The Modulation of CD40 Ligand Signaling by Transmembrane CD28 Splice Variant in Human T Cells

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Abstract

The role of CD40 ligand (CD40L)/CD40 signaling in T cell–dependent B cell differentiation and maturation has been amply documented. The mechanism of CD40 signaling in B cells has been well established, whereas the signaling mechanism of CD40L in T cell costimulation remains unknown. In this study we show that CD28i, a transmembrane splice variant of CD28 costimulatory receptor, complexes with CD40L in human T cells. The cross-linking of CD40L resulted in the coendocytosis of CD28i with CD40L. The tyrosine phosphorylation of CD28i followed the cross-linking of CD40L, and the overexpression of CD28i augmented the c-Jun NH2-terminal kinase, p21-activated kinase 2, and nuclear factor κB activation. These data indicate that CD28i, by functioning as a signaling adaptor, transduces CD40L signaling as well as CD28 signaling in human T cells.

Key words: CD40L • CD28 • CD28 variant • T cell costimulation

Introduction

Costimulation is a necessary requirement for T cells to achieve maximal activation by TCR engagement with Ag–MHC complexes. CD28 is a costimulatory receptor that plays a central role in stimulating naive T cells (1). Upon activation, T cells express a greater repertoire of costimulatory receptors including TNF receptor family 4–1BB, OX–40 (2, 3), the immunoglobulin family (CD28 and ICOS; reference 4), and TNF ligand family receptors (CD40 ligand [CD40L], FasL, and LIGHT; references 5–7). CD40L is a type II transmembrane protein of which interaction with CD40 on B cells triggers important signals for B cell differentiation, maturation, and apoptosis (5). The interaction of CD40L with CD40 expressed on APCs, such as macrophages and dendritic cells, promotes cell maturation and cytokine production (IL-12), which subsequently regulates the cell-mediated immunity by increasing Th1-type helper cell differentiation (8). Conversely, cross-linking of CD40L costimulates T cells, promoting secretion of many cytokines including IL-4, IL-10, interferon-γ, and TNF-α (9).

CD40L-deficient mice demonstrated that CD40L signaling is necessary for Ag-specific priming of CD4 T cells and negative selection of self-reactive T cells during thymic development (10, 11). The cytoplasmic tail of CD40L is 22 amino acids long and it lacks of known enzymatic activity (12). CD40L activates c-Jun NH2-terminal kinase (JNK)/p38 kinase, neutral sphingomyelinase, and protein kinase C (PKC) in human and murine T cells (13–15). Despite the compelling evidence of CD40L signaling in T cells, the proximal signaling mechanism of CD40L is not known.

We have previously characterized CD28i, which is one of several human CD28 splice variants (16–19). CD28i is unique among other isoforms as it retains an intact transmembrane region and cytoplasmic tail, but lacks the B7 ligand binding motif (16). CD28i associates with wild-type CD28 and cross-linking of CD28 transactivates CD28i. In this study, CD28i in CD40L+ T cells was investigated. Our data indicates that CD28i is a novel signaling adaptor protein that transduces and augments CD40L-mediated signaling.

Materials and Methods

Construction of Expression Vectors. CD28i was modulated with enhanced green fluorescent protein (EGFP; CLONTECH Laboratories, Inc.) or with hemophilus influenza hemagglutinin (HA)
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Detection of CD28, CD40L, CD28i, and Phosphotyrosine. The Abs specific for human CD28 (L293; BD Biosciences), human CD28 COOH-terminal (sc-1623; Santa Cruz Biotechnology, Inc.), human CD40L (sc-978, sc-9097; Santa Cruz Biotechnology, Inc. and CSA-186; StressGen Biotechnologies), and HA-tag (3F10; Roche and F-7; Santa Cruz Biotechnology, Inc.) were used. Cell extracts were prepared using lysis buffer containing 1% Nonidet P-40 (BDH Chemicals) or 1% digitonin (Sigma-Aldrich; reference 21). To immunoprecipitate CD28i-HA with anti-HA Ab (rat IgG1), protein A/G agarose and protein L agarose bead cocktail (sc-2336; Santa Cruz Biotechnology, Inc.) were used.

Assay of JNK, p21-activated Kinase (PAK), and Akt Activation in CD40L-stimulated D1.1 Transfectant Cells. Rabbit Abs specific for JNK, PAK, Akt, phospho-SAPK/JNK (Thr183/Tyr185), phospho-PAK1 (Ser144)/PAK2 (Ser141)/PAK3 (Ser139), and mouse Ab (587F11) specific for phospho-Akt (Ser473) were purchased from Cell Signaling Technology. D1.1 transfectant cells harboring inducible CD28i-HA were incubated with 0.5 μg/ml doxicyclin for 48 h. Cells were then serum starved for 6 h before being stimulated with 2 μg/ml anti-CD40L Ab. Whole cell lysates (5 × 10⁶ cell-derived 1% Nonidet P-40 extracts) were analyzed by Western blotting (16). Western blotting specific for each phosphor protein was performed first and then assay membrane was reproved with JNK, PAK, or Akt-specific Abs.

Laser Confocal Microscopy. CD28i-EGFP (16) was transfected into D1.1 cells by electroporation (300 V, 800 μF; Bio-Rad Laboratories). Cells were then incubated at 37°C overnight and stained with PE-conjugated Abs specific for CD40L, CD28, CD2, or CD3 (BD Biosciences) before confocal microscopy. To detect coendocytosis of CD40L and CD28i, CD28i-EGFP–transfected D1.1 cells were incubated with PE anti-CD40L Ab (TRAP1; BD Biosciences) for 30 min at 37°C.

Nuclear Factor (NF)-κB and IL-2 Promoter Reporter Assays. HEK293 cells were transfected with pcDNA3.1 harboring CD28i-HA and human CD40L using DEAE-dextran (16). The NF-κB reporter plasmid (κB-Luc; reference 22) or IL-2 promoter reporter plasmid (IL-2-luc; reference 23) were also transfected to HEK293 simultaneously or to D1.1 cells. After overnight culture at 37°C, cells were stimulated with anti-CD40L Ab for 6 h to perform NF-κB reporter luciferase assay (Promega). To assay IL-2 promoter activity, cells were stimulated with 10 ng/ml PMA plus 10 μg/ml PHA for 8 h in the presence of 2 μg/ml anti-CD40L Ab or 2 μg/ml anti-CD28 Ab. CD40L-specific goat Ab or CD28-specific mouse Ab was further cross-linked at the same time with 10 μg/ml anti–goat IgG Ab or anti–mouse IgG Ab, respectively, and then the prepared cell lysates were subjected to the luciferase assay. Data represent fold increase compared with nonstimulated cell response for NF-κB reporter assay or the PMA plus PHA response for IL-2 promoter reporter assay.

Figure 1. (a) The colocalization of CD28i with CD40L monitored by confocal microscopy. CD40L⁺Jurkat cell subline, D1.1 cell, was transfected with EGFP or CD28i-EGFP as indicated. In A, cells were surface stained with PE anti-CD40L Ab, PE anti-CD28 Ab, PE anti-CD2 Ab, or PE anti-CD3 as indicated below. In B, green fluorescence by EGFP or CD28i-EGFP expression was shown. In C, green fluorescence and red fluorescence were merged. (b) Anti-CD40L Ab induced coendocytosis of CD40L and CD28i. D1.1 cell was transfected with CD28i-GFP and incubated with PE anti-CD40L Ab at 37°C (top) or 4°C (bottom) for 30 min. Then cells were assayed by confocal microscopy. In panel a, CD40L and CD28i are merged. The magnified views of coendocytosis or control are shown in panel b. Panel c shows CD40L and panel d shows CD28i-EGFP staining.
Results and Discussion

**CD28i Colocalizes with CD40L in CD40L+ D1.1 Cells.** We investigated whether CD28i can associate with transmembrane receptors other than CD28. When examined by confocal microscopy, we found that CD28i-EGFP colocalizes with CD40L in the CD40L+ D1.1 Jurkat subline (Fig. 1a). In parallel, we also tested CD2, CD3, and CD43, but none of these receptors colocalized with CD28i in this cell line (only CD2 and CD3 are shown in Fig. 1a).

**CD40L Cross-linking Induces Coendocytosis of CD28i and CD40L.** To test a possibility of CD28i playing a role in CD40L signaling, we investigated whether the cross-linking of CD40L could stimulate endocytosis (24) that included both CD40L and CD28i. When D1.1 cells were transfected with CD28i-EGFP and stimulated with PE anti-CD40L Ab, we observed yellow cytoplasmic vesicles indicating green and red fluorescence cointernalization (Fig. 1b). The data provide evidence that CD40L and CD28i functionally/physically associate.

**CD40L Coimmunoprecipitates with CD28i.** To determine if CD40L and CD28i associate biochemically, we established a stable transfectant of D1.1 that expressed CD28i-HA. When characterizing the transfectant by performing CD40L-specific Ab or HA-specific Ab immunoprecipitation and Western blotting using anti-HA Ab or anti-CD40L Ab, we found that CD40L and CD28i-HA coimmunoprecipitated from 1% digitonin buffer cell extracts (Fig. 2a and b), in which weakly associated receptor complex can be retained (25). Similarly to what we observed in our previous study of the CD28 and CD28i complex (16), CD40L was not coimmunoprecipitated with CD28i from 1% Nonidet P-40 cell extracts, in which weakly associ-
ated receptor components dismembered. Thus, CD28i and CD40L form a weakly assembled complex in D1.1 cells.

To demonstrate the CD40L–CD28i complex in normal cells, we prepared PHA blasts from human peripheral blood mononuclear cells. Cells were lysed by 1% digitonin buffer and immunoprecipitated by CD40L or CD28 cytoplasmic tail-specific Abs. This experiment showed that the complex of CD40L and CD28i existed in normal T cell

Figure 3. (a) Tyrosine phosphorylation of CD28i induced by CD40L stimulation. CD28-HA–transfected D1.1 cells were serum starved for 6 h and incubated with anti-CD40L Ab for 30 min on ice. Anti–goat IgG was added to start stimulation at 37°C for 2, 5, and 10 min. Nonadhered P–P40 cell lysates of the stimulated cells were immunoprecipitated with anti-HA Ab. Immunoprecipitates were analyzed on Western blots. The top panel was blotted with anti-Phosphotyrosine (RC20; indicated as p-Tyr). The same membrane was reprobed with anti-HA Ab (indicated as CD28i-HA). L, Ig light chain; lane 1, nonstimulated control; lane 2, CD40L stimulated for 2 min; lane 3, CD40L stimulated for 5 min; lane 4, CD40L stimulated for 10 min. (b) Induction of CD28i-HA expression by doxycyclin in D1.1 transfectant cells. CD28i-HA (~23 kD) was expressed in D1.1 cells by doxycyclin-inducible promoter and whole cell lysates were assayed by HA-specific Western blotting. (−), doxycyclin-nontreated cells; (+), doxycyclin-treated cells (0.5 μg/ml for 48 h). (c) Overexpression of CD28i-HA enhances the activation of JNK and PAK2 in CD40L–stimulated D1.1 cells. Cells were stimulated with anti-CD40L Ab for periods of time indicated on top. Whole cell lysates were characterized with Western blotting specific for active-form JNK (represented by p46), PAK2 (~64 kD), and Akt (~60 kD), and indicated as pJNK, pPAK2, and pAkt on the left of each panel. To measure the level of proteins, the assay membranes were reprobed with JNK-, PAK-, or Akt-specific Abs and indicated as JNK, PAK2, or Akt on the left of each panel. Dox (−), cells not treated with doxycyclin; Dox (+), cells treated with doxycyclin.

Figure 4. (a) Enhanced induction of NF-κB activation by CD40L cross-linking in CD28i-HA–expressing D1.1 cells. CD28i-HA–induced (48 h) or –noninduced D1.1 cells were loaded with NF-κB reporter plasmid. Cells were stimulated with anti-CD40L Ab for 6 h and then the cell extracts were measured for NF-κB activity by luciferase assay. Results are representative of three experiments. Data are the average of triplicate cultures with SDs. (b) Enhanced induction of NF-κB activation by CD40L cross-linking in HEK293 CD28i-HA transfectant cells. HEK293 cells were transfected with CD28i and CD40L, and were loaded with NF-κB reporter plasmid. Cells were stimulated with anti-CD40L Ab for 6 h and then the cell extracts were measured for NF-κB activity by luciferase assay. Results are representative of two experiments. Data are the average of triplicate cultures with SDs.
mitogen blasts (Fig. 2, c and d). In addition, the assay revealed that wild-type CD28 (~44 kD) was not in association with CD40L (Fig. 2 c).

Cross-linking of CD40L Induces CD28i Tyrosine Phosphorylation. When D1.1 was incubated with anti-CD40L Ab, the cross-linking of CD40L resulted in the transient tyrosine phosphorylation of CD28i-HA (Fig. 2 c). The data suggests that CD40L transactivates CD28i in T cells by inducing tyrosine phosphorylation.

Expression of CD28i Enhances JNK and PAK2 Activation Induced by CD40L. CD40L signaling was shown to activate JNK/p38MAP kinase, sphingomyelinase, and PKC (13–15). PAK, p21-activated kinase, was also activated by CD28 signaling (21). Therefore, we investigated if the increased expression of CD28i alters the activities of these known kinases in CD40L-stimulated D1.1 cells. We used D1.1 transfectant in which CD28i-HA expression was inducible by doxicyclin (Fig. 3 b), but it did not alter CD40L expression levels (unpublished data). The expression of CD28i-HA in this transfectant cell in the absence of doxicyclin was at a nearly undetectable level. As shown in Fig. 3 c, the stimulation with CD40L-specific Ab significantly increased JNK phosphorylation in noninduced cells, which indicated JNK activation and confirmed the previously reported observation (13). When doxicyclin-treated D1.1 transfectant was tested, the expression of phosphorylated JNK increased in a greater extent than that observed in doxicyclin-nontreated cells. Thus, JNK activation induced by CD40L stimulation was enhanced by the increased expression of CD28i-HA. We also found that CD40L stimulation activated PAK2 (~64 kD) in both CD28i-HA–induced and –noninduced cells in 2–5 min. Nevertheless, PAK2 activation occurred in greater extent in doxicyclin-treated cells. Interestingly, neither PAK1 nor PAK3 was significantly activated by the stimulation of CD40L (unpublished data). One mechanism by which CD28i and CD40L interactions could enhance CD40L signaling might be enhancing the focal adhesion-like structure within T cells at T cell to B7+ APC binding sites via clustering a greater number of CD28-derived cytoplasmic tails as it was reported for CD28 (26). The enhanced activation of the PAK2-JNK pathway (Fig. 3 c) seems to support this possibility because it overlaps with Rac1, Cdc42-induced F-actin formation/cytoskeleton reorganization pathway (21, 26). The presence of CD28i may enhance the molecular mechanisms that contribute in establishing the immunological synapse (27) essential for the T cell costimulation. Finally, our study showed a weak induction of Akt activation in noninduced cells (Fig. 3 c). The activation of Akt was not detected in CD28i-HA–induced D1.1 cells by CD40L stimulation. The biological significance of this observation is not clear.

Expression of CD28i Increases the Activation of CD40L-induced NF-κB Stimulation. Previous studies indicated that CD40L stimulation activates PKC, which potentially activates NF-κB (15). Therefore, we extended our study by assaying NF-κB activity in CD40L-stimulated D1.1 transfec-tant cells expressing CD28i-HA. The induction of NF-κB reporter activity was significantly enhanced in CD28i-HA–

![Figure 5](image-url)

**Figure 5.** Poor induction of IL-2 promoter activity by cross-linking CD40L in D1.1 transfectant cells. CD28i-HA was induced (48 h) in D1.1 transfectant cells with doxicyclin treatment. Cells were loaded with an IL-2 promoter reporter plasmid for 6 h. Cells were then stimulated with PHA plus PMA in the presence of anti-CD40L Ab or anti-CD28 Ab for 6 h. Cell extracts were assayed for IL-2 promoter activity by luciferase assays. Results are representative of three experiments. Data are the average of triplicate cultures with SDs.
induced cells in comparison to that observed in noninduced cells (Fig. 4 a). Thus, it is indicated that CD40L activates NF-κB and the presence of CD28i enhances this signaling. To understand the interaction of CD40L and CD28i under a more critical setting in which wild-type CD28 is absent, we have investigated HEK293 (CD28−) cells transfected with CD40L and CD28i using NF-κB reporter assay (Fig. 4 b). The dual transfection of CD40L and CD28i into HEK293 cells resulted in >30% CD40L−Cd28i+ cells in which CD40L and CD28i-GFP colocalized (unpublished data). The study demonstrated that anti-CD40L Ab-induced NF-κB activation is CD28 independent and up-regulated by the presence of CD28i. Additional signaling mechanism that may result from trans-stimulating CD28i cytoplasmic tail by CD40L could be YMNMT motif-mediated survival signaling via PI3 kinase/NF-κB pathway (28). To verify this possibility, however, we may need additional study relevant to the PKC pathway as we found that Akt is not activated by CD40L stimulation in CD28i-HA-overexpressing D1.1 cells.

Study of IL-2 Promoter Activity in D1.1 Transfectant Cells Stimulated by CD40L. Finally, we investigated whether the stimulation of CD40L-augmented IL-2 promoter activity in CD28i-HA-induced D1.1 cells. The experiments showed only a modest augmentation of IL-2 promoter activity by the increased expression of CD28i-HA (Fig. 5). The stimulation of CD28 in the same cell efficiently induced IL-2 promoter activity, confirming our previous observation (16). A recent study showing that CD40L is not efficient in promoting IL-2 production in human PBL-T cells (29) supports our present findings. Accordingly, the association of CD28i does not simply combine the CD28 cytoplasmic tail signaling to CD40L, but selectively imparts CD28i-specific signaling activities, such as PKA2 activation, to CD40L stimulation. The difference in the effects CD28i plays on CD40L may result from a loose association between CD28i and CD40L, where the signal is not strong enough to activate IL-2 promoter. Alternatively, CD40L may transduce a signal via its own cytoplasmic tail or additional signaling adaptor molecules, and the cumulative signaling does not support IL-2 promoter activation.

In summary, this study identified CD40L as a new binding partner for CD28i. By increasing T cell adhesion with APCs and/or by increasing NF-κB signaling, CD28i co-clustering with CD28 and CD40L can function as a regulator of the T cell activation threshold.

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