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CD26 is a widely distributed 110-kDa cell surface glycoprotein with an important role in T-cell costimulation. We demonstrated previously that CD26 binds to caveolin-1 in antigen-presenting cells, and following exogenous CD26 stimulation, Tollip and IRAK-1 disengage from caveolin-1 in antigen-presenting cells. IRAK-1 is then subsequently phosphorylated to up-regulate CD86 expression, resulting in subsequent T-cell proliferation. However, it is unclear whether caveolin-1 is a costimulatory ligand for CD26 in T-cells. Using soluble caveolin-1-Fc fusion protein, we now show that caveolin-1 is the costimulatory ligand for CD26, and that ligation of CD26 by caveolin-1 induces T-cell proliferation and NF-κB activation in a T-cell receptor/CD3-dependent manner. We also demonstrated that the cytoplasmic tail of CD26 interacts with CARMA1 in T-cells, resulting in signaling events that lead to NF-κB activation. Ligation of CD26 by caveolin-1 recruits a complex consisting of CD26, CARMA1, Bcl10, and IκB kinase to lipid rafts. Taken together, our findings provide novel insights into the regulation of T-cell costimulation via the CD26 molecule.

CD26 is a 110-kDa cell surface glycoprotein with known dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) activity in its extracellular domain (1–3) and is capable of cleaving N-terminal dipeptides with either L-proline or L-alanine at the penultimate position (2). CD26 activity is dependent on cell type and the microenvironment, factors that can influence its multiple biological roles (reviewed in Refs. 4–8). Although CD26 expression is enhanced following activation of resting T-cells, CD4 + CD26high T-cells respond maximally to recall antigens such as tetanus toxoid (9, 10). Cross-linking of CD26 and CD3 with solid-phase immobilized monoclonal antibodies (mAbs) can induce T-cell costimulation and IL-2 production by CD26 + T-cells (2, 7, 10). In addition, anti-CD26 antibody treatment of T-cells enhances tyrosine phosphorylation of signaling molecules such as CD3ζ and p56lck (11, 12). Moreover, DPPIV activity is required for CD26-mediated T-cell costimulation (13). CD26 may therefore have an important role in T-cell biology and overall immune function. However, the costimulatory ligand of CD26 has not yet been identified, and the proximal signaling events following CD26 engagement in T-cell remain to be determined.

In our previous study (14), we identified caveolin-1 in antigen-presenting cells (APC) as a binding protein for CD26, and we demonstrated that CD26 on activated memory T-cells directly faces caveolin-1 on tetanus toxoid-loaded monocytes in the contact area, which was revealed as the immunological synapse for T-cell-APC interaction. Moreover, we showed that residues 201–211 of CD26 along with the serine catalytic site at residue 630, which constitute a pocket structure of CD26/DPPIV, contribute to binding to caveolin-1 scaffolding domain (14). More recently, we demonstrated that caveolin-1 binds to Tollip (Toll-interacting protein) and IRAK-1 (interleukin-1 receptor associated serine/threonine kinase 1) in the membrane of tetanus toxoid-loaded monocytes, and following exogenous CD26 stimulation, Tollip and IRAK-1 disengage from caveolin-1, with IRAK-1 being subsequently phosphorylated to up-regulate CD86 expression (15). It is conceivable that the interaction of CD26 with caveolin-1 on antigen-loaded monocytes results in CD26 up-regulation, therefore enhancing the subsequent interaction of CD86 and CD28 on T-cells to induce antigen-specific T-cell proliferation and activation. However, it is unclear whether caveolin-1 itself is the costimulatory ligand for T-cell CD26.

Recent studies have demonstrated that a newly identified membrane-associated guanylate kinase-like (MAGUK) molecule, CARMA1, is required for TCR/CD3-CD28 costimulation-
induced NF-κB activation and functions downstream of protein kinase Cθ (PKCθ) (16–18). CARMA1, which is predominantly expressed in thymus, spleen, and peripheral blood leukocytes, contains an N-terminal caspase-recruitment domain followed by a coiled-coil domain, a PDZ domain, an SH3 domain, and a guanylate kinase (GUK)-like domain (19, 20). After TCR/CD3-CD28 costimulation or PMA-CD28 stimulation, CARMA1 is phosphorylated by PKCθ, followed by association with Bcl10 and MALT1, and recruitment of these complexes into lipid rafts (21–23). The recruitment of the CARMA1-Bcl10-MALT1 complex activates 1κB kinase (IKK) through a ubiquitin-dependent pathway, leading to activation of NF-κB (24–27). However, it remains to be determined whether CARMA1 is associated with lipid rafts directly or is recruited to lipid rafts via undetermined lipid raft-interacting proteins in the immunological synapse of T-cells.

In this study, using recombinant immunoglobulin-caveolin-1 fusion proteins, we identify caveolin-1 as the costimulatory ligand for CD26, and we demonstrate that the N-terminal domain of caveolin-1 induces T-cell proliferation and cytokine production via CD26 costimulation. Furthermore, we show that CARMA1 is bound to the cytoplasmic tail of dimeric CD26 on T-cells and that this interaction of CD26 and CARMA1 plays a pivotal role in CD26-mediated T-cell costimulation. Our data hence identify a previously unknown ligand for CD26 as a costimulatory molecule while elucidating the mechanisms involved in CD26-mediated T-cell activation and differentiation.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Fc Proteins**—For initial attempts at expression of soluble forms of Fc fusion proteins, human IgG1 Fc cassette vector was made using pCAG-Eb6-MCS vector (28, 29). The 3′ portion of this cassette vector corresponding to human Ig G1 (Fcγ1) sequencing (comprising hinge + CH2 + CH3 regions) was made by PCR. All the primer information used in this study is described in the Supplemental Material. The 5′ portion of the cassette vector containing the signal peptide from human E-cadherin (huECDSp) was made by PCR. Final constructs were assembled by ligating both fragments of HindIII-Fcγ1-EcoRI and Sall-huECDSp-HindIII into Sall/EcoRI-cleaved pCAG-Eb6-MCS (pCAG-Eb6-huECDSp-Fcγ1). The N-terminal domain of human caveolin-1 (CavNT) was made by PCR and constructed into pCAG-Eb6-huECDSp-Fcγ1 (pCAG-Eb6-huECDSp-CavNT-Fcγ1). With the same methods, the N-terminal domain with deletion of the scaffolding domain (CavNTΔSCD) was made by PCR (pCAG-Eb6-huECDSp-CavNTΔSCD-Fcγ1). The Fc fusion protein containing amino acids 1–10 of human CD26 cytoplasmic tail (CD26 aa1–10) was constructed in identical fashion, using the primers described in the Supplemental Material (pCAG-Eb6-huECDSp-CD26 aa1–10-Fcγ1).

For expression of Fc fusion proteins, FreeStyle™293 expression system was used according to the manufacturer’s instruction (Invitrogen). The Fc fusion proteins expressed in the culture supernatant were then purified by affinity chromatography on protein A-Sepharose (Bio-Rad) followed by size-exclusion purification on Microcon® centrifugal filter devices (Millipore), and sterilized using inner diameter 0.22-μm filter microcentrifugation tube Spin-X (Corning Glass).

**Cells and Reagents**—HEK293FT human embryonal kidney, Jurkat T-cell line (JKTwt), and Jurkat T-cells stably transfected with human CD26 (J.CD26wt) were grown as described previously (2, 13, 14). CARMA1-deficient Jurkat T-cell line, JPM50.6, was developed as described elsewhere (18). Human peripheral blood T-cells were purified from peripheral blood mononuclear cells using MACS Pan T-cell isolation kit II (Miltenyi), collected from healthy adult volunteers and incubated according to the methods described previously (30). Informed consent was obtained from healthy adult volunteers. Biotinylation of recombinant proteins or antibody was generated using EZ-Link™ Sulfo-NHS-LC-Biotin reagents according to the manufacturer’s instruction (Pierce). Protease inhibitor mixture, phosphatase inhibitor mixture, and poly-L-lysine were from Sigma. Water-soluble digitonin was purchased from Wako Pure Chemicals Industries, Ltd.

**Biacore™ Analysis of Affinity of Caveolin-1-CD26 Interaction**—Experiments were carried out on a Biacore™ J (Biacore, Japan) using HBS buffer (25 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) supplied by the manufacturer (Biacore AB). Fcγ1, NT-Fc, or NTΔSCD-Fc was coupled in 10 mM sodium acetate (pH 5.0) to a research grade CM5 sensor chip (Biacore AB) using the amine coupling kit (Biacore AB), with an activating time of 5 min, resulting in immobilization of ~5,000–6,000 response units (RU). The surface of the chip was washed with 5 mM NaOH after coupling. NaOH (5 mM) was used also to regenerate immobilized Fcγ1, NT-Fc, or NTΔSCD-Fc chips after each experiment. Recombinant soluble CD26 (rsCD26), comprising the extracellular region of human CD26, was prepared as described previously (30, 31). rsCD26 at various concentrations (50, 25, 12.5, 6.3, 3.2, and 1.6 nM) was then injected for 120 s over immobilized Fcγ1, NT-Fc, or NTΔSCD-Fc chips. Equilibrium binding analysis was performed as described elsewhere (32), using the BIAevaluation software version 2.1 (Biacore AB).

**Generating Stable Transfectants**—The construct of V5-tagged full-length human CD26 (pEF6/V5-CD26wt) was made by PCR, using the primers described in the Supplemental Material. The amplified products were cloned into the pEF6/V5-His B vector (Invitrogen) at the BamHI/EcoRI site. The CD26-CD10 chimeric receptor was composed of the N-terminal cytoplasmic region of human CD10 (1–23-amino acid position) ligated to the transmembrane and extracellular regions of human CD26 (7–766-amino acid position), which were made by PCR. The construct of V5-tagged monomeric human CD26 (CD26H750E), which has histidine replacing glutamic acid as a point mutation at amino acid position 750, was made by site-directed mutagenesis method using pEF6/V5-CD26w as a template with the primers described in the Supplemental Material. After constructs were confirmed by DNA sequencing, plasmids were transfected to Jurkat T-cells using Nucleofector II device according to the manufacturer’s instruction (Amaza Biosystems). Two days after transfection of indicated plasmids, the cells were selected for blastocidin (1 μg/ml) resist-
ance for 4 weeks. Single clone cells expressing CD26wt (V5
-CD26wt), CD26-CD10 (V5-CD26 + CD10 cyto), and
CD26H750E were then selected using standard diluting
limitation method.

For rescue experiments, the CARMA1-deficient Jurkat cell
line JPM50.6 was transfected with expression vectors of CD26
and/or CARMA1. The constructs of Xpress-tagged CARMA1
and its deletion mutant (CARMA1wt, CARMA1-1(–742), or
CARMA1-1(–660), respectively) were made by PCR, using
primers described in the Supplemental Material. The PCR
products were ligated into pcDNA4/HisMax-TOPO (Invitro-
gen). After constructs were confirmed by DNA sequencing,
plasmids were transfected to JPM50.6 cells using the Nucleo-
fector II device according to the manufacturer’s instruction.
Two days after transfection of the indicated plasmids, the cells
were selected for blasticidin (1 μg/ml, for cells transfected
with pEF6/V5 vectors) or Zeocin (10 μg/ml, for cells transfected
with pcDNA4/HisMax vectors) resistance for 4 weeks. Single
cloned cells expressing CD26wt (JPM50.6/CD26wt, CARMA1wt
(JPM50.6/CARMA1wt), CD26wt and CARMA1wt (JPM50.6/
CD26wt + CARMA1wt), or CD26wt and CARMA1-(1–660)
(JPM50.6/CD26wt + CARMA1-(1–660)) were then selected
using standard diluting limitation method.

For stimulation experiments using the expression system,
CHO-K1 cells were transfected with GFP-fused full-length
caveolin-1 or SCD-deleted caveolin-1 expression plasmids,
with the constructs being described previously (14), using Lipo-
fectamine2000 reagent (Invitrogen). Two days after transfection
of the indicated plasmids, the cells were selected for G418
(500 μg/ml) resistance for 4 weeks. Single clone cells expressing
GFP and caveolin-1, detected by anti-caveolin-1 pAb (N20) rec-
oognizing the N-terminal region of caveolin-1 using flow cytom-
etry (FACSCalibur™), were then selected using standard diluting
limitation method.

Flow Cytometric Analysis—For assessment of J.CD26wt that
binds biotinylated NT-Fc or NTΔSCD-Fc, 1 × 10^6 cells were
washed in ice-cold phosphate-buffered saline and incubated
with Fcγ1 and mouse Ig isotypes (1 μg/ml) to block nonspecific
binding, followed by reaction with biotinylated NT-Fc or
NTΔSCD-Fc (1 μg/ml), and subsequently stained with FITC-
conjugated streptavidin (1:500). For blocking experiments, un-
labeled mouse IgG (20 μg/ml) or unlabeled anti-CD26 mAb
(20 μg/ml) was incubated with cells prior to reaction with bio-
tinylated NT-Fc or NTΔSCD-Fc. Flow cytometric analysis of
10,000 viable cells was conducted on FACSCalibur™. Each
experiment was repeated at least three times, and the results
were provided in the form of a histogram or dot plots of a rep-
resentative experiment.

Small Interfering RNA (siRNA) against Human CARMA1—
We selected two target sequences from nucleotides +305 to
+325 (ss1) and +792 to +802 (ss2) downstream of the start
codon of human CARMA1 mRNA (sense1 siRNA (ss1-siRNA),
5’ AAGAGGCCACUCGGGAGAUUUdTdT, and sense2
siRNA (ss2-siRNA), 5’ AACUGGAGCGGGGAUGAAAg
TdT). Moreover, mis-siRNA at four nucleotides was prepared
to examine nonspecific effects of siRNA duplexes (mis-siRNA,
5’ UAGUGGACACCGUGATTCdTdT). These selected
sequences also were submitted to a BLAST search against the
human genome sequence to ensure that only one gene of the
human genome was targeted. siRNAs were purchased from Qiagen. Transfection of siRNA into purified T-cells were con-
ducted using HVJ-E vector (GenomeONE™, kindly provided
by Ihihara Sangyo Kaisha Ltd.) as described previously (14).
After 48 h of transfection, cell were prepared for examination.

T-cell Proliferation and IL-2 Production Assay—For T-cell
proliferation assay, 1 × 10^5 purified T-cells were cultured in
96-well flat-bottomed plates (COSTAR) in a volume of 200 μl
of AIM-V medium (Invitrogen). For solid-phase stimulation,
anti-CD3 (OKT3, 0.05 μg/ml) and/or anti-CD26 mAb (5
μg/ml), anti-CD28 mAb (4B10, 5 μg/ml), or Fc fusion proteins
(5 μg/ml) were bound on the plates. For stimulation with
caveolin-1-transfected CHO cells, purified T-cells were cul-
tured in the presence of soluble anti-CD3 (OKT3, 0.05 μg/ml)
at 1 × 10^5 cells/well with varying amounts (T-cells: CHO =
800, 400, 200, 100, 50, 25:1 or no CHO cells as background
control) of CHO cell transfecants. Before coculturing with
T-cells, CHO transfectants were fixed with 0.05% glutaralde-
hyde for 30 s at room temperature, followed by washing three
times with phosphate-buffered saline. T-cell proliferation was
measured by [3H]TdR (ICN Radiochemicals) uptake. Cells were
incubated for 96 h and were pulsed with 1 μCi/well of [3H]TdR,
16 h prior to harvesting onto a glass fiber filter (Wallac), and the
incorporated radioactivity was quantified by a liquid scintilla-
tion counter (Wallac). For blocking experiments, cells were
treated with soluble anti-CD26 mAb (1F7), anti-CD28 (4B10),
or control mouse Ig (each at 20 μg/ml) before being cultured in
plates coated with stimulatory antibodies and/or Fc proteins.

For IL-2 production assay using Jurkat T-cell lines, JPM50.6,
or their transfectants, 5 × 10^5 cells/well in 200 μl of culture
media were incubated at 37 °C in the presence of the indicated
plate-bound antibodies and/or NT-Fc proteins. Cells were also
stimulated with PMA (10 ng/ml) in anti-CD2733-coated wells.
After 48 h of incubation, culture supernatants were pooled
from the triplicate wells and assayed for IL-2 content using
Human IL-2 Biotrack Easy ELISA (Amersham Biosciences)
according to the manufacturer’s instruction.

Two-dimensional PAGE—For two-dimensional PAGE anal-
ysis of cytotoxic proteins, Jurkat cells were lysed in TBS buffer
(50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 0.1%
diginiton, 10^2-fold diluted protease inhibitor mixture, 10^2-fold
diluted phosphatase inhibitor mixture), and then an aliquot (50
μg) of lyses was subjected to two-dimensional PAGE.
For pulldown by CD26 aa1–10-Fc, aliquots (1 mg) of lyses were
precleared by human IgG (2 μg) and protein A-Sepharose,
followed by immunoprecipitation with Fcγ1 (1 μg) or CD26 aa1–
10-Fc (1 μg). Total lyses or IPs were boiled at 95 °C for 3 min,
and supernatants were then resuspended in rehydration lysis
buffer (RHB; 8 M urea, 2 M thiourea, 4% CHAPS, 50 mM dithio-
thereitol, 0.5% ZOOM carrier ampholyte (pH range 3–10
(Invitrogen), 0.002% bromophenol blue). Two-dimensional
PAGE and peptide mass mapping were conducted as described
previously (15).

Preparation of Lysates or Lipid Raft Fractionation, Immuno-
blotting, and Western Blotting—Stimulated or unstimu-
lated cells were pelleted and lysed with TBS buffer (50 mM
Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 0.1% diginiton,
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10^2-fold diluted protease inhibitor mixture (Sigma), 10^2-fold diluted phosphatase inhibitor mixture (Sigma) and subjected to immunoprecipitation, followed by SDS-PAGE and Western blot analysis. To obtain the lipid raft fraction, purified T-cells (1 x 10^6) that were stimulated for 10 min with anti-CD3 alone or with anti-CD3 plus NT-Fc were lysed with 1 ml of 1% Triton X-100 and protease inhibitor mixture in ice-cold MNE buffer (25 mM MES (pH 6.5) (Sigma), 150 mM NaCl, 5 mM EDTA), and then fractionated by sucrose gradient centrifugation as described previously (33). For immunoprecipitation of the pooled lipid raft fraction, fractionated lipid rafts were lysed at 4 °C for 30 min with 1% Triton X-100 and subjected to immunoprecipitation experiment, followed by SDS-PAGE and Western blot analysis. Immunoprecipitation and Western blot analysis were conducted as described previously (14, 15, 33).

Nuclear Protein Extraction and DNA-binding Protein Assay—
Nuclear extracts were prepared from Jurkat cells or transfectants stimulated as indicated, and ELISA-based DNA-binding protein assays for NF-κB p65 were performed using Mercury TransFactor kits (BD Biosciences) as described previously (14).

Statistics—Student’s t test was used to determine whether the difference between control and sample was significant (p < 0.05 being significant).

RESULTS

We prepared soluble caveolin-1 protein consisting of the putative extracellular N-terminal region or the N-terminal region minus the SCD of human caveolin-1, fused with human IgG1 Fc (NT-Fc or NTΔSCD-Fc, respectively). The schematic diagrams of the full-length human caveolin-1 protein, NT-Fc, NTΔSCD-Fc and Fcγ1 are shown in Fig. 1A. As shown in Fig. 1B, where a band of the recombinant Fc portion of human IgG (Fcγ1) was observed at ~35 kDa under reducing conditions (lane 2), the NT-Fc and NTΔSCD-Fc proteins migrated under reducing conditions predominantly as single bands of 50 and 48 kDa, respectively (lanes 3 and 4). Because immunoglobulins are glycosylated posttranslationally, the recombinant Fc fusion proteins produced with mammalian cells had higher a molecular weight in SDS-PAGE than as calculated from their amino acid composition (34). In nonreducing conditions, Fcγ1, NT-Fc, and NTΔSCD-Fc were observed at ~60, 100, or 90 kDa, respectively (lanes 6–8 in Fig. 1B), indicating that they were expressed as a homodimer. Fcγ1, NT-Fc, and NTΔSCD-Fc were also evaluated by Western blot analysis using anti-human IgG antibody (Fig. 1C). We examined whether the generated NT-Fc fusion protein binds to CD26. For this purpose, we used the Jurkat T-cell line that was stably transfected with full-length human CD26 (J.CD26wt) as described under “Experimental Procedures.” As shown in Fig. 2A, by using lysates of J.CD26wt, CD26 was coimmunoprecipitated with NT-Fc (lane 2) but not with Fcγ1 (lane 1) nor with NTΔSCD-Fc (lane 3). We next evaluated binding of NT-Fc to cell surface CD26 using flow cytometry. As shown in Fig. 2B, cell surface CD26 of J.CD26wt was stained with anti-CD26-FITC mAb (peak 1) (whereas unlabeled CD26 mAb, but not control IgG, blocked staining with anti-CD26-FITC mAb (peak 2 and peak 3 of panel f in Fig. 2B). Staining with NT-Fc was blocked by unlabeled anti-CD26 mAb (peak 3 of panel e in Fig. 2B) but not control IgG (peak 4 of panel c in Fig. 2B). On the other hand, J.CD26wt was not stained with NTΔSCD-Fc (panel d in Fig. 2B). Moreover, native Jurkat
FIGURE 2. Fc fusion proteins of the N-terminal region of human caveolin-1 (NT-Fc) binds to CD26 and induces IL-2 production. A, lysates of J.CD26wt cells (50 μg) were precleared with Fcγ1 and protein A-Sepharose beads, and IP assays were conducted with Fcγ1 (lane 1), NT-Fc (lane 2), or NTΔSCD (lane 3) (each at 2 μg). IP complexes were then separated using 5–20% SDS-PAGE, followed by immunoblotting with anti-CD26 mAb (upper panel). An aliquot (50 μg) of the input lysate was also analyzed (lane 4). The membrane was stripped and reprobed with horseradish peroxidase-conjugated anti-human IgG (lower panel). Similar results were obtained in three independent experiments. B, J.CD26wt cells were used for binding activity of Fc fusion proteins. Panel a, forward and side scattergrams of the analyzed cells. Solid circle indicates the gated region for analysis. Panel b, cells were stained with FITC-conjugated control mouse IgG (peak 1) or FITC-conjugated anti-CD26 mAb (peak 2). For blocking assay, cells were first reacted with unlabeled anti-CD26 mAb (peak 3) or unlabeled control mouse IgG (peak 4), followed by staining as described in peak 2. Panel c, cells were stained with biotinylated Fcγ1 as control (peak 1) or biotinylated NT-Fc (peak 2), followed by reaction with FITC-conjugated streptavidin. For blocking assay, cells were first reacted with unlabeled anti-CD26 mAb (peak 3) or unlabeled control IgG (peak 4), followed by staining as described in peak 2. Panel d, cells were stained with biotinylated Fcγ1 as control (peak 1) or biotinylated NTΔSCD-Fc (peak 2), followed by reaction with FITC-conjugated streptavidin. For blocking assay, cells were first reacted with unlabeled anti-CD26 mAb (peak 3) or unlabeled control IgG (peak 4), followed by staining as described in peak 2. All four histograms in panel d were stacked in the same position. C, measuring the affinity of Fc fusion proteins to rsCD26 by equilibrium binding. Injections of rsCD26 at 25 °C started at 50 nM and were followed by five 2-fold dilutions (50, 25, 12.5, 6.3, 3.2, and 1.6 nM), flowing over Fcγ1 (panel a), NT-Fc (panel b), or NTΔSCD-Fc (panel c) immobilized at a concentration of 6032, 4996, or 4852 RU, respectively. The curves represent total specific binding after subtraction of the background responses observed in a control flow cell. D, native Jurkat (JKTwt) or J.CD26wt was stimulated with immobilized antibodies and/or Fc fusion proteins (anti-CD3, 1.0 μg/ml; anti-CD28, anti-CD26, Fcγ1, NT-Fc, NTΔSCD-Fc, each at 10 μg/ml). After culturing for 48 h, culture supernatants were pooled from the triplicate wells and assayed for IL-2 content. Values shown are means ± S.E. of determinations from triplicate cultures of three independent experiments. * and *** show points of significant increase (p < 0.05), whereas ** and # indicate points of no significant change compared with controls. E, following blocking with soluble anti-CD26, anti-CD28, or control mouse IgG, J.CD26wt cells were stimulated and IL-2 was measured as described in D. Values shown are means ± S.E. of determinations from triplicate cultures of three independent experiments. * and ** show results of significant inhibition obtained following blocking by anti-CD26 mAb (p < 0.05), and # and ## show results of no significant inhibition obtained following blocking by anti-CD28 mAb.
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T-cells were not stained with anti-CD26 mAb nor with NT-Fc (data not shown). These data suggested that the soluble N-terminal domain of caveolin-1 binds to cell surface CD26 and that the SCD of caveolin-1 is necessary for binding to CD26, as shown in our previous studies (14, 15).

To investigate the properties of binding of NT-Fc to CD26, we next examined the binding affinity with the Biacore system by injecting increasing concentrations of recombinant soluble CD26 (rsCD26) over each sensor surface containing recombinant Fc fusion proteins, Fcγ1, NT-Fc, or NTαSCD-Fc (Fig. 2C). rsCD26 did not bind to control recombinant Fcγ1 on a Biacore sensor chip (panel a in Fig. 2C). For each concentration of rsCD26 injected, the binding response at equilibrium was calculated by subtracting the response observed in NT-Fc, resulting in a $K_d$ value of $\sim 2 \times 10^{-5}$ M by equilibrium binding analysis (panel b in Fig. 2C). rsCD26 did not bind to NTαSCD-Fc on a Biacore sensor chip (panel c in Fig. 2C). These results clearly indicated that the N-terminal domain of caveolin-1 binds directly to CD26.

We next evaluated whether NT-Fc stimulation had a similar effect as anti-CD26 mAb on CD26-mediated T-cell costimulation in J.CD26wt (13). As shown in Fig. 2D, IL-2 production of J.CD26wt induced by plate-bound anti-CD3 plus NT-Fc was observed to be at a similar level as that induced by anti-CD3 plus anti-CD26 or by anti-CD3 plus anti-CD26 (*, **, and *** in the bar graph), whereas IL-2 production was not observed in JKTwt (which is CD26-negative) following stimulation by anti-CD3 plus anti-CD26 nor by anti-CD3 plus NT-Fc (* and *** in Fig. 2D). IL-2 production by J.CD26wt or JKTwt was not observed with the use of control recombinant Fcγ1 nor NTαSCD-Fc (# in Fig. 2D). To further investigate whether the T-cell costimulatory activity of NT-Fc was exerted via CD26, blocking experiments were...
conducted using CD26-specific mAb, which blocked binding of NT-Fc to J.CD26wt. As shown in Fig. 2E, IL-2 production induced by plate-bound anti-CD3 plus anti-CD26 was blocked by soluble anti-CD26 mAb but not by soluble anti-CD28 mAb (** and #). In this experimental condition, IL-2 production induced by plate-bound anti-CD3 plus NT-Fc was blocked by soluble anti-CD26 mAb but not by soluble anti-CD28 mAb (* and # in Fig. 2E). Control Fcγ1 or NTΔSCD-Fc did not have any T-cell costimulatory activity (Fig. 2E). Taken together, our results clearly indicated that caveolin-1 binds directly to CD26 and induces T-cell costimulation via CD26.

We next evaluated the ability of NT-Fc to reproduce the effects of anti-CD26 mAb on CD26-mediated T-cell costimulation (11, 35). As shown in Fig. 3A, T-cell proliferation induced by plate-bound anti-CD3 plus NT-Fc was observed to be at a similar level as that induced by anti-CD3 plus anti-CD28 or by anti-CD3 plus anti-CD26 (* in the bar graph), whereas T-cell proliferation was not observed using control recombinant Fcγ1 or NTΔSCD-Fc (** in the bar graph). Moreover, T-cell costimulation induced by NT-Fc was observed in a dose-dependent manner, whereas increasing doses of Fcγ1 or NTΔSCD-Fc did not induce T-cell proliferation (Fig. 3B).
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A

CD26 AA1-10 -Fc MKTPWKVLLG Fcγ1

B

Lane 1 2 3 4 5 6

C

(a) 20% of input lysate

(b) pulldown by Fcγ1

(c) pulldown by CD26 AA1-10-Fc

D

E

F

G
Further define the costimulatory activity of caveolin-1, we prepared CHO cells stably expressing human caveolin-1. As shown in Fig. 3C, we then showed that Cav-wt CHO cells expressed T-cell costimulatory activity in the presence of anti-CD3 mAb, an effect not observed with mock CHO cells nor Cav-ΔSCD CHO cells. The costimulatory activity of Cav-wt CHO cells was further observed in a cell number-dependent manner (* in Fig. 3C).

To further investigate whether the T-cell costimulatory activity of NT-Fc is exerted via CD26, blocking experiments were conducted using CD26-specific mAb which blocked binding of NT-Fc to J.CD26 (Fig. 2B). As shown in Fig. 3D, T-cell proliferation by plate-bound anti-CD3 plus anti-CD26 was blocked by soluble anti-CD26 mAb but not by soluble anti-CD28 mAb (*). On the other hand, T-cell proliferation by plate-bound anti-CD3 plus anti-CD28 was blocked by soluble anti-CD28 mAb but not by soluble anti-CD26 mAb (** in Fig. 3D). In this experimental condition, T-cell proliferation by plate-bound anti-CD3 plus NT-Fc was blocked by soluble anti-CD26 mAb but not by soluble anti-CD28 mAb (** in Fig. 3D). Control Fcγ1 or NTΔSCD-Fc did not have any T-cell costimulatory activity (Fig. 3D). Moreover, the blocking effect of CD26-specific mAb on NT-Fc costimulation was observed in a dose-dependent manner (* in Fig. 3E), and control IgG or anti-CD28 mAb at concentrations of 0–50 μg/ml did not block NT-Fc costimulation (Fig. 3E). Taken together with data shown in Figs. 1–3, these data suggested that NT-Fc functionally engages CD26 and not nonspecific proteins and that caveolin-1 has a costimulatory effect on T-cell proliferation via the TCR/CD3 pathway.

The proximal signaling molecules of CD26-mediated T-cell costimulation by caveolin-1 were next determined. We first examined whether the cytoplasmic tail of CD26 is responsible for T-cell costimulation by NT-Fc in the presence of anti-CD3 mAb. For this purpose, costimulation experiments were performed on Jurkat T-cells transfected with CD26-CD10 chimeric receptor. Moreover, whereas CD26 is reported to form dimers, CD26 was not detected in mock vector-transfected Jurkat (JKT/mock; panel a in Fig. 4C). Using theseJurkat transfectants, costimulation experiments by NT-Fc were conducted. As shown in Fig. 4D, IL-2 production was observed in CD26wt-transfected Jurkat T-cells by stimulation with anti-CD3 plus NT-Fc (* in panel a), but not in CD26-CD10 chimera nor in CD26 H750E-transfected Jurkat. Moreover, p65, one of NF-κB components, was activated in CD26wt-transfected Jurkat T-cells following stimulation with anti-CD3 plus NT-Fc (* in panel b) but not in CD26-CD10 chimera nor in CD26 H750E-transfected Jurkat. Furthermore, IL-2 production or p65 induction by stimulation with anti-CD3 plus PMA was equally observed in either of CD26wt, CD26-CD10 chimera, or CD26 H750E-transfected Jurkat (panels a and b of Fig. 4D). These data strongly suggested that the cytoplasmic tail of dimeric CD26, but not of monomeric CD26, is responsible for T-cell costimulation by NT-Fc in the presence of anti-CD3 mAb.

Because the cytoplasmic tail of CD26 appears to play a key role for CD26-mediated T-cell costimulation as shown in Fig. 4, we next explored signaling molecules associated with the cyto-

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**FIGURE 5.** The cytoplasmic tail of dimeric CD26 is associated with CARMA1. A, schematic diagram of Fc fusion proteins of the cytoplasmic tail of CD26 (CD26 aa1–10, MKTPWKLVLG depicts amino acid residues of human CD26 at 1–10 positions. At the 3′ portion, the hinge (H) and CH2 and CH3 domains of human IgG1, Fc are also indicated (Fcγ1). B, expression fusion proteins were purified as described under “Experimental Procedures.” Aliquots (5 μg) of control human IgG1 anti-TCR α/β (lanes 1 and 4), Fcγ1 (lanes 2 and 3), and CD26 aa1–10-Fc (lanes 3 and 6) were subjected to SDS-PAGE under reducing (+2 ME, lanes 1–3) or nonreducing (–2 ME, lanes 4–6) conditions. Molecular weight markers are depicted in M, Proteins were visualized by staining with Coomassie Brilliant Blue. C, an aliquot (50 μg) of Jurkat lysates was separated by two-dimensional PAGE using pH 3.0–10 nonlinear (NL) IPG (isoelectric focusing of proteins using immobilized pH gradient) striped in the first dimension and 4–12% SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue (panel a). Aliquots (1 mg) of lysates were precleared by human IgG (2 μg) and proteins A-Sepharose, followed by immunoprecipitation with Fcγ1 (1 μg) (panel b) or CD26 aa1–10-Fc (1 μg) (panel c). IPs were analyzed by two-dimensional PAGE, and six spots were clearly detected in IP complex of CD26 aa1–10-Fc (1–6 in panel c), * and ** were spots of Fcγ1 and CD26-Fc (aa1–10), respectively. Similar results were obtained in five independent experiments, and the panels shown are the representative results. D, J.CD26wt were lysed, and IP assays were conducted with anti-CARMA1 pAb (goat), anti-CD26 mAb (mouse (ms)), or control Ig (clgG). IP complexes as well as 10% of input lysates were then separated using SDS-PAGE, immunoblotted with indicated antibodies. Similar results were obtained in three independent experiments. E, 293FT cells were transiently transfected with VS-tagged full-length CD26 (CD26wt), CD26-CD10 chimeric receptor (CD26 + CD10 cyto), or CD26 containing mutation of histidine residue at amino acid 750 for glutamic acid (CD26 H750E), together with Xpress-tagged full-length CARMA1 (CARMA1wt). Cells were lysed with TBS/20% buffer and immunoprecipitated with anti-V5 mAb. IPs were separated using 5–20% SDS-PAGE and immunoblotted with anti-Xpress mAb (upper panel), followed by stripping and reprobing with anti-V5 mAb (lower panel). Similar results were obtained in three independent experiments. F, schematic diagrams of Xpress-tagged CARMA1 and its deletion mutants: CARMA1wt, Xpress-tagged full-length CARMA1; CARMA1(1–742), Xpress-tagged CARMA1 minus the SH3 + GUK domains; CARMA1(1–660), Xpress-tagged CARMA1 minus the PDZ + SH3 + GUK domains. G, 293FT cells were transiently transfected with Xpress-tagged CARMA1wt, CARMA1 with the SH3 + GUK domains deleted (residues 1–742), or CARMA1 with the PDZ + SH3 + GUK domains deleted (residues 1–660), together with V5-transfected CD26wt. Cells were lysed with TBS/20% buffer and immunoprecipitated with anti-V5 mAb. IPs were separated using SDS-PAGE and immunoblotted with anti-V5 mAb (upper panel), followed by stripping and reprobing with anti-Xpress mAb (lower panel). Similar results were obtained in three independent experiments.
plasmatic tail of dimeric CD26. For this purpose, we prepared Fc fusion protein containing the first 10 aa of the N-terminal residues of CD26 (CD26 aa1–10-Fc) (Fig. 5A). As shown in Fig. 5B, purified protein of CD26 aa1–10-Fc was observed at ~37 kDa in reducing conditions (lane 3) and at ~70 kDa in nonreducing conditions (lane 6), suggesting that CD26 aa1–10-Fc formed homodimers. Following pulldown by CD26 aa1–10-Fc of Jurkat T-cell lysates, molecules that interacted with CD26 aa1–10-Fc were analyzed by two-dimensional SDS-PAGE. The gel of two-dimensional PAGE using input lysates is shown in Fig. 5C (panel a). Compared with two-dimensional gel analyzing IP complex by control Fcγ1 (panel b in Fig. 5C), six spots were detected by pulldown assays with CD26 aa1–10-Fc (panel c in Fig. 5C). Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry, the proteins were determined to be as follows: spot 1, epidermal cytokeratin 2 (~66 kDa); spot 2, glutamyl-tRNA synthetase (~70 kDa); spot 3, tubulin (~50 kDa); spot 4, unnamed protein (~84 kDa); spot 5, CARMA1 (~120 kDa); and spot 6, HSP70 (~55 kDa), respectively (panel c in Fig. 5C). Following five independent repeats of these experiments with similar results, the unnamed protein (spot 4) was not identified more precisely by this procedure, and the other spots other than CARMA1 were ubiquitously expressed as housekeeping proteins. Therefore, CARMA1 was identified as an interacting protein with the cytoplasmic tail of CD26 and subjected to further examination.

For further confirmation, we next performed IP studies using lysates of J.CD26wt. As shown in Fig. 5D, CD26 was detected in a complex of lysates coprecipitated with anti-CARMA1 pAb (lane 2 of upper panel), and not coprecipitated with control goat IgG (lane 1 of upper panel). Moreover, CARMA1 was detected in a complex of lysates coprecipitated with anti-CD26 mAb (lane 4 of lower panel in Fig. 5D), and not coprecipitated with control mouse IgG (lane 3 of lower panel in Fig. 5D). These data suggested that CARMA1 binds to CD26 in cells. To determine the binding domain between CD26 and CARMA1, commmunoprecipitation assay was next performed using 293FT-cells cotransfected with Xpress-tagged human full-length CARMA1 (CARMA1wt) and with CD26wt, CD26 + CD10 cyto, or CD26 H750E. As shown in Fig. 5E, CARMA1 was coprecipitated with CD26wt (lane 2) but not with CD26 + CD10 cyto nor CD26 H750E (lanes 3 and 4). These data strongly suggested that the cytoplasmic tail and dimerization of CD26 are necessary to interact with CARMA1. We next explored the binding domain of CARMA1 to CD26. For this purpose, we prepared the C-terminal truncated deletion mutants of CARMA1 (Fig. 5F). As shown in Fig. 5G, CD26 was coprecipitated with CARMA1 wt or with CARMA1–(1–742) (lanes 2 and 3) but not with CARMA1–(1–600) (lane 4), suggesting that the PDZ domain in CARMA1 was necessary for binding to CD26.

To explore the role of CARMA1 in CD26-mediated T-cell costimulation, we used CARMA1-deficient Jurkat T-cell lines JPM50.6 to conduct rescue experiments (18). As shown in Fig. 6A, CARMA1 and CD26 were not detected in JPM50.6 (lane 3 of upper and lower panels), whereas CARMA1 was expressed in native Jurkat and J.CD26wt (lanes 1 and 2 of lower panel), and CD26 was expressed in J.CD26wt (lane 2 of upper panel) but not in native Jurkat (lane 1 of upper panel). We next generated the stable transfectants using JPM50.6 as described under “Experimental Procedures.” Fig. 6B shows that transfected Xpress-tagged CARMA1 was expressed in JPM50.6/CARMA1wt, JPM50.6/CD26wt + CARMA1wt, and JPM50.6/CD26wt + CARMA1–(1–660) and that transfected V5-tagged CD26 was expressed in JPM50.6/CD26wt, JPM50.6/CD26wt + CARMA1wt, and JPM50.6/CD26wt + CARMA1–(1–660). Fig. 6C shows the cell surface expression of CD3 and CD26 in JPM50.6 transfectants. CD3 was expressed at similar intensity among transfectants (horizontal axis of panels a–e in Fig. 6C). Although the intensity of cell surface CD26 expression was similar among JPM50.6/CD26wt, JPM50.6/CD26wt + CARMA1wt, and JPM50.6/CD26wt + CARMA1–(1–660) (panels b, d, and e in Fig. 6C), CD26 was not observed in JPM50.6/V (mock vector) and JPM50.6/CARMA1wt (panels a and c in Fig. 6C). Using these transfectants, IL-2 production and NF-κB activation assays were performed with stimulation by anti-CD3 alone or anti-CD3 plus NT-Fc. As shown in Fig. 6D, IL-2 production induced by anti-CD3 plus NT-Fc was clearly observed in JPM50.6/CD26wt + CARMA1 but not in JPM50.6, JPM50.6/CD26wt, JPM50.6/CARMA1, nor JPM50.6/CD26wt + CARMA1–(1–660) (panel a). Similarly, NF-κB activation induced by anti-CD3 plus NT-Fc was clearly observed in JPM50.6/CD26wt + CARMA1 but not in JPM50.6, JPM50.6/CD26wt, JPM50.6/CARMA1, nor JPM50.6/CD26wt + CARMA1–(1–660) (panel b in Fig. 6D). Furthermore, IL-2 production or NF-κB activation by stimulation with anti-CD3 plus PMA was equally observed in either of the transfectants (panels a and b of Fig. 6D). Taken together, these results suggested that CARMA1 is necessary to exert CD26-mediated costimulation by NT-Fc.

As shown above, costimulation of CD26 is observed to be exerted via interaction of CD26 with CARMA1 in the cytoplasm in Jurkat cells. To confirm this interaction more profoundly, we performed biochemical assays using human T-cells purified from healthy adult peripheral blood mononuclear cells (APB-T-cells). For this purpose, we first conducted IP studies using lysates of APB-T-cells. As shown in Fig. 7A, CD26 was detected in a complex of lysates coprecipitated with anti-CARMA1 pAb (lane 2 of upper panel), and not coprecipitated with control goat IgG (lane 1 of upper panel). These data strongly suggested that the cytoplasmic tail and dimerization of CD26 are necessary to interact with CARMA1. We next explored the binding domain of CARMA1 to CD26. For this purpose, we prepared the C-terminal truncated deletion mutants of CARMA1 (Fig. 5F). As shown in Fig. 5G, CD26 was coprecipitated with CARMA1 wt or with CARMA1–(1–742) (lanes 2 and 3) but not with CARMA1–(1–600) (lane 4), suggesting that the PDZ domain in CARMA1 was necessary for binding to CD26.

To explore the role of CARMA1 in CD26-mediated T-cell costimulation, we used CARMA1-deficient Jurkat T-cell lines JPM50.6 to conduct rescue experiments (18). As shown in Fig. 6A, CARMA1 and CD26 were not detected in JPM50.6 (lane 3 of upper and lower panels), whereas CARMA1 was expressed in native Jurkat and J.CD26wt (lanes 1 and 2 of lower panel), and CD26 was expressed in J.CD26wt (lane 2 of upper panel) but not in native Jurkat (lane 1 of upper panel).
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A, cell lysates of native Jurkat, J.CD26wt, or JPM50.6 were resolved in SDS-PAGE under reducing conditions, followed by immunoblotting with anti-CD26 mAb (upper panel) or anti-CARMA1 pAb (lower panel). B, JPM50.6 cells stably transfected with V5-tagged full-length CD26 (CD26wt) and/or Xpress-tagged full-length CARMA1 (CARMA1 wt) or CARMA1 with the PDZ + SH3 + GUK domains deleted (CARMA1-(1–660)) were generated as described under “Experimental Procedures.” Lysates were resolved in SDS-PAGE and immunoblotted with anti-Xpress mAb (CARMA1) (upper panel) or anti-V5 mAb (CD26) (lower panel). C, dot plots for cell surface expression of CD3 and CD26. % positive of CD3 is shown in mock vector-transfected JPM (panel a) and CARMA1-transfected JPM 50.6 (panel c), and % positive of CD3 and CD26 is shown in other transfec-tants (panels b, d, and e). D, JPM50.6 transflectants, which were described in B, were stimulated with plate-bound anti-CD3 in the presence or absence of plate-bound NT-Fc as described in Fig. 4D. Panel a, following 48 h of culture, IL-2 concentration of the culture supernatant was measured by ELISA. Values shown are means ± S.E. of determinations from triplicate cultures. * shows points of significant increase (p < 0.05) compared with control. Panel b, JPM50.6 transflectants were stimulated as described in panel a and harvested for nuclear extract. Each 5 μg of nuclear extract was subjected to ELISA-based DNA-binding protein assay. Binding activity to p65 NF-κB component was revealed by absorbance value at 450 nm. Data represent mean ± S.E. from triplicate experiments. * shows a point of significant increase (p < 0.05). Xpress vec. or V5 vec. depicts pcDNA4/HisMax or pEF6/V5 empty vector as a mock, respectively.

fractions of APB-T-cell lysates in the presence or absence of anti-CD3 alone. Moreover, time course analysis revealed that CD26, CARMA1, Bcl10, and IKKβ were not detected in the lipid raft fractions after stimulation with anti-CD3 plus NT-Fc (Fig. 7C), whereas CD26, CARMA1, Bcl10, and IKKβ were not detected after anti-CD3 treatment (data not shown). Furthermore, to examine whether CD26 and CARMA1 forms a complex with Bcl10 and IKKβ in lipid rafts, coprecipitation assay was performed using lipid raft fractions of APB-T-cell lysates from cells costimulated with anti-CD3 plus NT-Fc. As shown in Fig. 7D, CD26, CARMA1, Bcl10, and IKKβ were not detected in the lipid raft fractions (lane 4), although not detected in the lysates of APB-T-cells following stimulation with anti-CD3 alone (lane 2). Taken together, these data indicated that ligation of CD26 by caveolin-1 recruits a complex of CARMA1, Bcl10, and IKKβ to lipid rafts in normal T-cells.

To examine the role of CARMA1 on CD26-mediated T-cell costimulation more directly, we performed siRNA experiments in freshly isolated APB-T-cells. For this purpose, we prepared two sets of specific siRNA against CARMA1 as described under “Experimental Procedures,” and both of these siRNAs decreased CARMA1 expression in APB-T-cells, whereas the expression levels of CD26, TCR-β, or β-actin were not changed in the presence of control siRNA, ss1-siRNA, or ss2-siRNA (inside box of Fig. 7E). After transfection of these siRNAs into APB-T-cells, the proliferation assay was performed in the presence of anti-CD3 plus NT-Fc stimulation. As shown in Fig. 7E, T-cell proliferation stimulated with anti-CD3 plus NT-Fc was decreased in T-cells treated with siRNAs against CARMA1, whereas T-cell proliferation was observed in T-cells treated with control siRNA (* in Fig. 7E). These results suggested that CARMA1 plays an important role in signal transduction following CD26 binding to caveolin-1, leading to T-cell proliferation in normal T-cells.
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In this study, we showed that caveolin-1 is the costimulatory ligand for CD26, and that ligation of CD26 by caveolin-1 induces T-cell proliferation and NF-κB activation with costimulation of TCR/CD3. Moreover, we showed that the cytoplasmic tail of CD26 in T-cell interacts with CARMA1, resulting in signal transduction leading to NF-κB activation and that ligation of CD26 by caveolin-1 recruits a complex of CD26, CARMA1, Bcl10, and IKKβ to lipid rafts.

Enhancement of CD26 expression in autoimmune diseases may correlate with disease severity (40, 41), because patients with autoimmune diseases such as Grave’s disease and rheumatoid arthritis have increased levels of CD26 + T-cells in their peripheral blood as well as inflamed tissues, including thyroid and synovial fluids and membranes (9, 42). These findings imply that CD26 + T-cells play a role in the inflammation process and subsequent tissue destruction. Originally characterized as a T-cell activation antigen, human CD26 is preferentially expressed on the CD4+ memory T-cell subset and is up-regulated after T-cell activation (2, 3, 10). Along with its enhanced expression on activated T-cells, various lines of evidence have converged to demonstrate that CD26 is functionally associated with T-cell signal transduction processes relating to T-cell activation (2, 10, 11, 43). However, the precise mechanism involved in T-cell activation via CD26 in response to memory antigen such as tetanus toxoid remains to be clearly characterized, including the identification of its costimulatory ligand and the associated proximal signaling molecules.

Recently, we demonstrated that CD26 binds to caveolin-1 on APC and that residues 201–211 of CD26 along with the serine catalytic domain and subsequent tissue destruction. Originally characterized as a T-cell activation antigen, human CD26 is preferentially expressed on the CD4+ memory T-cell subset and is up-regulated after T-cell activation (2, 3, 10). Along with its enhanced expression on activated T-cells, various lines of evidence have converged to demonstrate that CD26 is functionally associated with T-cell signal transduction processes relating to T-cell activation (2, 10, 11, 43). However, the precise mechanism involved in T-cell activation via CD26 in response to memory antigen such as tetanus toxoid remains to be clearly characterized, including the identification of its costimulatory ligand and the associated proximal signaling molecules.

In this study, we showed that caveolin-1 is the costimulatory ligand for CD26, and that ligation of CD26 by caveolin-1 contains a caveolin-binding domain (ΦΨΦXXXΦΨΨΦ; Φ and Ψ depict aromatic residue and any amino acid, respectively), specifically WVYEEVFSAY in CD26 (2, 44). These
observations strongly support the notion that DPPIV enzyme activity is necessary to exert T-cell costimulatory activation via CD26 as demonstrated in our previous report using CD26 specific mAbs (13).

To examine the binding of caveolin-1 to CD26 in T-cells, we used soluble Fc fusion proteins containing the N-terminal domain of caveolin-1 (NT-Fc) (Fig. 1), and we found that NT-Fc binds specifically to CD26 to induce T-cell proliferation in the presence of TCR/CD3 costimulation (Figs. 2 and 3). Moreover, the binding affinity between caveolin-1 and CD26 ($K_d \sim 2 \times 10^{-5} \text{M}$), as determined by the Biacore system (Fig. 2C), is comparable with that of other costimulatory molecules with important roles in immune responses and their associated ligands, such as CD2-CD5 ($K_d \sim 10^{-6} \text{M}$), CD80-CD28 ($K_d \sim 10^{-7} \text{M}$), and CD86-CD28 ($K_d \sim 10^{-6} \text{M}$) (45–47). Until now, CD26-mediated T-cell costimulation was performed using anti-CD26 mAbs, resulting in various CD26 functions (4, 7, 48, 49). Assuming that the affinity between antigen and antibody is higher ($K_d \sim 10^{-9} \text{M}$) than that of a ligand-receptor system, and that ligand-specific conformations are capable of differentially activating distinct signaling partners (50), ligand-dependent pathways may be predicted to have different signals associated with the antigen-antibody system and ligand-receptor system.

We have demonstrated previously that ligation of CD26 by the anti-CD26 mAb 1F7 induces T-cell costimulation and IL-2 production by CD26-transfected Jurkat T-cell lines, while increasing tyrosine phosphorylation of signaling molecules such as ZAP70, p56lk, and CD3ζ was observed (2, 7, 12). In addition, we have shown that ligation of the CD26 molecules by the anti-CD26 mAb 1F7 increases the recruitment of CD26 molecules with CD45RO to lipid rafts, resulting in increased tyrosine phosphorylation of signaling molecules (33). However,
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The precise proximal signaling pathway of CD26 has not yet been identified, particularly in view of the fact that the cytoplasmic tail of CD26 contains only 6 amino acid residues without a common signaling motif structure. Moreover, it has been unclear whether the short cytoplasmic tail is responsible for signal transduction associated with CD26-mediated costimulation. In this study, using recombinant CD26-CD10 chimeric receptor, we showed that the cytoplasmic tail of CD26 is indeed responsible for T-cell costimulation induced by anti-CD3 plus caveolin-1 (Fig. 4D). Furthermore, to explore the proximal signaling molecules interacting with the cytoplasmic tail of dimeric CD26, we used proteomic analyses with Fc fusion proteins containing the cytoplasmic amino acid residues of CD26 (Fig. 5, A and B) to identify that CARMA1 binds to the cytoplasmic tail of dimeric CD26 (Fig. 5C). Moreover, we demonstrated here that a PDZ domain in CARMA1 is necessary for binding to CD26 (Fig. 5G). The importance of CARMA1 in CD26-mediated costimulation is also shown by rescue experiments using the CARMA1-deficient Jurkat T-cell line JPM50.6 (Fig. 6D) and using siRNA against CARMA1 in APB-T-cells (Fig. 7E). CARMA1, containing caspase-recruitment domain and MAGUK domains, plays an essential role in the NF-κB activation and IL-2 expression induced by CD3-CD28 or CD28-PMA stimulation (18, 22). After being phosphorylated, CARMA1 functions as a signaling intermediate downstream of PKCθ and upstream of IKK in the TCR signaling transduction pathway leading to NF-κB activation (39, 51). Because MAGUK domain-containing proteins are generally involved in the organization of multiprotein complexes at the interface of the cytoplasmic membrane (52), it is possible that CARMA1 associates with as yet undefined membrane proteins in the immunological synapse of T-cells. In this regard, our present data suggest a novel mechanism for CAMRA1 function as it complexes with Bcl10 and IKK to transduce CD26-costimulatory signals. Moreover, as shown Fig. 5C, cytoskeletal proteins were also observed in the complex in the pulldown assays with CD26 aa1–10-Fc. Because MAGUK domain-containing proteins are generally involved in the organization of multiprotein complexes in the cytoskeleton (52), the downstream signaling of CD26 may also be associated with cytoskeletal assembly via CARMA1. The association of CD26, CARMA1, and the cytoskeleton will be elucidated in future studies.

CD26/DPPIV is reported to exist as homodimers, a structural organization that allows access of substrates to DPPIV catalytic activity (36, 37). Although DPPIV activity is crucial for CD26-mediated T-cell costimulation (13, 30), the exact role played by DPPIV in this process is unclear. Our previous study showed that the enzymatic pocket structure of the DPPIV catalytic site is necessary for binding of CD26 to caveolin-1, leading to the up-regulation of CD86 expression on APC (14, 15). In this study, we found that monomeric CD26 H750E, which has a 300-fold decrease in catalytic activity (36), does not bind to CARMA1 (Fig. 5E), resulting in loss of CD26-mediated T-cell costimulation by anti-CD3 plus caveolin-1 (Fig. 4D). Therefore, dimerization of CD26 is not only necessary for binding to caveolin-1 but also serves as a scaffolding structure for the cytoplasmic signaling molecule CARMA1. The precise binding position of CARMA1 in the cytoplasmic domain of CD26 remains to be elucidated in future work, because PDZ domains bind primarily to specific C-terminal motifs (X(S/T)X(V/L), where X depicts any amino acid) or internal target motifs as well as other PDZ domains (52).

Based upon this study, we propose the following model to explain the sequence of events leading from CD26-CD3 costimulation to NF-κB activation (Fig. 8). In CD3-CD26 costimulation, TCR engagement by peptide-loaded major histocompatibility complex class II presented on APC activates phosphatidylinositol 3-kinase via phosphorylation of immunoreceptor tyrosine-based activation motifs in TCR, leading to recruitment of PKCθ and IKK complex in lipid rafts (16, 18, 25, 38). Concomitantly, CD26 ligation by caveolin-1 on APC recruits CD26-interacting CARMA1 to lipid rafts, resulting in the formation of a CARMA1-Bcl10-MALT1-IKK complex, and this membrane-associated Bcl10 complex then activates IKK through ubiquitination of the NF-κB essential modulator. This study involving Jurkat T-cell lines and human peripheral T-cells represents a different cellular system than those with murine T-cells, where other investigators previously described a role for CD26 in thymic development of murine T-cells (53, 54). Our objective with this study was to define a costimulatory ligand for CD26 and proximal signaling molecule of CD26 in human T-cells, with a future aim of analyzing the in vivo role of CD26-mediated T-cell immunity.

In conclusion, we have now demonstrated that CD26 on the T-cell surface binds to caveolin-1, hence identifying the first endogenously expressed CD26 costimulatory ligand in the immune system. Moreover, the caveolin-1–CD26 interaction results in strong T-cell costimulation as a result of the recruitment of a molecular complex consisting of CARMA1-Bcl10-MALT1-IKK in lipid rafts. Our findings will therefore serve as a foundation for future insights into the regulation of T-cell costimulation via the CD26 molecule.

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