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Biological Activity of Soluble CD100. I. The Extracellular Region of CD100 Is Released from the Surface of T Lymphocytes by Regulated Proteolysis

Abdellah Elhabazi, 2* Stéphanie Delaire, 2* Armand Bensussan, * Laurence Boumsell, * and Georges Bismuth 3†

CD100 is the first semaphorin described in lymphoid tissues, where it has been shown to be associated with a serine kinase activity. Semaphorins are molecules involved in axon pathfinding during nerve development and act as repellent guidance cues. In the nervous system semaphorins exist as either membrane-bound or secreted forms. We report here a spontaneous processing of membrane CD100, suggesting that it is also produced as a diffusable semaphorin from lymphoid cells. Monomeric and homodimeric forms of CD100 are expressed by T lymphocytes and CD100-transfected fibroblasts. We demonstrate that CD100 is released through a proteolytic process blocked by metalloprotease inhibitors. In T cells, only soluble CD100 dimers are produced, suggesting that CD100 dimerization is required for proteolysis. In agreement, we observe that increasing membrane dimers strongly favors shedding of the molecule. By expressing a CD100 molecule mutated at cysteine 674 into a COS cell system, we additionally demonstrate that this particular residue in the extracellular domain of the molecule is required for dimerization.

Finally, we show that staurosporine, a serine kinase inhibitor, enhances the membrane cleavage of CD100. Together these results demonstrate that membrane CD100 is cleaved by a metalloprotease-dependent process, which is probably regulated by phosphorylation. Mainly, these findings shed light on a possible function for the semaphorin region of CD100 as a long range guidance cue in the immune system. The Journal of Immunology, 2001, 166: 4341–4347.

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When indicated (see Results), reagents were added at the beginning of the incubation. At the end of the incubation cell suspensions were centrifuged, and the supernatants were collected. The cell pellets were washed twice in cold PBS and solubilized in a 1% Nonidet P-40 (v/v) detergent buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 50 μM aprotinin, and 1 mM leupeptin for 30 min at 4°C. After centrifugation at 12,000 × g to remove cellular debris, the lysates were preclarified with protein A-Sepharose CL-4B before immunoprecipitation with 5 μg of a mixture (1:1) of CD100 mAb BD16 and BB18 previously bound on protein A-Sepharose. Immunoprecipitation of biotinylated CD100 molecules in the supernatants was performed in parallel using the same procedure. Immune complexes were eluted from the beads by boiling the samples for 3 min in Laemmli buffer (500 mM Tris-HCl (pH 6.8), 10% SDS, 10% glycerol, and 10% bromophenol blue) supplemented (reducing conditions) or not (nonreducing conditions) with 5% 2-ME. Solubilized proteins were separated by SDS-PAGE and electrotransferred on polyvinylidene difluoride membranes (Amersham, Paris, France). Blots were probed using HRP-conjugated streptavidin and an enhanced chemiluminescence detection system (Amersham) followed by autoradiography on Cronex x-ray film (Du Pont de Nemours, Les Ulis, France). Scanning densitometry of the films was performed with Bio-Rad densitometer GS-670, and results were analyzed using Molecular Analyst/PC image analysis software (Bio-Rad, Ivry sur Seine, France).

**Results**

CD100 is spontaneously released as a dimer from the surface of Jurkat T lymphocytes

Jurkat T cells express a high level of membrane CD100 (mCD100) in this cell line, biotinylated cells were incubated in complete culture medium for 90 min. Cells and supernatants were recovered separately and analyzed for CD100 expression by immunoprecipitation with specific mAbs and blotting. CD100 has been reported to migrate as a 150-kDa homodimer under nonreducing conditions in PBMC (1, 2). However, under these experimental conditions in Jurkat T cells, the major band was approximately 150 kDa (Fig. 1A). A faint band was seen at 300 kDa (arrowhead at the top of Fig. 1), and a third band was around 280 kDa. Under reducing conditions, we observed a single protein at 150 kDa. These findings suggested the predominant expression of monomeric CD100 at the membrane of Jurkat cells. As shown in Fig. 1A, a protein reacting with CD100 mAb was recovered in parallel from the cell supernatant. This soluble form of the molecule (sCD100) had an apparent m.w. of 220 kDa under nonreducing conditions and 120 kDa in reduced gels. Similar results were consistently observed with other CD100-positive T cell lines, but also with fibroblasts transfected with the molecule (see Fig. 3). Time-course experiments were also performed (see Fig. 1B). They showed a progressive release of CD100 in the supernatant. A parallel decrease in the molecule at the cell surface was noticeable, and we found a good correlation between the appearance and accumulation of biotinylated CD100 in the medium and its disappearance from the cell surface. Taken together, these results demonstrate the existence of a soluble form of CD100 spontaneously released from the surface of the Jurkat T cell line as a homodimer. They also suggest the requirement for a previous dimerization of the molecule at the membrane to be cleaved. The 150-kDa monomeric form present at the membrane may give rise to the 150- to 150-kDa homodimer that undergoes membrane cleavage, releasing the homodimeric 120- to 120-kDa sCD100.

**Shedding of CD100 is inhibited by metalloprotease inhibitors EDTA and EGTA**

To assess the nature of the protease that cleaves CD100, cells were treated with a set of inhibitors for various protease subclasses. Table I summarizes the results. We observed that none of the inhibitors completely blocked CD100 shedding. Only the light metal chelators, EDTA and EGTA, blocked up to 50% of initial CD100 shedding. Serine or cysteine protease inhibitors were not or were

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### Materials and Methods

**Cells, reagents, and Abs**

Jurkat T cells and NIH 3T3 fibroblasts stably expressing human CD100 were grown in RPMI 1640 medium supplemented with 10% FCS, antibiotics, and glutamine. COS cells, cultured in the same medium, were also used for transient transfection experiments. The CD100-transfected fibroblasts were provided by Dr. G. J. Freeman (Dana-Farber Cancer Institute, Boston, MA).

Different protease inhibitors were used in this study: serine protease inhibitors (aprotinin, chymostatin, soybean trypsin inhibitor, and 1,1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone) (4), metalloprotease inhibitors (EDTA, EGTA, phosphoramidon, and 1,10-phenanthroline), cysteine and cysteine-serine protease inhibitors (E64, HgCl₂, leupeptin, and PMSF), and aspartic protease inhibitor (pepstatin). Staurosporine (a cell-permeant, broad spectrum inhibitor of serine/threonine kinases), okadaic acid (a serine/threonine phosphatase inhibitor), chloroquine (an inhibitor of endocytosis), Brefeldin A (an inhibitor of exocytosis and protein transport), and iodoacetamide (IAA, an alkylating agent) were also used. All these reagents were purchased from Sigma (St. Louis, CA). Matrix metalloprotease (MMP) inhibitor GM 6001 and MMP-3 inhibitor II were purchased from Calbiochem (Meudon, France).

mAbs BD16 and BB18 recognized different epitopes of the extracellular domain of CD100 (2). They were produced as ascitic fluid and purified by affinity chromatography on protein A- or G-Sepharose CL-4B (Pharmacia, Uppsala, Sweden), as previously reported (1).

**Constructs and cell transfection**

A full-length cDNA encoding human CD100 in pCDM8 vector was a gift from Dr. G. J. Freeman (Dana-Farber Cancer Institute, Boston, MA). The open reading frame was PCR-amplified from a Jurkat T cell cDNA library using mutagenic oligonucleotides as primers (29). The following mutagenic oligonucleotides were used: 5'-CCGCACTGCCTGGAAGCAAAAAG (GTC-3') and 5'-GCTTTTGGTTGCGCAGGC (GCCG-3'), according to the manufacturer’s protocol of the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) and using wild-type CD100 in pCDM8 vector as a template. The mutation was verified by DNA sequencing.

COS cells cultured in six-well culture plates (80% confluence) were transfected with CD100 constructs in pCDM8 using Lipofectamine (Life Technologies, Cergy Pontoise, France) and 2 μg of plasmid DNA according to the manufacturer’s conditions. Transfected cells were cultured for 48 h before use. CD100 expression was monitored by indirect immunofluorescence using BD16 mAb and a goat anti-mouse FITC-conjugated secondary Ab (Sigma).

**Cell surface labeling and measurements of CD100 shedding**

Biotinylation of cell surface proteins was performed as described previously (29). Briefly, after a wash in PBS, cells were labeled in 1 ml of ice-cold PBS containing 2 mg of Immunopur sulfatecinimidyl-6-(biotinamido) hexanoate (Pierce Europe, Oud-Beijerland, The Netherlands) for 15 min. The reaction was stopped by adding an excess of ice-cold RPMI 1640 medium containing 10% FCS for 10 min, followed by rapid centrifugation.

For shedding experiments, after biotinylation, cells were resuspended in six-well plates at a cell density of 10⁷/ml in RPMI medium containing 10% FCS, and the cells were incubated at 37°C for various periods of time.
CD100 dimers in the supernatant was not affected. The drug con-

less efficient even at higher concentrations (not shown). As shown in Fig. 2A the cleavage of membrane CD100 was efficiently inhibited with azide and completely when cells were incubated at 4°C, showing that an enzymatic process was probably involved. The partial inhibition of the shedding of sCD100 by EDTA and EGTA shows that a metalloprotease activity may account for the cleavage of membrane CD100. Thus, proteolysis may occur below this position, suggesting that the cleavage site of the molecule lies close to the cell membrane. To further assess their involvement in the shedding of CD100, we therefore used a sulfhydryl oxidizing agent such as IAA to acetylate the free thiols. Shown in Fig. 3 are nonreducing gels from an experiment performed with murine fibroblasts stably expressing CD100 and Jurkat cells. As found with Jurkat, both monomers and dimers of CD100 were expressed at the membrane of fibroblasts, but dimers were much more abundant in this cell type (compare with Fig. 1). The 220-kDa dimer of sCD100 was present in the cell supernatant, and its release was also inhibited by EDTA. In both cell types IAA had a dramatic effect on the fate of CD100. We expected a reduced dimerization of the molecule, but, surprisingly, IAA treatment strongly reduced membrane monomers (even suppressed them in Jurkat) while at the same time it increased both the 300-kDa dimeric form of mCD100 and the 220-kDa form of sCD100. Note the shift in the M₉ of the different forms of CD100 after IAA treatment. Therefore, IAA strongly increased the dimerization of mCD100, leading to increased cleavage of sCD100. This suggests that conformational changes and folding, consisting in the model-

Cysteine residue 674 in the extracellular domain of CD100 is required for dimerization

To further investigate the role of cysteine residues in CD100 dimerization, we used a mutational analysis method. We found that CD100 was mainly released as a 220-kDa homodimer. This suggests that the cleavage site of the molecule lies close to the cell membrane. Importantly, there is a unique cysteine residue at position 674 in this region of CD100, between Ig-like and transmembrane domains. Thus, proteolysis may occur below this position,

Table 1. Effect of various protease inhibitors on sCD100 release

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Specificity</th>
<th>Percent Inhibition</th>
<th>Tested at</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>Serine proteases</td>
<td>25</td>
<td>200 KUV/ml</td>
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<tr>
<td>Chymostatin</td>
<td>Serine proteases (chymotrypsin)</td>
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<td>20 μM</td>
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<td>E-64</td>
<td>Cysteine proteases</td>
<td>30</td>
<td>10 μM</td>
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<td>Metalloproteases</td>
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</tr>
<tr>
<td>EGTA</td>
<td>Metalloproteases</td>
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<td>5 mM</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>Cysteine proteases</td>
<td>0</td>
<td>0.5 mM</td>
</tr>
<tr>
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<td>Serine/cysteine proteases</td>
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<td>50 μg/ml</td>
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<td>Aspartic proteases</td>
<td>0</td>
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<tr>
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<td>5 mM</td>
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<tr>
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<td>50 μM</td>
</tr>
<tr>
<td>PMSF</td>
<td>Serine/cysteine proteases</td>
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<td>2 mM</td>
</tr>
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<td>Soybean trypsin inhibitor</td>
<td>Serine proteases (trypsin)</td>
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<td>TPCK</td>
<td>Serine proteases (chymotrypsin)</td>
<td>0d</td>
<td>50 μM</td>
</tr>
</tbody>
</table>

a Biotinylated Jurkat cells were cultured for 90 min in complete culture medium at 37°C supplemented with various protease inhibitors. Supernatants were then collected and CD100 was immunoprecipitated with specific mAbs. sCD100 release was evaluated by scanning densitometry after SDS-PAGE, blotting with HRP-conjugated streptavidin and ECL detection.

b Cells incubated in medium alone were used as control (0% inhibition). Results are representative of three independent experiments.

c Standard effective concentrations of inhibitors were used. Lack of toxicity was determined by trypan blue staining and the percentage of viable cells was always >90%.

d These inhibitors enhanced sCD100 release.
after the formation of a disulfide bridge on this particular cysteine residue. To explore this possibility we mutated this residue into an alanine. COS cells were transfected with this construct or with a construct encoding the wild-type form of CD100, and 48 h later cell supernatants were analyzed for CD100 release after cell biotinylation. CD100 expression controlled by immunofluorescence was identical with both constructs (data not shown). Fig. 4 clearly shows the lack of CD100 dimers in the supernatant of COS cells transfected with the mutant. Contrasting with the results obtained in Jurkat cells, significant levels of monomers were observed, suggesting that some direct proteolysis of CD100 monomers at the cell membrane may occur in this cell system. Note that, as in Jurkat cells, the dimer was poorly expressed at the membrane in the COS cell system (not shown). We can conclude from this experiment that cysteine 674 is required for CD100 dimerization and also that membrane cleavage probably occurs below this residue.

CD100 is cleaved at the plasma membrane

Turnover of cell surface molecules can be driven by membrane processing or by internalization. However, many membrane structures are shed after recycling through endosomal/lysosomal compartments, where they are subjected to some proteolytic degradation by acid cysteine proteases. Among these molecules, CD71 cleavage was reported to take place in this compartment (30). This was assessed in the case of CD100 by treating the cells either with chloroquine, a lysosomotopic agent, or with E64, an inhibitor of lysosomal cysteine proteases. The results (see Fig. 5) indicate that both failed to clearly block CD100 cleavage (E64 had a slight

FIGURE 2. A proteolytic pathway is probably responsible for CD100 release. A, Biotinylated Jurkat cells were incubated for 90 min at 37°C in complete culture medium supplemented with various protease inhibitors used at the concentration indicated in Table I. A cell aliquot was maintained at 4°C. CD100 immunoprecipitates were performed with specific mAbs as in Fig. 1 in cell lysates (M) and supernatants (S) and migrated in nonreducing conditions. B, Jurkat cells were incubated as described in A with the two MMP inhibitors, GM 6001 (5 nM) and MMP-3 inhibitor II (MMP-3i; 1 μM). Supernatants were subjected to CD100 immunoprecipitation followed by migration in nonreducing conditions.

FIGURE 3. Iodoacetamide increases membrane CD100 dimerization and shedding. Biotinylated CD100-transfected 3T3 fibroblasts and biotinylated Jurkat cells were incubated for 90 min at 37°C in complete culture medium either alone or with IAA (5 mM) or EDTA (5 mM) as indicated. CD100 immunoprecipitates were performed with specific mAbs as described in Fig. 1 in cell lysates (M) and supernatants (S), and the precipitates were analyzed in nonreducing conditions. In fibroblasts the Mo lane represents CD100 expression in the cell lysate at time zero of the experiment.

FIGURE 4. Cysteine residue 674 in the extracellular domain of CD100 is required for dimerization. COS cells were transfected with a pCDM8 vector encoding wild-type CD100 or a CD100 molecule mutated at cysteine 674 into an alanine (C674A). Forty-eight hours later cells were biotinylated and incubated for 90 min at 37°C in complete culture medium. CD100 immunoprecipitates were performed with specific mAbs in supernatants as described in Fig. 1, and the precipitates were analyzed in nonreducing and reducing conditions.

FIGURE 5. CD100 is cleaved at the plasma membrane. Biotinylated Jurkat cells were incubated either alone or with the indicated reagents for 90 min at 37°C in complete culture medium. Concentrations of Brefeldin A, chloroquine, E64, vanadate and staurosporine were 10 μg/ml, 100 μM, 5 μg/ml, 100 μM, and 500 nM, respectively. Biotinylated CD100 molecules in membranes and supernatants were then immunoprecipitated and analyzed in nonreducing conditions.
Staurosporine enhances sCD100 release

We previously demonstrated the association of the cytoplasmic tail of CD100 with a cellular serine kinase that can phosphorylate the molecule and also with CD45, a protein tyrosine phosphatase (9–11). To investigate whether these associations were involved in cleavage and release of sCD100, we used staurosporine, a broad spectrum, cell-permeant inhibitor of serine kinase, and vanadate, an inhibitor of protein tyrosine phosphatases. As shown in Fig. 5, staurosporine dramatically enhanced sCD100 release. No obvious change in the amount of sCD100 was observed with vanadate. This observation was further confirmed by incubating biotinylated cells with increasing amounts of staurosporine. We observed an increased shedding of CD100, well correlated with its down-modulation from the membrane. A plateau was reached at about 500 nM (Fig. 6). As found with untreated cells, the increased shedding of CD100 observed with staurosporine was strongly reversed in the presence of EDTA. This suggests that the half-life of the dimer at the cell surface is rather short. Importantly, we also show in the present report by mutational analysis that cysteine 674 of CD100 is necessary for dimerization and, thus, that it may form one intermolecular disulfide bridge. Owing to the size of the released CD100 fragments and the position of this residue, near the membrane and outside the sema domain, it is likely that cleavage occurs just below this cysteine, releasing the whole sema and Ig-like domains of CD100. Quite interestingly, mutation of a single cysteine residue found at a similar position in semaphorin D, a secreted class III semaphorin, also prevents dimerization and abolishes the biological activity of the molecule (31).

The mechanism responsible for the shedding of CD100 is as yet unclear. Zinc metalloproteases are frequently involved in the cleavage of membrane-anchored proteins. We found, however, that the zinc chelator 1,10-phenanthroline, a very good inhibitor of this kind of metalloprotease, did not block sCD100 release (see Table I). Intriguingly, we even repeatedly found an increase in CD100 release with 1,10-phenanthroline, but also PMSF and L-phenylsulfonyl-2-phenylethyl chloromethyl ketone. It is noteworthy that all these reagents contain an aromatic group that may interfere with some aliphatic residues, thus inducing changes in the conformational structure of the CD100 molecule that may favor the shedding process. CD100 shedding was also not altered by MMP inhibitors such as GM 6001, which inhibits many MMPs in the subnanomolar range. Taken together, these results suggest that CD100 is released via a particular proteolytic pathway and that the mechanism involved in the cleavage of the molecule is probably different from those as yet described (18, 32).

The proteolytic cleavage of surface molecules often involves structural requirements and post-translational changes, such as phosphorylation and modification of the glycosylation state of the molecule (33–35). Staurosporine, a cell-permeable, broad range inhibitor of serine/threonine kinases, has an activating effect on sCD100 release. Once again this observation is unique and differs from what has been reported for several surface molecules, such as TNF receptor (36). This result underlines the importance of serine phosphorylation events in regulating CD100 cleavage and ultimately suggests the possible involvement of the serine kinase activity associated with CD100 in the regulation of its cell membrane expression and release. We previously reported that one CD100-specific mAb, termed BB18, stabilized the association of CD100 with its associated serine kinase, in contrast to another CD100
mAb, mAb BD16, which is directed against a physically distinct and functionally different epitope of the molecule (2, 37). Interestingly, we also reported that only BD16 increased CD100 shedding, suggesting that the kinase association could prevent CD100 cleavage. It should be noted that internalization of CD100 from the cell surface has also been reported in the CEM T cell line (38). This is not contradictory to our present report, since the two mechanisms (internalization and shedding) have been described for other surface molecules, such as CD71 and TNF receptor (30, 39).

The chemorepulsive activity of the secreted semaphorin III/D has been recently reported to be a consequence of the covalent dimerization of native monomers (31, 40). In a parallel work the chemorepulsive activity of semaphorin III/D was also reported to be enhanced by a furin-dependent proteolytic processing (41). This secreted semaphorin III/D is essential for normal patternning and growth of various tissues and cells, including nerve, bone, and heart (42). This raises the possibility that the induction of CD100 function may be mediated by the dimerization of monomeric mCD100 and its subsequent release as an active soluble factor. In the nervous system diffusible semaphorins can act as a long range guidance cue on the growth and migration of axon tips during nerve development (4). These molecules mediate their effects in the nervous system by interacting with complexes of neuropilins andplexins, a family of semaphorin receptors (43–48). We show in the companion paper (49) that sCD100 exerts an inhibitory role on immune cell migration. We also demonstrate that H-semaIII semaphorin, another diffusible semaphorin in the nervous system (44), inhibits immune cell migration, and that in both cases inhibitions would be due to interaction between the two soluble semaphorins with a receptor distinct from neuropilins. We additionally demonstrate that only dimers of soluble CD100 mediate this effect, underlying the biological interest of the results presented herein about the dimerization process of the molecule and its release as a soluble factor.

References


