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Enhanced Fas-associated death domain recruitment by histone deacetylase inhibitors is critical for the sensitization of chronic lymphocytic leukemia cells to TRAIL-induced apoptosis

Satoshi Inoue, Nick Harper, Renata Walewska, Martin J.S. Dyer, and Gerald M. Cohen

MRC Toxicology Unit, University of Leicester, Leicester, United Kingdom

Abstract

Chronic lymphocytic leukemia (CLL) is an incurable disease characterized by failure of mature lymphocytes to undergo apoptosis. CLL cells are inherently resistant to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL). Pretreatment with histone deacetylase inhibitors (HDACi) sensitizes CLL cells to TRAIL-mediated apoptosis primarily via TRAIL-R1 and offers a novel approach for the therapy of CLL and other malignancies. Depsipeptide (romidepsin), a HDACi, did not enhance TRAIL binding to TRAIL-R1, TRAIL-R1 aggregation, or internalization of TRAIL-R1, but it enhanced Fas-associated death domain protein (FADD) recruitment to TRAIL-R1 in the death-inducing signaling complex. Cotreatment with phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, dramatically inhibited the HDACi-mediated increase in FADD recruitment and sensitization to TRAIL-induced apoptosis and both of these were reversed by PKC inhibitors. Thus, enhanced FADD recruitment is a critical step in HDACi-mediated sensitization of CLL cells to TRAIL-induced apoptosis and this step is differentially affected by HDACi and phorbol 12-myristate 13-acetate. Using biotinylated TRAIL and streptactin-tagged TRAIL, we have identified several novel TRAIL receptor interacting proteins, including PKCβ, lymphocyte-specific protease-1, Lyn, and Syk. These molecules may play an as yet unappreciated role in TRAIL signaling in CLL cells and inhibition of one or more of these kinases/phosphatases may provide a novel target to overcome TRAIL resistance. [Mol Cancer Ther 2009;8(11):3088–97]

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Requests for reprints: Gerald M. Cohen, MRC Toxicology Unit, University of Leicester, Hodgkin Building, P.O. Box 138, Lancaster Road, Leicester, LE1 9HN, United Kingdom. Phone: 44-116-2525601; Fax: 44-116-2525616. E-mail: gmc2@le.ac.uk

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Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent form of adult leukemia in the western world and is characterized by the accumulation of $CD5^+CD19^+$ cells in the blood, bone marrow, and lymph nodes. In some patients, the disease may remain stable for many years, whereas in others it progresses rapidly and new treatments are urgently required. There has been much interest in the exploitation of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) as a novel treatment for cancer, as it induces apoptosis in many tumor cell lines but not in most normal cells (1). Interaction of TRAIL with its two membrane-bound death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5/TRICK2), results in the recruitment of the adaptor molecule Fas-associated death domain (FADD)/MORT1, which in turn recruits and activates caspase-8 in the death-inducing signaling complex (DISC) to induce apoptosis (2–4). Resistance to TRAIL is an important therapeutic problem that may be circumvented by combination treatments including the use of histone deacetylase inhibitors (HDACi; reviewed in ref. 5). Several HDACi have recently entered clinical trials and HDACi exert their antitumor effects by a combination of differing effects including the induction of growth arrest, differentiation, and apoptosis (6–8). HDACi may sensitize cells to TRAIL-induced apoptosis by various proposed mechanism(s) including increased expression of death receptors and their ligands, redistribution of TRAIL receptors into lipid rafts, or decreases in antiapoptotic molecules including c-FLIP, cellular inhibitor of apoptosis protein-2, X-linked inhibitor of apoptosis protein, and phosphoprotein enriched in diabetes (PED; refs. 9–14). In addition to their ability to sensitize to death receptor–induced apoptosis, HDACi alone can induce apoptosis (15). However, the mechanism by which HDACi sensitize to TRAIL is clearly different from the mechanism whereby they induce apoptosis with ~300-fold lower concentrations of HDACi required to sensitize to TRAIL-induced apoptosis than to induce apoptosis (16).

Primary cells from patients with various tumors, including CLL and non-Hodgkin's lymphoma, are highly resistant to TRAIL (17, 18) and this may be overcome in CLL cells by prior treatment with HDACi (19). In this regard, it should be noted that HDACi can potentiate TRAIL-induced apoptosis in several cell lines, including K562, U937, DU145, and U266 cells (5, 11, 20). However, in all these cell lines, higher concentrations of TRAIL alone (500 ng/mL) can induce apoptosis, in marked contrast to primary CLL cells, which are completely resistant to TRAIL alone even at concentrations as high as 2,000 ng/mL (18). Thus, it is imperative to use primary CLL cells to understand the "pure

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sensitization effect" of HDACi on TRAIL-induced apoptosis to differentiate it from the enhancement effect by HDACi observed in cell lines. The sensitization of CLL cells occurs primarily through TRAIL-R1 and inhibition of HDAC class I but not class II (21, 22).

In this study, we show the critical importance of increased recruitment of FADD to the TRAIL DISC in HDACi-mediated sensitization to TRAIL-induced apoptosis. Protein kinase C (PKC) β, the tyrosine kinases Lyn and Syk, and the tyrosine phosphatase SHP-1 were detected in the TRAIL DISC, and these interactions could be important for the inherent resistance of CLL cells to TRAIL.

Materials and Methods

Lymphocyte Purification and Culture

CLL cells obtained with patient consent and local ethical committee approval were purified and cultured as described (19, 22).

Reagents

Media and serum were from Life Technologies. Mouse anti-Lyn monoclonal antibody (mAb), mouse anti-Syk mAb, mouse anti-SHP-1 mAb, mouse anti-RACK1 mAb, mouse anti-PKCβ mAb, and mouse anti-flotillin-1 mAb were from BD Transduction Laboratories. Rabbit anti-phospho-FADD antibody, rabbit phospho-Src family (Y416) antibody, rabbit phospho-Lyn (Y507) antibody, rabbit PED/PEA-15 antibody, and phospho-PED/PEA-15 (S104) antibody were from Cell Signaling Technology. Rabbit anti-phospho-PED/PEA-15 (S116) antibody was from Biosource. Mouse anti-IgM antibody was from Jackson Immuno Research. Lambda phosphatase was from New England Biolab. Mouse lymphocyte-specific protease-1 mAb was kindly provided by Dr. K. Pulford (23). Bisindolylmaleimide I, Go6976, PP2, SU6656, piceatannol, valproate, and NSC-87877 were from Calbiochem (Merck Biosciences). Depsipeptide (romidepsin) and LBH589 (panobinostat) were kindly provided by Dr. E. Sausville (National Cancer Institute) and Dr. P. Atadja (Novartis Pharmaceuticals), respectively. Other reagents, including other HDACi and antibodies to TRAIL-R1, TRAIL-R2, FADD, and caspase-8, were from previously described sources (21) or from Sigma.

Preparation of Recombinant TRAIL

Biotinylated human recombinant TRAIL (b-TRAIL) was prepared as described previously (24). Strep-II–tagged TRAIL (ST-TRAIL) was produced as follows. A Strep-II tag was introduced into pet-TRAIL by replacing the NH_2-terminal 6× His and T7 epitopes with a Strep-II tag plus linker region (ASWSHPQFEKGA). The construct was verified by sequencing and protein produced in *Escherichia coli* as described previously (24). ST-TRAIL was purified using streptactin-Sepharose beads (IBA) using standard protocols (25).

Quantification of Apoptosis and Western Blot Analysis

Apoptosis was quantified either by phosphatidylserine externalization in the presence of propidium iodide or by measurement of the mitochondrial membrane potential (19). Samples for Western blot analysis were prepared and caspases were detected as described previously (18, 19, 21).

DISC Analysis

DISC precipitation was done using either b-TRAIL or ST-TRAIL as described (18). CLL cells (2.5×10^8 per treatment) were either cultured alone or with depsipeptide (10 nmol/L) for 16 h. Cells were then treated with either b-TRAIL or ST-TRAIL (500 ng/mL) and DISC formation was examined. Western blotting was done using 30 μL eluted complexes from streptavidin or streptactin beads representing DISC precipitated from 3×10^7 cells. DISC complexes isolated with ST-TRAIL could be eluted from streptactin beads using a biotin conjugate, desthiobiotin (Molecular Probes), as described (25). Use of ST-TRAIL together with the mild elution conditions allows for isolation of DISC components with a reduction in nonspecific protein binding.

Analysis of TRAIL Receptor Aggregation and Two-Dimensional Gel Electrophoresis

TRAIL DISCs were isolated as described above and subjected to gel electrophoresis in the absence of reducing agents (26). Two-dimensional gel electrophoresis from isolated TRAIL DISC samples was carried out as described (26).

Isolation of Lipid Rafts

CLL cells (400×10^6) were exposed to depsipeptide for 16 h followed by b-TRAIL (500 ng/mL) for 0.5 h and then washed twice with ice-cold PBS supplemented with Na_3VO_4 (2 mmol/L). Lipid rafts were isolated using lysis conditions and floatation on discontinuous sucrose gradients as described (27). After separation on discontinuous sucrose gradients, 12 × 1 mL fractions were collected from the top of the gradient and subjected to Western blotting.

Detection of External and Internalized TRAIL-R1

To detect external TRAIL-R1, CLL cells (200×10^6) were transferred to ice for 15 min followed by exposure to b-TRAIL (500 ng/mL) for a further 60 min on ice. Cells were then washed twice in ice-cold PBS to remove unbound b-TRAIL followed by lysis for 30 min on ice in buffer [20 mmol/L Tris (pH7.5), 20 mmol/L NaCl, 10% (v/v) glycerol, 1% Triton X-100 containing protease inhibitors (Roche)]. TRAIL receptors from the lysate were isolated as described previously (19).To determine internalized TRAIL-R1, we used a biochemical method using cleavable NHS-SS-biotin as described recently (28).

Results

Depsipeptide Does Not Enhance TRAIL-R1 Receptor Aggregation or Receptor Internalization

Previously, we have shown that HDACi enhanced the recruitment of FADD into the TRAIL DISC and this was not due to HDACi-mediated upregulation of TRAIL-R1 or TRAIL-R2 (19, 20). Furthermore, as TRAIL-mediated apoptosis of CLL cells occurs almost exclusively through signaling via TRAIL-R1 (22), we examined if depsipeptide, a HDACi, modified TRAIL-R1 aggregation. On receptor ligation, CD95/Fas monomers rapidly aggregate to form SDS-stable higher molecular weight complexes primarily dimers and trimers, which can be resolved by SDS-PAGE

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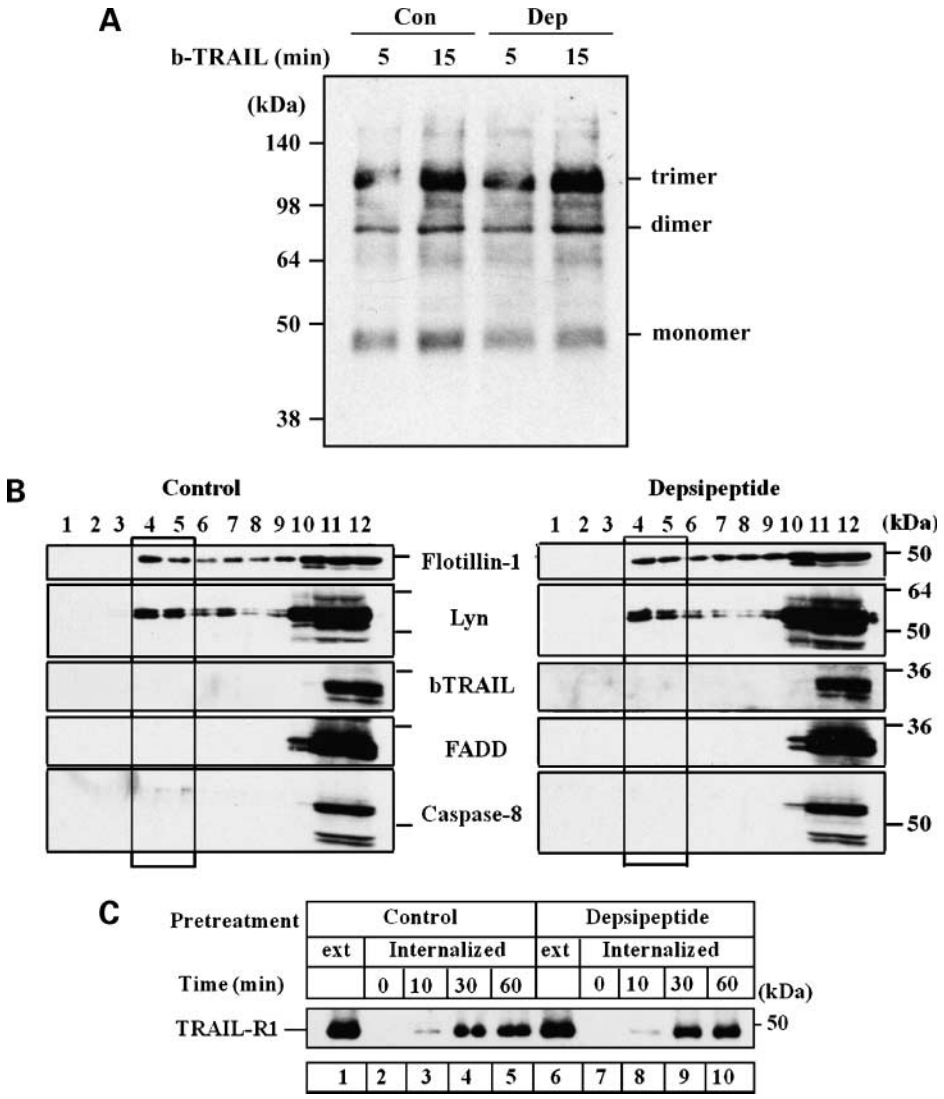

Figure 1. Depsipeptide does not enhance TRAIL-R1 aggregation and the redistribution of DISC components to lipid rafts. CLL cells were cultured for 16 h either alone (*Con*) or with depsipeptide (*Dep*; 10 nmol/L) and then (**A**) exposed to TRAIL (500 ng/mL) for the indicated times. Aggregation of TRAIL-R1 was analyzed by Western blotting under nonreducing conditions or (**B**) exposed to b-TRAIL (500 ng/mL) for 0.5 h. Lipid rafts were isolated and fractions (1 mL) were separated by discontinuous sucrose gradients and analyzed by Western blotting. **C,** CLL cells were treated as in **A** and then labeled with cleavable biotin as described in Materials and Methods. Cells were then exposed to TRAIL (500 ng/mL) for the indicated times, and following removal of cell surface biotin, internalization of TRAIL-R1 was detected by Western blotting.

in the absence of reducing agents (2). A critical role for aggregation of death receptors has been proposed to initiate apoptotic signaling (29–31). Exposure of CLL cells to TRAIL resulted in a rapid aggregation of TRAIL-R1 primarily to dimers and trimers, which was unaltered by depsipeptide (Fig. 1A), suggesting that the ability of HDACi to enhance apoptosis occurs after TRAIL-R1 aggregation.

Lipid rafts are liquid-ordered domains that float freely in the liquid disordered bilayer of cell membranes (32). Glycosylphosphatidylinositol-anchored proteins and Src family tyrosine kinases, such as Lyn and Lck, and palmitoylated and myristoylated proteins, such as flotillins, preferentially partition into raft domains (32, 33). Some reports have proposed a role for these membrane microdomains in death receptor signaling (34). It was proposed that the redistribution of TRAIL-R1 and TRAIL-R2 into membrane lipid rafts was important for the HDACi-mediated TRAIL sensitization (11, 14). To examine this possibility, CLL cells were exposed to depsipeptide followed by b-TRAIL and raft fractions were isolated. The insoluble fractions (Fig. 1B, fractions 4 and 5) comprised the lipid raft enriched fractions as evidenced by the enrichment of flotillin-1. FADD, caspase-8, and b-TRAIL were detected in the soluble (Fig. 1B, *lanes 10-12*) but not the raft fractions in both control and depsipeptide pretreated cells. Due to low levels of TRAIL-R1 and TRAIL-R2, we were unable to determine whether the receptors redistributed to raft fractions. However, we have also used U266 cells, a human myeloma cell line, which expresses TRAIL-R1 but not TRAIL-R2. In these cells, TRAIL-R1 and caspase-8 were detected in the "non-raft" but not in the "raft" fractions following exposure of the cells to depsipeptide or LBH589 (Supplementary Fig. S1). We next examined whether HDACi affected the internalization of TRAIL-R1. Exposure to TRAIL resulted in a time-dependent internalization of TRAIL-R1, which was

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not significantly altered by depsipeptide (Fig. 1C). Taken together, our data strongly suggest that HDACi-mediated facilitation of TRAIL-induced apoptosis was due neither to partitioning of FADD nor caspase-8 into lipid membrane rafts following TRAIL exposure nor altered internalization of TRAIL-R1.

FADD Recruitment to TRAIL-R1 Is Critical for HDACi-Mediated Sensitization

We and others have shown that phorbol 12-myristate 13-acetate (PMA), a PKC activator, can inhibit CD95/Fas- and TRAIL-induced apoptosis through inhibition of FADD recruitment to the DISC (26, 35–37). Bisindolylmaleimide I, a broad-range PKC inhibitor, reverses the PMA-induced inhibition of FADD recruitment and resensitizes HeLa cells to TRAIL-induced apoptosis, supporting a critical role for FADD recruitment in TRAIL-mediated apoptosis (26). We therefore examined the effects of PKC modulators on HDACi-mediated sensitization to TRAIL. CLL cells, exposed to depsipeptide for 16 h, were sensitized to TRAIL-induced apoptosis and this sensitization was completely blocked following PMA treatment for 30 min before TRAIL and this block was totally reversed by prior exposure to bisindolylmaleimide I (Fig. 2A, *lanes 13-16*).

Examination of the native TRAIL DISC in the presence or absence of PMA revealed that none of the treatments resulted in major changes to the amount of TRAIL-R1 in the DISC or an increase in the availability of cell surface TRAIL-R1 (Fig. 2B), suggesting that these treatments did not change the affinity between TRAIL-R1 and the ligand. In untreated cells, treatment with TRAIL resulted in the recruitment of a small amount of FADD and caspase-8 to the DISC (Fig. 2B, *lane 1*), which was not markedly altered in untreated cells exposed to either bisindolylmaleimide I or Go6976, a more selective cPKC inhibitor (38), in the presence or absence of PMA (Fig. 2B, *lanes 2-6*). Pretreatment with depsipeptide caused a marked increase in the recruitment of FADD and caspase-8, and the caspase-8 was processed to its p43/41 forms as well as its p18 catalytically active large subunit (Fig. 2B, *lane 7*), in agreement with our previous findings (19). Exposure to PMA for only 30 min before isolation of the DISC abrogated the depsipeptide-induced increased recruitment of FADD and caspase-8 (Fig. 2B, *lane 10*) and this inhibition was reversed by exposure to bisindolylmaleimide I or Go6976 (Fig. 2B, *lanes 11* and *12*). These data show that the protective effects of PMA completely override the HDACi-mediated increased FADD recruitment, supporting the suggestion that FADD recruitment to TRAIL-R1 is critical for HDACi-mediated sensitization to TRAIL-induced apoptosis. These data also suggest that FADD recruitment appears to be a focal point where both HDACi and PKC activators (PMA) modulate TRAIL signaling.

HDACi Did Not Alter the Phosphorylation of TRAIL-R1 and FADD

As our data suggested the possible involvement of PKCs or related kinases, we examined if components of the TRAIL DISC, such as TRAIL-R1 or FADD, were modified following HDACi or PMA pretreatment. Using two-dimensional gel electrophoresis, no major changes were observed in TRAIL-R1 in the isolated DISC from control cells or those exposed to depsipeptide for 16 h, PMA for 30 min, or the combination (Fig. 3A), suggesting that these treatments did not modify the post-translational status (e.g., phosphorylation) of TRAIL-R1 in the DISC. Examination of the effects of HDACi on FADD revealed that two main species of FADD were detected in the lysate and these were largely unaffected by any of the treatments (Fig. 3B, *right, L1* and *L2*). In the isolated DISC from control cells, one major species of FADD (D1) was detected (Fig. 3B, *top left*). Following exposure to depsipeptide, a second species (D2) was also detected and the appearance of this species was abrogated by PMA (Fig. 3B, *left, third* and *fourth panels*), suggesting that either HDACi could post-translationally modify FADD in the DISC or post-translational modification was a consequence of more rapid DISC formation. To evaluate the latter possibility, we examined FADD modification following TRAIL exposure at 25°C to slow down the kinetics of DISC formation. At early times (15 min) following exposure of untreated control cells to TRAIL, only one major species of FADD (D1) was detected in the isolated TRAIL DISC, whereas at 45 min a second species (D2) was also observed (Fig. 3C, *left*), suggesting that D1 has a higher affinity than D2 for the TRAIL receptor. Following exposure to depsipeptide and then TRAIL, two species of FADD were detected at the earliest time point measured (5 min) and the D2/D1 ratio increased in a time-dependent manner (Fig. 3C, *right*). These results supported the possibility that the presence of two species of FADD in the depsipeptide-treated DISC samples (Fig. 3B, *left*) was due to the more rapid kinetics of DISC formation.

FADD is known to be phosphorylated (39, 40). To further characterize the FADD species precipitated with the DISC, we analyzed FADD in the presence of λ phosphatase, which removes phosphate groups from serine, threonine, and tyrosine and would result in a net basic shift on two-dimensional gels. Two species of FADD were again detected in both lysates (L1 and L2) and isolated DISC (D1 and D2) from depsipeptide pretreated cells. Exposure to λ phosphatase resulted in the disappearance of D2 and L2 and an increase in D1 and L1, supporting the suggestion that D1/L1 and D2/L2 represented unphosphorylated FADD and phospho-FADD, respectively (Fig. 3D). To further characterize these species, we used a specific phospho-S194-FADD antibody. Both D2/L2 species appeared to be predominantly S194-FADD, as they disappeared following exposure to λ phosphatase (Fig. 3D, *bottom*). Thus, FADD is phosphorylated primarily at S194 in both the lysate and DISC of CLL cells, and HDACi pretreatment resulted in an increase in phospho-FADD in the DISC, which associated more slowly with the TRAIL receptor than unphosphorylated FADD.

PKCβ and Tyrosine Kinases/Phosphatase Associate with the TRAIL DISC

The effects of PMA and the differences in FADD phosphorylation in the DISC led us to examine whether any protein kinases were associated with the TRAIL DISC (Fig. 2).

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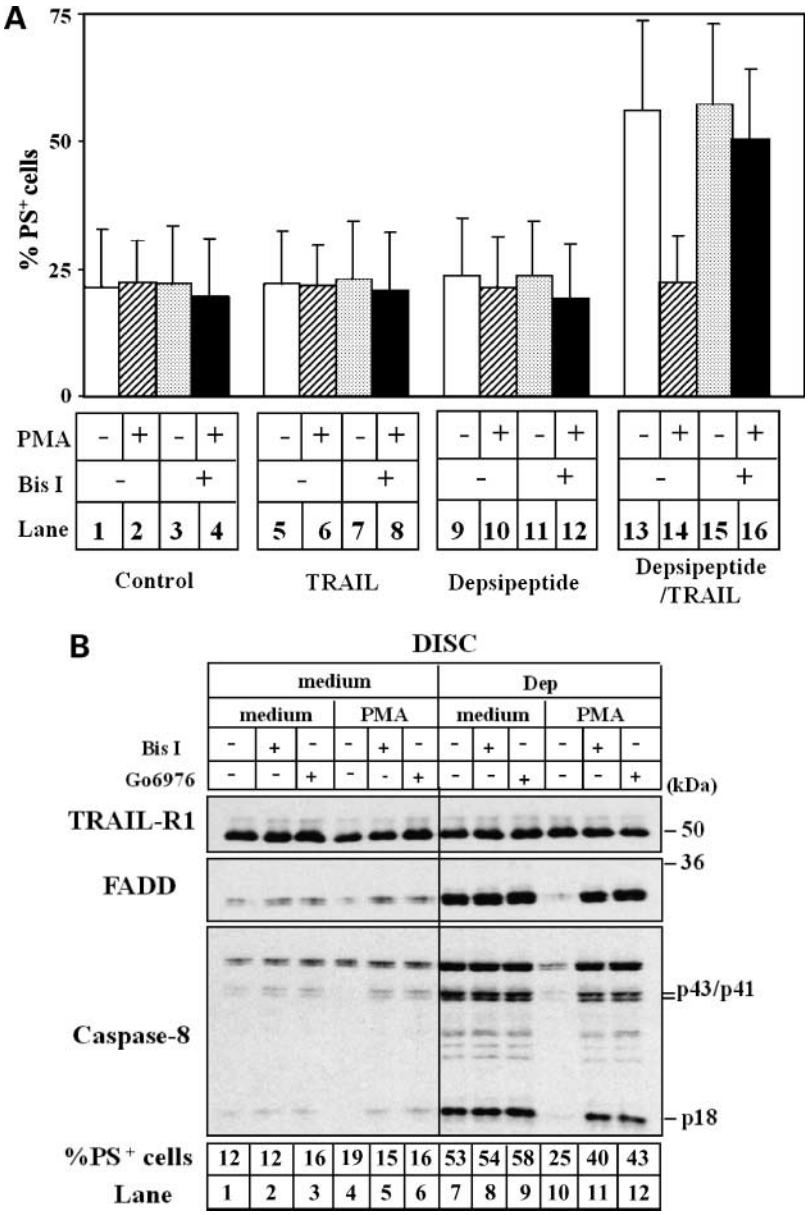

Figure 2. Recruitment of FADD is critical for HDACi-mediated sensitization to TRAIL-induced apoptosis. **A,** CLL cells were incubated for 16 h either alone or in the presence of depsipeptide (10 nmol/L). Cells were exposed to PMA (20 ng/mL) for 30 min and/or bisindolylmaleimide I (*Bis I*; 1 μmol/L) for 60 min before TRAIL (500 ng/mL) for a further 4 h when cells were assessed for apoptosis by phosphatidylserine (*PS*) externalization. *Columns,* mean (n = 9); *bars,* SD. **B,** CLL cells were treated as in **A**, except that cells were also exposed to Go6976 60 min before TRAIL exposure, and apoptosis was assessed as in **A**. Cells were also exposed to b-TRAIL (500 ng/mL) for 15 min and the DISC was isolated as described in Materials and Methods and examined by Western blotting.

In these studies, the DISC was pulled down using ST-TRAIL and streptactin-agarose beads and then eluted with desthiobiotin (20 mmol/L) for 2 h at 25°C. This resulted in a mild elution in the absence of any detergent, therefore minimizing the elution of nonspecific proteins bound to the beads. Pretreatment with depsipeptide again caused a more rapid recruitment of FADD to the DISC (Fig. 4A). Some variation was observed in the degree of associated proteins before sensitization with depsipeptide (Fig. 4A), which may be due to interindividual variation in spontaneous apoptosis or in the kinetics of DISC formation. Next, we examined the DISC for the presence of PKCβ, which is abnormally expressed in CLL and is also a B-cell receptor (BCR) signaling molecule (41). Extensive amounts of PKCβ but not PKCα were detected in the lysate from CLL cells compared with cell lines (Supplementary Fig. S2), in agreement with a previous study (41). Both PKCβ and lymphocyte-specific protease-1, a PKCβ1-associated protein and a major PKC substrate in CLL (23, 42), but not RACK1, a PKCβ2-associated protein, were detected in the isolated DISC and these were increased following depsipeptide (Fig. 4A). We also investigated whether other components of the BCR complex were recruited to the DISC (Fig. 4B). In addition to FADD and caspase-8, more PKCβ was detected in the isolated TRAIL DISC following pretreatment with depsipeptide (Fig. 4B), raising the possibility that the TRAIL DISC also associates with PKCβ-associated proteins. At early times, more of the tyrosine kinases (Lyn and Syk) and the tyrosine phosphatase

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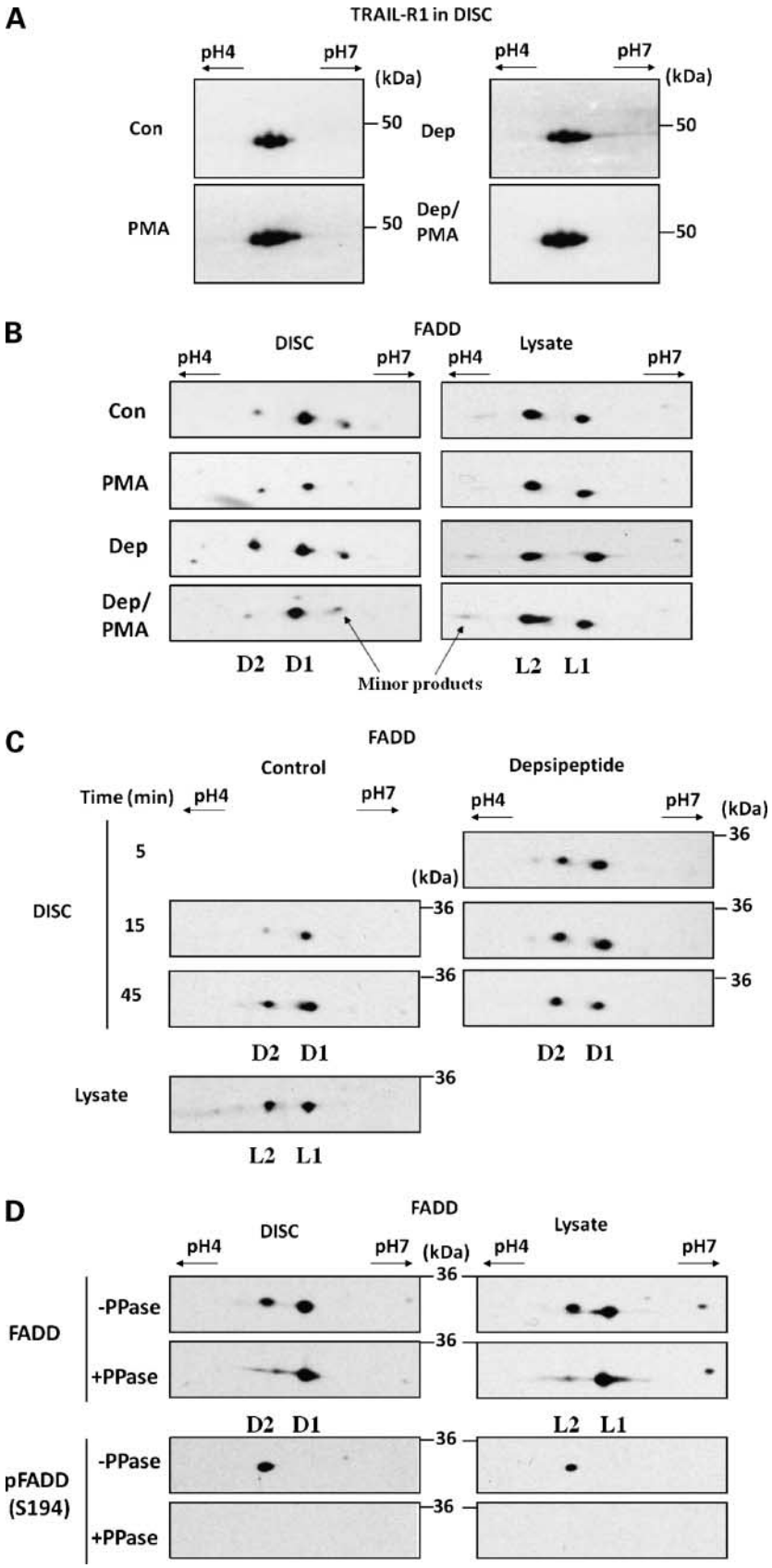

Figure 3. Pretreatment with depsipeptide results in an increase in phospho-FADD in the DISC. CLL cells were exposed either to depsipeptide (10 nmol/L) for 16 h or PMA (20 ng/mL) for 30 min or a combination of both followed by b-TRAIL (500 ng/mL) at 37°C for 15 min. The TRAIL DISC was isolated and two-dimensional gel electrophoresis from either isolated DISC or lysate samples was carried out. **A,** TRAIL-R1 was detected in the isolated DISC. **B,** FADD was detected in the DISC and lysate. **C,** control CLL cells or cells exposed to depsipeptide (10 nmol/L) for 16 h were then treated for the indicated times with b-TRAIL (500 ng/mL) at 25°C to slow down DISC formation. The isolated DISC samples and the lysate were subjected to two-dimensional gel electrophoresis and FADD was detected by Western blotting. **D,** CLL cells were exposed to depsipeptide (10 nmol/L) for 16 h followed by b-TRAIL for 15 min. The TRAIL DISC and the lysate samples were incubated alone or in the presence of λ phosphatase at 30°C for 30 min and analyzed for the presence of FADD or phospho-FADD (S194).

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(SHP-1) were detected in the TRAIL DISC in depsipeptide pretreated compared with control cells (Fig. 4B, compare *lanes 1* and *4*). To further investigate whether these kinases/phosphatase were associated with the TRAIL receptors, the receptors were pulled down from unstimulated cells. Lyn, Syk, SHP-1, and PKCβ, but not FADD or caspase-8, precipitated with the TRAIL receptors (Fig. 4C, *lanes 3* and *6*; Supplementary Fig. S3, *lanes 3* and *4*), suggesting that these kinases/phosphatase were constitutively associated with TRAIL receptors and these constitutive interactions were unaffected by depsipeptide (Fig. 4C, compare *lanes 3* and *6*).

As BCR signaling molecules appeared to predominantly coprecipitate with unstimulated TRAIL receptors, we wished to explore the potential importance of BCR signaling in relation to both TRAIL-induced apoptosis and HDACi-mediated sensitization to TRAIL. Stimulation of the BCR with anti-IgM antibody neither induced apoptosis nor sensitized to TRAIL-induced apoptosis nor inhibited depsipeptide-mediated TRAIL-sensitization (Fig. 5A), suggesting that there was no functional cross-talk between BCR and TRAIL signaling in CLL cells. Next, we examined if the kinase activity of the tyrosine kinases associated with the BCR complex and the TRAIL DISC could modulate TRAIL-induced apoptosis. In contrast to depsipeptide, neither PP2 nor SU6656 (both Src family protein tyrosine kinase inhibitors) nor piceatannol (inhibitor of Syk and ZAP-70) sensitized to TRAIL-induced apoptosis (Fig. 5B), although these concentrations of PP2 clearly inhibited Src but not Lyn phosphorylation (Fig. 5C, *lanes 9-11*). Similarly, NSC-87877, a SHP-1/2 inhibitor, did not sensitize to TRAIL-induced apoptosis (data not shown). PP2, SU6656, and piceatannol also did not abrogate PMA-mediated reversal of HDACi-mediated sensitization to TRAIL (Supplementary Fig. S4).

As PED downregulation was proposed to be important for HDACi-mediated sensitization to TRAIL in

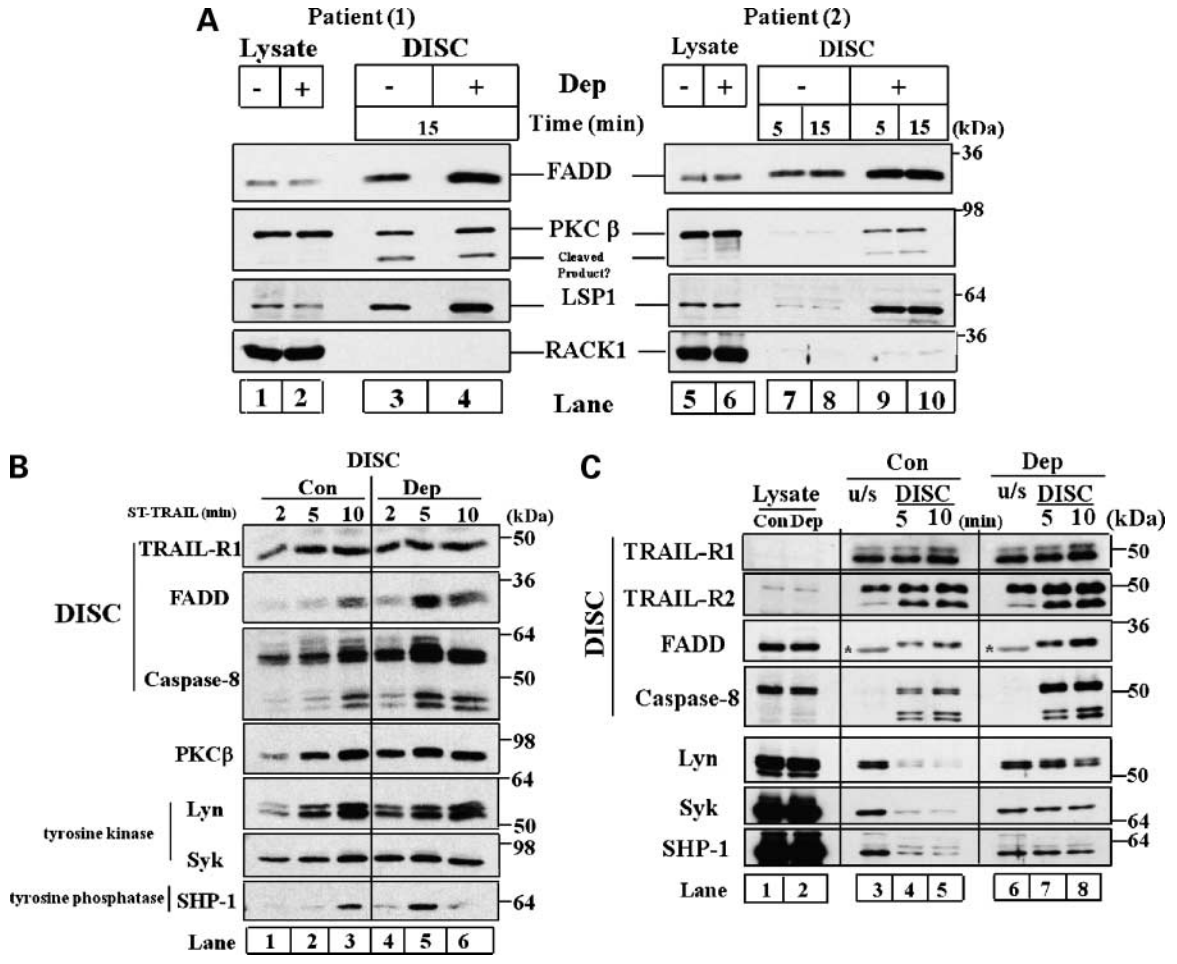

Figure 4. PKCβ, lymphocyte-specific protease-1 (*LSP1*), Lyn, Syk, and SHP-1 are detected in the isolated DISC. CLL cells were exposed to depsipeptide (10 nmol/L) for 16 h followed by ST-TRAIL (500 ng/mL) at 37°C and the TRAIL DISC was isolated. **A,** protein samples of both lysate and the DISC from two patients were analyzed by Western blotting. **B,** protein samples of the isolated DISC from control or depsipeptide-exposed cells at the indicated times were analyzed. **C,** protein samples of the lysate and the receptor pulled down from unstimulated (*u/s*) cells as well as the isolated DISC from control or depsipeptide-exposed cells were analyzed.

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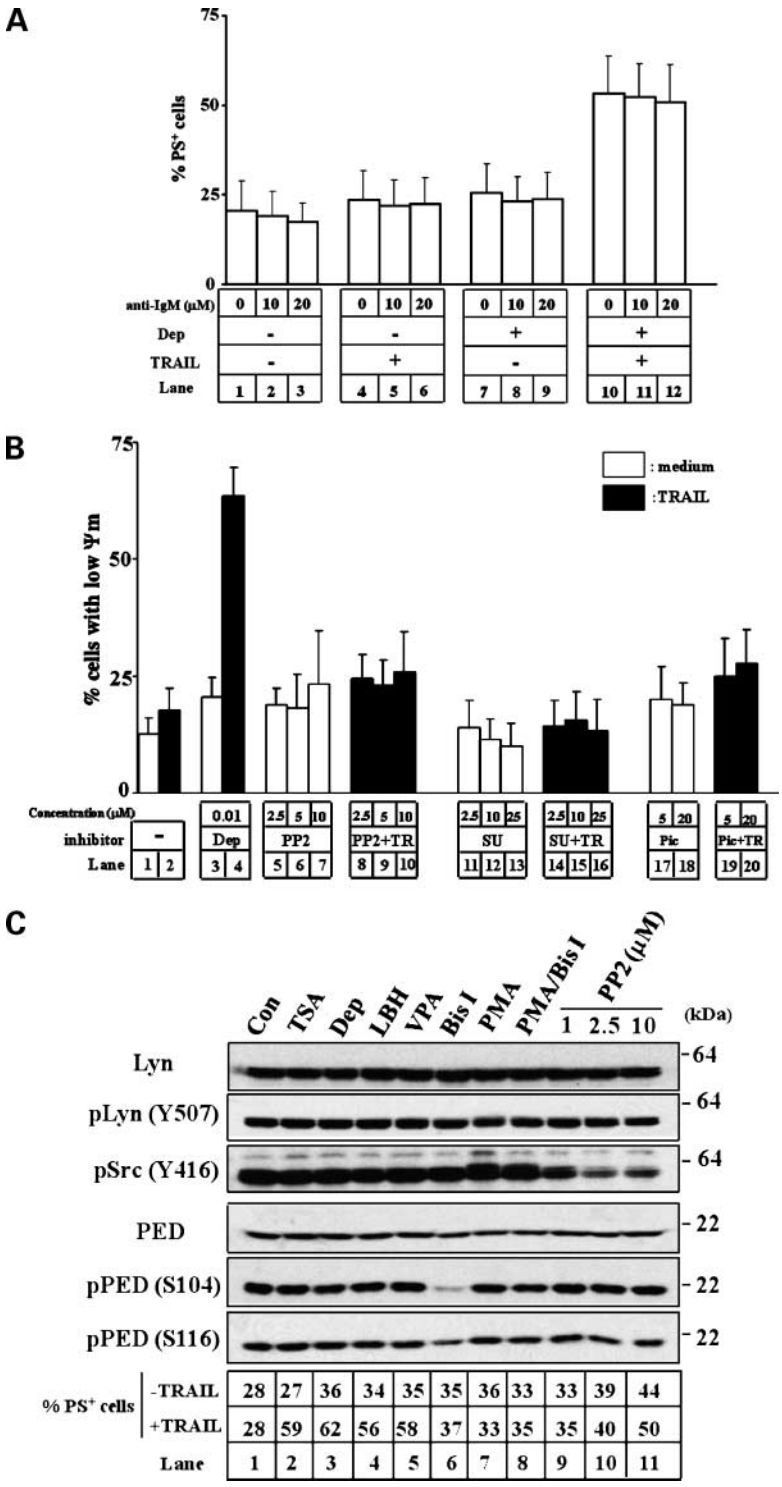

Figure 5. BCR signaling and tyrosine kinase activity are not important for TRAIL sensitivity. **A,** CLL cells were incubated for 16 h either alone or with depsipeptide (10 nmol/L). Cells were then exposed to anti-IgM antibody for 60 min before TRAIL (500 ng/mL) for a further 4 h and apoptosis was assessed. *Columns,* mean (n = 8); *bars,* SD. **B,** CLL cells were exposed as indicated either for 16 h to depsipeptide (*lanes 3* and *4*) or for 2 h to either PP2 (*lanes 5-10*), SU6656 (*SU*; *lanes 11-16)*, or piceatannol (*Pic*; *lanes 17-20*). Cells were then exposed to TRAIL (*TR*; 500 ng/mL) for 4 h and apoptosis was assessed. *Columns,* mean (n = 5); *bars,* SD. **C,** CLL cells were exposed for 16 h either alone (*lane 1*) or to HDACi [trichostatin A (*TSA*); 0.25 μmol/L; *lane 2*], depsipeptide (10 nmol/L; *lane 3*), LBH589 (*LBH*; 10 nmol/L; *lane 4*), or valproate (*VPA*; 2.5 mmol/L; *lane 5*). Cells were also exposed to bisindolylmaleimide I (1 μmol/L; *lanes 6* and *8*) for 1 h, PMA (20 ng/mL; *lanes 7* and *8*) for 30 min, or PP2 (1-10 μmol/L; *lanes 9-11*) for 2 h. Protein samples were prepared before TRAIL exposure and analyzed by Western blotting. Cells were also further incubated in the presence or absence of TRAIL (500 ng/mL) for 4 h and apoptosis was assessed.

CLL (9), we investigated the effects of HDACi on PED (Fig. 5C). Preexposure to four different HDACi (TSA, depsipeptide, LBH589, and valproate) did not cause any decrease in either the levels or phosphorylation status of PED (Fig. 5C, *lanes 2-5*), whereas bisindolylmaleimide I (1 μmol/L) decreased PED phosphorylation at S104 but did not potentiate TRAIL-induced apoptosis (Fig. 5C, *lane 6*). These results do not support the hypothesis that PED is an important factor in mediating TRAIL sensitization.

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Discussion

We have reported previously that HDACi sensitized CLL cells to TRAIL primarily through TRAIL-R1 by facilitating formation of an active DISC (19, 22). In the present study, PMA prevented increased FADD recruitment to the DISC, thereby protecting against HDACi-enhanced apoptosis in response to TRAIL, and both these effects were reversed by PKC inhibitors (Fig. 2). These results strongly support the hypothesis that enhanced FADD recruitment is critical for HDACi-mediated sensitization to TRAIL in CLL cells. PKCβ, Lyn, Syk, and SHP-1 were also detected in the isolated DISC (Fig. 4) in contrast to many cell lines that we have screened. These results emphasize the importance, wherever possible, of using primary cells from patients rather than cell lines to obtain the most meaningful data.

We observed a HDACi-mediated increase in phospho-FADD in the DISC and this seemed to be due to a more rapid association of FADD with the TRAIL receptor (Fig. 3). In contrast to CLL cells, we did not see any difference in FADD phosphorylation status induced by HDACi in the DISC from DU145 cells (data not shown), suggesting that the phosphorylation status of FADD is not important for the HDACi-mediated TRAIL sensitization effect. By contrast, FADD phosphorylation at S194 is important in cell cycle progression (43). Both c-Jun NH_2-terminal kinase and casein kinase 1 have been reported to phosphorylate FADD (39, 40, 44). We have also detected casein kinase-1, but not casein kinase-2, in the DISC (Supplementary Fig. S5) presumably associated with FADD. We could not readily discern the importance of these kinases in CLL cells, as both casein kinase inhibitors (CKI-7 and D4476) and the c-Jun NH_2-terminal kinase inhibitor SP600125 were toxic but had little effect on either FADD phosphorylation status or TRAIL sensitivity (data not shown).

The abrogation by PMA of the HDACi sensitization to TRAIL is cell type specific. PMA reverses the sensitivity in CLL, DU145, U266, and HeLa cells but not in K562, Jurkat, and U937 cells (Fig. 2; data not shown; ref. 26). The cell type specificity could be due to different expression profiles of PKC isoforms or other kinases or to PMA preferentially affecting TRAIL-R1 not TRAIL-R2 signaling. Both bisindolylmaleimide I and Go6976, a broad-range and selective cPKC inhibitor, respectively, reversed the effects of PMA (Fig. 2), suggesting that cPKC (α, β, and γ) was the likely target of PMA. However, PKCβ is more highly expressed than PKCα in CLL cells (Supplementary Fig. S2; ref. 41). Furthermore, enzastaurin, a PKCβ-specific inhibitor, did not reverse the PMA abrogation effect (data not shown), raising the possibility that a tyrosine kinase (s), rather than a non-cPKCs, may be important. Tyrosine kinases, including Src family kinases, play important roles in intracellular survival signaling and are abnormally overexpressed and activated in some cancers. In CLL cells, Lyn is both abnormally overexpressed and constitutively activated and inhibition of its activity results in apoptosis (45). Although Lyn, Syk, and SHP-1 were detected in the DISC, inhibition of their activities (Fig. 4B and Supplementary Fig. S4) did not reverse PMA effects, suggesting that the activity of these tyrosine kinases/phosphatase is not responsible. Our preliminary data suggest that PMA preferentially affects cells signaling through TRAIL-R1 (CLL, U266, and HeLa) rather that TRAIL-R2 (K562, Jurkat, and U937; data not shown) and this may in part explain the cell type specificity of PMA.

Using both b-TRAIL and ST-TRAIL, PKCβ, Lyn, Syk, and SHP-1 were detected in isolated DISC (Fig. 4B; data not shown), suggesting that these kinases/phosphatase are associated with the DISC, most likely primarily through the TRAIL receptors (Fig. 4C), although we cannot exclude direct association with TRAIL. Although the kinase activity of Lyn was not required for HDACi sensitization to TRAIL, (discussed above), its physical interaction with TRAIL receptors may interfere with FADD recruitment. Overexpressed Lyn in CLL cells shows atypical cytoplasmic localization and is found in a high molecular complex (~600 kDa), containing hematopoietic lineage cell-specific protein 1, SHP-1 and Hsp90, and this complex is dissociated by geldanamycin, a Hsp90 inhibitor, early in apoptosis, suggesting that Lyn could be a relevant clinical target in CLL (45, 46). As Lyn and SHP-1 were in the isolated DISC (Fig. 4), they may also be associated with the Lyn-containing complex. HDACi could affect the composition of the complex, thereby facilitating FADD association with TRAIL receptors. To investigate this possibility, we have attempted unsuccessfully to knock down Lyn and SHP-1 in CLL cells using methods we have established to knockdown inducible proteins, including Bfl-1/A1 and Bcl-x (47).

In summary, we propose that CLL cells are inherently resistant to TRAIL due to a low efficiency of FADD recruitment to TRAIL-R1. Sensitization to TRAIL by HDACi is mediated by enhanced FADD recruitment, which is inhibited by PMA. This suggests that both HDACi and PMA differentially affect a critical event in TRAIL DISC formation, that is, FADD recruitment. The identification of several novel TRAIL receptor interacting proteins, PKCβ, lymphocyte-specific protease-1, Lyn, and Syk, suggests that these molecules may play an as yet unappreciated role in TRAIL signaling in CLL cells. Inhibition of one or more of these kinases/phosphatases may provide a novel target to overcome TRAIL resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Satoshi Inoue, Nick Harper, Renata Walewska, et al.

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