An essential role for SKAP-55 in LFA-1 clustering on T cells that cannot be substituted by SKAP-55R

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Lymphocyte function-associated antigen (LFA)-1 clustering, which is needed for high avidity binding to intercellular adhesion molecule (ICAM)-1 and -2, regulates T cell motility and T cell–antigen-presenting cell (APC) conjugation. In this study, down-regulation of SKAP-55 by small interfering RNAs (siRNAs) identified an essential role for this adaptor molecule in the T cell receptor (TCR)–mediated "inside-out signaling" that is needed for LFA-1 clustering and T cell–APC conjugation. In contrast, down-regulation of SKAP-55 had no effect on TCR–CD3 clustering. Furthermore, the expression of the related protein SKAP-55R failed to compensate for the loss of SKAP-55 in LFA-1 clustering, indicating that SKAP-55 has a unique function that cannot be replaced by this closely related protein. Our findings therefore indicate that SKAP-55, unlike SKAP-55R, is specifically tailored as an essential component of the inside-out signaling events that couple the TCR to LFA-1 clustering and T cell–APC conjugation.

The interaction of T cells with MHC-bearing APCs initiates signaling events for proliferation and effector T cell function. Conjugation is mediated by lymphocyte function-associated antigen (LFA)-1, which binds to intercellular adhesion molecule (ICAM)-1 and -2 on APCs. The antigen–T cell receptor complex (TCR–CD3) and integrins are temporarily and spatially regulated at a specialized region known as the immunological synapse (IS; references 1–3). The IS is characterized by the formation of a supramolecular activation cluster (SMAC) that is composed of a central cluster (c-SMAC) with TCR–CD3 and costimulatory receptors, such as CD4, CD2, and CD28, and a peripheral ring (p-SMAC) with LFA-1 and the cytoskeletal protein talin. Kinases such as lck and fyn also colocalize in the c-SMAC. Although tyrosine phosphorylation and phosphatidylinositol 3 kinase signaling can occur outside the SMAC (4–6), the remodeled SMAC has been postulated to contribute to sustained signaling and to the internalization of postengaged receptors (7). SMAC formation involves directed viral transmission (8), but is not necessary for cytolitic T cell function (9).

LFA-1 binding to ICAM-1 and -2 is needed for the initiation and maintenance of conjugate formation (10, 11). The conversion of LFA-1 from low to intermediate or higher affinity forms involves initial conformation changes combined with an increase in avidity caused by clustering. The identity of the signaling pathways that regulate this conversion and enhanced cell–cell adhesion is the subject of much interest. The guanine nucleotide exchange factor Vav-1, the GTP-binding protein Rap1, its binding partner RapL (regulator of cell adhesion and polarization enriched in lymphoid tissues), and the adaptors, SLP-76 (76-kD src homology 2 domain–containing leukocyte phosphoprotein), ADAP (adhesion and degranulation–promoting adaptor protein), and SKAP-55 (55-kD src kinase–associated phosphoprotein) have been implicated in LFA-1 clustering (12–18). Transgenic mice overexpressing Rap1 show enhanced LFA-1 binding (19), whereas a dominant negative form of RapL inhibits clustering and conjugation (20). Furthermore, the retroviral expression of SKAP-55 enhances LFA-1 clustering and adhesion (17), whereas adap−/− T cells show defects in LFA-1 clustering (13, 14). In addition, a form of ADAP that is defective in binding to SLP-76 interferes with p-SMAC formation (21). RapL and ADAP also colocalize with the LFA-1/talin–enriched p-SMAC (20, 21).
SKAP-55 is an adaptor with a unique NH$_2$-terminal region followed by a pleckstrin homology domain and a COOH-terminal SH3 domain (22, 23). It is predominately expressed in T cells and has been implicated in LFA-1 clustering, T cell–APC conjugation, and the activation of extracellular signal–regulated kinase (17, 24). The SKAP-55 SH3 domain binds to a proline-rich region in ADAP (23, 25), whereas the ADAP SH3 domain can bind to a tyrosine-based RKxxYxxY motif in SKAP-55 (26). In addition to SKAP-55, a related protein termed SKAP-55–related (SKAP-55R), or SKAP-55Hom (Scap2), has been cloned (23, 27). SKAP-55R/Hom and SKAP-55 share 44% identity, with the greatest conservation occurring in the pleckstrin homology domain (residues 115–214) and in the SH3 domain (residues 303–307; references 23, 27). The tyrosine motif YEEL in SKAP-55 exists as YEEL in SKAP-55R/Hom, both of which potentially serve as src kinase SH2 domain binding sites. Adjacent to this motif in SKAP-55R/Hom also exists a region of charged residues (i.e., EEEED; residues 266–290). The NH$_2$-terminus in the full-length SKAP-55R/Hom has multiple residues that could serve as phosphorylation sites. These include YLPPL (residues 11–14), YLQEE (residues 59–62), YDDP (residues 75–79), and YDKD (residues 93–97). The one structural difference between SKAP-55 and SKAP-55R/Hom is in the NH$_2$-terminal region where SKAP-55R/Hom showed the presence of a well-defined coiled coil (residues 20–75; references 23, 27). Both SKAP-55 and SKAP-55R/Hom share an ability to bind to ADAP and as such have been postulated to mediate similar functions (23, 27).

A major question concerns the function of SKAP-55 in the regulation of T cell immunity and whether SKAP-55R/Hom plays a redundant role in regulating the same events. A previous study using retroviral gene transfer identified a role for SKAP-55 (Scap1) in LFA-1 clustering (17). Here, in a complementary approach using small interfering RNAs (siRNAs) to down-regulate SKAP-55 expression, we clearly demonstrate that SKAP-55 expression is essential for TCR induction of LFA-1 clustering and optimal T cell–APC conjugation using two different antigen presentation systems. In contrast, the loss of SKAP-55 had no apparent effect on TCR–CD3 capping. In addition, the loss of SKAP-55 had no effect on the expression of SKAP-55R/Hom, indicating that this homologue cannot compensate for the loss of SKAP-55 in T cells. Our findings therefore indicate that SKAP-55, unlike SKAP-55R/Hom, is specifically tailored as an essential component in the “inside-out signaling” events that couple the TCR to LFA-1 clustering and T cell–APC conjugation.

RESULTS AND DISCUSSION

Previous overexpression studies by retroviral gene transfer have identified a role for SKAP-55 in LFA-1 clustering and T cell–APC conjugation (17). In a complementary approach, siRNA was used to down-regulate SKAP-55 expression (Fig. 1; see Materials and methods). Using three distinct duplexes, different clones of T8.1 T cells were identified where siRNA reduced SKAP-55 expression by >90% as shown by immunoblotting (Fig. 1 A, compare lanes 2 and 3 with 1). At the same time, the expression of other proteins such as Vav-1 (Fig. 1 A, compare lanes 5 and 6 with 4), SKAP-55R/Hom (Scap2) (Fig. 1 A, compare lanes 8 and 9 with 7), ADAP (Fig. 1 A, compare lanes 11 and 12 with 10), and actin (Fig. 1 A, compare lanes 14 and 15 with 13) was unaffected. With this, wild-type and SKAP-55 siRNA knockdown cells (SKAP-55KD) were contrasted for their ability to support T cell–APC conjugate formation (Fig. 1 B). A conjugation assay was initially used in which T8.1 cells expressed an antigen receptor for tetanus toxoid peptide (Ttox) in the context of HLA-DR*1102. These cells were co-cultured with the APC L625.7 expressing the appropriate HLA-DR antigen with ICAM-1 and -2 (17, 28). Adhesion was measured by the use of a 3–(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and required the presence of adherent cells because the binding of T cells to tissue culture plates without adherent cells was negligible (unpublished data). Although Ttox increased T cell adhesion to APCs in mock control cells, the increased conjugation was impaired by 40–50% in the SKAP-55KD Z14 and Z19 cells. The final level of conjugation was similar to or lower than that observed in mock control cells cultured in the absence of peptide agonist (Fig. 1 B, top). Some antigen-independent conjugation was also inhibited by the down-modulation of SKAP-55. Concurrent with the loss of conjugation was a loss of interleukin 2 production in response to Ttox (Fig. 1 B, bottom). IL-2 production was comparable to mock control cells cultured without Ttox. A third siRNA, ZKW2, led to the derivation of other stable transfectants (Z24 and Z29) that showed the same change in adhesion and cytokine production (unpublished data). These observations indicate that SKAP-55 expression is needed for the optimal ability of T cells to form conjugates with APCs.

Similar results were obtained using a conjugation assay between Jurkat and Raji cells cultured in the presence of the superantigen staphylococcal enterotoxin E (SEE; Fig. 1 C). Distinct RNAi duplexes (one similar to that used in T8.1, cells and another unique duplex) reduced SKAP-55 expression in different clones as monitored by anti–SKAP-55 immunoblotting (Fig. 1 C, inset). Although SEE increased conjugation between Jurkat and Raji cells, it was reduced by >75% in the SKAP-55KD W1–34 and W3–37 cells relative to mock-transfected T cells (Fig. 1 C, top). The level of conjugation was lower than that observed in mock control cells cultured in the absence of the SEE agonist. Concurrent with reduced conjugation was an impairment of TCR-induced NFAT/AP1 transcriptional activity (i.e., >90%; Fig. 1 C, bottom). Using two different T cell systems in response to two distinct antigens (Ttox and SEE), our findings therefore show that a reduction in SKAP-55 expression by siRNA has considerable effects on T cell–APC conjugate formation and the resultant IL-2 gene expression.
The requirement for SKAP-55 in optimal T cell–APC conjugation suggested that the adaptor mediates LFA-1–mediated adhesion. The conversion of LFA-1 from low to intermediate or higher avidity forms involves LFA-1 clustering (10, 11). To assess whether SKAP-55 expression was needed for LFA-1 clustering, anti-CD3 was initially used to activate cells followed by the staining of cells with anti–LFA-1 conjugated to Texas red as previously described (17). Anti–LFA-1 capping was defined by the presence of a discrete polarized cap. Anti-CD3 increased the percentage of T8.1 cells forming LFA-1 caps from 20 to 45% (i.e., relative to vector mock transfected control cells; Fig. 2 A, top, mock panels, right). In contrast, Z14 and Z19 cells showed a marked impairment of LFA-1 clustering (Fig. 2 A, top [Z14 and Z19 panels, right] and bottom). Clustering was reduced by >90% in SKAP-55KD cells.

In another approach, the clustering of LFA-1 was imaged at the T cell–APC interface in conjugates formed by T8.1/L625 cells cultured in the presence of Ttox over 30 min (Fig. 2 B). Anti-CD11a was used to bind cells followed by Alexa Fluor 568 conjugated anti–rat staining. Imaging of the IS among individual conjugates (40) was conducted by laser scanning confocal microscopy (17, 29). Although LFA-1 could readily be visualized at the interface of T cell–APC-stimulated conjugates, the clustering of Z14 cells at the IS was markedly reduced (Fig. 2 B, top right [b and d] and bottom). The level of clustering was similar to that observed in the mock control that was cultured without agonist. Overall, these observations indicate that SKAP-55 plays an essential role in the avidity modulation or clustering of LFA-1 on the surface of T cells.

Despite this striking dependency of LFA-1 clustering on SKAP-55, TCR–CD3 clustering occurred normally on SKAP-55KD cells (Fig. 3 A). For this, anti-CD3 that was conjugated to Texas red was also exposed to cells for 30 min and visualized by immunofluorescence microscopy (17). Unlike the results observed with LFA-1 clustering, the clustering of the anti-CD3 was unaffected by the reduction of SKAP-55 expression (Fig. 3). In both cases, ~50–60% of cells underwent capping in response to binding to the surface of cells (Fig. 3 A, top [c and d] and bottom). The slight difference was not statistically significant as shown by overlapping standard deviations (P > 0.05). Our findings show that SKAP-55 is needed for anti-CD3–induced LFA-1 clustering, but not TCR–CD3 capping on the surface of T cells.

Previous studies have implicated Vav-1, ADAP, and Rap1/RapL in the regulation of LFA-1 clustering (12–21). Retroviral-induced SKAP-55 overexpression can also modulate LFA-1 clustering and T cell–APC conjugation (17). However, uncertainties still existed regarding the degree to which these findings reflected overexpression and whether interleukin 2-dependent NFAT transcriptional activity is impaired in W1-34 and W3-37 SKAP-55KD cells. Jurkat/SEE/Raji conjugate assay and NFAT transcription assay was conducted as described in Materials and methods. Results are representative of three independent experiments.
the function of endogenous SKAP-55 can be mimicked by the endogenous homologue SKAP-55R/Hom. The findings of this study unequivocally establish endogenous SKAP-55 as an essential component in the signaling cascade that couples the TCR–CD3 complex to LFA-1 clustering in T cells. The use of three distinct double-stranded RNAi complexes makes it highly improbable that their ability to alter function was caused by cross-interference with the expression of other proteins. Similarly, even though SKAP-55 expression was reduced by $>90\%$, the expression of neither Vav-1, actin, ADAP, nor SKAP-5R/Hom was affected (Fig. 1 A). The reduction in T cell–APC conjugation was observed in two different T cells with TCRs for two distinct antigens (Ttox and SEE). Conjugation in the SKAP-55KD cells was reduced

Figure 2. LFA-1 clustering is impaired in SKAP-55 RNAi knockdown T8.1 cells. (A) Mock, Z14, and Z19 cells were incubated with anti-CD3 (2C11) and biotinylated anti–hamster IgG followed by visualization of LFA-1 surface expression as described in Materials and methods. LFA-1 was stained with anti-CD18 and isotype-specific Cy3-conjugated anti–hamster as previously described (reference 23). (top) Examples of LFA-1 clustering in mock (left), Z14 (middle), and Z19 (right) with and without prior anti-CD3 ligation; (bottom) histogram with the mean percentages ($\pm SD$) of T cells that have formed polarized LFA-1 caps. At least 500 cells were counted for each sample using fluorescence microscopy. Results are representative of three independent experiments. (B) LFA-1 confocal imaging at the T cell–APC interface was performed as described in Materials and methods. (top) Examples of LFA-1 clustering in conjugates formed by mock (left) and Z14 (right) cells, with and without prior exposure to Ttox; (bottom) histogram with mean percentages ($\pm SD$) of T cells with LFA-1 clustering at the IS.
essential role for SKAP-55 in the bifurcation of TCR-mediated signals that leads to LFA-1 clustering. The selective effect on LFA-1 clustering in SKAP-55KD T cells is similar to that observed in ADAP−/− T cells (13, 14), and places SKAP-55 in the inside-out signaling cascade linking the TCR–CD3 complex to the LFA-1 clustering and adhesion (Fig. 3 B). The cascade includes Vav-1, SLP-76 binding to ADAP, and possibly the binding of ADAP to SKAP-55. A different set of signals that includes Vav-1/WASP is needed for TCR aggregation (30).

SKAP-55R/Hom is closely related to SKAP-55, binds to ADAP, and has been postulated to play a similar role in adhesion. However, our findings using siRNA to selectively reduce SKAP-55 expression show that the residual expression of endogenous SKAP-55R/Hom cannot substitute for SKAP-55 in the regulation of LFA-1 clustering. Although it is formally possible that the inability of SKAP-55R/Hom to substitute for SKAP-55 might be related to the need for a threshold of SKAP-55–SKAP-55R/Hom protein expression (i.e., expression below threshold), blotting showed that endogenous SKAP-55R/Hom was expressed at a level comparable to Vav-1, SKAP-55, and ADAP (Fig. 1). A more likely interpretation is that the unique structure of SKAP-55 tailors the protein to participate in a selective manner in the inside-out signaling events that couple the TCR to LFA-1 clustering. This must be related to an event other than ADAP binding because both SKAP-55 and SKAP-55R/Hom bind to this adaptor (23, 25, 26). This selective involvement of SKAP-55 is consistent with its preferential expression of SKAP-55 in T cells (22). SKAP-55R/Hom is more broadly expressed in other cells and, as such, may play a more general role in adhesion events related to other cell types. Future studies will be needed to establish the molecular basis for the selective connection of SKAP-55 to LFA-1 function in T cells.

**MATERIALS AND METHODS**

Construction of psiRNA vectors for generation of siRNA. The template for human-mouse–SKAP-55 siRNAs were generated by ligating the annealed primers 5′-ACCTCACATTGGACAGGACAGCTCTGTCAAGAGCAGA GCTGTCCTGT CCAATGTTT-3′ and 5′-CAAAAACATTGGACAGGACAGCTCTGCTCTTGACA GACGCTGTC TGTCCAAATGTG-3′ (for W1 and Z1), and 5′-CCTCATAACGTAATGCAAGGATTCAAGAGAT CCTTGCTTGATTACGTTATTT-3′ and 5′-CAAAAAAT AACGTTAACGGAAGGAT CTCTTGAACGTCATTGATTACGTTATG-3′ (for W3 and Z3). siRNA for the ZKW2 target (nt positions 264–284) was 5′-AGGACAGCTCTGATGATA ATC-3′. For cloning of the third ZKW2 target, the primers 5′-ACCTCAGGACACGTCATTGCTGTCCCTCCTG-3′ and 5′-CAAAAAAGGACAGCTCTGATGATAATCCTCTTGAGAT TATCATCAGAGCTGTCCTG-3′ were used. Designed RNA

**Figure 3.** TCR–CD3 clustering is normal in SKAP-55 RNAi knock-down T8.1 cells. (A) Mock and Z14 cells were incubated with anti-CD3 (2C11) and a Cy3-conjugated secondary antibody as described in Materials and methods. (top) Examples of the clustering of anti-CD3 in mock and Z14 T8.1 cells; (bottom) histogram with mean percentages (±SD) of T cells with anti-CD3 caps in the mock and Z14 T8.1 cells. (B) Schematic of pathways from the TCR–CD3 complex that regulate LFA-1 clustering and adhesion. The proposed pathway involves VAV-1, SLP-76, ADAP, and SKAP-55. The homologue SKAP-55R/Hom cannot substitute for SKAP-55 function. In contrast, Vav-1 and WASP are needed for TCR clustering on the surface of T cells.

to the level of vector controls in the absence of peptide agonist. The residual conjugation may be related to other adhesion receptor–ligand pairs such as CD2–CD58. LFA-1 clustering in response to anti-CD3 was reduced by >90–95% without any apparent effect on TCR clustering. This indicates an essential role for SKAP-55 in the bifurcation of TCR-mediated signals that leads to LFA-1 clustering. The selective effect on LFA-1 clustering in SKAP-55KD T cells is similar to that observed in ADAP−/− T cells (13, 14), and places SKAP-55 in the inside-out signaling cascade linking the TCR–CD3 complex to the LFA-1 clustering and adhesion (Fig. 3 B). The cascade includes Vav-1, SLP-76 binding to ADAP, and possibly the binding of ADAP to SKAP-55. A different set of signals that includes Vav-1/WASP is needed for TCR aggregation (30).

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oligonucleotides were blasted against the GenBank/EMBL/DBJ database to ensure gene specificity. This nonrelevant control has no homology with human and mouse SKAP-55 genes.

Cells and antibodies. The murine hybridoma T8.1-expressing human CD4 and a chimeric human mouse TCR specific for Ttox (830–843), as well as L625.7 cells, were gifts of O. Acuto (Instituto Pasteur, Paris, France) and were cultured as previously described (17, 21). Anti-SKAP-55 and anti-ADAP were purchased from Transduction Laboratories; anti-Vav1 was purchased from Upstate Biotechnology; anti-SKAP-55 SR was purchased from Santa Cruz Biotechnology; anti-human CD3 (American Type Culture Collection), anti-mouse CD3 (2C11; hamster anti-mouse CD3), and anti-CD11a (anti-LFA-1) were purchased from BD Biosciences; and Alexa-488-conjugated secondary Abs were purchased from Molecular Probes. Ttox (residues 830–843) was obtained from Research Genetics, and SEE exa-488–conjugated secondary Abs were purchased from Molecular Probes. Ttox was obtained from Toxin Technology.

Transfection and immunoblot assays. Full-length human SKAP-55 cDNA were cloned into the pSRα expression vector and cloned in-frame with the NH2 terminus of the GFP gene in the pSRα–GFP vector (Promega). Transfection assays were performed as previously described (21). IL-2 was measured by an IL-2 ELISA kit (BD Biosciences) according to the manufacturer’s instructions. NFAT transcriptional activity and immunoblotting were performed as previously described (17).

APC–T cell conjugate assay. The antigen-induced T8.1 cell adhesion was quantified by using a colorimetric assay for measuring intracellular succinate dehydrogenase content with MTT as previously described (17, 21). For conjugating Jurkat T cells and Raji B cells, we incubated 106 Jurkat T cells with the same number of Raji cells at 37°C for 10 min and fixed them in PBS with 6% paraformaldehyde. Conjugates were visualized by microscopy as described previously (21).

Clustering and immunofluorescence microscopy. Clustering and capping experiments were conducted as previously described (17, 21). In brief, cells were incubated with 5 µg/ml anti-CD3 (clone 148–2C11) on ice for 30 min. Cells were then washed twice with cold medium and cross-linked by incubating with 10 µg/ml goat anti–hamster IgG antibody (Jackson ImmunoResearch Laboratories) at 37°C for 30 min. Samples were then stained for anti-CD18 and isotype specific Cy3-conjugated anti–hamster as previously described (21). TCR capping on various cells was conducted as previously described (17, 21). At least 500 T cells were counted for cap formation in each experiment. Immunofluorescence microscopy was conducted as described previously (17, 29). Cells were incubated with anti-HA antibody, followed by a FITC-labeled secondary antibody and tetramethylrhodamine isothiocyanate–labeled phalloidin (Sigma–Aldrich). Image acquisition was performed with a microscope (model Eclipse E800; Nikon) and a digital camera (model RT-Slider; Diagnostics, Inc.). For LFA-1 confocal imaging at the T cell–APC interface, L625.7 cells were seeded on 12-mm coverslips in 24-well plates overnight and pulsed with 2.5 µg/ml Ttox cells followed by an incubation with anti-CD11a (1 µg/250 ml) as described previously (17). Cells were viewed with a 63× oil immersion objective using a laser scanning confocal microscope (model TCS SP2; Leica) equipped with argon/krypton and helium/neon lasers using excitation wavelengths of 488, 568, and 633 nm as described previously (29).

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