

## A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells

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**ABSTRACT** CD40 is a B-cell surface molecule that has been shown to induce B-cell growth upon ligation with monoclonal antibodies. This report shows that triggering via CD40 is essential for the activation of resting B cells by helper T cells ( $T_h$ ). A soluble fusion protein of CD40 and human immunoglobulin, CD40-Ig, inhibited the induction of B-cell cycle entry, proliferation, and differentiation by activated  $T_{h1}$  and  $T_{h2}$ . The ligand for CD40 was identified as a 39-kDa membrane protein that was selectively expressed on activated  $T_h$ . A monoclonal antibody specific for the 39-kDa protein inhibited CD40-Ig binding and also inhibited the activation of B cells by  $T_h$ . These data indicate that the 39-kDa membrane protein expressed on activated  $T_h$  is a binding protein for CD40 and functions to transduce the signal for  $T_h$ -dependent B-cell activation.

Studies by Mitchison, Benacerraf, and Raff first suggested that physical interactions between helper T cells ( $T_h$ ) and B cells were essential in the development of humoral immune responses. Later studies documented that  $T_h$  formed physical conjugates with class II major histocompatibility complex-compatible, antigen-presenting B cells (1) and that it was the B cells within these conjugates that responded to  $T_h$  (2). With the discovery that  $T_h$ -derived lymphokines exerted potent growth and differentiative effects on B cells, it was proposed that soluble factor(s) released in proximity by activated  $T_h$  mediated the activation of the interacting B cells. However, none of the molecularly cloned lymphokines, alone or in combination, manifested the ability to induce B-cell cycle entry. Unlike soluble factors, plasma membrane fractions from activated  $T_h$  induced B-cell cycle entry (3–5). Studies using purified plasma membranes from activated  $T_h$  ( $PM^{Act}$ ) suggested that a protein expressed on the membrane of activated  $T_h$  cells was responsible for initiating humoral immunity (5, 6).

$PM^{Act}$  have been used to investigate the nature of this effector function (4, 5).  $PM^{Act}$  from activated  $T_h$ , but not purified plasma membranes from resting  $T_h$  ( $PM^{Rest}$ ), expressed an activity that induced B-cell cycle entry in an antigen-nonspecific, class II-unrestricted manner. Because of the lack of antigen specificity and class II restrictions, it was proposed that nonpolymorphic membrane protein(s) on activated  $T_h$  mediated the activation of interacting B cells. In addition, the activity expressed by  $PM^{Act}$  required 4–6 hr of activation and *de novo* RNA synthesis and was protein in nature (6). Here we show that activated  $T_h$  express a 39-kDa protein that binds CD40. Blocking the binding of this ligand to CD40 inhibited  $T_h$ -dependent B-cell activation. These data suggest that the binding of the 39-kDa protein on activated  $T_h$  to CD40 on B cells initiates thymus-dependent humoral immune responses.

## MATERIALS AND METHODS

**Mice.** Female DBA/2J mice (The Jackson Laboratory) were used for the preparation of filler cells to support the growth of  $T_h$  clones and in the preparation of resting B cells.

**$T_h$  Clones.** D1.6, an I-A<sup>d</sup>-restricted, rabbit Ig-specific  $T_{h1}$  clone, and CDC35, an I-A<sup>d</sup>-restricted, rabbit Ig-specific  $T_{h2}$  clone, were obtained from David Parker (University of Massachusetts, Worcester). In this paper, D1.6 will be referred to as  $T_{h1}$  and CDC35 as  $T_{h2}$  (7).

**Activation of  $T_h$  by Anti-CD3.**  $T_{h1}$  or  $T_{h2}$  were cultured ( $8 \times 10^6$  per well) in cluster wells (six-well, Corning) coated with anti-CD3 (40  $\mu$ g/4 ml of phosphate-buffered saline per well) for 16 hr, as described (5).

**Preparation of  $T_h$  Plasma Membranes.** Plasma membranes were prepared by discontinuous sucrose gradient sedimentation (5).

**Preparation of Resting B Cells.** Resting splenic B cells were prepared by sedimentation on discontinuous Percoll gradients (8). Cells isolated from the 70–75% (density, 1.087–1.097 g/ml) Percoll interface were typically >95% membrane Ig-positive, had a uniform, low degree of near forward light scatter, and were unresponsive to Con A.

**Monoclonal Antibodies (mAbs).** The following mAbs were purified by ion-exchange HPLC from ascites grown in mice that had been irradiated and bone marrow-reconstituted: anti-CD3, 145-2C11 (9); anti- $\alpha\beta$  T-cell antigen receptor (TCR), H57-597 (10); anti-CD4, GK1.5 (11); anti-ICAM-1, YN1/1.7.4 (12); anti-LFA-1, FD441.8 (13); and anti-rat/hamster Ig  $\kappa$  chain, RG-7 (14).

**Preparation of the CD40 Recombinant Globulin (CD40-Ig).** A plasmid containing a cDNA encoding the CD40 antigen (15) was digested with the restriction enzymes *Pst* I and *Sau*3A1. The *Pst* I–*Sau*3A1 fragment was subcloned into the same plasmid digested with *Pst* I and *Bam*HI. This allowed the preparation of a DNA fragment encoding a CD40 protein that was truncated upstream from the transmembrane domain. The fragment encoding the truncated CD40 was then subcloned into the Ig fusion plasmid (16) by using an *Mlu* I and *Bam*HI digest. The CD40-Ig fusion protein was produced by transient transfection in COS cells and purified on a protein A column (16).

**Lymphokines.** Recombinant mouse interleukin 4 (IL-4) was generously provided by C. Maliszewski and K. Grabstein (Immunex, Seattle). Recombinant mouse IL-5 was purchased from R&D Research (Sorrento, CA).

**Induction of B-Cell RNA Synthesis by  $PM^{Act}$ .** Resting B cells ( $3 \times 10^4$ ) were cultured in 50  $\mu$ l of complete RPMI medium (RPMI 1640 plus 10% fetal bovine serum and 50  $\mu$ M 2-mercaptoethanol) in A/2 microtiter wells (Costar). To these

wells, 0.5  $\mu\text{g}$  of  $T_{\text{h}1}$  or  $T_{\text{h}2}$  membrane protein was added. Forty-two hours later 2.5  $\mu\text{Ci}$  (92.5 kBq) of [ $^3\text{H}$ ]uridine (New England Nuclear) was added to each well. After 6 hr the cells were harvested, and the radioactivity was determined by liquid scintillation spectrometry. Results were expressed as cpm per culture (mean  $\pm$  SD).

**Induction of B-Cell Ig Secretion by  $\text{PM}^{\text{Act}}$  and Lymphokines.** Resting B cells were cultured as described above. To each culture well, 0.5  $\mu\text{g}$  of  $T_{\text{h}1}$  membrane protein, IL-4 (10 ng/ml), and IL-5 (5 ng/ml) were added. On day 3 of culture, an additional 50  $\mu\text{l}$  of complete RPMI was added. On day 6 of culture, supernatants from individual wells were harvested and quantitated for IgM and IgG1 (5).

**Induction of B-Cell Proliferation by Activated  $T_{\text{h}}$  and IL-4.** Resting B cells ( $4 \times 10^4$ ) were cultured in 50  $\mu\text{l}$  of complete RPMI in A/2 microtiter wells (Costar). To each well, IL-4 (10 ng/ml) and  $10^4$  resting or activated irradiated (500 rads; 1 rad = 0.1 Gy)  $T_{\text{h}1}$  were added. On day 3 of culture, cells were incubated with 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine, and incorporation was determined as described (5).

**Production of mAbs Specific for Membrane Proteins Induced on Activated  $T_{\text{h}1}$ .** Hamsters were immunized intraperitoneally with  $5\text{--}10 \times 10^6$  activated  $T_{\text{h}1}$  (D1.6) at weekly intervals for 6 weeks. When the serum titer against murine  $T_{\text{h}1}$  was  $>1:10,000$ , the hamster splenocytes and NS1 mouse myeloma cells were fused in the presence of polyethylene glycol. Supernatants from wells containing growing hybridomas were screened by flow cytometry for reactivity with resting and activated  $T_{\text{h}1}$ . One particular hybridoma, which produced a mAb that selectively recognized activated  $T_{\text{h}}$ , was further tested and subcloned to derive MR1. The MR1 mAb was produced in ascites and purified by ion-exchange HPLC.

**Flow Cytofluorometric Analysis of Activation Molecules Expressed on  $T_{\text{h}}$ .** Resting and activated  $T_{\text{h}}$  (16 hr with anti-CD3) were harvested and incubated at  $10^5$  cells per 50  $\mu\text{l}$  with fusion protein for 20 min at  $4^\circ\text{C}$ , followed by fluorescein-conjugated goat anti-human IgG (25  $\mu\text{g}/\text{ml}$ ; Southern Biotechnology Associates, Birmingham, AL). Propidium iodide was added (2  $\mu\text{g}/\text{ml}$ ) to all samples. Flow cytofluorometric analysis was performed on a Becton Dickinson FACScan. After positive gating of cells by forward vs. side scatter, and by red negativity (for propidium iodide exclusion), the green fluorescence (logarithmic scale) of viable cells was ascertained. At least 5000 viable cells were analyzed for the determination of percent positive cells. Staining with MR1 employed fluorescein-conjugated RG7, a mouse anti-rat/hamster  $\kappa$  chain mAb.

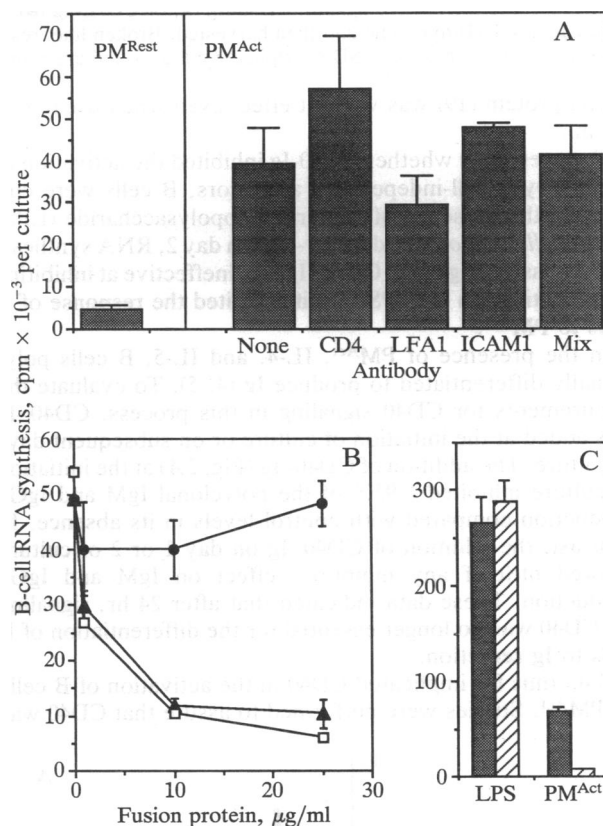
**Biosynthetic Labeling, Immunoprecipitation, SDS/PAGE, and Fluorography.**  $T_{\text{h}1}$  were rested or were activated with insolubilized anti-CD3 for 16 hr. Resting and activated  $T_{\text{h}}$  ( $20 \times 10^6$  per ml) were labeled with 1 mCi of [ $^{35}\text{S}$ ]methionine/cysteine for 1 hr, washed twice in RPMI-1640 with 10% fetal bovine serum, and lysed in extraction buffer (17). Purified antibodies or fusion proteins (1–10  $\mu\text{g}$ ) were added to 500  $\mu\text{l}$  of lysate ( $5 \times 10^6$  cell equivalents) at  $4^\circ\text{C}$  for 16 hr. At that time, the lysates were transferred to tubes containing 50  $\mu\text{l}$  of packed protein A-Sepharose. The pelleted protein A-Sepharose was resuspended and tubes were incubated at  $4^\circ\text{C}$  for 1 hr with agitation. The samples were then washed three times with high-stringency wash buffer. The pelleted protein A-Sepharose was suspended in 30  $\mu\text{l}$  of SDS sample buffer and run in a 10% polyacrylamide gel. After electrophoresis, the proteins were fixed in the gel and fluorography was performed.

## RESULTS

**Effect of mAbs on the Induction of B-Cell RNA Synthesis by  $\text{PM}^{\text{Act}}$ .** To define the cell surface molecules that mediate the induction of B-cell cycle entry by  $\text{PM}^{\text{Act}}$ , mAbs to  $T_{\text{h}}$  mem-

brane proteins were added to cultures of  $\text{PM}^{\text{Act}}$  and B cells. As previously published (5),  $\text{PM}^{\text{Act}}$  induced B-cell RNA synthesis 8-fold over that observed with  $\text{PM}^{\text{Rest}}$  (Fig. 1A). The addition of anti-LFA-1, anti-CD4, or anti-ICAM-1 alone, or in combination, did not inhibit induction of B-cell RNA synthesis by  $\text{PM}^{\text{Act}}$ .

**CD40-Ig Inhibits  $T_{\text{h}}$ -Induced B-Cell Cycle Entry, Differentiation, and Proliferation.** In the human system, it had been shown that anti-CD40 mAb induced B-cell proliferation (18), thereby implicating CD40 as an important triggering molecule for B cells. To determine whether CD40 was involved in the induction of B-cell RNA synthesis by  $\text{PM}^{\text{Act}}$ , a soluble fusion protein of the extracellular domains of human CD40 and the Fc domain of human IgG1 (CD40-Ig) was added to cultures of  $\text{PM}^{\text{Act}}$  and B cells.  $\text{PM}^{\text{Act}}$  derived from  $T_{\text{h}1}$  and  $T_{\text{h}2}$  were prepared and used to stimulate B-cell RNA synthesis. The addition of CD40-Ig to culture caused a dose-dependent inhibition of B-cell RNA synthesis that was induced by  $\text{PM}^{\text{Act}}$  from  $T_{\text{h}1}$  and  $T_{\text{h}2}$  (Fig. 1B). Half-maximal inhibition of B-cell RNA synthesis induced by  $\text{PM}^{\text{Act}}$  from  $T_{\text{h}1}$  and  $T_{\text{h}2}$  was achieved with about 5  $\mu\text{g}$  of CD40-Ig per ml. A CD7E-Ig



**FIG. 1.** Effect of mAbs and CD40-Ig on the induction of B-cell RNA synthesis by  $\text{PM}^{\text{Act}}$ . (A) Resting B cells were cultured with  $\text{PM}^{\text{Rest}}$  or  $\text{PM}^{\text{Act}}$  from  $T_{\text{h}1}$ . Individual mAb (anti-CD4, anti-LFA-1, or anti-ICAM-1, 25  $\mu\text{g}/\text{ml}$ ) or a combination of all the mAbs (each at 25  $\mu\text{g}/\text{ml}$ ) (Mix) was added. B-cell RNA synthesis was assessed from 42 to 48 hr of culture. Results presented are the arithmetic means  $\pm$  SD of triplicate cultures and are representative of five such experiments. (B) Resting B cells were cultured with  $\text{PM}^{\text{Act}}$  from  $T_{\text{h}1}$  (●, ▲) and  $T_{\text{h}2}$  (□). To these cultures, no fusion protein was added or CD40-Ig (Δ, □) or CD7E-Ig (●) was added at 1–25  $\mu\text{g}/\text{ml}$ . B-cell RNA synthesis was assessed as in A. Results are the arithmetic means  $\pm$  SD of triplicate cultures and are representative of three such experiments. (C) Resting B cells were cultured with LPS (50  $\mu\text{g}/\text{ml}$ ) or  $\text{PM}^{\text{Act}}$ . CD40-Ig (25  $\mu\text{g}/\text{ml}$ ; hatched bar) or CD7E-Ig (25  $\mu\text{g}/\text{ml}$ ; stippled bar) was added. B-cell RNA synthesis was assessed as A. Results are the arithmetic means  $\pm$  SD of triplicate cultures and are representative of three such experiments.

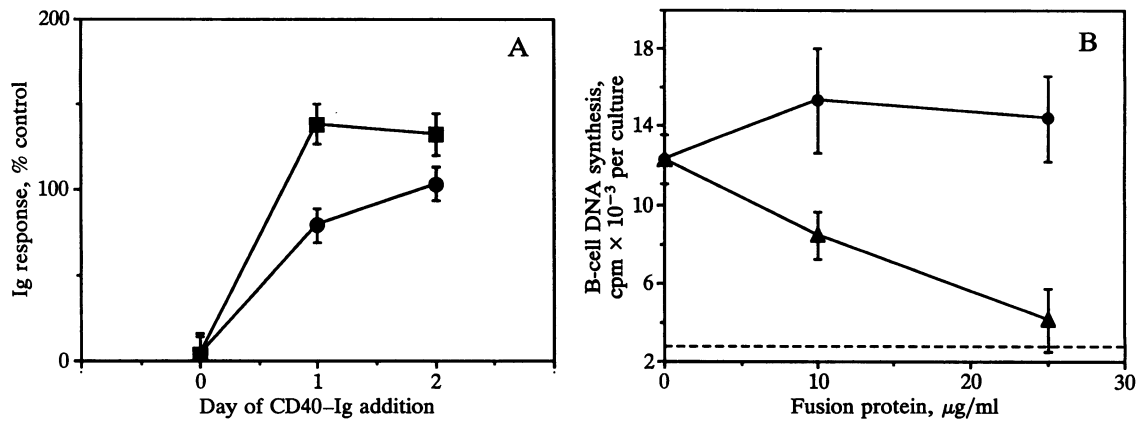


FIG. 2. CD40-Ig inhibits B-cell differentiation and proliferation. (A) Resting B cells were cultured with PM<sup>Act</sup>, IL-4 (10 ng/ml), and IL-5 (5 ng/ml). Either at the initiation of culture, or on day 1, 2, or 3 after initiation of culture, CD40-Ig or CD7E-Ig (25 µg/ml) was added. On day 6 of culture, supernatants from individual wells were harvested and quantitated for IgM (●) and IgG1 (■) by anti-isotype-specific ELISA (5). In the presence of PM<sup>Act</sup>, IL-4, and IL-5 (in the absence of added CD40-Ig), IgM and IgG1 were 4.6 µg/ml and 126 ng/ml, respectively. Cultures that received CD7E-Ig (25 µg/ml) on day 0 produced 2.4 µg/ml and 89 ng/ml of IgM and IgG1, respectively. The control of 100% is based on the response in the presence of CD7E-Ig. In the absence of IL-4 and IL-5, no IgM or IgG1 was detected. Results are representative of three such experiments. (B) T<sub>h</sub>1 were rested or activated with anti-CD3 for 16 hr, irradiated, and cultured (10<sup>4</sup> per well) with resting B cells (4 × 10<sup>4</sup>) in the presence of IL-4 (10 ng/ml). CD40-Ig (▲) or CD7E-Ig (●) was added at 0–25 µg/ml. From 66 to 72 hr of culture, cells were incubated with 1 µCi of [<sup>3</sup>H]thymidine and then harvested. Broken line, response of B cells to resting T<sub>h</sub> without the addition of fusion protein. Results are the arithmetic means ± SD of triplicate cultures and are representative of two such experiments.

fusion protein (19) was without effect even when used at 25 µg/ml.

To investigate whether CD40-Ig inhibited the activation of B cells by T-cell-independent activators, B cells were cultured in the presence of bacterial lipopolysaccharide (LPS; *Salmonella typhosa*) and CD40-Ig. On day 2, RNA synthesis was assessed (Fig. 1C). CD40-Ig was ineffective at inhibiting B-cell activation by LPS, yet it inhibited the response of B cells to PM<sup>Act</sup>.

In the presence of PM<sup>Act</sup>, IL-4, and IL-5, B cells polyclonally differentiated to produce Ig (4, 5). To evaluate the requirements for CD40 signaling in this process, CD40-Ig was added at the initiation of culture or on subsequent days of culture. The addition of CD40-Ig (Fig. 2A) at the initiation of culture inhibited >95% of the polyclonal IgM and IgG1 production compared with control levels in its absence. In contrast, the addition of CD40-Ig on day 1 or 2 of culture showed little if any inhibitory effect on IgM and IgG1 production. These data indicated that after 24 hr, signaling via CD40 was no longer essential for the differentiation of B cells to Ig secretion.

Data thus far implicated CD40 in the activation of B cells by PM<sup>Act</sup>. Studies were performed to assure that CD40 was

also involved in the activation of B cells by intact, viable, activated T<sub>h</sub>. T<sub>h</sub>1 were activated for 16 hr with insolubilized anti-CD3, harvested, and irradiated. The irradiated T<sub>h</sub>1 were cultured with B cells in the presence of IL-4 and B-cell proliferation was determined on day 3 of culture. An exogenous source of IL-4 was required to achieve B-cell proliferation because T<sub>h</sub>1 do not produce IL-4 (20). CD40-Ig inhibited the induction of B-cell proliferation by irradiated, activated T<sub>h</sub> in a dose-dependent manner, similar to that observed with PM<sup>Act</sup> (Fig. 2B). The negative control, CD7E-Ig, exerted no effect.

**CD40-Ig Detects a Molecule Expressed on Activated T<sub>h</sub> But Not on Resting T<sub>h</sub>.** To investigate whether activated T<sub>h</sub>1 expressed a binding protein for CD40, resting and activated (16 hr) T<sub>h</sub>1 were stained with CD40-Ig or CD7E-Ig, followed by fluorescein-conjugated anti-human IgG. Binding of CD40-Ig was assessed by flow cytometry (Fig. 3). Activated T<sub>h</sub>1, but not resting T<sub>h</sub>1, stained 56% positive with CD40-Ig, but not with the control CD7E-Ig. To identify the CD40-Ig-binding protein, T<sub>h</sub>1 proteins were biosynthetically labeled with [<sup>35</sup>S]methionine/cysteine and proteins were immunoprecipitated with CD40-Ig or CD7E-Ig. The immunoprecipitated proteins were resolved by SDS/PAGE and fluorography (Fig.

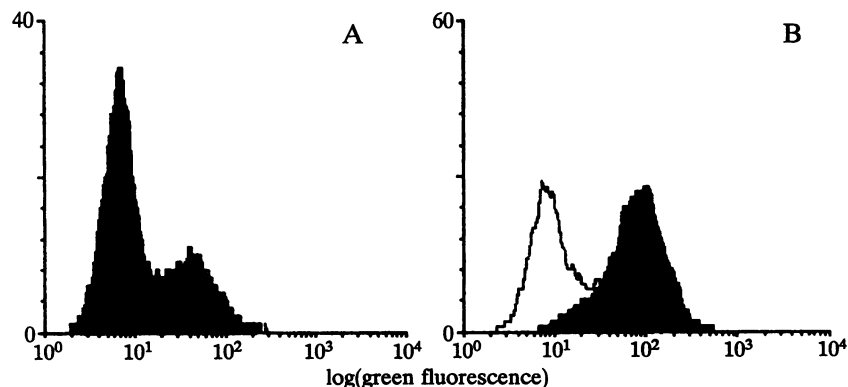


FIG. 3. CD40-Ig detects a molecule expressed on activated T<sub>h</sub> but not resting T<sub>h</sub>. Resting (A) or activated (B) T<sub>h</sub> were incubated with fusion proteins for 20 min at 4°C, followed by fluorescein-conjugated goat anti-human IgG (25 µg/ml). Percent positive cells (ordinate) was determined by the analysis of at least 5000 cells per sample. The threshold for positive cells was set at channel 85. Results are representative of six such experiments. On resting T<sub>h</sub>, staining with CD40-Ig and staining with CD7E-Ig are completely overlapping and identical in distribution.

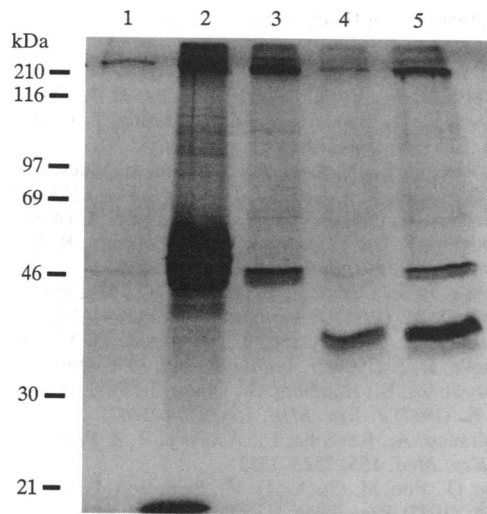


FIG. 4. CD40-Ig immunoprecipitated a 39-kDa protein from the lysate of activated T<sub>h</sub>1. T<sub>h</sub>1 were activated with insolubilized anti-CD3 for 16 hr. <sup>35</sup>S-labeled proteins from resting or activated T<sub>h</sub> were immunoprecipitated without addition (lane 1), with purified anti-class I antibody (2.5 µg; lane 2), with CD7E-Ig fusion protein (10 µg; lane 3), or with CD40-Ig fusion protein (1 µg and 10 µg; lanes 4 and 5). Gel profile is representative of three such experiments.

4). A prominent band at 39 kDa was immunoprecipitated with 1 and 10 µg of CD40 per sample in a dose-dependent manner. As controls, anti-class I mAb immunoprecipitated bands at 55 kDa and a low molecular mass band, β<sub>2</sub>-microglobulin. In the absence of mAb, no prominent bands were visible. A 39-kDa band was also immunoprecipitated from activated T<sub>h</sub> that were vectorially labeled with <sup>125</sup>I, confirming that the 39-kDa protein was a membrane protein (data not shown). The immunoprecipitated 39-kDa band was identical in size when resolved by SDS/PAGE under nonreducing conditions, indicating that the CD40-binding protein was a single-chain molecule (data not shown).

**A mAb Specific for the 39-kDa T<sub>h</sub> Membrane Protein Inhibits the Induction of B-Cell RNA Synthesis by PM<sup>Act</sup>.** mAbs specific for antigens selectively expressed on activated vs. resting T<sub>h</sub> were developed to identify T<sub>h</sub> molecule(s) potentially responsible for T<sub>h</sub> effector phase activity. One such mAb, MR1, recognized an antigen that was selectively expressed on activated T<sub>h</sub>1. To investigate whether MR1 and CD40-Ig recognized the same molecule, flow cytometry and blocking studies were performed. CD40-Ig and MR1 stained approximately 56% and 61%, respectively, of activated T<sub>h</sub>1, but not resting T<sub>h</sub>1 (Table 1). MR1, but not another hamster

Table 1. mAb MR1 and CD40-Ig compete for the same protein on activated T<sub>h</sub>

Staining mAb or fusion protein	Blocking mAb (conc., µg/ml)	% positive cells	
		Resting T <sub>h</sub>	Activated T <sub>h</sub>
MR1	—	5.7	61.8
CD40-Ig	—	4.9	56.5
	MR1 (5)	ND	49.5
	MR1 (10)	ND	30.3
	MR1 (25)	ND	20.7
	MR1 (50)	5.0	11.7
	Anti-αβ TCR (50)	ND	63.0

Blocking studies were performed with flow cytometric analysis. Cells were incubated with hamster mAb MR1 (50 µg/ml) followed by fluorescein-conjugated anti-hamster κ chain or with CD40-Ig (50 µg/ml) in the presence of blocking mAb as indicated, followed by fluorescein-conjugated goat anti-human IgG1. Results are representative of three such experiments.

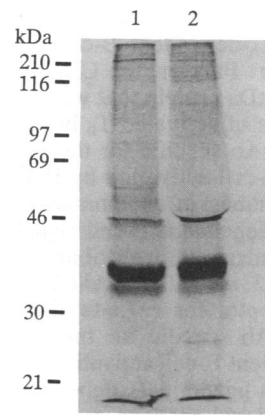


FIG. 5. MR1 and CD40-Ig recognize the same molecule in lysates of activated T<sub>h</sub>. Proteins from [<sup>35</sup>S]methionine-labeled activated T<sub>h</sub> were immunoprecipitated with MR1 (10 µg per sample) (lane 2) or CD40-Ig (10 µg per sample) (lane 1) and resolved by PAGE and fluorography. Results are representative of two such experiments.

anti-T cell mAb, anti-αβ TCR, blocked the staining of activated T<sub>h</sub>1 with CD40-Ig, in a dose-dependent manner. These data suggested that CD40-Ig and MR1 recognized overlapping or identical epitopes on the 39-kDa T<sub>h</sub> protein. To further investigate whether CD40-Ig and MR1 recognized the same molecule, the antigen that bound MR1 was identified by immunoprecipitation of radiolabeled proteins from T<sub>h</sub> lysates. Both CD40-Ig and MR1 immunoprecipitated a 39-kDa protein (Fig. 5). Finally, immunoprecipitation of the 39-kDa protein with CD40-Ig removed the antigen recognized by MR1 from radiolabeled lysates of activated T<sub>h</sub> (data not shown), supporting the idea that the MR1 antigen and the CD40 binding protein were identical.

Functional studies were performed with MR1 to address whether this mAb neutralized the activity expressed by PM<sup>Act</sup>. PM<sup>Act</sup> and B cells were cultured alone or in the presence of hamster mAbs or CD40-Ig. Two hamster mAbs, anti-αβ TCR and anti-CD3, did not inhibit the activation of resting B cells by PM<sup>Act</sup>. In contrast, MR1 or CD40-Ig inhibited B-cell activation (Fig. 6). MR1 did not inhibit LPS-induced B-cell RNA synthesis. B cells cultured with LPS incorporated 163,446 ± 3654 cpm per culture (<sup>3</sup>H]uridine) and in the presence of MR1 (25 µg/ml) incorporated 168,284 ± 8027 cpm per culture.

DISCUSSION

Blocking of prominent T<sub>h</sub> surface molecules (LFA-1, CD4, ICAM-1, CD3, αβ TCR) with mAbs did not impede the capacity of activated T<sub>h</sub> to induce B-cell cycle entry. In

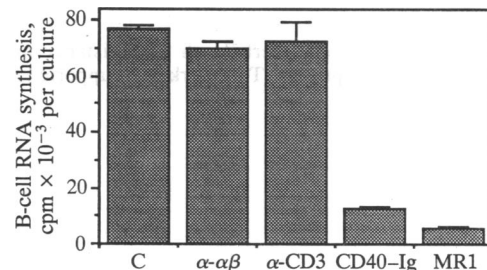


FIG. 6. A mAb specific for the induced 39-kDa T<sub>h</sub> membrane protein inhibits the induction of B-cell RNA synthesis by PM<sup>Act</sup>. Resting B cells and PM<sup>Act</sup> were cultured without addition (control, C) or with anti-αβ TCR (α-αβ), anti-CD3 (α-CD3), CD40-Ig, or MR1 (10 µg/ml). RNA synthesis was determined as described in Fig. 1. Results are the arithmetic means ± SD of triplicate cultures and are representative of three such experiments.

contrast, CD40-Ig or a mAb specific for the CD40-binding protein blocked  $T_h$ -dependent B-cell activation in a dose-dependent manner. Further, the CD40-binding protein was identified as a 39-kDa protein that was selectively expressed on the membranes of activated  $T_h$  but not resting  $T_h$ . Both CD40-Ig and a mAb specific for the 39-kDa CD40-binding protein blocked B-cell activation by  $PM^{Act}$ .

Although a number of membrane proteins have been implicated in  $T_h$ -dependent B-cell signaling, evidence presented herein dismisses the contribution of some molecules (LFA-1, CD4, CD3,  $\alpha\beta$  TCR, ICAM-1) and implicates CD40 as the B-cell receptor for cognate signaling by  $T_h$ . Either CD40-Ig or a mAb specific for the CD40-binding protein inhibits  $T_h$ -dependent B-cell activation. CD40 was suspected to be an important ligand in  $T_h$ -dependent B-cell activation, since many functional responses of B cells to anti-CD40 mAbs and to activated  $T_h$  are similar. For example, anti-CD40 and  $PM^{Act}$  induce B-cell cycle entry (21–23). Further, anti-CD40 and activated  $T_h$  in the presence of IL-4 induce potent IgE production from resting B cells (21, 24–26). Finally, anti-CD40 and  $PM^{Act}$  induce homotypic B-cell aggregation (27). Therefore, both direct and indirect evidence implicates CD40 as the B-cell receptor for cognate help.

The ligand for CD40 is a 39-kDa protein that is expressed on activated, but not resting  $T_h$ . The 39-kDa protein is a single-chain molecule, since electrophoretic migration was not influenced by reducing agents (data not shown). Based on the functional studies presented in this study and preliminary biochemical studies (data not shown), activated  $T_{h1}$  and  $T_{h2}$  express the 39-kDa CD40-binding protein. This is consistent with the functional studies showing that both  $T_{h1}$  and  $T_{h2}$  induce B-cell cycle entry. To further characterize the 39-kDa protein, cDNA encoding CD proteins in the size range of 39 kDa (CD53 and CD27) were transiently transfected into COS cells and the cells were tested for CD40-Ig binding. None of the transfected COS cells expressed proteins that bound CD40-Ig. It is therefore suspected that the 39-kDa protein is not one of these CD proteins.

The biochemical basis for signal transduction between  $T_h$  and B cells has been elusive. The identification of CD40 as the signal-transducing molecule for T-cell help focuses attention on specific biochemical pathways known to be coupled to the CD40 molecule. CD40 is a member of the nerve growth factor receptor family by virtue of the presence of four cysteine-rich motifs in its extracellular region. Signaling through CD40 by mAb has been shown (28) to involve the activation of tyrosine kinases resulting in the increased production of inositol trisphosphate and the activation of at least four distinct serine/threonine kinases. Based on information obtained from signaling through other members of the nerve growth factor receptor family, it is thought that interaction between activated  $T_h$  and B cells results in many of the same biochemical processes.

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