TcR and TcR-CD28 Engagement of Protein Kinase B (PKB/AKT) and Glycogen Synthase Kinase-3 (GSK-3) Operates Independently of Guanine Nucleotide Exchange Factor VAV-1

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TcR/CD3 and TcR/CD3-CD28 signaling requires the guanine nucleotide exchange factor (GEF) Vav-1 as well as the activation of phosphatidylinositol 3-kinase, protein kinase B (PKB/AKT), and its inactivation of glycogen synthase kinase-3 (GSK-3). Whether these two pathways are connected or operate independently of each other in T-cells has been unclear. Here, we report that anti-CD3 and anti-CD3/CD28 can induce PKB and GSK-3α phosphorylation in the Vav−/− Jurkat cell line J. Vav.1 and in primary CD4-positive Vav−/− T-cells. Reduced GSK-3α phosphorylation was observed in Vav−/−,2−,3−/− T-cells together with a complete loss of FOXO1 phosphorylation. Furthermore, PKB and GSK-3 phosphorylation was unperturbed in the presence of GEF-inactive Vav-1 that inhibited interleukin-2 gene activation and a form of Src homology 2 domain-containing lymphocytic protein of 76-kDa (SLP-76) (35, 36). These residues each reside within a YESP motif, both of which are phosphorylated upon receptor ligation. The Dbl homology (DH) domain has

T-cell activation is induced by ligation of the antigen-receptor (TcR/CD3) as well as co-receptors such as CD28. TcR/CD3 and CD4/CD8-lck initiate tyrosine phosphorylation, while TcR/CD3 and CD28 initiate the production of D-3 lipids (1, 2). CD28 co-signals are needed for optimal cytokine production, proliferation, and effector function (3, 4). CD28-deficient mice have reduced responses to antigen, highlighting the capacity of CD28 to lower the threshold of signaling (5). Primary responses exhibit more of a dependence on CD28 than do secondary responses, and the co-receptor can influence the differentiation of T helper 2 (Th2) versus T helper 1 (Th1) cell, increase cell survival, and prevent the induction of T-cell anergy (3, 6, 7).

The molecular basis of TcR/CD3 and CD28 signaling in T-cells has been the subject of much investigation. TcR/CD3 engagement with CD4/CD8-p56lck leads to the recruitment of ZAP-70 and the phosphorylation of multiple adaptors (8, 9). TcR and CD28 ligation can increase the expression of lipid rafts or microdomains (10, 11). Furthermore, CD28 can be distinguished from the TcR by virtue of the fact that it directly interacts with phosphatidylinositol 3-kinase (PI3K)2 by means of classic p85 Src homology 2 (SH2) domain binding to a cytoplasmic YMNFM motif (12–14). Additional proline residues of CD28 mediate supplemental binding to the Src homology 3 (SH3) domains of Grb-2 and p56lck (15–16). Mutations in both sets of residues attenuate CD28-mediated signaling (16–20).

In vivo reconstitution studies have provided mixed results in the role of the YMNFM motif in interleukin-2 (IL-2) production and graft versus host responses (17, 18, 21).

The production of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate lipids by PI3K leads to the membrane recruitment and activation of protein kinase B (PKB/AKT) that in turn can phosphorylate and inactivate glycogen synthase kinase-3 (GSK-3) (22, 23). In resting T-cells, GSK-3 negatively regulates IL-2 transcription by phosphorylating nuclear factor of activated T-cells (NFATs), leading to its translocation from the nucleus (24, 25). CD28 ligation can potentiate PKB and/or GSK-3 phosphorylation (26, 27). In this context, PKB has been implicated in the regulation of IL-2 production but not Th2 cytokines (28). PI3K-PKB also regulate Fas-mediated apoptosis (29), glucose uptake, and glycolysis in T-cells (30).

In addition to activating the PI3K pathway, TcR/CD3 and CD28 signaling is dependent on the guanine nucleotide exchange factor, Vav-1. It has a calponin homology domain, an acidic motif, a zinc finger-like region, two SH3 domains, and a SH2 domain (31–34). The SH2 domain of Vav-1 binds tyrosine residues within the adaptor SH2 domain-containing lymphocytic protein of 76 kDa (SLP-76) (35, 36). These residues each reside within a YESP motif, both of which are phosphorylated upon receptor ligation. The Dbl homology (DH) domain has

2 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; SH, Src homology; IL, interleukin; PKB, protein kinase B; GSK, glycogen synthase kinase-3; NFAT, nuclear factor of activated T-cell; DH, Dbl homology; JNK, c-Jun N-terminal kinase; WT, wild-type; FACS, fluorescence-activated cell sorter; RmM, rabbit anti-mouse; RbH, rabbit anti-hamster; GEF, guanine nucleotide exchange factor; FITC, fluorescein isothiocyanate.
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GEF activity for the activation of the small GTPases Rac1 and Cdc42 (37). Vav-1-deficient T-cells show defects in TcR coupling and the induction of cytokine production (38, 39). Phosphorylation of Vav-1 by CD28 depends on residues 173–181 of the receptor (15, 40). CD28 has been reported to boost TcR signaling via Vav-1-SLP-76 cooperation (41). Furthermore, co-expression can drive NFAT translocation into the nucleus of COS cells in response to CD28 ligation (36). In addition, CD28 engagement can activate IkB kinase (IKK) complex and NF-κB activation in a Vav-1 dependent fashion (42). In keeping this, confocal microscopy has shown that endogenous VAV-1 and IKKα co-localize in response to CD28 stimulation (42). Vav-1 has been reported to cooperate with protein kinase C theta to activate c-Jun kinase (JNK) (43), while Vav-1-deficient Jurkat cells (termed J.Vav1) show defects in Ca2+ mobilization as well as in the activation of JNK and transcription factors needed for interleukin-2 transcription (44).

Given that TcR and TcR-CD28 generate signals that depend on Vav-1 and PI3K, an important question is whether these pathways are interconnected or operate independently of each other. Observations have been mixed in other systems. Vav-1 GEF activity has been reported to be dependent on inositol lipids due to pleckstrin homology domain binding and localization (45). By contrast, inhibition of PI3K does not inhibit Vav-3 phosphorylation in Vav-3-deficient cells (46). Other studies have reported a partial or full role for Vav-1 in activating PI3K (47–49). BcR activation of protein kinase B phosphorylation occurs normally in Vav-1-deficient B-cells (49).

In this study, we report that anti-CD3 and anti-CD3/CD28 can induce PKB and GSK-3 phosphorylation in the Vav-1−/− Jurkat cell line J.Vav.1 and in primary CD4-positive Vav-1−/− T-cells. Reduced but significant GSK-3 phosphorylation was also observed in Vav-1,2,3−/− T-cells, despite a complete loss of FOXO1 phosphorylation. Furthermore, PKB and GSK-3 phosphorylation was unperturbed in the presence of a GEF inactive form of Vav-1, a mutant that markedly inhibits IL-2 gene activation. Wild-type levels of phosphorylation were also observed in the presence of a form of SLP-76 that is defective in mobilization as well as in the activation of JNK and transcription factors needed for interleukin-2 transcription (44).

Materials and Methods

Cells and Reagents—The Jurkat T-cell line was maintained using standard cell culture techniques. The J.Vav1 Jurkat cell line (clone 15–11 (Vav-1+/+) and clone 14-9 (Vav-1−/−)) were a kind gift from Dr. Robert Abraham, Burnham Institute, La Jolla, CA. WT and Vav-1−/− BALB/c mice were a kind gift from Dr. Victor Tybulewicz, National Institute for Medical Research, London, UK. Vav-1,2,3−/− B10.BR mice were kindly provided by Dr. Martin Turner, Babraham Institute, Cambridge, UK. CD4+ T-cells were purified from spleenocytes freshly isolated from WT and Vav-1−/− BALB/c mice using Mouse CD4 (L, T), Dynabeads (Dynal Biotech, UK). This technique resulted in the purification of a population of T-cells that were greater than 95% positive for CD4 as determined by FACS staining. Splenocytes were used for the analysis of the Vav-1,2,3−/− mice.

Cytochalasin D was purchased from Calbiochem (UK); SP600125 (JNK inhibitor) was purchased from Tocris (UK). Antibodies anti-human CD3 (OKT3) (American Type Culture Collection hybridoma) (2 μg/ml) and anti-human CD28 (9.3) (Bristol Meyers Squibb) (8 μg/ml) were cross-linked with rabbit anti-mouse (RoM) antibody (Southern Biotechnology), at concentrations that were half the sum of total primary stimulatory antibodies. For control RoM-only stimulations, 5 μg/ml was used. Anti-mouse CD3 (2C11) (2 μg/ml) and anti-mouse CD28 (PV-1) (8 μg/ml) from BioExpress were cross-linked with rabbit anti-hamster (RhH) antibody (Sigma, UK), at concentrations that were half the sum of total primary stimulatory antibodies or 5 μg/ml for the RhH-only control stimulations.

Plasmids—pCMV3-myc-hVav (WT) and pCMV3-hVav-L213Q (GEF-inactive) (Martin Turner, Babraham Institute), 3×NFAT-AP1-Luc reporter construct (Steve Burakoff, Dana-Faber Institute of Cancer, Boston, MA), wild-type SLP-76 and Y113F (Paul Findell, Roche Biosciences, Palo Alto, CA) were cloned into SIα.

Transfection—For transfections, 50 × 10^6 Jurkat cells were co-transfected with 50 μg of the indicated constructs and 25 μg of 3xNFAT-AP1-Luc reporter, using a BTX PrecisionPulse electroporator (250 V, 800 μF, and 200 Ω). Cells were incubated for 18 h in RPMI 1640 medium (Invitrogen) containing 10% fetal calf serum at 37 °C, prior to stimulation.

 Luciferase Assay—For the luciferase assays, 0.5 × 10^6 Jurkat cells were incubated in 100 μl of RPMI 1640 medium containing 5% fetal calf serum plus the appropriate antibodies for 6 h at 37 °C in a 96-well plate. Cells were lysed, and luciferase activity was determined using a Luminat LB9507 luminometer (EG&G Berthold) and the luciferase assay system protocol from Promega.

Analysis of GSK-3 and PKB Phosphorylation—1 × 10^6 Jurkat cells (standard or the J.Vav1 Jurkat cell line) or 3 × 10^6 CD4+ T-cells purified from spleens of WT or Vav-1−/− BALB/c mice were suspended in RPMI 1640 medium containing 2% fetal calf serum. Cells were incubated on a rotor with the appropriate primary stimulatory antibodies at 4 °C for 30 min and then incubated with cross-linking antibody (RoM or RhH) at 37 °C for the indicated times. Stimulations were terminated by addition of cold RPMI 1640 medium. Whole cells were pelleted, boiled in 3× SDS sample buffer, and proteins were separated by SDS-PAGE. The following antibodies were used in immuno-blotting: anti-phospho-GSK-3α/β (serines 21/9) (Cell Signaling Technology) (1:1000), anti-GSK-3α/β (BIOSOURCE) (1:1000), anti-Vav-1 (Upstate Biotechnologies) (1:1000), anti-SLP-76 (hybridoma from American Type Culture Collection) (1:1000), anti-phospho-PKB (Thr-308) (Cell Signaling Technology) (1:1000), anti-PKB (Cell Signaling Technology) (1:1000), anti-phospho-FOXO1 (Cell Signaling Technology) (1:1000), and anti-mouse/rabbit horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) (1:5000). Proteins were visualized using the enhanced chemiluminescence (ECL) system (Amersham Biosciences). Quantification of GSK-3α and PKB phosphorylation was done using ImageJ (NIH) densitometry software. Readings were expressed as a percent of the phosphorylated band of maximum intensity. Phosphorylation levels were normalized to total protein.
expression levels by plotting the ratio (percent phosphorylation)/(% total) × 100.

Anti-CD3 Surface Clustering—Jurkat cells were either untreated or preincubated with the indicated concentration of cytochalasin D for 1 h. Cells were then resuspended in 1 ml cold FACS buffer (1% bovine serum albumin and 0.01% sodium azide in phosphate-buffered saline, pH 7.0) and incubated with 2 μg/ml anti-CD3 (OKT3) for 30 min at 4 °C.
Cells were then washed twice with cold (0 h samples) or prewarmed (1-h samples) FACS buffer and FITC-conjugated anti-mouse IgG (Sigma) was added, followed by incubation immediately on ice or at 37 °C for 1 h. Cold FACS buffer was added to terminate the stimulations, and cells were fixed with 2% paraformaldehyde and then mounted on coverslips. TcR distribution was visualized by fluorescence microscopy. At least 200 T-cells were counted for TcR cap formation in each experiment.

F-actin Content—Jurkat cells were treated as above except RαM was used to cross-link anti-CD3 (OKT3) for 1 h at 37 °C. Cold FACS buffer was added to terminate the stimulations, and cells were fixed with 2% paraformaldehyde and then mounted on coverslips. TcR distribution was visualized by fluorescence microscopy.

RESULTS

To investigate the relationship between the PI3K and Vav-1 pathways in response to TcR and TcR-CD28 ligation, we initially wanted to confirm that anti-CD3 and anti-CD3/CD28 induced PKB and GSK-3 phosphorylation (Fig. 1). Anti-CD3, anti-CD28, and anti-CD3/CD28 antibodies were used for cross-linking of receptors on Jurkat cells for various times at 37 °C. This was followed by immunoblotting of cell lysates with a monoclonal antibody to phosphorylated Thr-308 of PKB (upper panels) or to phosphorylated Ser-21 and Ser-9 sites on GSK-3α/H9251 and -β/H9252, respectively (middle panels). Anti-CD3 increased PKB phosphorylation by 5 min relative to the RαM control (Fig. 1, upper panel, lane 2 versus lane 1). Phosphorylation was enhanced with anti-CD28 co-ligation (lane 4 versus lane 2). Anti-CD28 alone also induced PKB phosphorylation to levels as high or higher than observed with anti-CD3 (lane 3 versus lane 2). Blotting with an antibody to non-phosphorylated PKB confirmed equal levels of protein expression (lower upper panel, lanes 1–16). In terms of the kinetics, phosphorylation was rapid with the majority of phosphorylation having occurred by 5 min (lanes 2–4 versus lanes 5–16). The RαM control lane at 5, 15, 30, and 60 min was indicative of phosphorylation levels at time 0.

Similar results were obtained with anti-GSK-3α/β blotting (Fig. 1, middle panels). Of the two GSK-3 isoforms, GSK-3α was
preferentially phosphorylated relative to the GSK-3β isoform in T-cells. As with PKB phosphorylation, anti-CD3 increased GSK-3α phosphorylation relative to the RαM control (middle panel, lane 2 versus lane 1). This phosphorylation was increased with anti-CD28 co-ligation (lane 4 versus lane 2). Anti-CD28 also induced GSK-3α phosphorylation (lane 3). Blotting using an antibody against non-phosphorylated GSK-3α/β showed the level of protein expression (lanes 1–16). The observations confirm that anti-CD3, anti-CD28, and anti-CD3/CD28 induce rapid phosphorylation of PKB and GSK-3α in T-cells.

To assess whether Vav-1 expression was required for PKB and GSK-3α phosphorylation, blotting was conducted in the Vav-1-deficient Jurkat cell line J.Vav.1 (Fig. 1) (44). Anti-CD3, -CD28, and -CD3/CD28 induced levels of PKB or GSK-3α phosphorylation in Vav-1−/− cells that were comparable with that observed in WT cells (upper and middle panels, lanes 17–32 versus lanes 1–16). Densitometric readings of the phosphorylated bands documented similar levels of increased phosphorylation (histograms in the lower panels). The absence of Vav-1 was confirmed by anti-Vav-1 blotting (lowest panel, lanes 17–32 versus lanes 1–16). These data show that anti-CD3 and anti-CD3/CD28 induction of PKB and GSK-3α phosphorylation in T-cells can occur independently of Vav-1 expression. The independence of PKB/GSK-3 phosphorylation on Vav-1 expression contrasts with the previously reported defects in Ca2+ and JNK signaling in the same cells (44).

To assess whether this pathway was intact in freshly isolated primary Vav-1−/− T-cells, we also assessed PKB and GSK-3 phosphorylation in CD4+ T-cells purified from spleens of WT or Vav-1−/− BALB/c mice. Cells were stimulated for 30 min with anti-CD3, anti-CD28, or anti-CD3/CD28 followed by measurement of PKB (Fig. 2, upper panels, lanes 1–4 versus lanes 5–8) and GSK-3α phosphorylation (middle panels, lanes 1–4 versus lanes 5–8). The absence of Vav-1 was confirmed by blotting (lowest panel, lanes 5–8 versus lanes 1–4). The experiments showed no difference in the levels of phosphorylation. Occa-
sionally, an experiment showed a slight reduction in PKB and GSK-3 phosphorylation in Vav-1−/− T-cells; however, the reduction was never more than 10% less compared with WT cells. By contrast, Vav-1,2,3-deficient T-cells showed a consistent partial loss of GSK-3 phosphorylation (lower panels). A 40–50% reduction in phosphorylation was generally observed. This indicates that GSK-3 phosphorylation is partially dependent on Vav family members and that there is a second pathway that is Vav independent. Interestingly, this partial effect contrasted with the transcription factor FOXO1 where phospho-
CD28 also induced similar levels of phosphorylation in vector, level of total PKB and GSK-3 mutant. Anti-CD3 induced phosphorylation of PKB and GSK-3 activates the GEF activity of the DH domain. Jurkat cells were transcribed occurs independently of the PKB-GSK-3 pathway.

Vav-1 has also been shown to interact with the adaptor protein SLP-76 (50–52), an interaction mediated by the Vav-1 SH2 domain binding to two specific YESP sites on SLP-76 (52). Previous studies have shown that Vav-1 and SLP-76 can cooperate in the induction of IL-2 transcription, an effect that is lost with substitution of the YESP sites (Y113F) (50–52). We therefore next assessed whether PKB and GSK-3 phosphorylation could operate independently of the Vav-1 binding to SLP-76. Neither WT SLP-76 nor DN Y113F SLP-76 had an effect on anti-CD3, anti-CD28, or anti-CD3/anti-CD28 induced phosphorylation of PKB and GSKα (Fig. 4A, upper and middle panels, lanes 2–4 versus lanes 6–8 versus lanes 10–12). SLP-76 overexpression was confirmed by blotting (lowest panel, lanes 5–8 and 9–12 versus lanes 1–4). By contrast, as noted previously (35), while SLP-76 expression potentiated IL-2 promoter activity as measured by luciferase assay, the loss of the Vav-1 binding site (i.e. Y113F) failed to support this increase (Fig. 4B). These findings confirm that PKB and GSK-3α phosphorylation can occur without an interaction between Vav-1 and SLP-76.

Although the above data showing an independence of anti-CD3 and anti-CD3/CD28 up-regulation of PKB-GSK-3α from Vav-1, we also assessed whether the pathway could operate without the activation of downstream targets of Vav-1. Vav-1 can regulate JNK activation and actin cytoskeleton remodelling (31, 32). While the regulation of these events could have branched prior to the regulation of PKB-GSK-3 on Vav-1, it was of interest to assess whether the pathway could occur in the absence of these fundamental events. To assess the role of JNK, cells were treated with the inhibitor SP600125 at concentrations that have previously been shown to inhibit JNK activity (53). In this instance, we limited ourselves to an examination of GSK-3 phosphorylation (Fig. 5). Anti-CD3/anti-CD28 induced normal levels of GSK-3α phosphorylation in drug-treated cells (upper panel, lane 2 versus lane 4 and lower histogram). Anti-GSK-3α/β blotting confirmed equal levels of GSK-3 protein (lower panel).

A similar level of normal GSK-3α phosphorylation was observed in cells that had been treated with cytochalasin D (cyt.D) (Fig. 6). Treatment resulted in a shift of F-actin staining (i.e. reduced staining) indicative of its disruptive effect on the cytoskeleton (52) (Fig. 6A). In addition, anti-CD3-induced clustering on the cell surface was affected by cytochalasin D treatment (Fig. 6B, compare upper and lower images and histogram). Despite this, anti-CD3/CD28 continued to induce normal levels of GSK-3α phosphorylation over a range of cytochalasin D concentrations (Fig. 6C). In fact, in three experiments a slight increase in phosphorylation was observed at the higher concentration of the drug. These data document the unusual observation that neither the inhibition of JNK nor the disruption of the cytoskeleton with cytochalasin D interfered with the TcR/CD28 induction of GSK-3α phosphorylation.

**DISCUSSION**

The question of the relationship between the Vav-1- and PI3K-mediated pathways has been the topic of mixed observations. Vav-1 GEF activity has been reported to depend on inositol lipids (45), while others have found a role for Vav-1 in activating PI3K (46–49). By contrast, it has been reported that the inhibition of PI3K does not inhibit Vav-3 phosphorylation
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in Vav-3-deficient cells (47), while BcR activation of PKB occurs normally in Vav-1-deficient B-cells (50). With this background, it was important to establish how these pathways interrelate in T-cells. This is especially important in the context of CD28 co-signaling since the receptor recruits and activates PI3K and is also influenced by Vav-1 (12–21, 30, 36, 41).

Our findings show that anti-CD3 and CD3/CD28 induced wild-type levels of PKB and GSK-3α phosphorylation in Vav-1−/− Jurkat cells and primary CD4-positive T-cells (Figs. 1 and 2). This indicates that Vav-1 is not specifically required for the activation of the PKB-GSK-3 pathway in T-cells. Similarly, wild-type levels of phosphorylation were observed in the presence of a Vav-1 DH domain mutant that is well established in its ability to potently inhibit IL-2 transcription (Fig. 3). This indicates that the DH domain has an involvement in TcR driven IL-2 transcription that is independent of the PKB-GSK-3 pathway. Similarly, the lack of an effect of the Vav-1 binding mutant of SLP-76 indicated a separation of Vav-1-SLP-76 and the PKB-GSK-3 pathways. Our observations in T-cells are similar to those recently observed in B-cells where BcR activation of PKB occurs normally in the absence of Vav-1 (49).

Vav-1 is the dominant regulatory isoform of the Vav family in T-cells. Previous studies have shown that Vav-1−/− cells showed defects in TcR clustering, sustained Ca2+ mobilization, JNK activation, and IL-2 production (38, 39, 34, 44). These defects were evident despite the expression of other Vav isoforms indicating that Vav-1 can play a specialized or dominant role in regulating these events. This differs from our findings with the PKB-GSK-3 pathway. Unlike in the case of Vav-1−/− T-cells, Vav-1,2,3−/− cells showed a partial loss (i.e. <50%) of GSK-3 phosphorylation. This indicates that the involvement of the Vav family in a portion of the signaling that leads to GSK-3 phosphorylation. In this manner, other Vav isoforms (i.e. Vav-2,3) may substitute for Vav-1 in the Vav1−/− T-cells to allow for normal PKB and GSK-3 phosphorylation. At the same time, significant residual (i.e. >50%) GSK-3 phosphorylation was observed in the absence of Vav family members. This contrasted with the complete loss of FOXO phosphorylation in these cells (Fig. 2). The nature of the pathway linked to this residual activation remains to be elucidated. Similarly, whether other Vav family members transmit signals along a single pathway (i.e. their loss affects the threshold of signaling) or whether they constitute a separate pathway from that which mediates the remaining activation of GSK-3 awaits further studies.

FIGURE 6. TcR/CD3-CD28-induced GSK-3 inactivation operates independently of cytochalasin D disruption of the actin cytoskeleton. A, cytochalasin D treatment disrupts the actin cytoskeleton as detected by FACS. Jurkat cells were untreated (unt.) or incubated with 10 μM cytochalasin D (cyt. D) for 1 h at 37 °C. FACS analysis was used to determine F-actin content by staining permeabilized cells with FITC-Phalloidin. B, cytochalasin D treatment interferes with TcR clustering. Cells were untreated (unt.) or treated with 10 μM cytochalasin D (cyt. D). Following incubation with anti-CD3, cells were cross-linked with FITC-anti-mouse IgG and either incubated immediately on ice (0-h samples) or at 37 °C for 1 h (1-h samples), and then the TcR distribution was visualized by fluorescent microscopy. TcR clustering (white arrow) was scored. Histogram, quantification of TcR clustering: an average of 200 cells was scored for each condition. C, cytochalasin D treatment does not interfere with anti-CD3/CD28-induced GSK-3 phosphorylation. Jurkat cells were incubated with the indicated dose of cytochalasin D for 1 h, followed by stimulation with either rabbit anti-mouse alone (RAM) or with the combination anti-CD3/anti-CD28 (CD3/CD28) for 30 min. Upper panels, anti-phospho-GSK-3α/β blot; lower panel, anti-GSK-3α/β blot. Histogram shows the level of phosphorylation of GSK-3α.
The independence of PKB/GSK-3 activation from Vav-1 was observed with both anti-CD3 and anti-CD3/CD28 activation. We had expected to observe a differential dependence on Vav-1 with CD28 mediated co-stimulation; however, this was not the case. Co-stimulation was clearly demonstrated by the ability of anti-CD28 to potentiate PKB and GSK-3 phosphorylation as well as IL-2 production. Despite this, a similar increase in anti-CD3- and anti-CD3/CD28-induced PKB and GSK-3 phosphorylation was observed in WT and Vav-1 Δ/- cells (Figs. 1 and 2). The increase in PKB phosphorylation was mimicked by an increase in GSK-3 phosphorylation. The similarity in the level of increased phosphorylation with different modes activation was surprising given the existence of other pathways that regulate PKB and GSK-3. These include PKB autophosphorylation (55), phosphorylation with integrin-linked kinase (56, 57), and unidentified kinases in lipid rafts (58).

Another noteworthy observation was that GSK-3α was found to be the main target of phosphorylation in T-cells (Figs. 1–3). This occurred, despite similar levels of GSK-3α and β expression in Jurkat and primary T-cells. This was observed with both anti-CD3 and anti-CD3/CD28 co-ligation. The GSK-3α isoform therefore appears to be specifically localized, or conformationally altered, to facilitate its preferential phosphorylation in T-cells. This observation contrasts with other mammalian cells (i.e. chondrocytes) where the β-isoform of GSK-3 is the primary target of phosphorylation (24, 25). Our observation also implies that GSK-3α is the principle effector in the regulation of events such as NFAT localization in the nucleus of T-cells.

One apparent discrepancy is the difference between our results and those reported in a study on Vav-1-deficient double positive thymocytes (48). Vav-1-deficient thymocytes were found to show defects in PKB activation (48). Although the basis of this discrepancy is unclear, it could be related to a difference in the degree of surface receptor cross-linking or a difference between T-cells and thymocytes. For example, it is conceivable that lower degrees of receptor ligation are more dependent on Vav-1 expression than high degrees of co-engagement. It might also reflect different growth conditions etc. that may alter Vav-2 or -3 expression. Lower levels of Vav-2,3 may in turn increase the dependence on Vav-1.

Last, in addition to these direct assays, we examined the effect of inhibiting JNK or disrupting the cytoskeleton on PKB and GSK-3 phosphorylation. Although it could be argued that Vav-1 signaling may branch prior to the potential engagement of PKB/GSK-3, it might also have been linear in its connection to the kinases. Previous studies showed that JNK activity is either normal or defective in Vav-1Δ/- T-cells, while expression of dominant negative PI3K did not block c-Jun DNA binding mediated by JNK (44, 59). Inhibition of JNK activity had no effect on anti-CD3 or anti-CD3/CD28 phosphorylation of GSK-3 (Fig. 5). Similarly, disruption of the actin cytoskeleton with cytochalasin D had no effect on GSK-3 activation (Fig. 6). This latter result is especially surprising given the number of signaling events that are thought to depend on an intact cytoskeleton. In this way, the cytoskeleton is thought to serve as a docking region that assembles signaling proteins for activation. Despite this, PKB and GSK-3 phosphorylation was normal in over four experiments where cells were treated with concentrations of cytochalasin D that clearly disrupted the cytoskeleton. This observation suggests that an intact cytoskeleton is not needed for the ability of TcR/CD3 × CD28 co-ligation to induce GSK-3α phosphorylation and agrees with a previous study that found intact GSK-3 phosphorylation in response to PMA/ionomycin in cytochalasin D-treated cells (54). Further studies will be needed to establish the full range of pathways regulated by PI3K that operate independently of Vav-1.

Acknowledgments—We thank Drs. Victor Tybulewicz (National Institute for Medical Research, London) and Martin Turner (Babraham Institute) for providing mice deficient in Vav-1 and Vav-1,2,3, respectively.

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