for the coupling of CD38 with the PI 3-kinase signaling pathway. Other studies used MβCD to investigate the role of cholesterol in signaling by the TCR-CD3 complex. Thus, Xavier et al. (12) and Moran and Miceli (13) have shown that pretreatment of T cells with MβCD inhibits TCR-mediated Ca²⁺ mobilization and tyrosine phosphorylation, respectively, providing evidence for an important role for cholesterol in the function of the TCR-CD3 complex. In contrast, the Erk results suggest that normal levels of cholesterol may negatively regulate some downstream signaling events as the Ras/Erk pathway. The data are in agreement with previous results by others demonstrating that MβCD treatment of T cells strongly stimulates the CD3-mediated Ras/Erk pathway (52). The enhanced CD38- or CD3-mediated Erk activation observed after cholesterol depletion could be due to the contribution of additional factors involved in the activation of the Ras/Erk pathway in T cells. Thus, it has been demonstrated that MβCD alone causes protein kinase C-θ translocation from the cytosol to the plasma membrane, which in turn may contribute to fully activate the Ras/Erk pathway (52).

There are striking functional parallelisms between CD38 and other raft-resident components. Thus, clustering of the glycosphingolipid GM1 by cholera toxin cross-linking can stimulate signaling events that are similar to those induced by TCR stimulation and induce co-clustering of the TCR, LAT, and Lck (14, 64). Likewise, CD38 clustering by extensive cross-linking with anti-CD38 mAb and a secondary antibody induces co-clustering of the TCR at the same capping structure (29). Moreover, GM1, glycosphosphatidylinositol-anchored proteins, and CD38 require TCR-CD3 surface expression for signaling (14, 23, 24, 65, 66). Last, the raft-resident co-stimulatory molecules, including murine CD48, CD2, or CD5, cooperate with the TCR-CD3 to induce additional recruitment of raft-associated proteins and lipids to the TCR contact cap within the immune synapse (13, 67, 68). This might facilitate the stability of protein tyrosine phosphorylation through the exclusion of CD45 phosphatase activity. In this sense, we have shown that upon CD38 cross-linking, CD3-ζ becomes more resistant to detergent solubilization, which may facilitate the recruitment of signaling molecules such as ZAP-70 to the raft fraction.

The above studies suggest that ligation of raft-resident molecules induces the passive (Ab-induced) raft redistribution or coalescence facilitating the physical interaction with key signaling partners. Alternatively, individual receptors with weak raft affinity could oligomerize on ligand binding, and this would lead to an increased residency time in rafts (10, 11, 14, 49). We propose that CD38 signaling is mediated by TCR-CD3 and PTK molecules that are recruited into the same membrane raft domain induced by clustered CD38. In summary, the data of this study provide new insight into the signaling mechanism of CD38, suggesting that the signaling capacity of CD38 may derive from its ability to induce and stabilize the formation of supramolecular assemblies comprising all of the components, including the TCR-CD3 and PTKs, necessary for generating cellular responses.

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