Human Native Soluble CD40L Is a Biologically Active Trimer, Processed Inside Microsomes*

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CD40 ligand (CD40L) is a glycoprotein expressed on the surface of activated helper T cells, basophils, mast cells, and eosinophils. Binding of CD40L to its receptor CD40 on the B cell surface induces B cell proliferation, adhesion, and immunoglobulin class switching. We have identified soluble cleavage products of human CD40L in the supernatant of a stimulated human T cell clone. Subcellular fractionation experiments have shown that the transmembrane CD40L is processed inside the microsomes and that its cleavage is stimulation-dependent. The native human soluble CD40L is trimeric and, when used in conjunction with interleukin-4, induces B cell proliferation.

CD40L is a type II surface protein expressed by T cells, basophils, mast cells, and eosinophils (1-5). CD40L can induce B cell proliferation and is involved in the control of immunoglobulin (Ig) class switching (1-3, 6, 7). Patients expressing CD40L in their serum have been identified as a cause of immune complex diseases (1-3, 6, 7). CD40L is a receptor expressed on the surface of activated helper T cells, basophils, mast cells, and eosinophils (1-5). CD40L can induce B cell proliferation, adhesion, and immunoglobulin class switching. We have identified soluble cleavage products of human CD40L in the supernatant of a stimulated human T cell clone. Subcellular fractionation experiments have shown that the transmembrane CD40L is processed inside the microsomes and that its cleavage is stimulation-dependent. The native human soluble CD40L is trimeric and, when used in conjunction with interleukin-4, induces B cell proliferation.

MATERIALS AND METHODS

Induction of CD40L Expression—The human T cell clone JF7 was maintained in Iscove's medium supplemented with transferrin (20 μg/ml), insulin (5 μg/ml), rIL-2 (100 units/ml), and fetal calf serum. Every 3 weeks, cells were diluted to a density of 2 x 10^6 cells/ml and stimulated with phytohemagglutinin (0.2 μg/ml, Wellcome) and irradiated peripheral blood mononuclear cells (5 x 10^6 cells/ml). For induction of soluble CD40L production, 2 x 10^6 cells at a density of 1 x 10^6/ml were stimulated for 16 h with ionomycin (1 μm) and phorbol myristic acetate (10 ng/ml) in Iscove's medium supplemented with transferrin, insulin, and rIL-2.

Detection of CD40L Surface Expression—Aliquots of cells (4 x 10^6 in 40 μl) were either incubated with soluble CD40L-Fc (1 μg/ml), anti-CD40L mAb (1 μg/ml), kindly provided by R. Noelle, Darmouth Medical School, Lebanon, NH), or isotype-matched controls, followed by fluorescein isothiocyanate-labeled sheep anti-mouse IgG (Biochrome, Munich, Meudon, France) and propidium iodide (2 μg/ml) and analyzed by flow cytometry.

Preparation of CD40L-containing Supernatants—Cells were harvested from culture dishes by two centrifugations at 5000 g for 20 min, filtered through a 0.2-μm cellulose acetate membrane, and concentrated 10-fold using Amicon membranes with a 5-kDa cutoff.

Subcellular Fractionation of T Cells—The human T cell clone JF7 was used in immunoblotting and Western blotting. Cell supernatants were collected and analyzed by SDS-PAGE and Western blotting. Briefly, half of each fraction was taken and the proteins contained within were precipitated with 10% trichloroacetic acid. The remaining half of each fraction was cleared from sucrose by dialysis through a 5-kDa membrane cutoff and further tested for its ability to induce B cell proliferation.

Subcellular Fractionation of T Cells—For detection of CD40L in unstimulated or stimulated cells, 2 x 10^6 unstimulated or stimulated cells were lysed in 100 μl of SDS gel loading buffer for subcellular fractionation. Around 3 x 10^6 cells, resuspended in 5 μl of PBS, were passed 20 times through a ball homogenizer (17). Nuclear and intact cells were separated from the other cell fractions by centrifugation at 1000 g for 20 min.

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RESULTS AND DISCUSSION

Identification of Human Soluble CD40L in Activated T Cell Clone (JF7) Supernatant—In an attempt to detect soluble forms of the human CD40L released by activated CD4+ T cells, we stimulated the T cell clone JF7 (19) with PMA and ionomycin for 16 h. This led to expression of surface CD40L, easily...
detectable by flow cytometry with anti-CD40L mAb (Fig. 1) or soluble CD40-Fc (data not shown). To identify soluble forms of the CD40L released into the cell culture medium, supernatant from unstimulated T cells or supernatant from T cells stimulated with PMA and ionomycin was cleared from cell debris, concentrated by ultrafiltration, and analyzed for the presence of CD40L by Western blotting assays using anti-CD40L mAb. Three soluble forms of CD40L were observed (Fig. 2). Two forms migrated as a doublet with an apparent molecular mass of 18 kDa (Fig. 2). The third form migrated with an apparent molecular mass of 15 kDa when subjected to SDS-PAGE (Fig. 2). The nature of the protein detected by Western blotting was checked by sequencing of the NH₂ ends. The Edman degradation was successful only for the 18-kDa band. The sequence obtained (MQKGD) indicated that the 18-kDa forms corresponded to the form described by Graf et al. (16).

To determine whether the soluble forms corresponded to a cleavage of the membrane form on the T cell surface or to an intracellular processing, we isolated microsomal fractions from unstimulated and stimulated JF7 cells and analyzed the fractions by Western blot assays with anti-CD40L mAb (Fig. 2). Whereas in the total cell extract and the microsomal fractions of the unstimulated T cell clone only the 33-kDa membrane form of CD40L could be detected, in activated cells the membrane-bound form and the 18- and 15-kDa forms were detectable (Fig. 2). These data suggest that the soluble forms released into the supernatant are the result of an intracellular cleavage event dependent on an enzymatic activity only present in stimulated cells. Based on the known IL-1β and the TNF-α processing, one can imagine that the enzyme responsible for the CD40L cleavage belongs to a convertase family of enzymes (13, 20). The existence of a soluble form of CD40L allowed us to postulate two different pathways of CD40L regulation in the immune response. These soluble forms could represent inactive by-products generated during the down-regulation of CD40L expression. On the other hand, they could represent alternative forms of CD40L displaying biological activities. Previous work has shown that the soluble recombinant form of CD40L is trimeric (15). We therefore examined whether CD40L released by activated T cells was monomeric or multimeric.

Physical characterization of the native human soluble CD40L—Soluble forms of CD40L were subjected to sedimentation through a sucrose gradient. The 18-kDa form sedimented as a monomeric species with an apparent molecular mass of 56 ± 5 kDa. The 15-kDa form sedimented with an apparent molecular mass of 51 ± 5 kDa. This suggested that the two forms were multimeric and may be trimeric (Fig. 3). The sedimentation technique used does not allow us to make the distinction as to whether soluble forms of CD40L are homo- or heterotrimers. As trimeric forms of recombinant CD40L are active (15), we examined whether the native soluble forms of CD40L could also participate in the induction of B cell proliferation.

Biological activity of the native human soluble CD40L—When tested in a B cell proliferation assay, the sucrose gradient fraction 19 (containing soluble CD40L, Fig. 3) was able to induce a 7-fold increase in [³H]deoxythymidine incorporation in P3HR1 B cells cultured in the presence of IL-4 (Fig. 4). A smaller B cell proliferation was also induced by fraction 3, which does not contain soluble CD40L detectable by Western blot analysis (data not shown). To show that the activity in the sucrose gradient (fraction 19) was indeed mediated by soluble CD40L, soluble CD40-Fc was added as a competitor. The proliferation induced by fraction 19 was partially inhibited, around 30% of inhibition, whereas CD40-Fc had no effects on the proliferation induced by fraction 3 (Fig. 4). These data suggest that fraction...
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Fig. 4. Biological activity of the native human soluble CD40L. Purified tonsillar B cells were incubated either alone (lane 1), with rIL-4 (lane 2), with rIL-4 and anti-CD40 mAb (lane 3), or with rIL-4 and aliquots of either sucrose gradient fraction 3 or fraction 19 (from lane 4 to 7), and then the [3H]deoxythymidine incorporation was measured. Results are expressed in counts/min (mean ± S.D.). Lanes 4 and 5, B cells were incubated with 20 μg/ml of the sucrose gradient fraction 19 in the absence or presence of 10 μg/ml CD40-Fc, respectively. Lanes 6 and 7, B cells were incubated with 20 μg/ml of the sucrose gradient fraction 3 in the absence or presence of 10 μg/ml CD40-Fc, respectively.

19 contains active soluble CD40L, whereas the activity detected with fraction 3 could correspond to an activity resulting from the presence of cytokines such as IL-1, IL-2, and TNF-α produced by T cells (21).

Whereas it remains to be determined if all the identified soluble forms of CD40L are biologically active, our data suggest that cleavage of CD40L does not simply represent an alternative way to down-regulate the expression of this surface molecule by T cells. It seems unlikely that one of the two forms of the soluble CD40L acts as an antagonist since we have identified them as trimers. Soluble forms produced by intracellular cleavage share activities with the membrane form of CD40L and might therefore be involved in the control of B cell activation by helper T cells. Whereas the 33-kDa membrane form could be detected intracellularly on unstimulated CD44+ T cell clone cells by Western blotting assays, surface expression was undetectable by flow cytometry, indicating a post-transcriptional control of surface expression (22). This would appear to suggest that a preformed CD40L is stored inside unstimulated cells to be readily available in case of an immediate need. The absence of surface expression was paralleled by the CD40L cleavage, suggesting that the release of soluble CD40L by T cells is also tightly regulated.

CD40L is involved in the induction of a large variety of events in the immune system as indicated by the pleiotropic effects of the mutations observed in hyper-IgM syndrome patients (8, 23). The complex physiology of CD40L might be partially linked to the existence of multiple forms of the protein.

The existence of a membrane-bound and a soluble form of CD40L suggests that this molecule might transmit signals in two different ways. The two different forms could share some activities, but they could also display specific functions. The membrane molecule could be implicated in a cell-cell interaction process, which presents some physical limits of the signal propagation. In contrast the soluble form with its “cytokine-like activity” could represent a quick and diffusible way for T cells to transmit the signal.

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