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Pre-assembly of the extracellular domains of CD40 is not necessary for rescue of mouse B cells from anti-immunoglobulin M-induced apoptosis

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SUMMARY

CD40 is a tumour necrosis factor receptor (TNFR) family member of central importance for the adaptive immune system. To elucidate the functional role of the different extracellular domains of CD40, we have created a set of truncated CD40 molecules where domains, or parts of domains, have been removed. These CD40 proteins, which contain a peptide tag in the N-terminal end, have been expressed in a murine B-cell line, WEHI 231. It was found that ligation of these engineered CD40 proteins via the peptide tag, was sufficient to rescue as well as to promote proliferation of apoptotic WEHI 231 cells, even when all the extracellular domains of CD40 were absent. Our results suggest that pre association of CD40 in the cell membrane plays no critical role for the CD40 signalling pathway. Furthermore, our data imply that conformational changes initiated in the extracellular domains of CD40 are not essential for signal transduction.

INTRODUCTION

CD40 is a receptor that belongs to the tumour necrosis factor receptor superfamily (TNFR-SF) and is of central importance to the adaptive immune response.[1] It is expressed on a variety of cells in the immune system, such as B cells, dendritic cells and monocytes, but also on epithelial cells, endothelial cells and fibroblasts.[1] As all the members of the TNFR-SF, the extracellular domains of CD40 consist of cysteine-rich domains, where each domain consists of two modules.[2] Signal transduction via CD40 involves several signalling pathways, including activation of protein tyrosine kinases, phosphoinositide-3 kinase, serine/threonine kinases and nuclear factor-κB. Although the functional consequence of CD40 signalling depends on cell type and differentiation stage[1] it also seems to depend on the mode of activation, where the minimal requirement is the formation of a receptor dimer.[3] Haswell *et al*.[4] suggest that the strength of the receptor-mediated signal depends on the valency of the ligand. In addition, ligation via trimeric CD40L expressed on a cell membrane seems to be required in order to activate some signalling pathways.[5,6]

Members of the TNFR-SF have been shown to preassemble in the membrane via their preligand-binding assembly domain (PLAD),[7–9] which seems to be physically distinct from the ligand-binding domains. It has, however, been shown that PLAD is necessary for binding of the corresponding ligand.[7,8] It has been suggested that initiation of TNFR-SF signalling is not merely the result of receptor di- or trimerization, but rather of receptor rearrangement or supercluster formation.[10] Recently, it has also been shown that CD40 is recruited to membrane rafts after ligation[11–14] and that CD40 depends on the microenvironment in the raft for optimal signalling.[15]

It has previously been shown that it is possible to exchange the extracellular and transmembrane domain of CD40 with the extracellular and transmembrane domain of CD4[16] or CD8[17] and still retain the CD40 intracellular signalling capacity of these fusion proteins. However, CD4/CD8 form dimers in the membrane *in vivo*[18,19] and may substitute the role of the extracellular part of CD40. Here, we have analysed the functional role of the different extracellular domains of CD40, using a set of transfected murine B-cell lines that express different, extracellularly truncated CD40 constructs that have distally been fused to a peptide tag. The ability to rescue B cells that express these CD40 variants from immunoglobulin M (IgM)-induced apoptosis and growth inhibition has been investigated and our results demonstrate no direct link between the extracellular domain structures of CD40 and their functional activities.

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Abbreviations: TNF, tumour necrosis factor; TNFR, TNF receptor; PLAD, preligand assembly domain.

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MATERIALS AND METHODS

Reagents and cell cultures

Dulbecco's modified Eagle's minimal essential medium (DMEM) and supplements were purchased from Life technologies (Life Technologies, Paisley, UK) and fetal calf serum (FCS) was obtained from HyClone Laboratories, Inc., UT. Dr Mats Ohlin kindly provided the human anti-AD2 antibody ITC88.[20] The anti-CD40 antibodies, A2-54[21] and F33[22] were obtained as a single chain and A2-54 was converted to an IgG format. Fluoroscein isothiocyanate (FITC)-labelled rabbit anti-human IgG $(F(ab)_2$, was purchased from DAKO (DAKO A/S, Glostrup, Denmark). Goat anti-mouse IgM and Goat anti-human IgG were acquired from Jackson (Jackson ImmunoResearch Laboratories, Inc., PA). The M2 anti-FLAG antibody was obtained from Sigma (Sigma Chemical Co., St. Louis, MO).

Phoenix and 3T3 cells were cultured in D10 medium (DMEM supplemented with 10% FCS, 2 mM L-glutamine and 1% non-essential amino acids). WEHI cells were grown in D10 supplemented with 1 mM mercaptoethanol. Puromycin (1 μg/ml) was added to the medium in order to obtain a pure population of retrovirally transfected cells. Phoenix cells (packaging cell) and 3T3 cells were a generous gift from Stefan Carlsson (Lund, Sweden), whereas the WEHI cell line was obtained from the American Type Culture Collection (Rockville, MD).

Construction of chimeric CD40 receptors

Truncated CD40 genes, where extracellular modules have been removed one by one, were cloned into a retroviral vector, pBabe.[23] These CD40 constructs, which have a short sequence encoding the AD2 peptide[24] fused to the N-terminal, were initially made in pcDNA3.1.[22] The fusion genes were transferred to pBabe by cutting the original vector with *Nhe*I and *Xba*I *and* subsequently treating the gene fragment with DNA polymerase I to generate blunt ends. Thereafter it was ligated into the *Sna*BI site of pBabe. AE11, a scFv version of ITC88[25] was amplified by polymerase chain reaction (PCR), using primer A and B (Table 1) and cloned into the *Sna*BI/*Eco*RI sites of the pBabe vector. The transmembrane and cytosolic part of CD40 was then amplified, using primer G and H (Table 1) and cloned into the *Eco*RI/*Sal*I site of the resulting vector (Fig. 1). An additional construct (AE11-Z, Fig. 1) was made, in which a leucine-rich zipper (GCNpII)[26] fused to a hinge (from human IgG3[27]) was inserted into the *Eco*RI site of the original AE11 construct. The zipper/hinge was constructed by overlap extension PCR, using primer D and E (Table 1) followed by a reamplification step using primer C and F (Table 1).

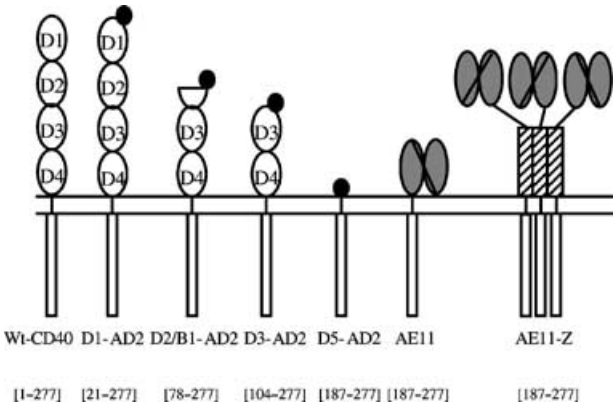

Figure 1. Structure of the different CD40 constructs. All the constructs contain the transmembrane and cytosolic part of CD40. The numbering of the different extracellular domains of CD40 is indicated in the figure. The filled circle indicates the recognition epitope (AD2) that was used for detection and stimulation. AE11 is a single-chain antibody fragment that recognizes the AD2 epitope. The hatched squares indicate the GCN4-pII (zipper) homotrimerization domain of the AE11-Z construct. A short hinge peptide was inserted between the scFv and the zipper. The amino acid residues from human CD40 that are used in the different constructs are given in brackets.

Generation of stable cell-lines

Stable cell lines were generated mainly as described by Krebs *et al.*[28] Briefly, 1 μg pBabe DNA was transiently transfected into a Phoenix packaging cell line (2×10^6 cells, on day 1), using Lipofectamine according to the manufacturer's protocol (Life Technologies). The medium was changed on day 3 and 10 hr later the virus-containing supernatants were collected and passed through a 0·22-μm filter. Polybrene (2·5 μl, 10 mg/ml) was added to the supernatant before it was added to the WEHI cells or the 3T3 cells. The cells were washed 24 hr post-infection and then, in order to select for stable transfectants, cultured for 2 weeks in medium containing 1 μg/ml puromycin (Sigma-Aldrich Sweden AB, Stockholm, Sweden).

Table 1. Primers used for the amplification of the CD40 variants

Primer		
A	AE11 3′ (*Eco*RI)	GCGGAATTCTTTGATCTCCACCTTGGT
B	AE11 5′ (*Sna*BI)	GAATTCTGATACGTAATGGTTCGTCTGCCT
C	3′-GCN-pII-zipper-reamp	GCGGAATTCGCGCTCGCCGATCAGCTT
D	5′-GCN-pII-zipper	CGCATGAAGCAGATCGAGGACAAGATCGAGGAGATCCTGTCCAAGATCTACCACATCGAG
E	3′-GCN-pII-zipper	GCGCTCGCCGATCAGCTTCTTGATGCGGGCGATCTCGTTCTCGATGTGGTAGATCTTGGA
F	5′ hinge + GCN-pII-zipper	GCGGAATTCACCCCCTTGGGCGACACCACCCACACCTCCGGCCGCATGAAGCAGATCGAG
G	3′CD40 (*Sal*I)	GCACGCGTCGACTCACTGTCTCTCCTGCACTGAGA
H	5′CD40 t/c (*Eco*RI)	CCGCCGGAATTCGGTCCCCAGGATCGGCTGA

For detection of surface expression, AD2-containing CD40 variants were incubated with anti-AD2 antibody (IT88), the wt-CD40 expressing cells were incubated with an anti-CD40 antibody (A2-54) and the AE11 expressing cells were incubated with biotinylated AD2. The cells were washed once and then incubated with FITC-labelled rabbit anti-human IgG ($F(ab)_2$; DAKO) 1 : 250 or streptavidin–phycoerythrin (PE)/Cy5 (DAKO) 1 : 250, respectively. After washing, the cells were analysed using FACScan (Beckton Dickinson, San Diego, CA).

Proliferation assay

Apoptosis was induced by addition of anti-IgM antibodies (2 μg/ml) to the WEHI 231 culture medium. The cells were rescued by addition of antibodies or scFv fragments against the different CD40 variants. The scFv was cross-linked with M2 anti-FLAG antibody. Anti-human IgG was used to further cross-link the antibodies. Alternatively, the WEHI 231 cells expressing the different CD40 variants were rescued by coculturing them with radiated 3T3-cells expressing AE11. The ability of the WEHI 231 cells to proliferate after rescue from apoptosis was measured after 3 days using a 16-hr [methyl-^{3}H]thymidine pulse (0·5 μCi/well) (Pharmacia Biotech, Uppsala, Sweden) in a 96-well plate.

Detection of apoptotic cells

Transfected WEHI 231 cells were cultured with or without anti-IgM antibodies (2 μg/ml). WEHI cells displaying different CD40 variants were rescued by addition of a anti-CD40 scFv (F33) together with a cross-linking antibody (M2 anti-FLAG). Alternatively, the transfected cells were rescued, using irradiated 3T3 cells that displayed AE11. In this case, 3T3 cells that displayed an irrelevant peptide were used as negative control. The cells were stained with annexin–FITC after 16 hr, according to the protocol provided by the manufacturer (R&D Systems, Inc., Minneapolis, MN) and analysed by flow cytometry.

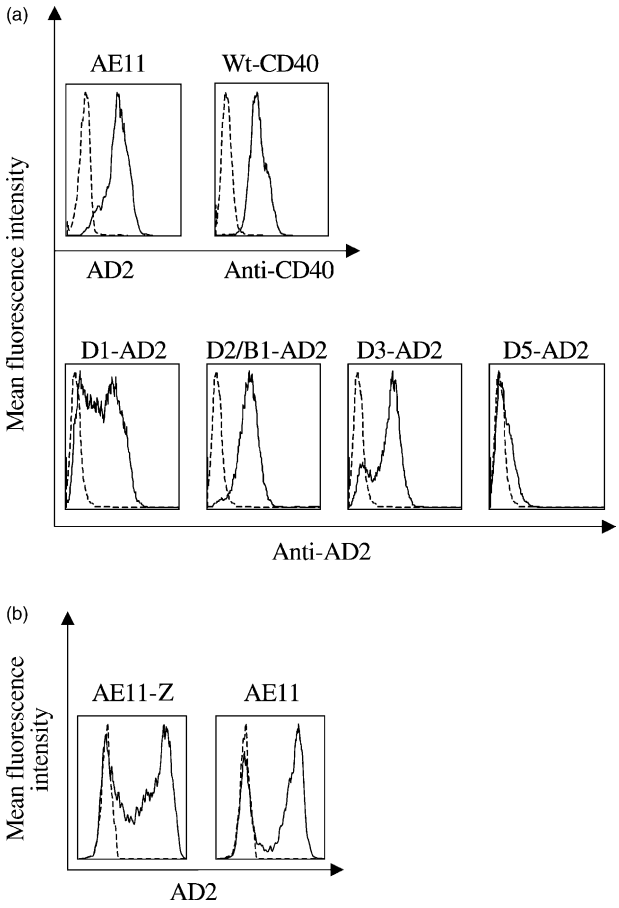

Figure 2. Expression profiles of the different transfected constructs. Surface expression of the stable transfectants (a) WEHI 231 variants and (b) 3T3 variants were detected, using biotinylated AD2 for the AE11 variants, ITC88 (anti-AD2 monoclonal antibody) for the AD2 containing variants and A2-54 (anti-CD40 antibody) for wt CD40. The dashed line indicates untranfected cells, used as negative control.

RESULTS AND DISCUSSION

Generation of stable mouse cell transfectants

A set of CD40 variants was constructed to investigate the role of the extracellular parts of CD40 for stimulation of naive B cells (Fig. 1). Different parts of the extracellular domains of CD40 were deleted and a peptide tag (AD2) was fused to the N-terminus. These constructs contained variants were either whole domains (D3-AD2 and D5-AD2) or one domain and one module (D2/B1-AD2) had been deleted. Furthermore, a scFv variant[25] of the anti-AD2 antibody ITC88[20] was fused 5′- to a hinge sequence and a leucine-rich zipper (GCN4pII[26]) at the transmembrane/cytosolic part of CD40 in order to anchor it in the membrane (AE11-Z, Fig. 1). The gene product of the modified GCN4 sequence is known to specifically homotrimerize,[26] and the construct thereby mimics the natural CD40L, which is expressed as a trimer on the surface of activated T cells *in vivo*.[1] The CD40 variants (wt-CD40, D1-AD2, D2/B1-AD2, D3-AD2 and D5-AD2) were transfected into WEHI 231 cells and the AE11 variants were transfected into 3T3 cells, using a retroviral vector system. Surface expression of the different CD40 variants was confirmed by FACScan analysis, even though the absolute levels differ somewhat (Fig. 2).

Although human CD40 differs somewhat from the murine CD40 sequence, it has been shown to be functional also in murine cells.[29] In fact, it has been shown that cross-linking of CD40 variants, in which all of the intracellular part of CD40 except the residues that are fully conserved between mice and man has been removed, is sufficient to support full proliferation of anti-IgM-treated WEHI cells.[16]

Signalling via CD40 can be achieved without any involvement of the extracellular domains

The ability of the different membrane expressed CD40 variants to rescue WEHI 231 cells from apoptosis and growth arrest, and thereby promote proliferation, after treatment with anti-IgM antibodies was compared, using a thymidine incorporation assay. Both wild type CD40 (wtCD40) and the full length

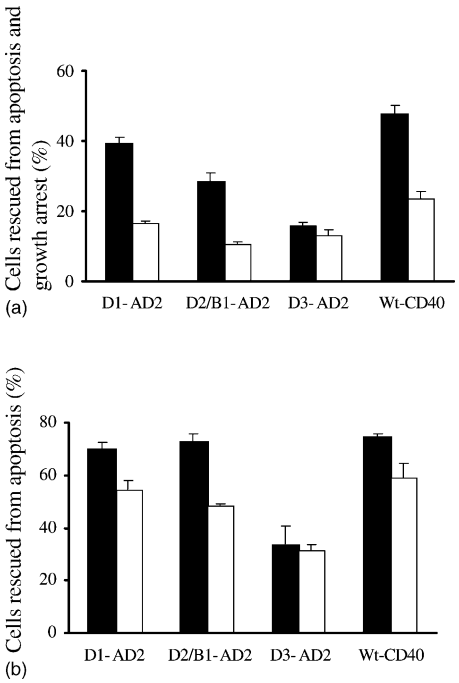

Figure 3. Stimulation of truncated CD40 constructs using an anti-CD40 antibody. An anti-CD40 antibody (F33) that recognize the D2/B1-AD2 CD40 variant was used to stimulate the different stable transfected WEHI-cells that had been pretreated with anti-IgM antibodies. Closed bars indicate cells treated with anti-CD40 antibody, whereas open bars indicate cells treated with medium (control). (a) The ratio of proliferating cells compared to untreated cells was determined after 3 days, using a thymidine incorporation assay. Closed bars indicate cells cultured with F33 and open bars cells cultured with medium. (b) The ratio of viable cells of treated, compared to untreated cells were determined after 16 hr, using an annexin-FITC staining and FACScan analysis. Data represent mean of three experiments and error bars represent SD of three experiments.

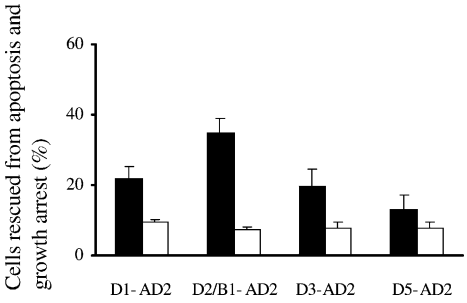

Figure 4. Proliferation of apoptotic WEHI cells containing truncated CD40 constructs. WEHI 231 cells expressing different truncated CD40 constructs were treated with or without anti-IgM. They were subsequently cultured with (closed bars) or without (open bars) anti-AD2 antibody (ITC88) together with anti-IgG. The ratio of proliferating cells compared to untreated cells was determined after 3 days using a thymidine incorporation assay. Closed bars indicate cells treated with ITC88 and open bars indicate cells treated with medium (control). Data represent mean of three experiments and error bars represent SD of three experiments.

CD40 construct that contain a peptide tag (D1-AD2) were able to rescue from apoptosis and growth arrest upon stimulation with an anti-CD40 antibody (Fig. 3a), which indicates that D1-AD2 is functionally equivalent to wtCD40. Although the absolute rescue level differs somewhat between the different constructs, the rescue level in relation to background proliferation is similar. Furthermore, the D2/B1-AD2 variant was also able to support proliferation when stimulated with F33 (Fig. 3a), which indicates that CD40 signalling is independent of domain 1 and the first module of domain 2. F33 does not bind to the D3-AD2 variant[22] and consequently this construct could not support proliferation when stimulated with this antibody.

Anti-IgM antibodies induce both apoptosis and cell growth arrest, which are two separate events, and it has been shown that it is possible to rescue cells from apoptosis without simultaneously promoting proliferation.[30,31] Consequently, we analysed whether the requirements to rescue the transfected WEHI 231 cells from apoptosis were less stringent than the requirements to induce proliferation. The level of rescued cells after anti-IgM treatment was measured, using annexin staining. The results from this assay (Fig. 3b) indicate that the requirements for rescue from apoptosis and from growth arrest is similar in WEHI 231 cells for these constructs, although the absolute level of rescued cells differed somewhat.

To investigate if the D3-AD2 and D5-AD2 variants were able to support proliferation, they were ligated with an anti-AD2 antibody (ITC88) after induction of apoptosis, using anti-IgM antibodies. When comparing the ability of the different membrane expressed CD40–AD2 variants to mediate rescue from apoptosis and to induce proliferation (Fig. 4), it was evident that all the CD40 variants were able to support rescue and proliferation. Thus, the extracellular part of CD40 is not necessary for the signal transmission through CD40 to support WEHI cell proliferation, which points to a complete redundancy of the extracellular domains of the CD40 molecule. D5-AD2, however, seems to induce a lower level of proliferation, compared to the other CD40 variants, but this may be due to the lower expression level or inaccessibility to the detecting antibody, which is also indicated by the lower signal in the FACS analysis (Fig. 2).

In TNFR1 and TNFR2 the PLAD is located in the distal domain of the extracellular part of the molecule.[7] This domain has been shown to mediate assembly of the receptor in the membrane prior to ligand binding and seems to be necessary for ligand binding. By analogy[32] the CD40 PLAD might be located in the N-terminal domain (D1) and we have previously shown that this domain is necessary for binding of the CD40 ligand (Malmborg Hager *et al*. manuscript in preparation). However, our results suggest that there is no requirement for PLAD-mediated pre-association of CD40 for functional signalling, as D2/B1-AD2 and D3-AD2 are as potent as the D1/AD2 construct at inducing proliferation (Figs 4 and 5). It is thus likely that formation of supercluster[10] via the CD40 PLAD after CD40 ligation is not critical for signalling. It may be suggested that the function of the PLAD is merely to allow CD40L binding and increase the avidity of the interaction.

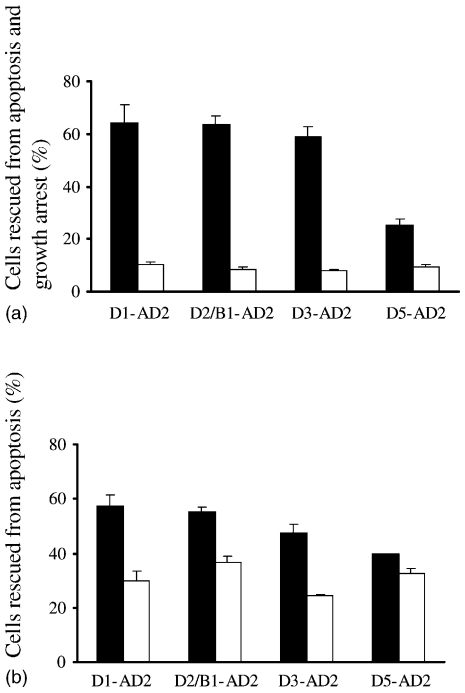

Figure 5. Rescue of transfected WEHI 231 cells containing truncated CD40 constructs with 3T3 cells transfected with AE11 from apoptosis and growth inhibition. WEHI 231 cells expressing different truncated CD40 constructs were treated with or without anti-IgM. They were subsequently cultured with 3T3 cells that express the AE11 construct (closed bars) or with 3T3 cells used as negative control (open bars). (a) The ratio of proliferating cells compared to untreated cells was determined after 3 days, using a thymidine incorporation assay. (b) The ratio of viable cells of treated, compared to untreated cells, were determined after 16 hr, using annexin-FITC staining. Data represent mean of three experiments and error bars represent SD of three experiments.

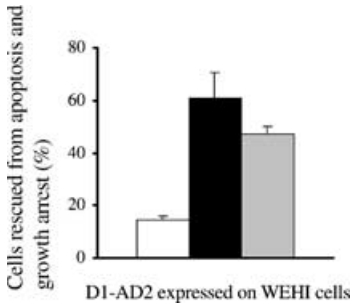

Figure 6. D1-AD2 expressing WEHI cells proliferate equally well when stimulated with the AE11 constructs (Fig. 1) as with the AE11-Z constructs, which contain a trimerization domain (Fig. 1). Open bars indicate cells treated with 3T3 cells (negative control), closed bars indicate cells treated with 3T3 cells that express the AE11 construct and grey bars indicate cells treated with 3T3 cells that express the AE11-Z construct. Data represent mean of three experiments and error bars represent SD of three experiments.

Single chain antibodies expressed on fibroblasts can promote rescue and proliferation

In order to investigate the effects of further receptor cross-linking a membrane-associated, more natural, model system was used (AE11-Z and AE11, Fig. 1). The ability of these membrane-associated scFv fragments to stimulate WEHI-cells that express the D1-AD2 variant was analysed. Interestingly, the AE11-Z variant, which contains a homotrimerization zipper did not promote proliferation more effectively than the monomeric AE11 variant, which lacks the zipper (Fig. 6), despite the fact that CD40 require cross-linking for activation. The mechanism behind this effect remains to be elucidated. However, it may be suggested that in the 3T3 cells the CD40 constructs become self-activated and thereby form lipid raft-associated complexes, which can induce oligomerization of the corresponding receptor on WEHI 231 cells. In fact, it has recently been shown that active formation of CD40L cluster is required for subsequent formation of CD40 cluster,[33] which is central for initiation of CD40 signalling.[16]

The ability of the AE11 variant (displayed on 3T3 cells) to stimulate proliferation of the different truncated CD40 variants (displayed on WEHI cells) was also analysed (Fig. 5a), and the result from this assay correlates well with the results obtained after stimulation with the anti-AD2 antibody. The transfected WEHI 231 cells did, however, proliferate more extensively after stimulation with the membrane-associated ligand, which also agrees with previous studies[5,6] showing that a membrane-associated ligand was necessary to activate some particular signalling pathways. Moreover, the D5-AD2 construct displayed a lower potential to promote proliferation, in agreement with the previous results, where a soluble anti-AD2 antibody was used for stimulation. This is most likely explained by the differences in expression levels (Fig. 2).

We further confirmed, using a annexin–FITC staining assay, that the none of the extracellular domains of CD40 is necessary for CD40 signalling. The ratio of viable cells was determined for cells that had been cultured with or without anti-IgM. As can be seen in Fig. 5(b), all the truncated CD40 variants are able to rescue WEHI 231 cells from apoptosis. This further reinforces the observation that the extracellular domains of CD40 do not play any critical role for CD40 signalling. As in the thymidine assay, the D5-AD2 variant mediates a somewhat lower level of signalling compared to the other truncated CD40 variants. Hence, the requirement for rescue from apoptosis and from growth arrest is similar.

It has been suggested that the CD40L and agonistic antibodies induce conformational changes of CD40, which is involved in signal transmission[34–36] and CD40s increased affinity for lipid rafts and TNFR-associated factors.[15] A conformational change induced by the CD40L is likely to depend on some key residues in the CD40L binding epitope. Since all our CD40 constructs have the capacity to induce proliferation, it seems unlikely that conformational changes induced by ligand binding are essential for CD40 signalling. However, the results from the apoptosis assay may indicate that conformational changes can have some, although not critical, effect on rescue from growth inhibition.

In summary, we have demonstrated a molecular redundancy of the CD40 receptor showing that neither PLAD-mediated preassembly nor conformational changes mediated through the extracellular part of CD40 are essential for CD40 signalling.

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