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*J Immunol* 2011; 186:3547-3555; Prepublished online 2 February 2011;
doi: 10.4049/jimmunol.1003156
http://www.jimmunol.org/content/186/6/3547

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OX40 Complexes with Phosphoinositide 3-Kinase and Protein Kinase B (PKB) To Augment TCR-Dependent PKB Signaling

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T lymphocyte activation requires signal 1 from the TCR and signal 2 from costimulatory receptors. For long-lasting immunity, growth and survival signals imparted through the Akt/protein kinase B (PKB) pathway in activated or effector T cells are important, and these can be strongly influenced by signaling from OX40 (CD134), a member of the TNFR superfamily. In the absence of OX40, T cells do not expand efficiently to Ag, and memory formation is impaired. How most costimulatory receptors integrate their signals with those from Ag through the TCR is not clear, including whether OX40 directly recruits PKB or molecules that regulate PKB. We show that OX40 after ligation by OX40L assembled a signaling complex that contained the adapter TNFR-associated factor 2 as well as PKB and its upstream activator phosphoinositide 3-kinase (PI3K). Recruitment of PKB and PI3K were dependent on TNFR-associated factor 2 and on translocation of OX40 into detergent-insoluble membrane lipid microdomains but independent of TCR engagement. However, OX40 only resulted in strong phosphorylation and functional activation of the PI3K–PKB pathway when Ag was recognized. Therefore, OX40 primarily functions to augment PKB signaling in T cells by enhancing the amount of PI3K and PKB available to the TCR. This highlights a quantitative role of this TNFR family second signal to supplement signal 1. The Journal of Immunology, 2011, 186: 3547–3555.

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Received for publication September 22, 2010. Accepted for publication January 5, 2011.

This work was supported by National Institutes of Health Grants AI49453 and CA91837 (to M.C.).

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Abbreviations used in this article: LAT, linker of activated T cells; MCC, moth cytochrome c; MHC, major histocompatibility complex; NP-40, Nonidet P-40; PDK1, phosphoinositide-dependent kinase 1; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PIP₃, phosphatidylinositol(3,4,5)triphosphate (PIP₃). The localization of PIP₃ at the inner leaflet of the plasma membrane of T cells recruits pleckstrin homology (PH) domain containing signaling molecules, such as Akt, known also as protein kinase B (PKB) (2–4). PKB is phosphorylated at threonine 308 and serine 473, resulting in its fully active form. This can promote several cellular responses, including controlling growth, cell cycle entry, survival, and glucose metabolism. Thus, the PI3K–PKB axis is essential for clonal expansion, differentiation, and longevity of T cells (5–8).

Several costimulatory receptors in the Ig superfamily have been described to target directly PI3K and result in PKB activation. The first- and best-characterized costimulatory molecule, CD28, which is constitutively expressed on T cells, directly recruits the p85 regulatory subunit of PI3K through a pYMFM motif located in the cytoplasmic tail (9, 10). Mutational analyses of CD28 by replacing the tyrosine of the YMMN motif with phenylalanine revealed that the CD28–PI3K axis transmitted survival signals, but this was not necessary for the production of IL-2 or proliferation (11–13). ICOS (CD278), which is not constitutive on T cells but is expressed on activated effector T cells, also recruits p85α and p50α of PI3K through a pYMFM motif (14, 15). A mutational analysis in the cytoplasmic tail by replacing the tyrosine with phenylalanine showed that the ICOS–PI3K axis controlled IL-4 and IL-21 production, which is critical for follicular Th cell differentiation and humoral immunity (16, 17).

The TNFR family molecule OX40 (CD134), which is induced on a T cell after recognition of Ag, can also regulate the overall level of PI3K–PKB activity in a T cell. CD4 T cells that lacked OX40 did not sustain PI3K activity and PKB signaling over time after Ag encounter, which correlated with impaired expansion and survival of effector cells and poor generation of T cell memory (18, 19). Moreover, the phenotype exhibited by OX40-deficient T cells included defective expression of Bcl-xL, Bcl-2, Bfl-1, and survivin, and this was rescued by introducing a constitutively active form of PKB into the Ag-responding OX40⁺/− T cells (18, 19). However, in contrast to CD28 and ICOS, OX40 does not have the consensus YXXM motif in its cytoplasmic tail that might recruit PI3K. It is therefore not clear how OX40 regulates the PI3K–PKB pathway and whether the activity exhibited by OX40 is direct.

In this study, we show that engagement of OX40L expressed on an APC resulted in OX40 moving into cholesterol- and sphingolipid-rich detergent-insoluble membrane lipid microdomains (DIM) and the assembly of a signaling complex that contained TNFR-associated factor (TRAF) 2, PKB, and the p85 subunit of PI3K. The association of PKB and p85/PI3K with OX40 was preceded by recruitment of TRAF2 and was dependent on TRAF2 and movement into DIM. However, the complex containing p85/PI3K and PKB was not dependent on the TCR or Ag recognition. In contrast, the OX40 signalosome only augmented total cellular PKB phosphorylation and PKB activity when Ag was presented. Thus, OX40 can recruit PI3K and PKB, but it regulates activation through a conventional role as a true co-signal, quantitatively enhancing the TCR-initiated signal 1.
Materials and Methods

Cells and constructs

CD4 T cells from AND (Tg[TxtRyAND])53Hed × ox40−/− (tnfrsf4−/−) TCR-transgenic mice (Vβ3/Vα11) on a B10.BR-H2K-D2 H-2B1a/SgSn/1 background were activated with mtohyctochrome c (MCC) peptide (MCC 88-103), and primary effector T cells fused with a thymoma cell line, BW5147, using polyethylene glycol. A representative IL-2–producing clone was selected. N-terminally c-Myc–tagged mouse OX40 was subcloned into the pEF1 vector (Invitrogen). T hybridoma cells were transfected with control or c-Myc–OX40 vector and selected with G418 (Invitrogen). MSCV- LTRmR3-Poly(3-LMP) vector (Open Biosystems) containing the microRNA–adapted short hairpin RNA (shRNA) against mouse TRAF2 was provided by Dr. Hideki Sanjo (20). Retrovirus production was as described (19). A fibroblast DCEK cell line expressing endogenous CD80 was transfected with I-Eκ and OX40L (21). T cells (5 × 105 cells per well) were stimulated with DCEK cells (1.5 to 10 cells per milliliter) and various concentrations of TlO2S, a superagonist MCC peptide. In general, 10 μM peptide was used. For DIM depletion, T cells were preincubated in varying concentrations of the cholesterol-depleting drug methyl–β-cyclooctestrin (MβCD) for 1 h. Inhibition of sphingolipid and cholesterol bio-synthesis was performed (22) culturing in serum-containing complete medium for 48 h in the presence of 25 μM myriocin and then serum-starved for 14 h in the presence of 5 μM zaragozic acid and 25 μM myriocin.

Peptides, chemicals, and Abs

TlO2S (aa 88–103; ANERADLQIYKQASK) was synthesized by Abgent (San Diego, CA). LY294002 (no. 440202) was from Calbiochem. Sucrose (no. 84097), MβCD (C555), HRP-conjugated cholera toxin B (C3741), myriocin (M1177), and zaragozic acid (Z2626) were from Sigma. Anti–c-Myc (R950-25) and Dynabeads Protein G (100.04D) were from Invitrogen. Brij-58 (no. 28356), Nonidet P-40 (NP-40; no. 28324), and n-dodecyl-β-maltoside (no. 89903) were from Thermo Scientific. Protein G Sepharose (17-0618-01) was from GE Healthcare. Anti-CD80 (16-10A1, no. 104710) and anti-OX40 (OX86, no. 119408) were from Biolegend. Anti–c-Myc (9B11, no. 2233 and no. 2276), anti–p-Erk (no. 9101), anti–p-PI3K p85 (no. 84097), M peptide was used. For DIM depletion, T cells were preincubated in 50 μM myriocin and then serum-starved for 14 h in the presence of 25 μM myriocin and 25 μM myriocin.

IL-2 production

IL-2 was measured by ELISA with Abs JES6-1A12 (no. 554424) and biotin-JES6-5H4 (no. 554426), from BD Pharmingen.

Western blotting

For total lysates, cells were lysed in ice-cold RIPA buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM Na3VO4) containing protease inhibitor mixture for 30 min. Cells were disrupted by passages (10 times) through a 22-gauge needle on a 1-mL syringe. Lysates were centrifuged at 1000 × g for 10 min at 4°C, and supernatants were mixed with 1 μL 80% v/v sucrose in the same lysis buffer and overlaid with 2 mL 30% sucrose followed by 1 mL 5% sucrose. Samples were ultracentrifuged in a Beckman SW50Ti rotor at 200,000 × g for 16 h at 4°C. Twelve fractions (0.4 mL fraction) were collected from the top of the gradient. For visualization of GM1, fractions were dot blotted on a nitrocellulose membrane and detected using HRP-conjugated cholera toxin B. Proteins from each fraction were precipitated with TCA before immunoblotting. Alternatively, the DIM fractions were prepared by a two-step separation method. Postnuclear lysates in 1% Brij-58 lysis buffer were prepared and ultracentrifuged at 100,000 × g for 50 min at 4°C. Cell pellets were collected and dissolved in 1% SDS containing ice-cold RIPA buffer. After vortexing for 60 min at 4°C, the supernatants containing extracted DIM proteins were recovered by centrifugation at 13,000 rpm for 10 min at 4°C.

Immunoprecipitation

Cells were lysed in ice-cold RIPA or 1% n-dodecyl-β-maltoside for 30 min. Cells were disrupted by passage (30 times) through a 22-gauge needle on a 1-mL syringe. Lysates were centrifuged at 1000 × g for 10 min at 4°C to remove insoluble material, followed by preclearing with Protein G Sepharose beads for 30 min at 4°C. Lysates were immunoprecipitated with primary Abs, c-Myc (9B11), and TRAF2 (M112-3), and protein G beads at 4°C for overnight. After washing with RIPA or 1% n-dodecyl-β-maltoside, beads were incubated at 70°C in 4× LDS sample buffer for 10 min. After collection of immunoprecipitated lysates from beads and dilution with ultrapure water, those samples in 2× LDS buffer were further reduced with DTT at 70°C for 10 min for immunoblotting.

Results

OX40 co-signal supports TCR-dependent PKB activation

Using OX40-deficient TCR-transgenic T cell systems, we previously demonstrated in Ag-responder effecter T cells that OX40 was required for optimal activation of the PI3K–PKB pathway, and this coordinately regulated T cell proliferation, survival, and cytokine secretion (18). To understand how OX40 regulates PI3K–PKB, we established an MCC peptide-specific T cell hybridoma, derived from effecter T cells obtained from OX40-deficient and Vo11/Vβ3 TCR-transgenic mice. These T cells were then transfected with a c-Myc–OX40 construct to allow OX40 to be efficiently immunoprecipitated via the Myc tag. These T cells were stimulated with a fibroblast APC that expresses B7 (CD80) and OX40L and that can present MCC peptide on L-E12 (21). Without Ag, the T cells did not produce any IL-2 even in the presence of OX40–OX40L and CD28–CD80 interactions (Fig. 1A). OX40 ligation strongly promoted IL-2 when Ag was presented. Some of this was independent and a proportion dependent on CD28, shown by blocking CD80. In contrast, Ag and CD28 signals resulted in only modest IL-2 production (Fig. 1A). In Jurkat human T cell lines, IL-2 production induced by the TCR and CD28 is insensitive to the PI3K inhibitors wortmannin and LY294002, in part due to loss of expression of phosphatase and tensin homologue (23–25). In our murine T cell hybridoma, LY294002 suppressed Ag- and OX40L-dependent IL-2 production in a dose-dependent manner (Fig. 1B), indicating PI3K activity. In accordance with this, when the p85 subunit of PI3K was immunoprecipitated after Ag and OX40L stimulation from APCs, we found that it was strongly tyrosine phosphorylated (Fig. 1C). Furthermore, PIP3 was elevated in Ag/OX40L-stimulated T cells (Fig. 1D).

During initial T cell activation in the presence of Ag, phosphorylation of PKB (T308 and S473) and p85 (Y458) was induced...
in OX40-deficient cells (control vector), but these activities were strongly upregulated and maintained at later times by engagement of OX40 with OX40L (c-Myc–OX40 T cells) (Fig. 1E). Similar to our previous results (18), when primary effector CD4 T cells were stimulated with Ag, phosphorylation of T308 and S473 on PKB was severely impaired over time in the absence of OX40 (Fig. 1F), showing endogenous OX40 provides the same signals as transfected OX40 in the hybridoma cells. Consistent with data in Fig. 1B, LY294002 completely inhibited PKB phosphorylation mediated by Ag and OX40L in the hybridoma (Fig. 1G). Thus, PI3K controls PKB activation, and OX40 contributes strongly to activation of this PI3K–PKB pathway when Ag is recognized.

To address the role of the TCR, cells were stimulated without Ag. OX40–OX40L interactions alone could not induce efficient phosphorylation of PI3K and PKB (Fig. 1C and 1H, respectively). This indicates that OX40 facilitates TCR-dependent PI3K ac-
tivation and resulting PKB phosphorylation. Phosphorylation of Erk was markedly induced by Ag but unaffected by OX40 signals, demonstrating some selectivity (Fig. 1E–H). Therefore, OX40 represents a dominant stimulus for promoting the PI3K–PKB axis in effector-like T cells but does so only as a co-signal to augment Ag/TCR-induced activity.

**OX40 recruits TRAF2, PI3K, and PKB**

To understand how OX40 augments TCR-dependent PKB activation, OX40 was immunoprecipitated via the c-Myc tag, and associated proteins were examined by immunoblotting. The adapter protein TRAF2 has been shown to associate with OX40 (26, 27). Before stimulation with OX40L expressed on APC, OX40 did not appreciably interact with TRAF2 or other signaling molecules. After stimulation with OX40L, OX40 was strongly associated with endogenously expressed TRAF2 (Fig. 2A–C). Moreover, p85 and PKB were found within this immunoprecipitate (Fig. 2A–C). In cultures where T cell–APC contact was not forced and likely increased over time, prolonged complex formation was observed (Fig. 2A); however, 10-min stimulation was sufficient to detect the

**FIGURE 2.** OX40 recruits PI3K, PKB, and PDK1, independent of Ag. A–E, c-Myc–OX40 or control vector T cells were stimulated with B7+ OX40L+ APC, with or without Ag, as in Fig. 1 (A–C, E) or were stimulated with an anti-OX40 agonistic Ab (D). Cells were lysed in RIPA buffer containing NP-40 (A–E) or in dodecyl-maltoside (E), and OX40 was immunoprecipitated. Samples were analyzed for the indicated proteins at 0–3 h (A), 3 h (B, D, E), or 10 min (C). The asterisk in E shows H chain bands. F, c-Myc–OX40 T cells were stimulated with APC with or without Ag for 1 h and lysates immunoprecipitated with anti-phospho-serine/threonine–PDK1 docking motif mAb. Bef. Stim. represents OX40 immunoprecipitated from unstimulated T cells. All results are representative of at least two experiments.
complex (Fig. 2C). Furthermore, it was not dependent on TCR engagement, occurring regardless of peptide presentation (Fig. 2B). The complex was also induced with an agonist Ab to OX40 in the absence of APC (Fig. 2D). We evaluated any possible recruitment of the TCR, CD3, or CD28, but failed to detect these molecules and to detect the proximal kinases, ZAP70, Lck, or SLP-76 in the immunoprecipitates (data not shown), suggesting that OX40 forms a unique signalosome separate from the TCR and CD28 signalosomes.

Notably, a low level of phosphorylated p85, and serine-, but not threonine-, phosphorylated PKB was found in the OX40 signalosome, and again this was also Ag-independent (Fig. 2B). Activation of PI3K induces relocalization of phosphoinositide-dependent kinase 1 (PDK1) to the phospholipid-enriched plasma membrane. PDK1 is activated on the membrane and phosphorylates substrate kinases such as PKB. We then investigated whether PDK1 was recruited to OX40. We did not find PDK1 in the standard immunoprecipitation conditions using RIPA, but when n-dodecyl-β-maltoside was used, a detergent that preserves membrane protein structure, we found a low level of PDK1 (bands of 58–68 kDa) precipitated after OX40 was ligated by OX40L (Fig. 2E). Again, this was independent of TCR engagement (Fig. 2E). This implied that the small amount of phosphorylated PKB present in the Ag-independent OX40 signaling complex was likely due to this minor amount of recruited PDK1. We also immunoprecipitated PDK1 but failed to detect OX40 (data not shown), also suggesting that the quantity of total cellular PDK1 that complexes with OX40 is small. As shown before, the majority of total cellular phosphorylated p85 and both serine- and threonine-phosphorylated PKB were dependent on Ag recognition (Fig. 1C, 1H). This further implied that PDK1 that might promote this activity was primarily engaged by TCR signaling, suggesting that the role of OX40 is to recruit both PI3K and PKB and make them available for further or continued phosphorylation when the TCR is engaged. To correlate this with the activity of PDK1, T cell lysates were prepared before and after stimulation with Ag/OX40L, and proteins were immunoprecipitated with mAb specific for phosphorylated substrate peptide sequences of PDK1 (PDK1 docking motifs). As demonstrated in Fig. 2F (upper panel), Ag/OX40L stimulation induced strong phosphorylation in an ~60 kDa cytoplasmic protein, but without an Ag signal, this was marginal. The protein was found to be PKB (Fig. 2F, middle panel). Thus, OX40 ligation by OX40L in the context of Ag presentation likely provides increased amounts of PKB that can then be targeted by PDK1 activity that primarily results from engagement of the TCR.

TRAF2 plays a critical role for PKB activation by OX40

An important question is how OX40 recruits PKB. By immunoprecipitating TRAF2, we found that both OX40 and PKB were pulled down, but only after OX40–OX40L interaction (Fig. 3A). This correlates with some studies that have suggested that certain

![Figure 3](http://www.jimmunol.org)
members of the TRAF family adapter proteins could directly or indirectly interact with PKB (1, 28). To investigate the functional importance of TRAF2 to the signalosome, expression was reduced by treatment with short hairpin RNA (shRNA) in c-Myc–OX40 T cells. Approximately 70% loss of TRAF2 was observed, but OX40 and c-Myc expression were unaltered (Fig. 3C, 3D). Inhibiting TRAF2 expression blocked IL-2 production in response to Ag presented on B7/OX40L APC, almost to the level secreted by OX40-negative T cells (Fig. 3B). In parallel, activation of total cellular PKB was impaired after Ag and OX40L stimulation (Fig. 3C). Correspondingly, the association of OX40 with PKB was severely reduced when TRAF2 was knocked down (Fig. 3D). Unlike CD28 and ICOS, OX40 does not contain consensus phospho-tyrosine motifs in the cytoplasmic tail to interact with PI3K (29). Notably, the association of OX40 with p85 was also strongly reduced when TRAF2 was knocked down (Fig. 3D), suggesting that TRAF2 directly or indirectly plays a pivotal role for recruitment of PI3K as well as PKB.

Detergent-insoluble lipid microdomains are essential for PKB activation

An important step in PKB activation is its translocation from the cytosol to the plasma membrane. We then focused on DIMs that might be necessary for efficient intracellular signaling (22, 30). Ag/APC-stimulated c-Myc–OX40 T cells were lysed in buffer containing Brij-58, a weak nonionic detergent that preserves DIM, and analyzed after sucrose gradient ultracentrifugation. Activation with Ag/OX40L induced strong translocation of OX40 into the DIM (Fig. 4A). The levels initially decreased to ~60% of the starting level within 1 h then further increased up to ~300% at 3–4 h (data not shown). OX40 moving into DIM was accompanied by TRAF2 (Fig. 4A). A proportion of p85, PKB, and PDK1 was expressed in the DIM before and after T cell stimulation suggesting these molecules were available to OX40 that moved into this compartment. Importantly, OX40 that translocated into the DIM associated with TRAF2, p85, and PKB (Fig. 4B), implying

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** OX40 translocation into DIM is associated with activation of PKB. A and B, c-Myc–OX40 T cells were stimulated with APC in the presence of Ag for 3 h. A, Fractions from sucrose gradient of lysates of unstimulated (0 h) and stimulated T cells were immunoblotted. LAT and GM1 are markers of the DIM. CD71 (transferrin receptor) represents a non-DIM marker. B, OX40 was immunoprecipitated from pooled fractions 1–5 in A. C, Control vector and c-Myc–OX40 T cells were stimulated with APC in the presence of Ag for 30 min as in A. The DIM and non-DIM (soluble) fractions were obtained by a two-step separation method, and protein levels were evaluated by immunoblotting. Results are representative of at least two experiments.
that the DIM environment is important to initiate the PI3K–PKB pathway. After stimulation, the DIM fractions recovered from Brij-58-insoluble pellets of c-Myc–OX40 T cells contained phosphorylated PKB, but this was dependent on Ag recognition and not seen after OX40L interaction in the absence of Ag (Fig. 4C and data not shown). These results suggested that OX40 might recruit PKB into its signaling complex in the DIM, which then allows PKB to be available for phosphorylation triggered by TCR-related signaling events.

To investigate the importance of the membrane microdomain environment, the cholesterol-depleting drug MβCD was used. Cholesterol depletion completely blocked Ag and OX40L-dependent IL-2 production (Fig. 5A), and correlating with this, it strongly inhibited phosphorylation of PKB (Fig. 5B). Suppression of sphingolipid and cholesterol synthesis, by the inhibitors myriocin and zaragozic acid, respectively, which also cause DIM disruption, similarly resulted in impaired PKB phosphorylation (Fig. 5C). Importantly, OX40 association with TRAF2 was not altered after DIM disruption, whereas that with p85 and PKB was essentially abolished (Fig. 5D). Thus, OX40 recruits TRAF2 outside the DIM, and then p85 and PKB are recruited into the DIM-resident OX40–TRAF2 complex. The DIM therefore serves as an important platform for OX40 to integrate with the TCR to enhance downstream PKB activation.

**Discussion**

In this study, we show that the TNFR family member OX40 forms a signaling complex that contains PI3K and PKB. Recruitment of these molecules is dependent on TRAF2 and occurs in detergent-insoluble membrane lipid microdomains after OX40 and TRAF2 translocate into this cellular compartment driven by ligation of OX40 by OX40L. We show this is a key event that strongly augments Ag/TCR-dependent activation of the PI3K–PKB pathway.

Physiological interactions between T cells and Ag-bearing APCs promote increased PIP3 in the T cell inner membrane over many hours (2, 3). It is known that after the TCR is cross-linked, PI3K generates PIP3 through phosphorylation of phosphatidylinositol 4,5-biphosphate. The PH domain of PKB recognizes PIP3, triggering PKB recruitment from the cytosol to the plasma membrane. PKB then can undergo phosphorylation on Thr308 and Ser473, in a process that is not completely understood but has been suggested to involve PDK1 and a second kinase called PDK2. PDK2 has been proposed to be the mammalian target of rapamycin complex 2 (22, 31). The two phosphorylation events on PKB promote its release into the cytosol, thereby allowing phosphorylation of downstream targets, of which there are many possible including GSK3, FoxO1, TSC, IKK, and Bad.

Although signal 1 from the TCR is critical for production of PIP3 and activation of the kinases at early time points, it has not been clear how kinase activation is maximized or maintained for long periods. CD28 has been known for many years to be distributed in the DIM (32–34), and recent studies showed that ligation of several receptors including CD28 resulted in accumulation of PIP3 in the plasma membrane and formation of functional hot spots in what were termed “raft nanodomains” that concentrated PKB in regions of the DIM and allowed sustained activation of the PI3K–PKB pathway (22). Furthermore, results from Saito and colleagues (35) and Davis and colleagues (36) demonstrated that TCR-initiated signaling occurred in “microclusters” or “islands” in the plasma membrane, and such spatially and temporally regulated protein structures were critical for T cell activation. These microclusters or islands were either found to be preformed or to be induced by TCR ligation and contained the TCR and several kinases and adapters, namely ZAP70, SLP-76, and LAT. After Ag recognition, they were also shown to link transiently or aggregate together. Collectively, these results promote the hypothesis that signal 2 from costimulatory receptors might support signal 1 by recruiting or maintaining a high concentration of PI3K and PKB in the vicinity of the TCR that is located in these membrane microclusters or nanodomains after peptide recognition. Our previous results (18), combined with our current results in this study, also could be interpreted into this model. We demonstrated that OX40 signals in the presence of Ag maximized and prolonged activation of PKB in a DIM-dependent fashion and that OX40 associates in a complex with both PI3K and PKB. However, OX40 did not appreciably or strongly affect the total amount of PI3K or PKB in the DIM. Therefore, it is likely that OX40 acts in very specific regions of the DIM and the primary role of OX40 is to promote highly concentrated depots of these molecules either in,
or close to, the TCR microclusters/nanodomains. Although this needs to be investigated in the future, OX40 may therefore increase the amount of PI3K and PKB available to these units.

Several other TNFR family members can activate the PI3K–PKB pathway. However, the proximal steps that are used by TNFRs to recruit or lead to activation of PI3K or PKB might vary and are not fully understood. Receptor activator for NF-kB (CD265), after binding receptor activator for NF-kB ligand (CD254), has been described to activate PI3K through recruiting TRAF6, Cbl-b, and PI3K into a complex, in a mechanism dependent on the kinase activity of c-Src (37, 38). CD40 induces recruitment of PI3K and c-Cbl (38), and deficiencies in Cbl-b, c-Cbl, and TRAF6 abrogated CD40L-dependent PKB phosphorylation (38, 39). TNF can also activate PI3K and PKB (40, 41). TRIFR1 constitutively forms a complex with PI3K, c-Src, and Jak2, and the tyrosine kinase activities of both c-Src and Jak2 were found to be important for activation of the PI3K–PKB pathway (41). Furthermore, TRAILR has been shown to interact with c-Src, C-cbl, and PI3K, with again c-Src activity being critical for TRAILR-dependent PI3K and PKB activation (42, 43). Our study shows that OX40 recruits PI3K and PKB in a manner dependent on TRAF2, but whether TRAF2 directly interacts with these molecules is not clear. c-Src appears to be common to the aforementioned receptors, but we failed to detect c-Src in the OX40 signalosome. Critical regulators for TRAF–PKB activation (42, 43). Our study shows that OX40 recruits PI3K and PKB after TNFR family engagement in T cells might be ubiquitin ligases in the TRAF family as well as Cbl. It has recently been reported that PKB undergoes lysine (K) 63-linked ubiquitination at K8 and K14 within its PH domain by TRAF6, and this can regulate PKB membrane recruitment and phosphorylation (28). We have not found TRAF6 in the OX40 signaling complex, but OX40L interaction with OX40 resulted in ubiquitination of OX40, which was also sensitive to MJMD treatment (T. So and M. Croft, unpublished observations), suggesting that this DIM-dependent ubiquitination event might also play a functional role for enhancing the molecular association of OX40 with p85 and PKB. Further experiments are then needed to understand the role of ubiquitination and whether other molecules might associate with the OX40 signalosome within the DIM to allow recruitment and activation of PI3K and PKB.

In summary, our results shed light on the mechanism by which the T cell costimulatory receptor OX40 promotes PKB activation. The data add to our knowledge of the molecular mechanisms by which ligation of OX40 amplifies TCR-driven T cell activation and provide further information that in part explains why OX40 is an important regulator of effector T cells.

Acknowledgments

We thank Dr. Jianxun Song and Dr. Hideki Sanjo for the DNA constructs. This is manuscript no. 1302 from the La Jolla Institute for Allergy and Immunology.

Disclosures

The authors have no financial conflicts of interest.


