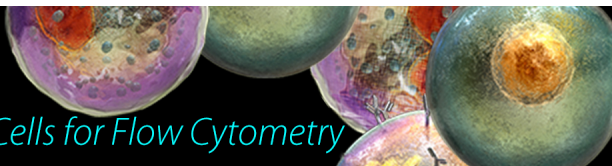


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# Distinct Regulation of Integrin-Dependent T Cell Conjugate Formation and NF- $\kappa$ B Activation by the Adapter Protein ADAP<sup>1</sup>

Brandon J. Burbach,\* Rupa Srivastava,\* Ricardo B. Medeiros,<sup>2\*</sup> William E. O’Gorman,\* Erik J. Peterson,<sup>†</sup> and Yoji Shimizu<sup>3\*</sup>

Following TCR stimulation, T cells utilize the hematopoietic specific adhesion and degranulation-promoting adapter protein (ADAP) to control both integrin adhesive function and NF- $\kappa$ B transcription factor activation. We have investigated the molecular basis by which ADAP controls these events in primary murine ADAP<sup>-/-</sup> T cells. Naive DO11.10/ADAP<sup>-/-</sup> T cells show impaired adhesion to OVAp (OVA aa 323–339)-bearing APCs that is restored following reconstitution with wild-type ADAP. Mutational analysis demonstrates that the central proline-rich domain and the C-terminal domain of ADAP are required for rescue of T:APC conjugate formation. The ADAP proline-rich domain is sufficient to bind and stabilize the expression of SKAP55 (Src kinase-associated phosphoprotein of 55 kDa), which is otherwise absent from ADAP<sup>-/-</sup> T cells. Interestingly, forced expression of SKAP55 in the absence of ADAP is insufficient to drive T:APC conjugate formation, demonstrating that both ADAP and SKAP55 are required for optimal LFA-1 function. Additionally, the ADAP proline-rich domain is required for optimal Ag-induced activation of CD69, CD25, and Bcl-x<sub>L</sub>, but is not required for assembly of the CARMA1/Bcl10/Malt1 (caspase-recruitment domain (CARD) membrane-associated guanylate kinase (MAGUK) protein 1/B-cell CLL-lymphoma 10/mucosa-associated lymphoid tissue lymphoma translocation protein 1) signaling complex and subsequent TCR-dependent NF- $\kappa$ B activity. Our results indicate that ADAP is used downstream of TCR engagement to delineate two distinct molecular programs in which the ADAP/SKAP55 module is required for control of T:APC conjugate formation and functions independently of ADAP/CARMA1-mediated NF- $\kappa$ B activation. *The Journal of Immunology*, 2008, 181: 4840–4851.

Naive T cells respond to Ag engagement of the TCR by rapidly increasing the functional activity of their integrin adhesion receptors, and by initiating molecular programs that promote proliferation and the gain of effector function. Cytosolic adapter proteins and enzymes control the initiation and transmission of these TCR-specific signals (1–3). One key adapter, the linker for activated T cells (LAT),<sup>4</sup> is rapidly tyrosine phosphorylated following TCR stimulation and provides docking sites for the Src homology 2 domain-containing leukocyte-specific

phosphoprotein of 76 kDa (SLP-76) and phospholipase C (PLC- $\gamma$ 1) (4). These proteins are critical for proximal signals generated by the TCR, as loss of LAT or SLP-76 expression severely disrupts T cell development (5–7). In contrast, disruption of proteins downstream of the LAT signalosome results in more selective defects in T cell activation.

Adhesion and degranulation promoting adapter protein (ADAP; formerly known as Fyb or SLAP-130) is expressed by T cells and other hematopoietic lineages except B cells. ADAP was originally identified by its TCR-dependent tyrosine phosphorylation and interaction with SLP-76 and the tyrosine kinase Fyn (8, 9). Like other adapters, ADAP contains a number of protein-protein and protein-lipid interaction domains (1, 10, 11). The C terminus of ADAP contains several tyrosine residues that are involved in TCR-dependent binding to SLP-76 and Fyn (12–14), as well as FPPP domains that have been implicated in interaction with the Ena/Vasp family of actin regulatory proteins (15). A central proline-rich domain between aa 326–426 of murine ADAP constitutively binds the Src homology 3 (SH3) domain of another adapter, SKAP55 (Src kinase-associated phosphoprotein of 55 kDa) (16–18). Immediately adjacent to this domain, aa 426–541 in ADAP contains an E/K-rich domain and part of an atypical helical SH3 domain (hSH3-N) that comprise a binding site for the adapter CARMA1 (caspase-recruitment domain (CARD) membrane-associated guanylate kinase (MAGUK) protein 1) that is critical for formation of the CARMA1/Bcl10 (B-cell CLL-lymphoma 10)/Malt1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1) signaling

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<sup>4</sup> Abbreviations used in this paper: LAT, linker for activated T cells; ADAP, adhesion and degranulation-promoting adapter protein; Bcl10, B-cell CLL/lymphoma 10; CARMA1, caspase-recruitment domain (CARD) membrane-associated guanylate kinase (MAGUK) protein 1; CBM, CARMA1/Bcl10/Malt1; HA, hemagglutinin; hCAR, human coxsackievirus and adenovirus receptor; hSH3, helical SH3; KO, knockout; Malt1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; OVAp, OVA aa 323–339; RIAM, Rap1-GTP-interacting adapter molecule; SH3, Src homology 3; SKAP55, Src kinase-associated phosphoprotein of 55 kDa; SLP-76,

Src homology 2 domain-containing leukocyte-specific phosphoprotein of 76 kDa; WT, wild type; wtADAP, wild-type ADAP.

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complex and NF- $\kappa$ B activation (19). This hSH3-N domain and a highly homologous hSH3-C located at the extreme C terminus of ADAP bind to phospholipids *in vitro* (20, 21). Finally, the N-terminal ~300 aa of ADAP binds HIP-55 (hematopoietic progenitor kinase 1-interacting protein of 55 kDa) (22), although the significance of this interaction is unclear.

Although initial overexpression studies reported both positive and negative roles for ADAP in T cell activation (8, 9, 12, 14), the production of ADAP<sup>-/-</sup> mice demonstrated that ADAP positively regulates T cell activation (23, 24). While ADAP<sup>-/-</sup> T cells mice show normal proximal TCR-dependent responses, including Ca<sup>2+</sup> mobilization and ERK phosphorylation, they exhibit impaired TCR and Ag-dependent integrin-mediated adhesion and subsequent T cell activation and survival (23–25). ADAP or SKAP55 overexpression enhances LFA-1-dependent T cell interactions with APCs in a manner dependent on the SKAP55 SH3 domain (26). Similarly, T cells from SKAP55 knockdown (27) or from SKAP55<sup>-/-</sup> mice (28) exhibit defects in LFA-1 function comparable to ADAP<sup>-/-</sup> mice. The ADAP proline-rich and SKAP55 SH3 domains are critical for TCR-dependent membrane targeting of Rap1 (29), a small GTPase that is important for T cell integrin activation and T:APC conjugate formation (30–32). Additionally, SKAP55 constitutively associates with RIAM (Rap1-GTP-interacting protein), which binds to the active form of Rap1 (29, 33). Thus, an ADAP/SKAP55/RIAM/Rap1 signaling arm appears to be required for control of T cell integrin activation. However, the identification of precise functions for ADAP has been complicated by the observation that stable SKAP55 expression requires a constitutive SH3 domain-mediated interaction of SKAP55 with ADAP (17, 34). The role of the ADAP/SKAP55 interaction in NF- $\kappa$ B activation, as well as the function of SKAP55 independent of ADAP expression, has not been investigated.

In the present study, we used adenovirus to express ADAP and ADAP mutant constructs in resting naive T cells from ADAP<sup>-/-</sup> mice expressing the hCAR (human coxsackievirus and adenovirus receptor) adenovirus receptor (35, 36) and the OVA aa 323–339 (OVAp)-specific DO11.10 transgenic TCR (25, 37). Using naive mouse T lymphocytes, we show that a small region of the ADAP proline-rich domain is required for rescue of SKAP55 expression by ADAP<sup>-/-</sup> T cells, as well as for optimal Ag-dependent T:APC conjugate formation and downstream T cell activation. However, SKAP55 expression alone in the absence of ADAP is not sufficient to restore T:APC conjugate formation. Furthermore, we demonstrate that formation of the ADAP/SKAP55 complex is not required for control of NF- $\kappa$ B activation and that SKAP55 is not found in the CARMA1/Bcl10 complex, suggesting differential control of integrin and NF- $\kappa$ B pathways by ADAP.

## Materials and Methods

### Mice

DO11.10/ADAP<sup>+/+</sup> and ADAP<sup>-/-</sup> mice on the BALB/c background have been previously described (25) and were crossed to the hCAR transgenic mice (35) (provided by Dr. C. Weaver, University of Alabama-Birmingham). Mice were housed in specific pathogen-free facilities at the University of Minnesota and were used between 8 and 12 wk of age. All experimental protocols involving the use of mice were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

### Cells

The Jurkat E6-1 human T cell line was obtained from the American Type Culture Collection and maintained at 37°C in RPMI 1640 supplemented with FCS (Atlanta Biologicals), L-glutamine, and penicillin/streptomycin and grown in 5% CO<sub>2</sub>.

### Abs and reagents

The DO11.10 TCR was detected with FITC- or biotin-conjugated mAb KJ1-26 (Caltag Laboratories). Anti-Thy1.1 allophycocyanin or PE, anti-B220-PE-Cy5.5, anti-CD69-FITC, and anti-CD25 allophycocyanin were from eBioscience. Sheep anti-murine ADAP has been previously described (38). The following Abs were purchased: rabbit anti-SKAP55 (Upstate Biotechnology), rabbit anti-CARMA1 (Alexis Biochemicals), goat anti-hemagglutinin (HA) agarose (Bethyl Laboratories), mouse anti-HA (clone 16B12) (Covance), and mouse anti-Bcl10 (331.3) and mouse anti-NF- $\kappa$ B p65 (F-9) (Santa Cruz Biotechnology). Alexa 488-conjugated donkey anti-sheep and goat anti-rabbit IgG were used for intracellular staining detection. For immunoblotting, Alexa Fluor 680 (Molecular Probes) goat anti-mouse and goat anti-rabbit, as well as IRDye 800-conjugated goat anti-rabbit, goat anti-mouse, and donkey anti-sheep IgG (Rockland Immunochemicals) were used.

### Recombinant DNA

The pENTR-HA-ADAP construct for adenovirus production encodes for full-length murine ADAP (130-kDa isoform) and is derived from pENTR-UP-IT (19). ADAP mutants were generated as previously described (19) using the QuickChange II XL site-directed mutagenesis kit (Stratagene) by introducing a stop codon at amino acid position 426 (mutants  $\Delta$ 426–819) or by using primers flanking both sides of the deleted site (mutants  $\Delta$ 1–327,  $\Delta$ 326–426,  $\Delta$ 338–358, and  $\Delta$ 426–541). FLAG-tagged SKAP55 was obtained from Dr. B. Schraven (Otto-von-Guericke University, Magdeburg, Germany) and was subcloned into pENTR-UP-IT by blunt ligation. GFP-Rap1 was generated by inserting Rap1 in frame in pEGFP-C1 (Clontech) and subcloning the GFP-Rap1 cassette into the *EcoRV* site of pENTR-UP-IT. All constructs were verified by DNA sequencing at the University of Minnesota Microsequencing Facility.

### Adenovirus production and transduction

Adenovirus expression plasmids were generated as previously described (19) using Gateway recombination between pAD/PL-DEST (Invitrogen) and either control pENTR-UP-IT or pENTR-HA-ADAP. Adenovirus was produced in AD-293 packaging cells (Stratagene), and viral particles were purified and titered as described previously (35, 39, 40). Freshly isolated, resting ADAP<sup>+/+</sup> and ADAP<sup>-/-</sup> lymph node T cells expressing the hCAR receptor were transduced with control, ADAP, or SKAP55 adenoviruses as described (35) and incubated at 37°C for 3 days in complete T cell medium containing 5 ng/ml mouse IL-7 (R&D Systems). Preliminary experiments were performed to optimize the length of incubation required to achieve peak ADAP or SKAP55 expression. Expression of HA-ADAP or FLAG-SKAP55 was confirmed in all experiments by intracellular flow cytometry for ADAP (38), HA, or SKAP55. Transduction of Jurkat T cells was performed similarly to the primary T cells (19). Briefly, 10 × 10<sup>6</sup> Jurkat cells at a density of 50 × 10<sup>6</sup>/ml in DMEM containing 10 mM HEPES (pH 7.4) were incubated with 500 × 10<sup>6</sup> infectious units of adenovirus (multiplicity of infection of 50) for 30 min at 37°C. Cells were washed and cultured in T cell medium at 0.5–1 × 10<sup>6</sup> cells/ml for 2 days. Flow cytometric analysis (data not shown) indicated that 80–90% of cells in these cultures were transduced and expressed the indicated ADAP construct.

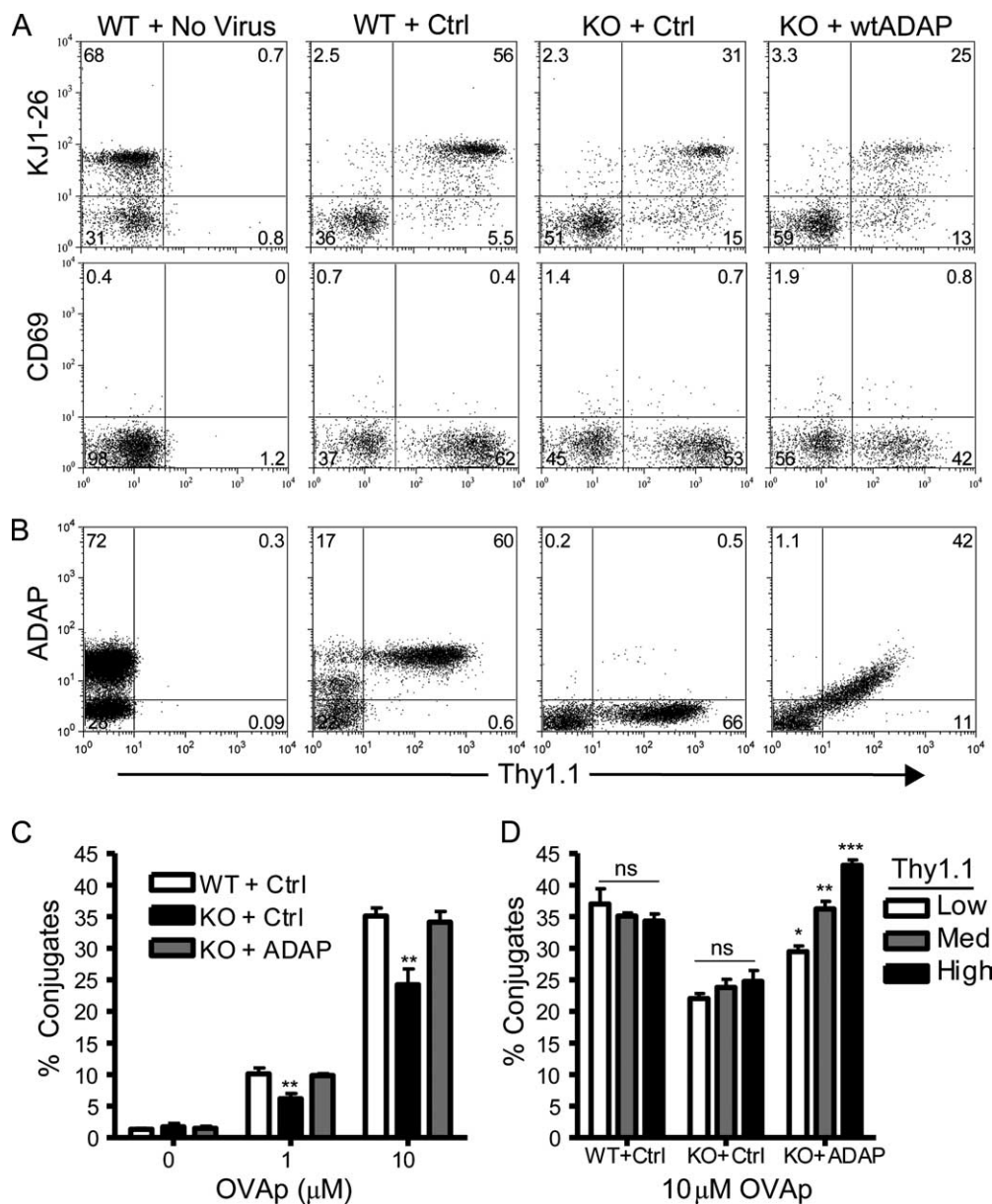
### Conjugate and activation assays

Flow cytometry-based conjugate assays were performed as previously described (25). Briefly, control wild-type (WT) and ADAP<sup>-/-</sup> DO11.10/hCAR bulk lymph node T cells were transduced with adenovirus as described above. Fresh nontransgenic BALB/c splenocytes were labeled with Cell Tracker Orange (Molecular Probes) and then left unpulsed or pre-pulsed for 30 min at 37°C with OVAp (Invitrogen) at the indicated concentrations. Transduced KJ1-26<sup>+</sup>Thy1.1<sup>+</sup> T cells were then combined at a 1:4 ratio with the peptide-loaded splenocytes, pelleted in a 96-well round-bottom plate, incubated for 10 min at 37°C, mixed for 20 s in a plate shaker, fixed with 1% paraformaldehyde, and stained for flow cytometry. Conjugates were defined as KJ1-26<sup>+</sup>Thy1.1<sup>+</sup> T cell events costaining with Cell Tracker Orange and B220. *In vitro* activation was performed similar to the conjugate assays except that 0.1 × 10<sup>6</sup> KJ1-26<sup>+</sup>Thy1.1<sup>+</sup> T cells were seeded into 96-well flat-bottom plates and activated by the addition of 0.2 × 10<sup>6</sup> unstained, peptide-loaded splenocytes. Cells were harvested at the indicated times and KJ1-26<sup>+</sup>Thy1.1<sup>+</sup> T cells were stained for CD25, CD69, and Bcl-x<sub>L</sub> as previously described (25).

### Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting for Bcl10, CARMA1, and endogenous ADAP was performed as previously described (19, 41). Briefly, 10 × 10<sup>6</sup> cells per condition in PBS containing 0.5% BSA were stimulated





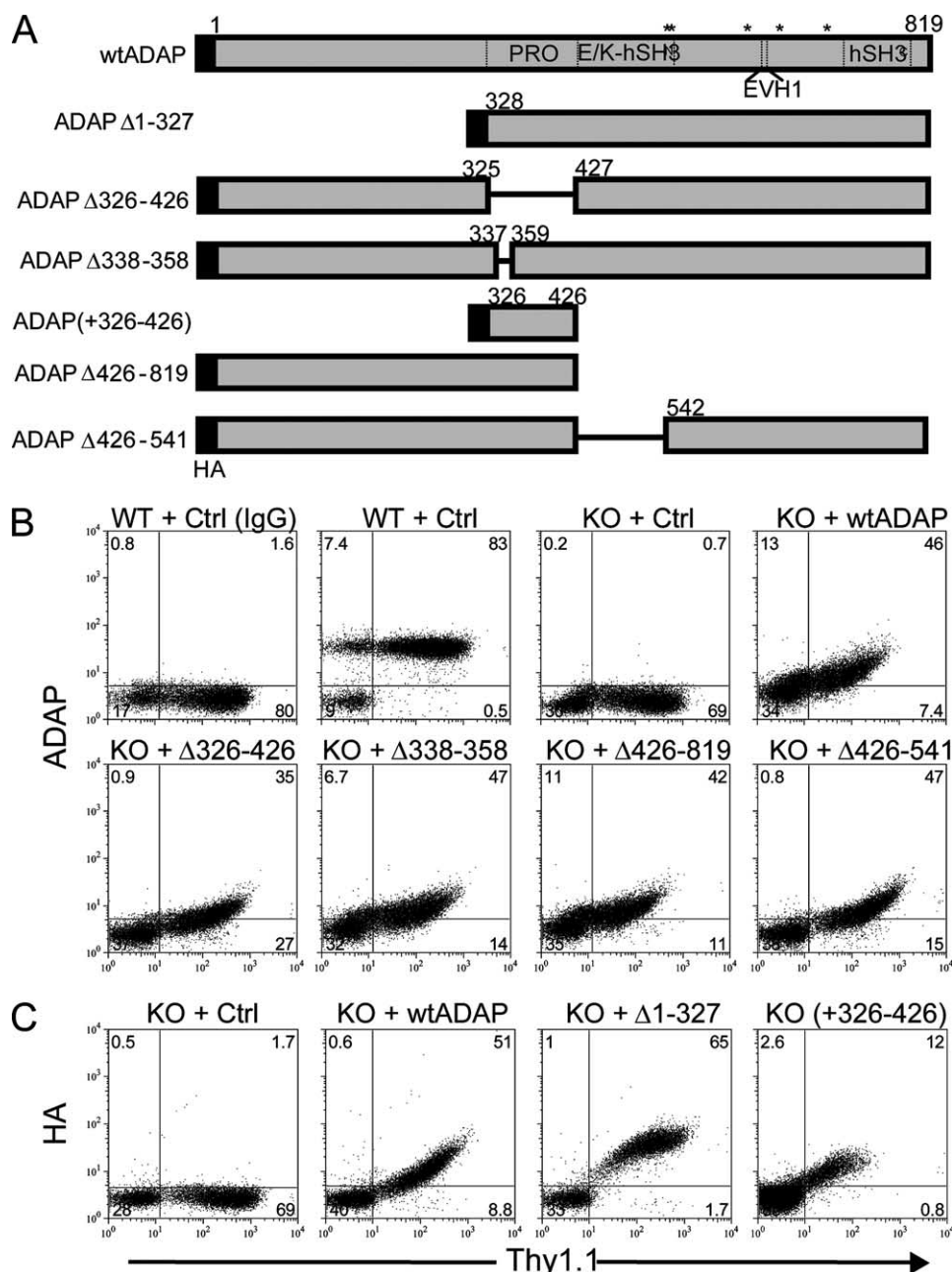
**FIGURE 1.** Rescue of Ag-dependent T:APC conjugate formation in ADAP<sup>-/-</sup> T cells following reconstitution of wtADAP. Freshly harvested, naive lymphocytes from transgenic DO11.10/hCAR/ADAP<sup>+/+</sup> (WT) or ADAP<sup>-/-</sup> (knockout (KO)) mice were transduced with adenovirus encoding Thy1.1 alone (Ctrl), Thy1.1 plus murine ADAP (wtADAP), or mock transduced (No Virus) and cultured *ex vivo* for 3 days as described in *Materials and Methods*. **A**, Cells were stained for Thy1.1 and either the DO11.10 TCR (KJ1-26) or CD69 and analyzed by flow cytometry. **B**, Cells were fixed and intracellular staining was performed with a sheep anti-ADAP Ab. **C**, T:APC conjugate formation between KJ1-26<sup>+</sup>Thy1.1<sup>+</sup> T cells and BALB/c splenocytes prepulsed with the indicated concentrations of OVAp (left panel) was performed as described in *Materials and Methods*. **D**, The same KJ1-26<sup>+</sup>Thy1.1<sup>+</sup> population of T cells was gated into three subpopulations expressing either low, medium, or high levels of Thy1.1, and T:APC conjugate efficiency with 10  $\mu$ M OVAp was determined. Results are representative of at least three independent experiments performed.

with PMA (50 ng/ml), lysed with an equal volume of 2 $\times$  lysis buffer (2% Nonidet P-40, 100 mM Tris-HCl (pH 7.6), 300 mM NaCl, 4 mM EDTA, 2 mM sodium vanadate, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 2 mM PMSF), and then cleared by centrifugation at 12,000 relative centrifugal force. For immunoprecipitation, individual Abs were adsorbed to protein A-Sepharose for 2 h at 4°C, washed with 0.2 M NaBO<sub>3</sub> (pH 9.0), resuspended in 20 mM dimethyl pimelimidate, and incubated for 30 min at room temperature, washed with 0.2 M ethanolamine (pH 8.0), and incubated for 2 h in 0.2 M ethanolamine. The cleared lysates were then incubated overnight at 4°C with the crosslinked bead/antibody complexes. The immune complexes were washed twice with 1 $\times$  lysis buffer containing 0.1% Triton-X 100 and separated by SDS-PAGE. After Western transfer of the proteins, polyvinylidene difluoride membrane was blocked with 0.2% casein for 1 h, primary Ab was incubated overnight in PBS/0.2% casein/0.2% Tween 20, washed with PBS containing 0.2% Tween 20, incubated for 1 h in secondary Ab in PBS/0.2% casein/0.2% Tween 20, and finally washed

with PBS/0.2% Tween 20/0.02% SDS. The membrane was imaged with an Odyssey infrared imager (LI-COR Biosciences). Coimmunoprecipitation of SKAP55 with HA-ADAP constructs was performed by lysing cells as described above, incubating the soluble lysate with anti-HA agarose for 3 h at 4°C, and immunoblotting for ADAP, HA, or SKAP55 as described above.

#### NF- $\kappa$ B nuclear translocation

Lymph node T cells isolated from ADAP<sup>+/+</sup> or ADAP<sup>-/-</sup> mice were transduced and cultured as described above. At the time of harvest, cells were washed into PBS/0.2% BSA and stimulated with 10  $\mu$ g/ml anti-CD3 (2C11) and 1  $\mu$ g/ml anti-CD28. Cells were then fixed in 2% paraformaldehyde, stained with anti-Thy1.1-PE, anti-p65 FITC, and 7-aminoactinomycin D (eBioscience), and then analyzed for NF- $\kappa$ B nuclear translocation using the ImageStream 100 multispectral imaging flow cytometer (Amnis)



**FIGURE 2.** Schematic and expression of ADAP mutant constructs. **A**, Scale diagram of ADAP expression constructs used in this study. Amino acid numbering is given for the murine ADAP p130 isoform. Abbreviations: PRO, proline-rich domain; E/K, glutamic acid and lysine-rich domain; hSH3, N-terminal (N) or C-terminal (C) helical SH3 domain; EVH1, Ena/Vasp homology domain. Asterisks are used to denote the position of tyrosines 547, 549, 584, 615, and 687 found in phosphorylation consensus motifs. **B**, Freshly harvested naive DO11.10/hCAR/ADAP<sup>+/+</sup> (WT) or ADAP<sup>-/-</sup> (KO) lymphocytes were transduced with adenoviruses encoding the indicated constructs or Thy1.1 control adenovirus (Ctrl) and fixed and stained with an anti-Thy1.1 Ab or either anti-ADAP Ab or nonimmune sheep serum (IgG) and analyzed by flow cytometry. **C**, Same as in **B** except the indicated constructs were stained with an anti-hemagglutinin (HA) Ab. Expression profiles are representative of at least three independent analyses performed for each construct shown.

as described previously (42). A minimum of 10,000 cells were collected and analyzed under each experimental condition. NF- $\kappa$ B p65 nuclear translocation was specifically assessed in cells expressing similar levels of Thy1.1.

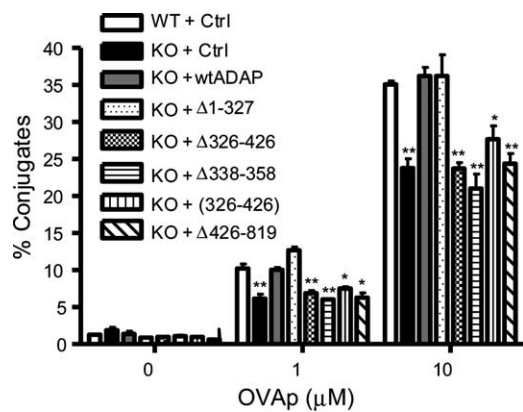
#### Bead conjugates

Five-micrometer latex beads (Interfacial Dynamics) were coated with anti-CD3 (2C11) in PBS for 3 h at 37°C, blocked with 3% BSA, and pelleted briefly at a 1:1 ratio with transduced T cells. After 2 min, the pellets were gently resuspended and incubated at 37°C for an additional 10 min. The T cell/bead complexes were then fixed with paraformaldehyde and processed for anti-SKAP55 intracellular staining or analyzed directly for GFP-Rap1

using the ImageStream cytometer as described above. Amnis Ideas 3.0 software was used to quantify the percentage of GFP-Rap1 or SKAP55 at the T cell/bead contact. First, a mask encompassing the bead area was dilated to include the adjacent T cell surface. Then, the percentage of recruited protein was defined as the intensity of GFP or SKAP55 within this mask, divided by the total intensity within the entire T cell/bead event ( $\times 100$ ).

#### Statistical analysis

Measures of statistical significance were determined with GraphPad Prism 5.0 software using an unpaired Student's *t* test. As indicated, \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; ns, not significant.



**FIGURE 3.** The ADAP proline-rich domain is required for T:APC conjugate formation. Naive DO11.10/hCAR/ADAP<sup>-/-</sup> (KO) or ADAP<sup>+/+</sup> (WT) lymphocytes expressing the indicated ADAP constructs were analyzed for T:APC conjugate formation between KJ1-26<sup>+</sup>Thy1.1<sup>+</sup> T cells and BALB/c splenocytes prepulsed with the indicated concentrations of OVAp. Results are shown for a single representative experiment and are representative of at least four independent experiments performed for each construct.

**Results**

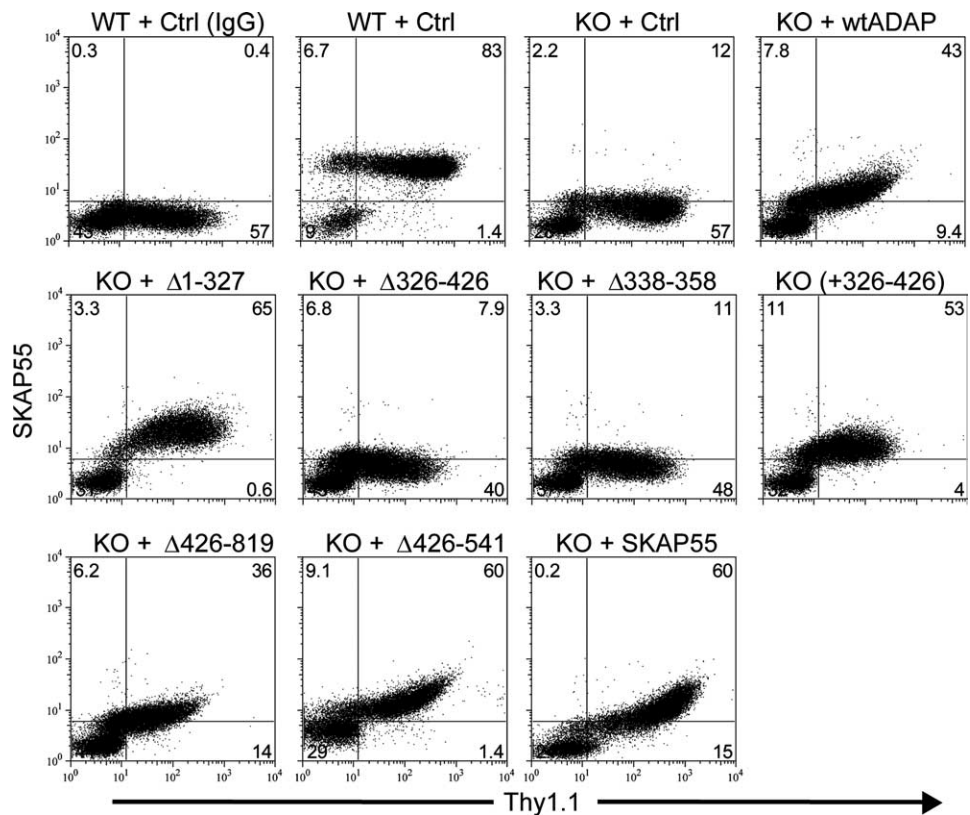
*Rescue of T:APC conjugate formation in naive ADAP<sup>-/-</sup> T cells by expression of WT ADAP (wtADAP)*

To investigate the ADAP functional domains that control integrin activation in naive primary murine T cells, we analyzed Ag-dependent T:APC conjugate formation by DO11.10/ADAP<sup>-/-</sup> T cells expressing either wtADAP or ADAP deletion mutants. We expressed the hCAR transgene with a T cell-specific promoter (35) on the DO11.10/ADAP<sup>-/-</sup> background (25) to permit efficient adenovirus-mediated transduction of T cells from bulk lymph node preparations (data not shown) (35). Recombinant adenoviruses en-

coding the WT murine ADAP gene and an IRES (internal ribosome entry site)-driven Thy1.1 (CD90.1) cell surface reporter (19) were used to reconstitute ADAP expression in freshly harvested, resting hCAR<sup>+</sup>/DO11.10/ADAP<sup>-/-</sup> T cells (Fig. 1). Thy1.1 expression was observed following adenovirus transduction (Fig. 1A, no virus vs control (Ctrl; Thy1.1 only) virus). Both ADAP<sup>+/+</sup> and ADAP<sup>-/-</sup> cells expressed similar levels of the DO11.10 TCR following incubation with adenovirus, as judged by equivalent intensity of the KJ1-26 staining (Fig. 1A, upper panels). There was no up-regulation of expression of either CD69 (Fig. 1A, lower panels) or CD25 (data not shown, see also Fig. 7B) in Thy1.1-expressing cells across these treatment groups, indicating that the cells were not being activated while resting in culture following adenovirus transduction. Intracellular staining with an anti-ADAP Ab (Fig. 1B) indicated the presence and absence of endogenous ADAP in the majority of Thy1.1<sup>+</sup> ADAP<sup>+/+</sup> and ADAP<sup>-/-</sup> cells, respectively. Flow cytometric analysis of ADAP<sup>-/-</sup> cells transduced with wtADAP demonstrates that Thy1.1 expression correlated with rescue of ADAP expression to levels approaching, but not exceeding, those of endogenous ADAP expression (Fig. 1B, right panel).

DO11.10/ADAP<sup>-/-</sup> T cells show an ~30–50% impairment in LFA-1-dependent T cell conjugate formation with OVAp-pulsed APCs at all Ag doses examined (19, 25). A similar defect in T:APC conjugate formation was observed following OVAp stimulation in this system. Background levels of T:APC conjugates in the absence of OVAp were low (<2%; Fig. 1C, 0  $\mu$ M OVAp). Following incubation with APCs loaded with 10  $\mu$ M OVAp, ~35% of control adenovirus-transduced ADAP<sup>+/+</sup> T cells interacted with APCs. In contrast, this conjugate efficiency was reduced to ~25% for control ADAP<sup>-/-</sup> T cells (Fig. 1C). Importantly, reconstitution of ADAP<sup>-/-</sup> T cells with wtADAP restored conjugate formation to a level similar to that found in ADAP<sup>+/+</sup> cells (Fig. 1C) (19). Because ADAP detection by intracellular staining

**FIGURE 4.** The ADAP proline-rich domain controls SKAP55 expression in ADAP<sup>-/-</sup> T cells. Naive DO11.10/hCAR/ADAP<sup>-/-</sup> (KO) or ADAP<sup>+/+</sup> (WT) lymphocytes expressing the indicated ADAP constructs, WT SKAP55, or the control adenovirus (Ctrl) were fixed and intracellular staining was performed with rabbit anti-SKAP55 Ab or control rabbit Ig (IgG) and analyzed by flow cytometry. Results are shown for a single representative experiment and are representative of at least four independent experiments performed for each construct.





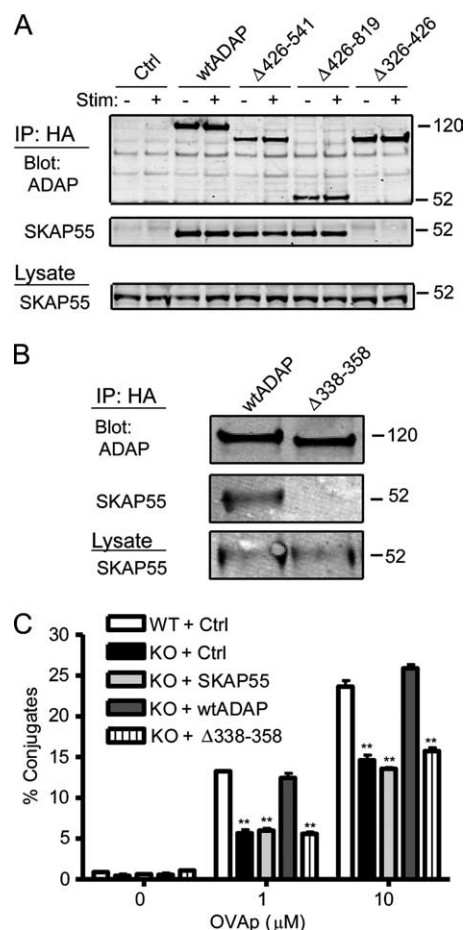
was sensitive enough to detect differences in ADAP expression based on the transduced level of Thy1.1 staining (43), we analyzed conjugate formation in ADAP<sup>-/-</sup> T cells gated for increasing ranges of Thy1.1 expression and found that the magnitude of this rescue depended on the level of ADAP reconstitution (Fig. 1D). For example, KJ1-26<sup>+</sup> cells falling within the lower third of Thy1.1 expression showed only modest rescue of adhesion, while cells within the medium or high Thy1.1 gate showed highly significant increases in adhesion compared with the ADAP<sup>-/-</sup> vector control (Fig. 1D). Subsequent analyses are presented using the top approximate third of Thy1.1<sup>+</sup> (Thy1.1<sup>high</sup>) cells expressing the wtADAP construct, with this gate being applied identically to all samples in the experiment to ensure consistency between the constructs used.

*The ADAP C terminus and proline-rich domains are required for efficient T:APC conjugate formation*

We next prepared a panel of ADAP mutants to identify the molecular domains in ADAP that are required for control of T:APC conjugate formation (Fig. 2A). The expression of constructs containing the N terminus of ADAP was demonstrated by intracellular staining with an anti-ADAP Ab that recognizes the N terminus (Fig. 2B). The ADAPΔ1–327 and ADAP(+326–426) constructs, which lack the N terminus, were detected by staining with an anti-HA Ab (Fig. 2C). As in Fig. 1B, Thy1.1 expression correlated with increased expression of all the ADAP constructs we examined. Wild-type ADAP and an ADAP mutant removing the first 327 aa (ADAPΔ1–327) both rescued conjugate formation (Figs. 1C and 3), indicating that the N terminus of ADAP is not essential for this response. However, deletion of the proline-rich domain of ADAP (Δ326–426) completely abolished rescue of conjugate formation, down to levels of control virus alone. Similarly, a restricted deletion within this proline-rich domain (Δ338–358) also failed to rescue T:APC adhesion, indicating that this 20-aa segment between aa 338–358 of ADAP is critical for control of integrin function (Fig. 3). However, expression of the proline-rich domain alone (+326–426) failed to rescue conjugate formation, indicating the requirement for a second domain in ADAP (Fig. 3). Indeed, removal of the C-terminal ~400 aa of ADAP (Δ426–819) also restricted rescue of conjugate formation, indicating that the C terminus of ADAP is also required for efficient conjugate formation.

*The ADAP proline-rich domain controls SKAP55 expression in naive T cells*

Analysis of ADAP-deficient Jurkat T cells (34) and ADAP<sup>-/-</sup> murine T cells (44) has demonstrated that SKAP55 expression is also severely impaired in the absence of ADAP, due to caspase and/or proteasome-mediated destabilization of free SKAP55 protein when it cannot interact with ADAP. We have confirmed this finding using intracellular flow cytometry of WT or ADAP<sup>-/-</sup> lymphocytes using an anti-SKAP55 Ab (Fig. 4) and Western blotting (data not shown). Wild-type T cells infected with control Thy1.1 adenovirus showed robust SKAP55 levels, while ADAP<sup>-/-</sup> T cells infected with the same control adenovirus demonstrated very low expression, consistently just above the baseline signal derived from control Ig (Fig. 4). Similar results were observed using a monoclonal anti-SKAP55 Ab for flow cytometry and Western blotting, and in freshly isolated, non-adenovirus-transduced lymphocytes (data not shown). Adenoviral-mediated reconstitution of ADAP expression allowed SKAP55 levels to accumulate (Fig. 4), with peak expression observed after ~3 days of infection (data not shown). This effect was positively correlated with the level of Thy1.1 expression, indicating that SKAP55 accumulation mirrors the level of ADAP reconstitution (see Fig. 1D).

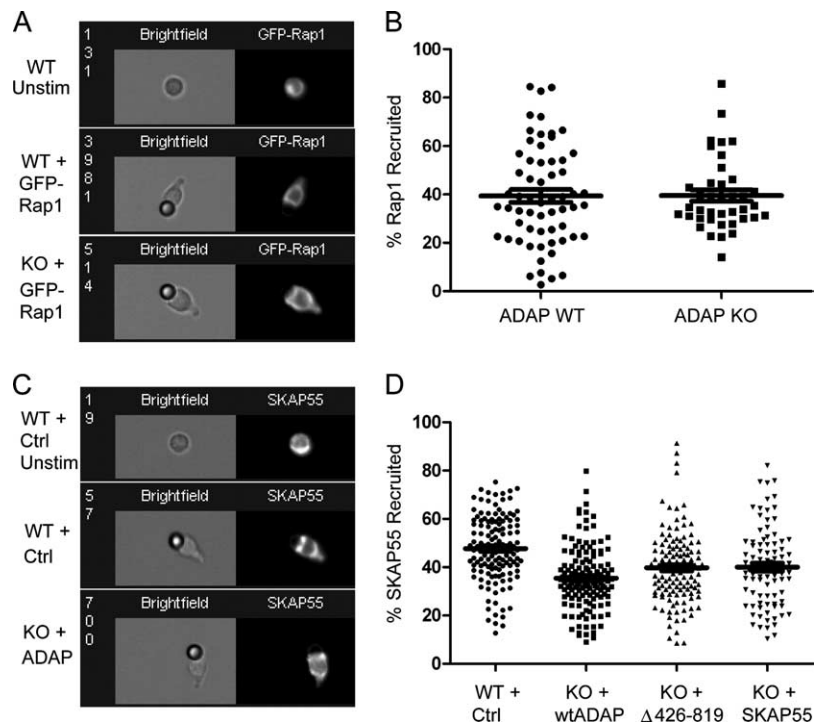


**FIGURE 5.** SKAP55 expression in ADAP<sup>-/-</sup> T cells fails to rescue T:APC conjugate formation. **A**, Jurkat T cells were transduced with control adenovirus (Ctrl) or with the indicated HA-tagged ADAP constructs. After 2 days, 10<sup>6</sup> cells were left unstimulated or stimulated for 5 min with anti-TCR mAb OKT3, lysed, and immunoprecipitated with agarose-conjugated anti-HA and Western blots for ADAP and SKAP55 were performed. The relative molecular mass of size standards is shown on the right. Similar results were observed in four independent experiments. **B**, Naive hCAR/ADAP<sup>+/+</sup> lymphocytes expressing endogenous levels of SKAP55 were transduced with either full-length murine ADAP (wtADAP) or ADAP lacking the restricted proline-rich domain (Δ338–358). Cells were lysed as described in *Materials and Methods*, immunoprecipitated with agarose-conjugated anti-HA Abs, and Western blots were performed with anti-HA or anti-SKAP55 Abs. **C**, Naive DO11.10/hCAR/ADAP<sup>-/-</sup> (KO) or ADAP<sup>+/+</sup> (WT) lymphocytes expressing the indicated constructs were analyzed for T:APC conjugate formation between KJ1-26<sup>+</sup>Thy1.1<sup>+</sup> T cells and BALB/c splenocytes prepulsed with the indicated concentrations of OVAp. Results are shown for a single representative experiment and are representative of at least four independent experiments performed for each construct.

Analysis of the ADAP domains required for SKAP55 stability demonstrated that neither the N terminus (aa 1–327) nor the C terminus (aa 426–819) of ADAP is essential. In contrast, removal of the central proline-rich domain of ADAP (Δ326–426) or the restricted deletion (Δ338–358) within this region precluded accumulation of SKAP55 (Fig. 4). Expression of the proline-rich domain alone (+326–426) was sufficient to stabilize SKAP55, even though this domain does not rescue conjugate formation (Fig. 4).

We next performed coimmunoprecipitation analysis to confirm that the interaction of ADAP with SKAP55 is dependent on the ADAP proline-rich domain. Jurkat T cells were transduced with the control virus, or with HA-tagged wtADAP, Δ426–541,

**FIGURE 6.** ADAP is not required for recruitment of Rap1 or SKAP55 to the contact site between T cells and anti-TCR beads. **A**, Naive DO11.10/hCAR/ADAP<sup>+/+</sup> (WT) or ADAP<sup>-/-</sup> (KO) lymphocytes were transduced with GFP-Rap1 and conjugates with anti-TCR (2C11)-coated beads were formed as described in *Materials and Methods*. GFP-Rap1-expressing cells were gated and photographed by image-scanning cytometry and a representative image from each sample is shown. An example of GFP-Rap1 expression in a cell absent of bead stimulation is also shown (Unstim). **B**, Graphical display of the percentage of total GFP-Rap1 signal in each cell that is concentrated against the anti-TCR-coated bead, quantified as described in *Materials and Methods*. **C**, Naive DO11.10/hCAR/ADAP<sup>+/+</sup> (WT) T cells expressing the control virus (Ctrl) or ADAP<sup>-/-</sup> (KO) lymphocytes expressing wtADAP, ADAP $\Delta$ 426–819, or SKAP55 were stimulated with anti-TCR-coated beads as described for Fig. 4. **D**, Graphical display of the percentage of total SKAP55 signal in each cell that is concentrated against the anti-TCR-coated bead.



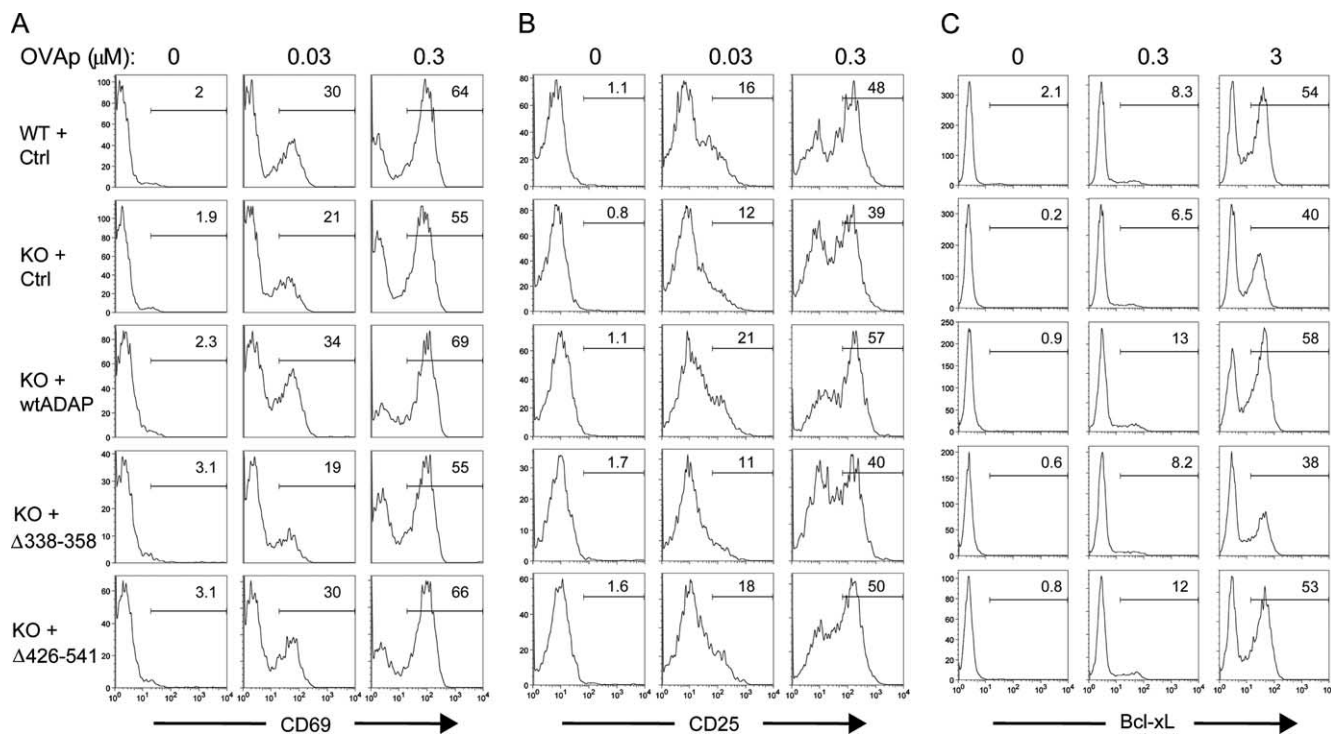
$\Delta$ 426–819, or  $\Delta$ 326–426, and stimulated with anti-TCR mAb OKT3 or left unstimulated. Following lysis and anti-HA immunoprecipitation, SKAP55 constitutively interacted with all constructs except the proline-rich domain mutant ( $\Delta$ 326–426) (Fig. 5A) and with the restricted proline deletion ( $\Delta$ 338–358, data not shown). To assess the dependence on this proline-rich domain in primary murine T cells, we transduced ADAP<sup>+/+</sup> T cells, which maintain endogenous SKAP55, with either HA-tagged wtADAP or HA-tagged ADAP $\Delta$ 338–358. Input whole-cell lysates from each sample contained equivalent levels of SKAP55, and the epitope-tagged ADAP construct was pulled down equally following anti-HA immunoprecipitation (Fig. 5B). Importantly, SKAP55 was detected in the HA immunoprecipitate from the wtADAP-transduced sample but not from the ADAP $\Delta$ 338–358 sample (Fig. 5B). Thus, the ADAP proline-rich motif is critical for association with and stability of SKAP55 in primary murine T cells.

#### SKAP55 expression is not sufficient for T:APC conjugate formation

Since cells expressing the ADAP proline-rich domain mutants lack normal levels of SKAP55, it remains possible that stable SKAP55 expression alone might be sufficient for T:APC conjugate formation. To directly test this model, we infected ADAP<sup>-/-</sup> T cells with adenovirus expressing SKAP55. Following transduction with the virus, SKAP55 was detected by intracellular flow cytometry (see Fig. 4, *final panel*) at levels approaching that of endogenous SKAP55 found in ADAP<sup>+/+</sup> cells. This level of SKAP55 is at or above the level of SKAP55 that accumulated following expression of wtADAP. Furthermore, the exogenously supplied SKAP55 was able to interact with ADAP when expressed in ADAP<sup>+/+</sup> T cells (data not shown). However, SKAP55 expression in DO11.10/ADAP<sup>-/-</sup> T cells failed to rescue T:APC conjugate formation (Fig. 5C). This result suggests that SKAP55 is not sufficient for this function and that both ADAP and SKAP55 are required for control of Ag receptor-dependent integrin function in naive T cells.

We additionally tested the dependence on ADAP for recruitment of Rap1 and SKAP55 to TCR-coated beads, as a measure of the membrane recruitment potential of SKAP55 and Rap1. ADAP has been shown to be important for TCR-induced membrane recruitment of Rap1 (29 and R. B. Medeiros and Y. Shimizu, unpublished observations). Due to limitations in cell numbers obtained from our ex vivo adenovirus cultures, we were unable to perform biochemical fractionation assays as previously described (29). Instead, we expressed GFP-Rap1 in WT or ADAP<sup>-/-</sup> T cells and monitored targeting of this construct to the interface of anti-TCR-coated beads using image-scanning cytometry. Although we did not see striking organization of Rap1 at the contact site in WT T cells (Fig. 6A), ~40% of the T cell Rap1 was within the bead contact site in WT T cells (Fig. 6B). Surprisingly, ADAP<sup>-/-</sup> T cells did not show a defect in Rap1 targeting to the TCR contact site (Fig. 6B,  $p = 0.96$ ), and there were no differences in this measure of Rap1 recruitment between ADAP<sup>-/-</sup> cells reconstituted with wtADAP, ADAP $\Delta$ 426–819, or ADAP $\Delta$ 326–426 (data not shown). The recruitment of SKAP55 was also monitored in ADAP<sup>+/+</sup> or ADAP<sup>-/-</sup> T cells expressing wtADAP,  $\Delta$ 426–819, or SKAP55 in the absence of ADAP (Fig. 6C). In contrast to Rap1, SKAP55 was tightly recruited to the T cell/bead interface and frequently adopted a bimodal staining as the T cell wrapped around the edges of the bead (Fig. 6C). Quantification of these observations indicated that ~40% of the SKAP55 in the cell was recruited to the bead interface in this assay. However, no differences in SKAP55 recruitment were observed between ADAP<sup>-/-</sup> cells reconstituted with wtADAP or ADAP $\Delta$ 426–819 (36 vs 39%; Fig. 6D). Similarly, we also noticed that 40% of exogenous SKAP55 expressed in the absence of ADAP was also recruited to the bead contact. This suggests that determinants within SKAP55 may be sufficient to drive membrane targeting in these assays. Interestingly, ADAP<sup>+/+</sup> cells were somewhat more efficient in their overall ability to recruit SKAP55 to the bead contact (48% of cellular SKAP55) compared with ADAP<sup>-/-</sup> cells expressing wtADAP, ADAP $\Delta$ 426–819, or SKAP55 alone ( $p < 0.0001$ ).





**FIGURE 7.** The ADAP proline-rich domain is required for Ag-dependent T cell activation. Naive DO11.10/hCAR/ADAP<sup>+/+</sup> (WT) or ADAP<sup>-/-</sup> (KO) lymphocytes expressing the indicated ADAP constructs were stimulated with fresh BALB/c splenocytes pulsed with the indicated doses of OVAp as described in *Materials and Methods*. *A* and *B*, Cells were stimulated for 18 h, stained for KJ1-26, Thy1.1, CD69, and CD25 as indicated, fixed, and analyzed by flow cytometry. *C*, Cells were stimulated for 48 h with OVAp, stained for KJ1-26 and Thy1.1, fixed, permeabilized with saponin, stained for anti-Bcl-x<sub>L</sub>, and analyzed by flow cytometry. *A–C*, The percentage of CD69<sup>+</sup>, CD25<sup>+</sup>, or Bcl-x<sub>L</sub><sup>+</sup> cells within the KJ1-26<sup>+</sup>Thy1.1<sup>+</sup> gate is shown on each histogram. Results are shown for a single representative experiment and are representative of at least four independent experiments performed for CD69 and CD25 and three experiments for Bcl-x<sub>L</sub>.

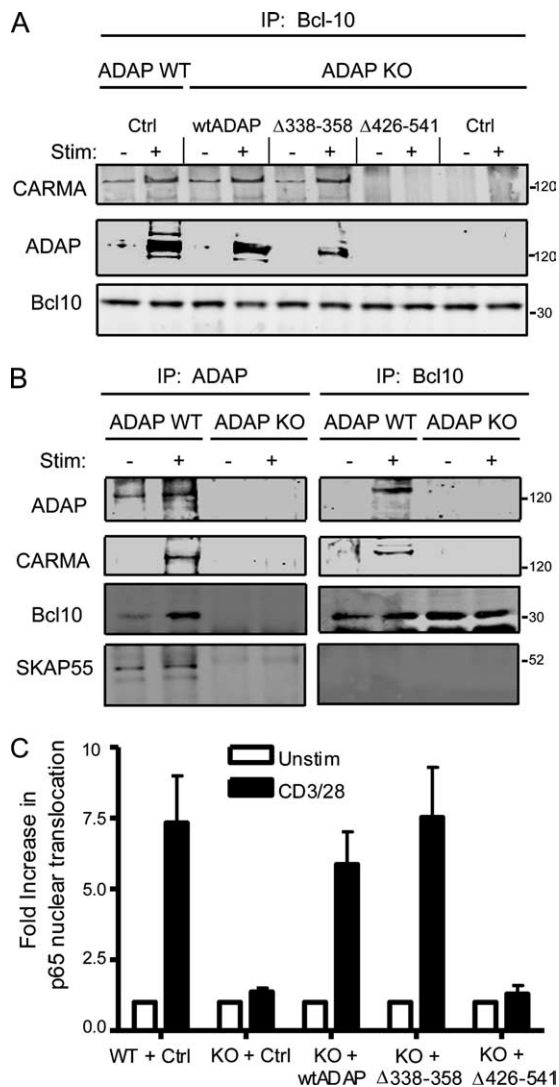
#### ADAP/SKAP55 interaction is required for optimal T cell activation

In addition to impaired integrin-mediated adhesion, ADAP is also important for TCR- and Ag-dependent T cell activation and clonal expansion, especially at low Ag concentrations (25). However, it is not known whether these reported defects in Ag-dependent T cell activation trace to impaired integrin-mediated adhesion at the onset of Ag stimulation, or to other ADAP-dependent signaling pathways. To determine which ADAP functional domains are important for T cell activation, we monitored expression of the early activation marker CD69, the IL-2 receptor (CD25), and the pro-survival protein Bcl-x<sub>L</sub> in ADAP<sup>-/-</sup> T cells expressing the wtADAP or ADAP mutants. Using primary naive DO11.10/hCAR/ADAP<sup>+/+</sup> or ADAP<sup>-/-</sup> cells reconstituted ex vivo with control adenovirus, we consistently observed a reduction in the percentage of ADAP<sup>-/-</sup> cells displaying CD69 and CD25 expression following 18 h of stimulation with OVAp (Fig. 7, *A* and *B*). Bcl-x<sub>L</sub> expression was also impaired in ADAP<sup>-/-</sup> cells after 48 h of stimulation (Fig. 7*C*), consistent with previous results (25). Reconstitution of ADAP<sup>-/-</sup> cells with wtADAP but not with the SKAP55-binding mutant ADAPΔ338–358 restored CD69, CD25, and Bcl-x<sub>L</sub> activation to levels at or above ADAP<sup>+/+</sup> T cells (Fig. 7), suggesting that the ADAP/SKAP55 interaction is important for optimal Ag-dependent T cell activation. Similarly, ADAP<sup>-/-</sup> cells expressing the ADAPΔ426–819 C-terminal mutant, or expressing SKAP55 alone in the absence of ADAP, were unable to activate CD69, CD25, or Bcl-x<sub>L</sub> as robustly as when intact wtADAP is expressed (data not shown). In contrast, ADAP<sup>-/-</sup> cells expressing the CARMA1-binding mutant ADAPΔ426–541 did not show appreciable defects in CD69, CD25, and Bcl-x<sub>L</sub> expression, espe-

cially when compared with ADAP<sup>+/+</sup> cells. These results indicate that ADAP-dependent conjugate formation influences the functional activation state of T cells.

#### ADAP/SKAP55 is not required for NF-κB activation

ADAP also regulates TCR-mediated activation of the transcription factor NF-κB in T cells via TCR-regulated binding of the NF-κB regulatory protein CARMA1 to aa 426–541 of ADAP, and subsequent assembly of the CARMA1/Bcl10/MALT1 complex (19). However, the ADAP mutant lacking CARMA1 binding capacity (ADAPΔ426–541) can restore efficient T:APC conjugate formation (19). In line with this finding, primary ADAP<sup>-/-</sup> T cells expressing ADAPΔ426–541 showed efficient accumulation of SKAP55, suggesting that the CARMA1-binding domain within ADAP is dispensable for SKAP55 stabilization (Fig. 4). This suggests that ADAP forms physically and/or functionally distinct complexes with SKAP55 and with CARMA1 in the cell. However, it is unclear whether the ADAP/SKAP55 complex is required for NF-κB activity. To address this question, ADAP<sup>-/-</sup> T cells expressing wtADAP, ADAPΔ426–541, or ADAPΔ338–358 were stimulated, and then cell lysates were prepared and subjected to immunoprecipitation for Bcl10 and Western blotting for CARMA1 and ADAP. Consistent with previous results (19), stimulation with PMA enhanced the association of CARMA1 with Bcl10 in control ADAP<sup>+/+</sup> cells and in ADAP<sup>-/-</sup> cells expressing wtADAP, but not in ADAP<sup>-/-</sup> cells expressing either control adenovirus or ADAPΔ426–541 (Fig. 8*A*). When ADAPΔ338–358 was expressed in ADAP<sup>-/-</sup> T cells, CARMA1 also associated with Bcl10 following PMA stimulation (Fig. 8*A*), indicating that neither the ADAP proline-rich domain nor SKAP55 is required for



**FIGURE 8.** The ADAP/SKAP55 interaction is not required for assembly of the CARMA1/Bcl10 complex or TCR induced NF- $\kappa$ B activation. **A**, Naive DO11.10/hCAR/ADAP<sup>-/-</sup> (KO) or ADAP<sup>+/+</sup> (WT) lymphocytes expressing the indicated ADAP constructs or the control (Ctrl) were left untreated (-) or stimulated with PMA (+) and lysed as described in *Materials and Methods*. Lysates were subjected to immunoprecipitation with an anti-Bcl10 Ab, and Western blots were performed with Abs to CARMA1, ADAP, or Bcl10. **B**, Freshly harvested resting ADAP<sup>+/+</sup> (WT) or ADAP<sup>-/-</sup> (KO) lymphocytes were left unstimulated or stimulated with PMA and lysed as in **A** and immunoprecipitated in parallel with Abs to either ADAP (*left panel*) or Bcl10 (*right panel*). Western blots were performed with Abs to ADAP, CARMA1, Bcl10, and SKAP55 as indicated between the panels. Results are representative of three (**A**) or two (**B**) independent experiments. **C**, Naive DO11.10/hCAR/ADAP<sup>-/-</sup> (KO) or ADAP<sup>+/+</sup> (WT) lymphocytes expressing the indicated constructs were stimulated with anti-CD3 plus anti-CD28 Abs as described in *Materials and Methods* or left untreated. The samples were fixed and stained with Abs to Thy1.1 and NF- $\kappa$ B (p65), and nuclei were stained with 7-aminoactinomycin D. Cells were analyzed on a multispectral image-scanning flow cytometer as described in *Materials and Methods*. Nuclear localization of p65 in unstimulated T cells was set to 1, and results show the increase in p65 nuclear translocation in stimulated relative to unstimulated Thy1.1<sup>+</sup> cells from three independent experiments.

this complex to assemble. Furthermore, ADAP was only found in the Bcl10 immunoprecipitates where the Bcl10/CARMA1 association formed (Fig. 8A).

To assess whether the cellular pool of ADAP assembled with Bcl10/CARMA1 is separate from that found with SKAP55, identical aliquots of stimulated lysates from ADAP<sup>+/+</sup> cells were subjected to immunoprecipitation with either anti-Bcl10 or anti-ADAP Abs. Assembly of Bcl10, CARMA1, and ADAP was readily observed by either immunoprecipitation strategy (Fig. 8B). Interestingly, while anti-ADAP immunoprecipitation showed SKAP55 association (Fig. 8B, *left panels*), SKAP55 was not detectable in Bcl10 immunoprecipitates (Fig. 8B, *right panels*). This suggests that SKAP55 and its binding domains in ADAP are not involved in TCR-dependent NF- $\kappa$ B activation. Consistent with this model, expression of the ADAP $\Delta$ 338-358 mutant rescued TCR-dependent NF- $\kappa$ B nuclear translocation to levels similar to that observed when wtADAP is expressed (Fig. 8C). In contrast, ADAP $\Delta$ 426-541, which fails to bind Bcl10 and CARMA1, did not rescue NF- $\kappa$ B activation (Fig. 8C). Taken together, these findings indicate that the ADAP proline-rich domain and assembly of the ADAP/SKAP55 complex are not important for ADAP-dependent NF- $\kappa$ B activity.

## Discussion

We have investigated the molecular mechanism of ADAP-dependent integrin and NF- $\kappa$ B activation in naive primary murine T lymphocytes. The development and analysis of ADAP<sup>-/-</sup> mice clearly demonstrated that ADAP positively regulates T cell activation,  $\beta_1$  and  $\beta_2$  integrin-dependent adhesion (23, 24), and peptide Ag-dependent T:APC conjugate formation (19, 25). However, initial analysis of ADAP function before the development of ADAP<sup>-/-</sup> mice utilized overexpression approaches and yielded results that were consistent with both a positive and negative function for ADAP in regulating TCR-dependent signaling (8, 9, 12, 14). Several recent investigations of ADAP function have also utilized overexpression approaches and Ab-mediated TCR stimulation in Jurkat T cells (20, 29, 33) or retroviral-mediated transduction and overexpression in activated primary T cells (26, 45). Given the concerns with interpreting functional effects of mutations under conditions where ADAP is overexpressed in cells, we reasoned that structure/function analysis of ADAP would be most informative under conditions where mutant ADAP constructs could be expressed in the absence of endogenous ADAP. To overcome the technical challenges of gene delivery into naive primary murine ADAP<sup>-/-</sup> T cells, we crossed the OVAp Ag-specific DO11.10/ADAP<sup>-/-</sup> mice (25) to mice bearing the hCAR transgene (35), which allows efficient adenoviral-mediated gene delivery into resting, naive T lymphocytes.

Using this system, we show that impaired peptide Ag-dependent T:APC conjugate formation in ADAP<sup>-/-</sup> T cells (25, 46) is rescued following expression of wtADAP to levels approaching that of endogenous ADAP. Our mutational analysis indicates that while the N-terminal 327 aa of ADAP is not required for the rescue of conjugate formation, the central proline-rich domain is necessary but not sufficient for rescue. We also found that this ADAP proline-rich domain is important for optimal functional T cell activation as measured by CD69, CD25, and Bcl-x<sub>L</sub> expression following Ag stimulation. Furthermore, the C-terminal half of ADAP (aa 426-819) is a second region critical for Ag-dependent integrin adhesive function. Thus, our results are consistent with recent work demonstrating a requirement for the ADAP proline-rich and C-terminal domains for TCR-induced adhesion to immobilized  $\beta_1$  and  $\beta_2$  integrin ligands (29, 33).

Several previous studies have outlined the physical and functional relationship between the T cell adapter proteins ADAP and

SKAP55. A direct association between the central proline-rich domain of ADAP and the SH3 domain of SKAP55 was first identified using two-hybrid screens and coimmunoprecipitation experiments with Jurkat T cells (17, 18). The link between the ADAP/SKAP55 complex and promotion of LFA-1 integrin function was demonstrated by overexpressing either protein in an Ag-specific T cell hybridoma line, or by retroviral-mediated overexpression of SKAP55 in activated primary murine T cells (26). Conversely, loss of SKAP55 by small interfering RNA-mediated gene knockdown (27, 29, 33) or in SKAP55<sup>-/-</sup> mice (28) decreased LFA-1-mediated adhesion. Detailed mechanistic studies on the role of ADAP and SKAP55 in T cell activation and integrin function have recently been confounded by the observation that ADAP<sup>-/-</sup> T cells are severely deficient in SKAP55 (28, 29, 34). This loss of SKAP55 in the absence of ADAP traces to the constitutive interaction between ADAP and the SH3 domain of SKAP55, which acts to protect SKAP55 from caspase- and proteasome-mediated degradation (34). Our studies show that the SKAP55 deficiency in ADAP<sup>-/-</sup> primary murine T cells is reversible upon reintroduction of intact ADAP protein. Furthermore, we show that a 20-aa region between aa 338–358 in the ADAP proline-rich domain is necessary and sufficient for stabilization of endogenous SKAP55. This region of murine ADAP is analogous to a 24-aa site defined in human ADAP (aa 340–364) (29), and contains a 12-aa LGPP-PPKPNRPP sequence that includes a canonical core PxxPxP motif capable of binding to several types of SH3 domains (47). There is high sequence identity in this region of ADAP isolated from human, mouse, monkey, dog, cow, chicken, and zebrafish, suggesting a conserved function for ADAP that is dependent on this proline-rich region.

Since cells expressing ADAP proline-rich domain mutants simultaneously fail to rescue conjugate formation and to reexpress SKAP55, it is unclear whether ADAP, SKAP55, or both are required for control of Ag-dependent integrin function. Indeed, we are unaware of any experiments to date that have examined the function of SKAP55 independent of ADAP expression. In our experiments, expression of the isolated proline-rich domain of ADAP (+326–426) or of the C-terminal deletion ( $\Delta$ 426–819) permits significant recovery of endogenous SKAP55 expression, while still failing to rescue conjugate formation. To directly test the model that free SKAP55 expression in the absence of any ADAP interaction could be sufficient for T:APC conjugate formation, we specifically expressed SKAP55 in ADAP<sup>-/-</sup> T cells. Our results show that even in ADAP<sup>-/-</sup> T cells containing SKAP55 similar to endogenous levels, T:APC conjugate formation was not enhanced. Thus, SKAP55 expression in the absence of ADAP is not sufficient to regulate TCR signaling to integrins. The presence of the proline-rich domain of ADAP, as well as the C terminus of ADAP, in combination with SKAP55 is necessary for optimal integrin function.

Regulation of  $\beta_1$  and  $\beta_2$  integrin function in T cells is dependent on both the activation and the membrane/synapse targeting of the small GTPase Rap1 (30, 41, 48). Recently, ADAP<sup>-/-</sup> T cells have been shown to be defective for membrane targeting of the active (GTP-bound) form of Rap1 (29), a finding consistent with the defects in integrin-mediated adhesion observed in ADAP<sup>-/-</sup> T cells. We attempted to test the role of our ADAP C-terminal mutations in controlling Rap1 membrane targeting, but we were unable to obtain enough transduced cells to perform these biochemical comparisons. To test whether ADAP controls recruitment of Rap1 to the TCR signaling complex, we transduced primary WT or ADAP<sup>-/-</sup> cells with GFP-Rap1 and monitored the recruitment of GFP-Rap1 to anti-TCR-coated beads. Using this approach, we did not detect any gross differences in the percentage of Rap1 recruited

to the T cell/bead interface. This apparent discrepancy between our results and the published results may trace to the ability of Rap1 to localize or become activated on intracellular membranes (49, 50), to rapid kinetics of Rap1 activation that we were unable to capture during imaging, or to the inherent differences between soluble anti-TCR stimulation vs T cell activation against an anti-TCR-coated bead, which is somewhat more similar to an APC.

Elucidation of the molecular pathways downstream of the ADAP/SKAP55 complex have implicated the involvement of the constitutive SKAP55-binding protein, RIAM (also known as PREL1), which contains a central RA-PH domain capable of binding to active Rap1 (33). RIAM has also been shown to control  $\beta_1$  and  $\beta_2$  integrin activation in T cells following TCR stimulation (51), and it has been implicated in binding Ena/Vasp proteins and the regulation of actin dynamics in T cells (52). RNA interference-mediated depletion of SKAP55 in Jurkat T cells results in impaired membrane localization of RIAM and Rap1 following TCR stimulation (33), consistent with the role of SKAP55 as a critical effector in TCR-mediated integrin signaling (28). By contrast, ADAP membrane targeting following TCR stimulation is unaffected by SKAP55 depletion (29, 33). While we were unable to detect the ADAP/SKAP55/RIAM complex in the present study (data not shown), we were able to monitor the targeting of SKAP55 to anti-TCR-coated beads. ADAP<sup>-/-</sup> cells expressing wtADAP or ADAP $\Delta$ 426–819 were equally able to recruit their rescued SKAP55 to the T cell/bead interface. Interestingly, SKAP55 was also recruited even when expressed in the absence of ADAP. These previous results and our findings suggest that both ADAP and SKAP55 may contain membrane targeting information that has the capacity to recruit the proposed ADAP/SKAP55/RIAM/Rap1-GTP complex to the immune synapse following TCR activation by APCs. Alternatively, the entire complex itself may complete a quaternary structure that promotes membrane targeting and/or integrin activation.

The precise role of the ADAP C terminus (aa 426–819) in promoting integrin function remains unclear. Although the extreme C-terminal SH3-like domain (hSH3c) of ADAP has been implicated in a secondary low-affinity interaction with SKAP55 that is released following TCR activation and SKAP55 phosphorylation (53, 54), this domain of ADAP is not absolutely required for enhanced integrin-mediated adhesion in mast cells (55). Indeed, we were able to detect robust TCR-independent interaction between ADAP and SKAP55 following removal of the entire ADAP C terminus (ADAP $\Delta$ 426–819), suggesting that the ADAP C terminus is not absolutely required for ADAP interaction with SKAP55. Additionally, the ADAP domain between aa 426 and 541, which is critical for interaction with CARMA1 and for NF- $\kappa$ B activation, is not required for T:APC conjugate formation (19) or for constitutive binding to SKAP55. However, since the ADAP C terminus still controlled the ultimate outcome of T:APC conjugate formation and T cell activation, it is thus likely that an ~200-aa segment between murine ADAP aa 542 and 750 is a secondary domain that controls T:APC conjugate formation. This region contains several tyrosine residues implicated in binding SLP-76 (12, 13). Presumably, ADAP tyrosine phosphorylation in this region is responsible for its recruitment to LAT-associated SLP-76 at the plasma membrane following TCR stimulation (1, 3, 11). Although a reduction in integrin activation has been reported following treatment with a SLP-76 inhibitor peptide (56), and an ADAP construct containing mutations in the ADAP tyrosine residues implicated in binding to the SH2 domain of SLP-76 shows impaired overexpression-induced T:APC conjugate formation (45), a direct role for SLP-76 in T cell integrin function has not yet been carefully defined. The



ADAP C terminus also contains EVH1 (Ena/Vasp homology domain 1) homology motifs that have been implicated in binding members of the actin-regulatory Ena/Vasp family proteins (15, 57), which could affect T cell integrin activity. Interestingly, the helical extension of the two noncanonical hSH3 domains of ADAP has been reported to influence integrin-dependent adhesion (20). These domains bind phospholipids in vitro (21) and are predicted to influence the overall conformation of ADAP in response to oxidative stress following T cell activation (58). Additionally, part of the N-terminal hSH3 domain of ADAP overlaps with the CARMA1 binding site in ADAP (19). Thus, future experiments will be required to further distinguish molecular signatures in the C terminus of ADAP required for T:APC conjugate formation.

In addition to controlling TCR-dependent integrin function, ADAP regulates TCR-dependent NF- $\kappa$ B activation (19). This novel function for ADAP is dependent on the ability of the central aa 426–541 in ADAP to bind the adapter CARMA1, which in turn allows the assembly of the CARMA1/Bcl10/Malt1 (CBM) signaling complex. This ADAP-CBM signaling complex is critical for phosphorylation and degradation of I $\kappa$ B, liberating NF- $\kappa$ B to translocate to the T cell nucleus and promote gene transcription. We previously reported that the CARMA1-binding domain in ADAP is dispensable for T:APC conjugate formation (19). In the present study we found that the CARMA1 binding function of ADAP was also not required for rescue of downstream activation markers of T cell function including CD69, CD25, and surprisingly the NF- $\kappa$ B-regulated gene Bcl-x<sub>L</sub>. This suggests that the initial integrin-dependent adhesion during T cell priming is a critical event in determining the activation status 1–2 days following Ag challenge.

The role of the ADAP proline-rich domain and the ADAP/SKAP55 complex in regulating NF- $\kappa$ B activation has not been previously investigated. We show herein that reconstitution of ADAP<sup>-/-</sup> T cells with ADAP lacking its proline-rich domain (which negates SKAP55 expression) is sufficient to rescue assembly of the CBM complex and NF- $\kappa$ B activation. Indeed, while ADAP immunoprecipitates from activated T cells contain Bcl10, CARMA1, and SKAP55, Bcl10 immunoprecipitates from activated T cells contain ADAP and CARMA1, but not SKAP55. This is consistent with reports that TCR-induced activation of a NF- $\kappa$ B reporter is unaffected by RNA interference-mediated suppression of SKAP55 expression (59). In summary, our data support a model where ADAP coordinates two distinct and physically segregated signaling pathways following TCR stimulation. One pathway involves recruitment of the ADAP/SKAP55/RIAM/Rap1-GTP complex to the membrane and leads to LFA-1 activation and clustering. A second pathway involves TCR-mediated protein kinase C  $\theta$  activation that promotes ADAP-dependent assembly of the CBM complex and subsequent NF- $\kappa$ B activation.

The relative contributions of ADAP-dependent T:APC conjugate formation and NF- $\kappa$ B activation toward T cell activation are currently not clear. While we found that the ADAP/CARMA1 interaction is not absolutely required for early T cell activation events in vitro, we were unable to assess Ag-dependent T cell clonal expansion, which occurs 2–3 days following activation, because of the transient nature of our adenovirus expression assay. Furthermore, it is clear that these ex vivo activation assays do not accurately recapitulate the in vivo microenvironment. In particular, there are defects in clonal expansion of DO11.10 ADAP<sup>-/-</sup> T cells in response to Ag challenge in vivo that are particularly pronounced when naive T cells are present at physiologically low precursor frequencies (25). Our results and those of others (28) suggest that impaired interactions of ADAP<sup>-/-</sup> T cells with APCs in vivo may lower TCR sensitivity to Ag, resulting in inefficient

delivery of activation signals required for activation and clonal expansion. Thus, our finding that ADAP also controls a separate T cell activation pathway involving NF- $\kappa$ B suggests that impaired clonal expansion of ADAP<sup>-/-</sup> T cells in vivo in response to Ag may also be due to impaired induction of NF- $\kappa$ B-dependent genes critical for T cell activation and survival. The combined functions of ADAP may explain the dramatically impaired clonal expansion of ADAP<sup>-/-</sup> T cells at low precursor frequencies in vivo, despite the fact that ADAP<sup>-/-</sup> T cells still exhibit some level of T:APC conjugate formation and activation in vitro. A role for ADAP in NF- $\kappa$ B signaling suggests that T cells capable of forming conjugates with APCs in the absence of ADAP may still not receive the proper array of signals necessary for optimal T cell activation. Indeed, a recent report found that while ADAP<sup>-/-</sup> CTLs have no defects in target cell killing, they exhibited impaired allograft-mediated rejection, consistent with the presence of underlying non-adhesion-dependent activation defects in the absence of ADAP (60). Future mechanistic studies in vivo will need to distinguish between concurrent defects in both ADAP-dependent integrin-mediated adhesion and NF- $\kappa$ B signaling.

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## Disclosures

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