The transmembranous domain of CD40 determines CD40 partitioning into lipid rafts

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Abstract **Stimulation of CD40 has been previously shown to result in a release of ceramide in small sphingolipid-enriched rafts in the cell membrane [Grassmé et al., J. Immunol. 168 (2002) 298–307]. Those rafts fused to larger signaling platforms that served to cluster CD40. Here, we defined molecular mechanisms of CD40 clustering in membrane platforms. To this end, we replaced the transmembranous domain of CD40 with that of CD45, a molecule known to be excluded from lipid rafts. Murine T cells were stably transfected with wild-type CD40 or chimeric CD40/CD45. Flow cytometry confirmed normal binding properties of the mutant to CD40 ligand. Stimulation with CD40 ligand resulted in clustering of wild-type CD40, while the chimeric CD40/45 receptor failed to cluster. This correlated with a deficiency of the chimeric receptor to activate JNK, p38 MAP kinase and SAPK, known signaling molecules of the intracellular pathway initiated by CD40. Forced crosslinking of the CD40/45 chimeric receptor restored, at least partially, these signaling events. The results suggest that the transmembranous domain of CD40 is central for the recruitment to and clustering of CD40 in membrane platforms.**

Key words: CD40; CD45; Transmembranous domain; Lipid raft; Receptor clustering

1. Introduction

Receptor clustering has been shown for many receptor molecules and seems to be central in the generation of transmembranous signals. Receptors shown to aggregate upon stimulation include the T cell receptor/CD3 complex [1,2], B cell receptor [3,4], CD40 [5–8], Fcε receptor 1 [9], CD95 [10,11], CD28 [12], tumor necrosis factor receptor [13], platelet-derived growth factor receptor [14], CD2, CD44, L-selectin or integrins [15,16]. Many receptors cluster in distinct domains of the cell membrane, termed lipid rafts or platforms. Lipid rafts are microdomains in the plasma membrane that are enriched in sphingolipids and cholesterol and separate from other phospholipids in the cell membrane [17–19]. This separation is mediated by a tight association of sphingolipids with each other and with cholesterol, which seems to function as a spacer between the bulky sphingolipids. Sphingolipid- and cholesterol-enriched microdomains are resistant to solubilization by non-ionic detergents at low temperatures [17,19], and, thus, have also been named detergent-insensitive glycosphingolipid-enriched microdomains.

Disruption of rafts has been shown to prevent signaling by many receptor molecules indicating a central function of lipid rafts for signal transmission into the cell. However, the molecular determinants of receptor clustering are unknown. Here, we employed the CD40 system to define some of the molecular requirements for receptor clustering. CD40 has been shown to be involved in a variety of immune responses, for instance modulation of T lymphocyte activation, B lymphocyte differentiation, immune regulation by dendritic cells, germinal center formation, immunoglobulin isotype switching and interleukin-12 production [20–24]. We and others have recently demonstrated a recruitment and clustering of CD40 in sphingolipid-rich rafts of dendritic cells [5,6] and B lymphocytes [7,8]. Stimulation of CD40 appeared to trigger the fusion of intracellular vesicles, which contain the acid sphingomyelinase, with the cell membrane resulting in exposure of the acid sphingomyelinase on the cell surface [8]. Activity of the acid sphingomyelinase mediated the release of ceramide in membrane microdomains and ceramide triggered the fusion of small membrane rafts to large signaling platforms that trapped CD40 [8]. CD40 trapping in ceramide-enriched platforms resulted in the formation of large CD40 aggregates, which were required for the initiation of CD40 signaling.

In contrast to CD40 and other receptor molecules that are trapped in sphingolipid- and ceramide-enriched membrane domains, the tyrosine phosphatase CD45 has been shown to be excluded from lipid rafts [4,25,26]. The mechanisms of this selective exclusion are unknown. Here, we employed this difference in membrane distribution to analyze molecular mechanisms of receptor–raft interactions and receptor clustering and replaced the membrane spanning region of CD40 with the transmembranous domain of the protein tyrosine phosphatase CD45. The data reveal that CD40 clustered in platforms, while the CD40/45 chimera was excluded from lipid rafts and platforms. Clustering of CD40 in membrane platforms correlated with the activation of intracellular signaling molecules and the failure of the CD40/CD45 chimera to cluster resulted in a deficiency to initiate CD40 signaling.

2. Materials and methods

2.1. Cell culture and antibodies

T cell hybridoma 3A9 cells were from murine thymoma fused with T cells. 3A9 T cell hybridoma and JY B lymphocytes were grown in RPMI 1640 medium, COS cells were grown in Dulbecco's modified Eagle's medium (DMEM). RPMI 1640 and DMEM were both sup-

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plemented with 10% fetal calf serum, 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin (all purchased from Gibco BRL-Life Technologies, Grand Island, NY, USA) and 50 μM 2-mercaptoethanol.

Surface CD40 was stained with the mouse monoclonal anti-human CD40 antibody 5C3 from Pharmingen (San Diego, CA, USA) or recombinant Flag-tagged CD40 ligand from Alexis (San Diego, CA, USA). Immunoprecipitation experiments and Western blots of CD40 were performed with a rabbit polyclonal anti-CD40 antibody (C-20) from Santa Cruz (Santa Cruz, CA, USA), c-Jun amino-terminal kinase (JNK-1) was detected with a rabbit polyclonal anti-JNK-1 antibody (C-17) from Santa Cruz. Recombinant, Flag-tagged CD40 ligand and enhancer were from Alexis. FITC-conjugated mouse anti-Flag antibodies were from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit anti-phospho-p38, and anti-phospho-SAPK/JNK were from New England Biolabs (Beverly, MA, USA).

2.2. Generation of chimeric receptor and transfectants

The transmembranous domains of CD40 and CD45 were determined according to the SwissProt database. The corresponding amino acid sequence of CD40 is ALVVIPIIFGILFAILLVLVFI, that of CD45 is ALIAFLAFLIIVTSIALLVVLY. CD40 and CD45 are both type I membrane proteins and both transmembranous domains contain 22 amino acids. The chimeric receptor of CD40 with the transmembranous region of CD45 was generated by overlapping PCR using extended primers. The extracellular domain was fused with the transmembranous part of CD40 by amplification with the 5′ primer GCT CTA GAA TGG TTC GTC TGC CTC TGC AG and the 3′ primer GTA GAG AAC AAC AAG CAG GGC TAT TGA TGT CAC AAT AAT CAG AAA TGC CAG AAA TGC TAT CAG TGC TCT CAG CCG ATC CTG GGG ACC ACA GAC AAC ATC. The intracellular domain was fused to the transmembranous domain using the 5′ primer GCA CTG ATA GCA TTT CTG GCA TTT CTG ATT ATT GTG ACA TCA ATA GCC CTG CTT GTT GTT CTC TAC AAA AAG GTG GCC AAG AAG CCA ACC AAT AAG GC and the 3′ primer GTG CCC GGG TCA CTG TCT CTC CTG CAC TGA GAT GC. These two overlapping fragments were then fused by PCR with the 5′ primer GCT CTA GAA TGG TTC GTC TGC CTC TGC AG containing a *Xba*I site and the 3′ primer GTG CCC GGG TCA CTG TCT CTC CTG CAC TGA GAT GC containing a *Sma*I site. The resulting chimera was subcloned into the expression vector pJK [27] using the *Xba*I and *Sma*I sites and verified by sequencing. 3A9 cells were transfected by electroporation with 40 μg each of the CD40 expression plasmid pJK-*cd40*, the chimeric receptor designated pJK-*cd40/45* or the (empty) control pJK plasmid. The pJK vector contains an elongation factor promoter to induce constitutive protein expression. Stable transfectants were selected using 3 mg/ml G418, transient transfections were performed in COS cells using FuGENE 6 (Roche, Indianapolis, IN, USA).

2.3. Receptor aggregation, fluorescence microscopy and flow cytometry (FACS)

1×10^6 3A9 cells per sample were centrifuged at 1200 rpm for 5 min, resuspended in 50 μl of pre-warmed RPMI 1640 and kept at 37°C for 8 min. Cells were stimulated with 1 μg/ml CD40 ligand plus 1 μg/ml enhancer to crosslink the Flag tag fused to the CD40 ligand. Stimulation was stopped by addition of 1 ml ice-cold washing buffer (phosphate-buffered saline supplemented with 2% fetal calf serum and 0.2% sodium azide). Samples were then incubated at 4°C for 45 min with 500 ng/ml of anti-CD40 antibody (Pharmingen), washed with ice-cold washing buffer, stained with a cyanine 3.18 (Cy3)-coupled anti-mouse Ig antibody and analyzed on a Zeiss (Axioplan 2) fluorescence microscope. Receptor aggregates were defined as an area of intense fluorescence on the cell surface, while unstimulated cells displayed a homogeneous distribution of fluorescence. In every sample at least 200 cells were screened for the presence of aggregates and results are given as percentage of cells positive for clusters at the indicated time. For FACS analysis cells were stained as described above or with recombinant Flag-tagged CD40 ligand coupled to a FITC anti-Flag antibody. FACS analysis was performed on a Becton-Dickinson Calibur.

2.4. JNK activity

For detection of JNK activity, 4×10^6 cells were incubated for 20 min with CD40 ligand (1 μg/ml) plus enhancer (1 μg/ml) and lysed in ice-cold lysis buffer containing 25 mM HEPES (pH 7.4), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM sodium fluoride, 10 mM Na_3VO_4, 10 mM sodium pyrophosphate, 10 mM EDTA, and 10 μg/ml each of aprotinin and leupeptin as previously described [28]. Lysates were centrifuged at $21\,000\times g$ for 10 min at 4°C, and JNK was immunoprecipitated from the supernatant at 4°C for 4 h using polyclonal rabbit anti-mouse JNK. Immunocomplexes were immobilized on protein A/G agarose (Santa Cruz Inc.), incubated for an additional 45 min at 4°C, washed twice in lysis buffer, twice in a buffer containing 132 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$, 1% NP40 and 2 mM Na_3VO_4, once in 100 mM Tris (pH 7.5), 100 mM LiCl, and finally twice in kinase reaction buffer consisting of 12.5 mM morpholinopropanesulfonic acid (pH 7.5), 12.5 mM γ-glycerophosphate, 0.5 mM EGTA, 7.5 mM $MgCl_2$, 0.5 mM NaF, 0.5 mM Na_3VO_4. Immunoprecipitates were finally resuspended in kinase reaction buffer supplemented with 10 μCi/sample of [^{32}P]γATP (6000 Ci/mmol, Amersham, Freiburg, Germany), 10 μM ATP, and 1 μg of glutathione *S*-transferase (GST)-c-JUN (amino acids 1–79). The samples were incubated at 30°C for 20 min, the kinase reaction was stopped by addition of 5 μl of boiling 5×SDS sample buffer and 5% 2-mercaptoethanol. Proteins were separated by 10% SDS–PAGE, electrophoretically transferred to nitrocellulose membranes (Hybond) and analyzed by autoradiography. To test for equal amounts of immunoprecipitated protein, the membranes were blocked with bovine serum albumin after autoradiography, probed with anti-JNK-1 antibodies, washed and developed using an alkaline phosphatase AP-coupled anti-rabbit antibody and the Tropix ECL system.

The substrate GST-c-JUN was expressed in DH5α bacteria by incubation with isopropyl-D-thiogalactopyranoside (200 μM) for 4 h. Bacteria were lysed in a solution containing 25 mM HEPES (pH 7.4), 0.2% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, and 10 μg/ml each of aprotinin and leupeptin. The GST fusion protein was purified by binding to glutathione Sepharose and eluted in kinase reaction buffer supplemented with 20 mM glutathione. The purity of the preparations was tested by SDS–PAGE and Coomassie staining. Additional titration experiments revealed a limit of JNK detection at approximately 0.5×10^5 cells/sample. The substrate GST-c-JUN was added in large excess to achieve linear assay kinetics.

2.5. Phosphorylation of p38K and SAPK/JNK

To determine serine/threonine phosphorylation of p38K [29–31] 0.5×10^6 3A9 cells per sample were washed, equilibrated at 37°C for 8 min and stimulated by addition of CD40 ligand and enhancer. The reaction was terminated by addition of 20 μl ice-cold lysis buffer consisting of 25 mM HEPES, pH 7.4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM NaF, 10 mM Na_3VO_4, 10 mM sodium pyrophosphate and 10 μg/ml each of aprotinin and leupeptin. Cell lysates were centrifuged and the supernatants were added to 5×SDS sample buffer and 5% 2-mercaptoethanol. Proteins were separated by 10% SDS–PAGE and the blots were probed with the monoclonal anti-phospho-p38K antibody 28B10 (Cell Signaling Technology, Beverly, MA, USA) following the instructions of the vendor. The anti-phospho-p38K antibody specifically detects phospho-Thr180 and phospho-Tyr182 for p38 kinase.

3. Results

3.1. CD40/45 mutants bind CD40 ligand

In order to identify mechanisms that determine the presence of receptor molecules in lipid rafts, we replaced the transmembranous domain of CD40, known to be present in ceramide-enriched platforms [8], with that of CD45, known to be excluded from rafts [24–26].

Murine T cell 3A9 hybridoma or COS cells were transfected with wild-type CD40, the CD40/45 chimeric receptor or the empty pJK vector that served as control. Flow cytometry analysis revealed a similar surface expression of CD40 and CD40/45 in 3A9 cells (Fig. 1A) or COS cells (not shown).

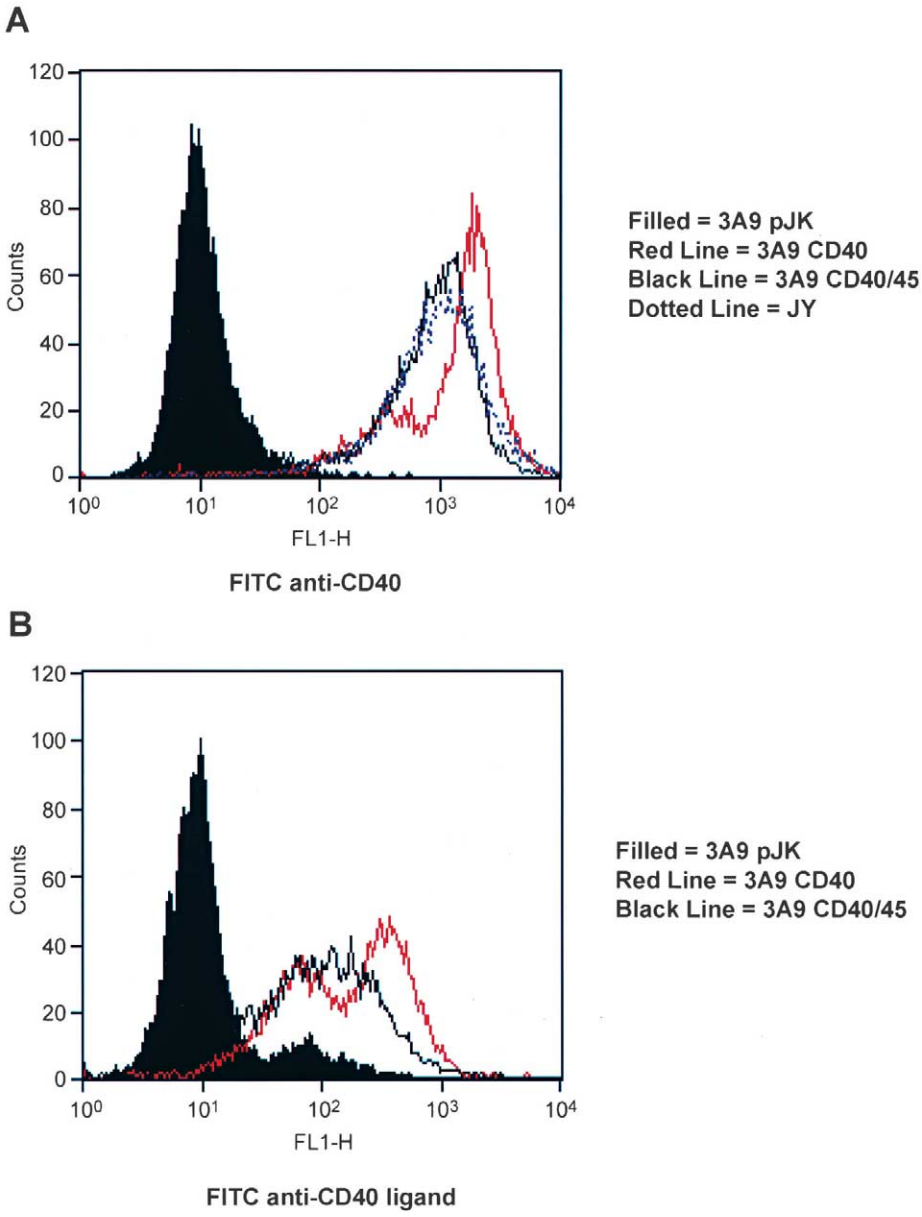

Fig. 1. Transfection of CD40 and CD40/CD45 chimeric constructs. A: Transfection of wild-type CD40 or the CD40/CD45 chimeric construct results in surface expression of both proteins. Surface expression of the receptor molecules was determined by flow cytometry upon staining of the cells with FITC-labeled anti-CD40 antibodies. The filled curve represents 3A9 cells transfected with empty control vector, the red line CD40 transfected cells, the black line 3A9 hybridoma transfected with chimeric CD40/45 and the dotted line is a control staining of JY cells known to endogenously express CD40. B: Wild-type CD40 and the mutant CD40/45 construct exhibit similar binding capacities to CD40 ligand. Cells were incubated with FITC-coupled anti-Flag antibodies and binding of the ligand was determined by flow cytometry. The filled curve represents binding to 3A9 cells transfected with empty vector, the red line binding to CD40 transfected cells, the black line binding to 3A9 hybridoma transfected with chimeric CD40/45. The data in A and B are representative of three flow cytometry studies each with very similar results.

The expression levels of both molecules were similar to that of CD40 in JY lymphoblastoid B cells (Fig. 1A), which are known to endogenously express CD40 and served as a control.

In addition, the CD40 and the CD40/45 transfectants showed similar affinity to CD40 ligand as determined by FACS analysis upon staining of the cells with a FITC-coupled CD40 ligand (Fig. 1B). This indicates that the switch of the transmembranous domain did not significantly alter the ligand binding capacity of the extracellular domain of CD40.

3.2. CD40/45 mutants fail to cluster upon stimulation

We employed these transfectants to analyze receptor localization and clustering in sphingolipid-enriched domains. We have recently shown that stimulation via CD40 alters rafts by acid sphingomyelinase-mediated release of ceramide in those rafts [8,10]. Ceramide triggered the fusion of rafts to large membrane platforms and, second, served to trap CD40 molecules in those platforms finally resulting in a clustering of CD40 [8]. To test whether the transmembranous domain of CD40 is involved in clustering of the receptor in raft-derived

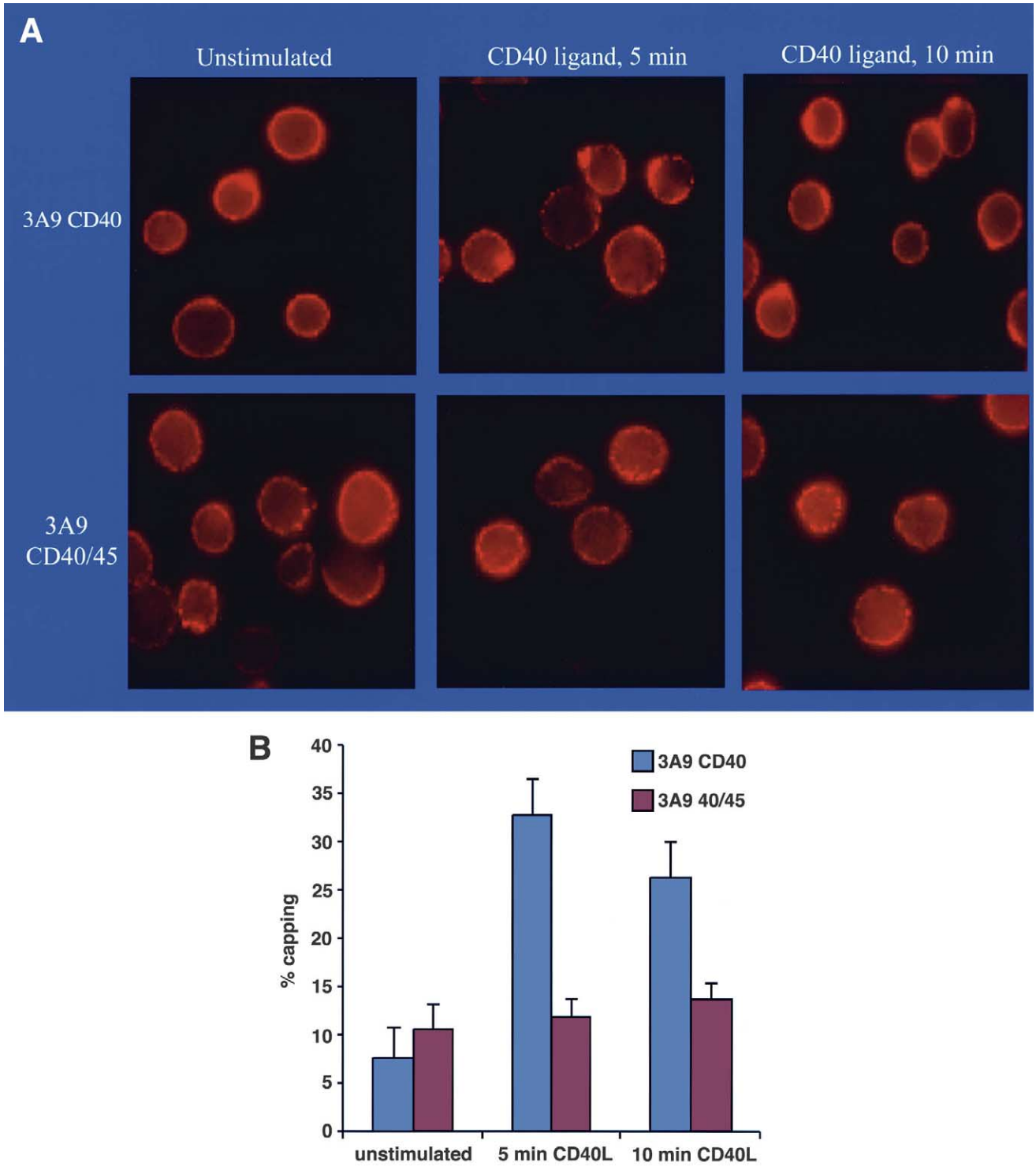

Fig. 2. CD40, but not the CD45/45 protein, clusters upon stimulation. A: Stimulation of 3A9 cells expressing CD40 results in rapid clustering of CD40, while the cells expressing CD40/45 fail to form receptor cluster upon stimulation. Clustering was analyzed by fluorescence microscopy. Shown is a typical result of three independent studies. B: The quantitative analysis of CD40 and CD40/45 clustering after stimulation with CD40 ligand indicates a failure of the CD40/45 mutant to cluster. Data (mean ± S.D.) were obtained from three independent experiments. The quantification of clustering was confirmed by a second independent observer. These independent studies revealed a variation of less than 5% in the determination of receptor clustering.

membrane platforms, we treated 3A9 cells expressing CD40 or CD40/45 chimera with recombinant CD40 ligand and measured clustering of the receptor by fluorescence microscopy. The data revealed that wild-type CD40 clustered within minutes after stimulation, while the chimeric CD40/45 molecule failed to cluster (Fig. 2). These data identify the transmembranous domain of CD40 as an important element for clustering of the receptor upon stimulation.

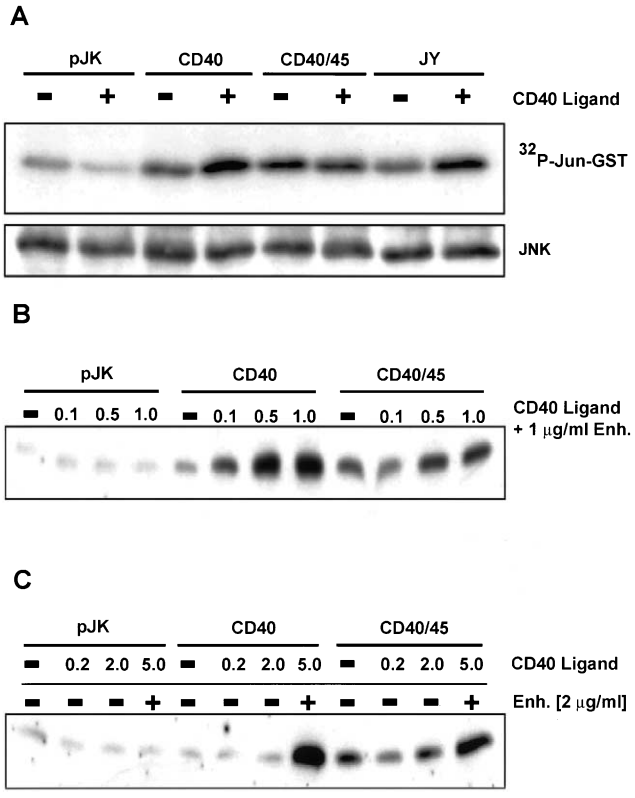

Fig. 3. Activation of JNK and p38K depends on receptor clustering. A: Stimulation of 3A9 cells expressing wild-type CD40 results in activation of JNK, while cells expressing the chimeric CD40/45 mutant failed to respond. Cells were stimulated for the indicated time with 1 μg/ml CD40 ligand and 1 μg/ml anti-Flag antibodies (= enhancer). The activity of JNK was determined by phosphorylation of the substrate GST-Jun. The autoradiography is representative of two experiments. B: Cells expressing CD40 ligand already respond to 0.1 μg/ml CD40 ligand plus 1 μg/ml enhancer with phosphorylation of p38K. Cells expressing the chimeric mutant fail to phosphorylate p38K at low doses of crosslinked CD40 ligand. Higher doses of CD40 ligand induce a limited phosphorylation of p38K in these cells. Cells were stimulated with the indicated dose of CD40 ligand crosslinked with 1 μg/ml enhancer. Phosphorylation of p38K was determined by Western blotting using phospho-specific anti-p38K antibodies. The studies were repeated four times with very similar results. C: Artificial crosslinking of CD40 using doses as high as 5 μg/ml of the CD40 ligand and 2 μg/ml of the enhancer restores, at least partially, phosphorylation of p38K. Soluble CD40 ligand without addition of enhancer is insufficient to initiate CD40 signaling. Phosphorylation of p38K was determined by Western blotting using phospho-specific anti-p38K antibodies. The data are representative of three experiments.

3.3. *The clustering deficiency of CD40/45 mutants correlates with a signaling defect*

Previous data suggested that disruption of rafts prevents cellular activation via CD40 [8]. We, therefore, tested whether the failure of the CD40/45 mutant to cluster correlates with a defect in the initiation of intracellular signals elicited by CD40. To this end we determined the phosphorylation and activation of p38 MAP kinase, SAPK and JNK, known targets of CD40 [32–35], in 3A9 T cells transfected with wild-type CD40 or the CD40/45 chimera. Furthermore, we analyzed whether CD40-mediated activation of intracellular signaling molecules requires the presence of the receptor in lipid domains or whether a clustering of the receptor is already sufficient to initiate at least some signaling. In the latter case, an artificial clustering of chimeric CD40/45 molecules should permit the activation of the cells. The results revealed that cells expressing the chimeric CD40/45 receptor failed to stimulate JNK or to phosphorylate p38K upon treatment with CD40 ligand (Fig. 3A,B). Quantitative analysis of the blots by laser scanning densitometry revealed a 5 ± 0.45-fold increase of JNK activity after stimulation of CD40-expressing cells (mean ± S.D., $n = 2$), while the activity of JNK after stimulation of CD40/CD45-transfected cells was unaltered (1.0 ± 0.15-fold change). The phosphorylation of p38K increased 12 ± 2-fold in CD40-positive cells, but only 1.2 ± 0.5-fold in the CD40/45-transfected cells (mean ± S.D., $n = 4$) after stimulation of the cells with 0.5 μg/ml CD40 ligand plus 1 μg/ml enhancer. Forced crosslinking of the CD40/45 mutant using higher concentrations of crosslinked CD40 ligand, i.e. up to 5 μg/ml CD40 ligand and 2 μg/ml enhancer, at least partially restored phosphorylation of p38K (Fig. 3C). Laser scanning densitometry revealed that stimulation with doses between 1 and 5 μg/ml CD40 ligand and 2 μg/ml enhancer triggered a 12.5 ± 3-fold and a 6 ± 1-fold increase of p38K phosphorylation in CD40-positive cells or CD40/45-expressing cells, respectively (mean ± S.D., $n = 3$). This indicates that the mutation did not abolish the ability of the receptor to transmit signals into the cell.

4. Discussion

Although many receptors have been shown to cluster in detergent-resistant membrane domains, molecular mechanisms mediating the preferential partitioning of receptors in those domains require definition. The present data identify the transmembranous domain of CD40 as an important element of the receptor to interact with sphingolipid-enriched rafts and to cluster in membrane platforms upon stimulation. The failure of the CD40/45 mutant to cluster is not caused by a simple conformational change or inactivity of the mutant protein, since forced crosslinking of the CD40/45 molecule is sufficient to trigger phosphorylation of p38K. Further, the transmembranous domains of CD40 and CD45 are both composed of 22 amino acids (CD40: ALVVIPIIFGILFAILLVLVFI and CD45: ALIAFLAFLIIVTSIALLVVLY) excluding a large difference in the length of the transmembranous domain as the cause of the inability of the CD40/45 mutant to cluster. A simple model might suggest that individual amino acid residues mediate the different abilities of the receptor molecules to cluster within lipid rafts. The transmembranous domain of CD40 contains only uncharged amino acids and three phenylalanine residues, which might facilitate integration of the receptor in hydrophobic, sphingolipid- and cholesterol-enriched membrane platforms. In contrast, the transmembranous domain of CD45 contains central threonine and serine residues with aliphatic hydroxyl side chains and a tyrosine residue at the interface to the cytosol. The hydrophilic amino acids may render the transmembranous domain of CD45 less hydrophobic and prevent integration into ceramide-enriched lipid platforms. However, this models fails to explain a selective recruitment of activated, CD40 ligand-bound CD40 molecules into lipid rafts. Therefore, we would like to suggest that the release of ceramide in rafts not only triggers fusion of those rafts to larger platforms [36], and alters the hydrophobicity of the membrane domain, but may also alter the diameter of the cell membrane in the platform area. Binding of the

ligand to CD40 may change the conformation of the receptor to fit into a ceramide-enriched membrane domain, while the presence in membrane parts with other diameters might be energetically unfavorable for the ligand–receptor complex. Vice versa, the transmembranous domain of CD45 might favorably fit into the cell membrane outside rafts, while its presence in ceramide-enriched platforms with other diameters might be energetically unlikely.

Our results demonstrate that rapid CD40 clustering depends on association with rafts and exclusion from these microdomains results in the inability to cluster and to signal upon stimulation. Forced crosslinking of the CD40/45 mutant at least partially restored signaling indicating that the clustering event per se is central for transmission of a signal via CD40 into the cell. This suggests that physiological clustering of CD40 in membrane platforms serves to initiate a high local concentration of receptor molecules that may permit the transactivation of molecules associated with CD40, e.g. JAK3 [37]. However, additional functions of CD40 clustering in membrane platforms that appear to contribute to full activation via CD40 might be a close contact of the receptor with signaling molecules present in lipid platforms, an exclusion of inhibitory signaling molecules, e.g. CD45, and/or a direct modification of CD40 signaling by ceramide. In a physiological situation, the presence of CD40 in ceramide-enriched membrane platforms might also limit lateral diffusion and promote binding to its ligand.

In conclusion, the present study indicates that the transmembranous domain of CD40 plays a central role for clustering of the receptor in membrane platforms that is required for efficient signaling of CD40.

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