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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 Bismuth3*

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.

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Investigating this issue, we report here the existence of a spontaneous soluble form of CD100 through spontaneous proteolytic shedding. CD100 dimers are predominantly released in T cells, and we show that proteolytic cleavage is favored by dimerization of the molecule at the cell membrane. We also report the essential role played by a particular cysteine residue in the extracellular domain of the molecule in the formation of the dimer. Our results finally suggest the regulation of CD100 shedding by phosphorylation events that probably account for ultimate structural changes in CD100 that render the molecule sensitive to the proteolytic pathway in its dimeric form. Thus, CD100 appears to be a unique semaphorin because it exists as both a transmembrane and a diffusible molecule in the immune system.

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 L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone) (4), metalloprotease inhibitors (EDTA, EGTA, phosphoramidon, and 1,10-phenanthroline), cysteine and cysteine/proximate protease inhibitors (E64, HgCl2, leupeptin, and PMSF), and aspartic protease inhibitor (pepstatin). Staurosporine (a cell-permeant, serine/threonine phosphatase inhibitor), chloroquine (an inhibitor of endocytosis), Brefeldin A (an inhibitor of exocytosis and protein transport), and small molecule inhibitors of T cell receptor (TCR) signaling, such as 5-fluorouracil (5-FU), pertussis toxin (PTX), and aspartic protease inhibitors (pepstatin) were used in this study. JC0174, a specific inhibitor of CD100 shedding, was provided by Dr. I. Pettengell (St. George’s Hospital Medical School, London, United Kingdom).

Results

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

Jurkat T cells express a high level of CD100. To study the fate 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 be shed in a soluble form as a homodimer. We observed a single band at around 150 kDa (Fig. 1A). This shedding was confirmed by a homogeneous 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 under reducing conditions. sCD100 was 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 150-150 kDa homodimer that undergoes membrane cleavage, releasing the homodimeric 120-/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
CD100 dimers in the supernatant was not affected. The drug con-
troller, and MMP-3 inhibitor II. As shown in Fig. 2 was also evaluated using GM6001, a broad spectrum MPP inhib-
sCD100 release. The sensitivity of the shedding to MMP inhibitors
EGTA shows that a metalloprotease activity may account for
The partial inhibition of the shedding of sCD100 by EDTA and
4°C, showing that an enzymatic process was probably involved.
SDS-PAGE in nonreducing (N-Red) or reducing conditions (Red). The labeled proteins were revealed by streptavidin peroxidase using an ECL detection method. B, Time-course analysis of sCD100 release by Jurkat cells was performed by incubating the biotinylated cells for different periods of time in complete culture medium before CD100 immunoprecipitation.

FIGURE 1. Soluble CD100 is produced as a dimer by the Jurkat T cell line. A, Biotinylated Jurkat cells were cultured for 90 min in complete culture medium at 37°C. Immunoprecipitations of CD100 in cell lysates (M) and cell supernatants (S) were performed with CD100-specific mAbs and protein A-Sepharose as described in Materials and Methods. Immunoprecipitates were migrated on 8% SDS-PAGE in nonreducing (N-Red) or reducing conditions (Red). The labeled proteins were revealed by streptavidin peroxidase using an ECL detection method. B, Time-course analysis of sCD100 release by Jurkat cells was performed by incubating the biotinylated cells for different periods of time in complete culture medium before CD100 immunoprecipitation.

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>
</tr>
<tr>
<td>Chymostatin</td>
<td>Serine proteases (chymotrypsin)</td>
<td>21</td>
<td>20 μM</td>
</tr>
<tr>
<td>E-64</td>
<td>Cysteine proteases</td>
<td>30</td>
<td>10 μM</td>
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<td>EDTA</td>
<td>Metalloproteases</td>
<td>65</td>
<td>5 mM</td>
</tr>
<tr>
<td>EGTA</td>
<td>Metalloproteases</td>
<td>55</td>
<td>5 mM</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>Cysteine proteases</td>
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<td>0.5 mM</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Serine/cysteine proteases</td>
<td>0</td>
<td>50 μg/ml</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>Aspartic proteases</td>
<td>0</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>Metalloproteases</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5 mM</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>Metalloproteases (CD10)</td>
<td>12</td>
<td>50 μM</td>
</tr>
<tr>
<td>PMSF</td>
<td>Serine/cysteine proteases</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 mM</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>Serine proteases (trypsin)</td>
<td>0</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>TPCK</td>
<td>Serine proteases (chymotrypsin)</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50 μM</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

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

<sup>c</sup> 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%.

<sup>d</sup> These inhibitors enhanced sCD100 release.

IAA treatment increases the dimerization of membrane CD100 and the release of its soluble form

The extracellular domain of CD100 has several cysteine residues. By forming a disulfide bridge some of them may be involved in the folding of monomeric mCD100 or in the protein homodimerization process of the molecule at the 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 M<sub>r</sub> 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 modeling of cysteine reactivity into CD100 structure, are needed for its dimerization and subsequent release.

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.
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
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-p-tosylamino-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). Stauroporine, 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 underscores 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-specific mAb, which decreased the association of CD100 with its associated serine kinase (37, 38). The mechanism by which these two mAbs induced a change in the CD100 association with its serine kinase was different. One mechanism involved the phosphorylation of serine/threonine residues of CD100, thus favoring the dimerization of CD100. This is in agreement with the results obtained by mutational analysis that cysteine 674 of CD100 is necessary for homodimerization (37, 38). The other mechanism involved the phosphorylation of the serine kinase that can phosphorylate the CD100 molecule. The phosphorylation of serine/threonine residues of CD100 may thus stabilize the association of CD100 with its cell-permeant, broad range inhibitor staurosporine.}

**FIGURE 6.** Soluble CD100 release is enhanced by the serine kinase inhibitor staurosporine. Biotinylated Jurkat cells were incubated with increasing amounts of staurosporine for 90 min at 37°C in complete culture medium. Membrane and supernatant fractions were then analyzed for the presence of CD100 in nonreducing conditions. The effect of EDTA (5 mM) is also shown.
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 chemorepellive 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 chemorepellive 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 patterning 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 and plbexs, 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 different from neuropilins. We additionally demonstrate that only dimers of soluble CD100 mediate this effect, underlining the biological interest of the results presented herein about the dimerization process of the molecule and its release as a soluble factor.

References


