Original Article

Identification and assignment of three disulfide bonds in mammalian leukocyte cell-derived chemotaxin 2 by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Summary **Mammalian leukocyte cell-derived chemotaxin 2 (LECT2) contains six evolutionarily conserved cysteine residues. To date, however, the presence of disulfide linkages between these residues has not been determined. To search for disulfide bonds, the protein was proteolitically digested and the resulting peptides were analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The analysis showed that murine and human LECT2 have three intramolecular disulfide bonds (Cys25-Cys60; Cys36-Cys41; Cys99-Cys142) and no free cysteine residues.**

Keywords: Leukocyte cell-derived chemotaxin 2, disulfide bonds, MALDI-TOF mass spectrometry, trypsin, Asp-N

1. Introduction

Leukocyte cell-derived chemotaxin 2 (LECT2) was originally named for its possible neutrophil chemotactic activity *in vitro* (*1*). Since the first identification of LECT2 in mammals, homologous genes have been identified in many vertebrates, including agnathans, teleosts, amphibians, crocodilians, and avians (Figure 1). LECT2 seems to be widely conserved in vertebrates. In avians, myb-induced myeloid protein-1 (Mim-1) consists of two imperfect repeats that are each homologous to LECT2 (*1*).

Murine and human LECT2 are expressed preferentially in the liver in a constitutive manner, and are secreted into the bloodstream (*2*). To elucidate the role of mammalian LECT2 *in vivo*, we generated LECT2 knockout mice and found that LECT2 plays an important role in pathological changes associated with hepatic injury and inflammatory arthritis (*3,4*). Other researchers reported that LECT2 could function as a growth-stimulating factor for chondrocytes and osteoblasts (*5,6*), as a Wnt signaling repressor (*7*), and as a renal amyloid protein (*8*). Overall, the accumulating evidence suggests that LECT2 is a pleiotropic protein, as are many cytokines. Characterization of this protein may provide insights of value for the therapeutic treatment of LECT2-related diseases, such as rheumatoid arthritis.

Murine and human LECT2 are both comprised of 151 amino acids that contain a signal peptide. The mature protein consists of 133 amino acids that include six completely conserved cysteine residues in all reported mammals (Figure 1). Interestingly, the six cysteine residues are present only in cyprinid fish and catfish; most teleost fish species lack the second and third cysteine residues of mammalian LECT2. To date, the assignment of disulfide linkages in LECT2 has not been reported.

In this study, we have determined the disulfide

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Figure 1. Multiple alignment of the deduced amino acid sequences of LECT2 of various species. Sequences were aligned using the ClustalX program. The black shading indicates the six conserved cysteine residues. Sequences for the comparisons were obtained from GenBank. Accession numbers: mouse, BAA33383; human, BAA23609; bovine, NP_776805; sea lamprey, CO553119; chicken Mim-1, NP_990809; American alligator, ES321039 (partial sequence); Chinese giant salamander, EG018563; carp, BAB16024; zebrafish, XP_695533; channel catfish, FD317410; rainbow trout, AF271114; barramundi perch, ABV66068. Identical residues are indicated with asterisks.

linkages in murine and human LECT2 using in-gel protease digestion and matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

2. Materials and Methods

2.1. *Purification of murine and human LECT2*

Recombinant murine LECT2 (GenBank accession number: BAA33383) and human LECT2 (BAA23609), produced by transfection of Chinese hamster ovary cells, were purified by the same procedures as described previously (*9*).

2.2. *Mass spectrometry*

To investigate whether murine and human LECT2 have free cysteine residues, the purified proteins were treated with 1 mM sodium iodoacetate in the dark at room temperature for 30 min. They were then dialyzed against 50 mM NaH_2PO_4-NaOH (pH 7.4) and analyzed with an ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). To identify the arrangement of disulfide bonds in murine recombinant LECT2, the protein was partially purified by CM-sepharose chromatography and separated by SDS-PAGE under non-reducing conditions. A gel slice containing murine LECT2 was excised, washed, and dried under reduced pressure. The dried gel slice was treated with a reducing solution containing 100 mM dithiothreitol and 10 mM Tris-HCl (pH 7.5) for 30 min at 57°C. Alkylation of the protein in the gel was carried out in a solution containing 100 mM iodoacetate and 10 mM Tris-HCl (pH 7.5) at 37°C for 30 min. The gel was then treated with sequencing grade modified trypsin (Promega) for 18 h at 37°C in 10 mM Tris-HCl buffer (pH 7.5) or endoproteinase Asp-N (Roche Diagnostics) for 18 h at 37°C in 10 mM Tris-HCl buffer (pH 7.5). The peptides produced in this final digestion were subjected to MALDI-TOF mass spectrometry.

Purified recombinant human LECT2 was directly digested with sequencing grade modified trypsin for 18 h at 37°C in 10 mM Tris-HCl buffer (pH 7.5). The digest was then treated with the reducing solution for 30 min at 57°C, followed by the alkylating solution at 37°C for 30 min. The resulting peptides were subjected to MALDI-TOF mass spectrometry.

3. Results

To identify possible disulfide linkages between the six cysteine residues in mammalian LECT2, we first treated murine LECT2 with iodoacetate to substitute a carboxymethyl group for the hydrogen of any thiol groups present in the cysteine residues. Treated and untreated proteins were then analyzed by MALDI-TOF mass spectrometry. The main peak of treated murine LECT2 was observed at *m/z* 14630.8 and that of untreated protein at *m/z* 14632.1. Since the estimated mass of murine LECT2 with three intramolecular disulfide bonds is *m/z* 14631.5 [$M+H^+$], our results indicate that most murine LECT2 is monomeric with three intramolecular disulfide bonds.

Next, we sought to determine the positions of the disulfide bonds. Iodoacetate-treated murine LECT2 was separated by SDS-PAGE and the gel slice containing the

protein was digested with trypsin and divided equally into two pieces. One of the gel pieces was reduced with dithiothreitol and then treated with iodoacetate. The non-reduced and reduced peptides mixtures were analyzed by MALDI-TOF mass spectrometry (Table 1, upper column). Two peaks at *m/z* 1536.5 and *m/z* 2222.8 were observed in the analysis of non-reduced fragments. These peaks correspond closely to the estimated mass of disulfide peptides with a linkage between Cys36 and Cys41. After the reducing treatment, we did not detect peaks for a peptide with the disulfide linkage Cys36-Cys41. In place of these peaks, we observed two peaks at *m/z* 1654.7 and *m/z* 2341.0 that correspond to peptides containing carboxymethylated Cys36 and carboxymethylated Cys41, respectively. We also analyzed murine LECT2 fragments produced by endoproteinase Asp-N digestion (Table 1, lower column). A peak at *m/z* 2847.2 was observed in the mass spectra of non-reduced peptides. This corresponds to the estimated mass of a peptide with a disulfide linkage between Cys36 and Cys41. After Asp-N digestion of reduced and carboxymethylated fragments, this peak was replaced by two new peaks in the mass spectra at *m/z* 2052.8 and *m/z* 2304.9. These correspond to a carboxymethylated Cys36-containing peptide and a carboxymethylated Cys41-containing peptide. Furthermore, in the mass spectrometric analysis of the non-reduced peptides resulting from trypsin or Asp-N digestion, we did not observe any peptide peaks corresponding to disulfide linkages associated with Cys36 or Cys41 except for the Cys36-Cys41 disulfide bond. Overall, these data indicate that Cys36 and Cys41 would form a disulfide bond in murine LECT2.

Under the non-reducing conditions of the trypsin digest, a peak was also observed at *m/z* 3095.3. This peak corresponds to the estimated mass of a disulfide peptide with a linkage between Cys99 and Cys142 (Table 1, upper column). In the mass spectra of the trypsin digest, we did not detect any peaks corresponding to other disulfide linkages associated with Cys99 or Cys142 except for the Cys99-Cys142 disulfide bond. Under the reducing and carboxymethylating conditions, the disulfide peptide with the linkage between Cys99 and Cys142 detected under non-reducing conditions could theoretically be resolved into two smaller peptides with mass of *m/z* 2603.2 and *m/z* 611.3. These peaks correspond to a carboxymethylated Cys142-containing peptide and a carboxymethylated Cys99-containing peptide. The former peptide was clearly identifiable at *m/z* 2603.2, but the latter peptide was too small for unambiguous assignment. Overall, these results indicate that Cys99 and Cys142 would form a disulfide bond.

As described above, it was shown that murine LECT2 does not have free cysteine residues, and would have Cys36-Cys41 and Cys99-Cys142 disulfide bonds. We sought to detect the disulfide link between Cys25 and Cys60 by a mass spectrometric analysis of an endoproteinase Asp-N digest (Table 1, lower column). We observed a peak in the analysis of non-reduced fragments at *m/z* 1867.8. This corresponds to the mass of a peptide fragment consisting of Gly19-Asn31 and Asp57-Ser61 formed by a disulfide bond between Cys25 and Cys60. We could not detect any peaks corresponding to other disulfide linkages associated with Cys25 or Cys60 except for the Cys25-Cys60 disulfide bond. In the analysis of the reduced and carboxymethylated endoproteinase Asp-N digest, this peak was replaced by two new peaks at *m/z* 1392.6 and *m/z* 2052.8. These correspond to peptides containing carboxymethylated Cys25. These results clearly indicate that Cys25 and Cys60 would form a disulfide bond.

Table 1. Assignments of cysteine-containing fragments produced by trypsin or Asp-N digestion of murine LECT2

	Observed mass [M+H$^+$]	Expected mass [M+H$^+$]	Peptide	Cysteine residues, S–S bonds
Trypsin				
	Non-reduced and iodoacetate-treated LECT2			
	1536.5	1536.6	Thr35-Arg48	Cys36-Cys41
	2222.8	2222.9	Ser29-Arg48	Cys36-Cys41
	3095.3	3095.4	Gly97-Lys101 + Ile129-Leu151	Cys99-Cys142
	Reduced and carboxymethylated LECT2			
	1654.7	1654.7	Thr35-Arg48 + 2 Cm	Cys36, Cys41
	2341.0	2341.0	Ser29-Arg48 + 2 Cm	Cys36, Cys41
	2603.2	2603.2	Ile129-Leu151 + 1 Cm	Cys142
Asp-N				
	Non-reduced and iodoacetate-treated LECT2			
	1867.8	1867.8	Gly19-Asn31 + Asp57-Ser61	Cys25-Cys60
	2847.2	2847.3	Glu32-Val56	Cys36-Cys41
	Reduced and carboxymethylated LECT2			
	1392.6	1392.6	Gly19-Asn31 + 1 Cm	Cys25
	2052.8	2052.9	Gly19-Cys36 + 2 Cm	Cys25, Cys36
	2304.9	2305.0	Asp37-Val56 + 1 Cm	Cys41

Cm: carboxymethyl group

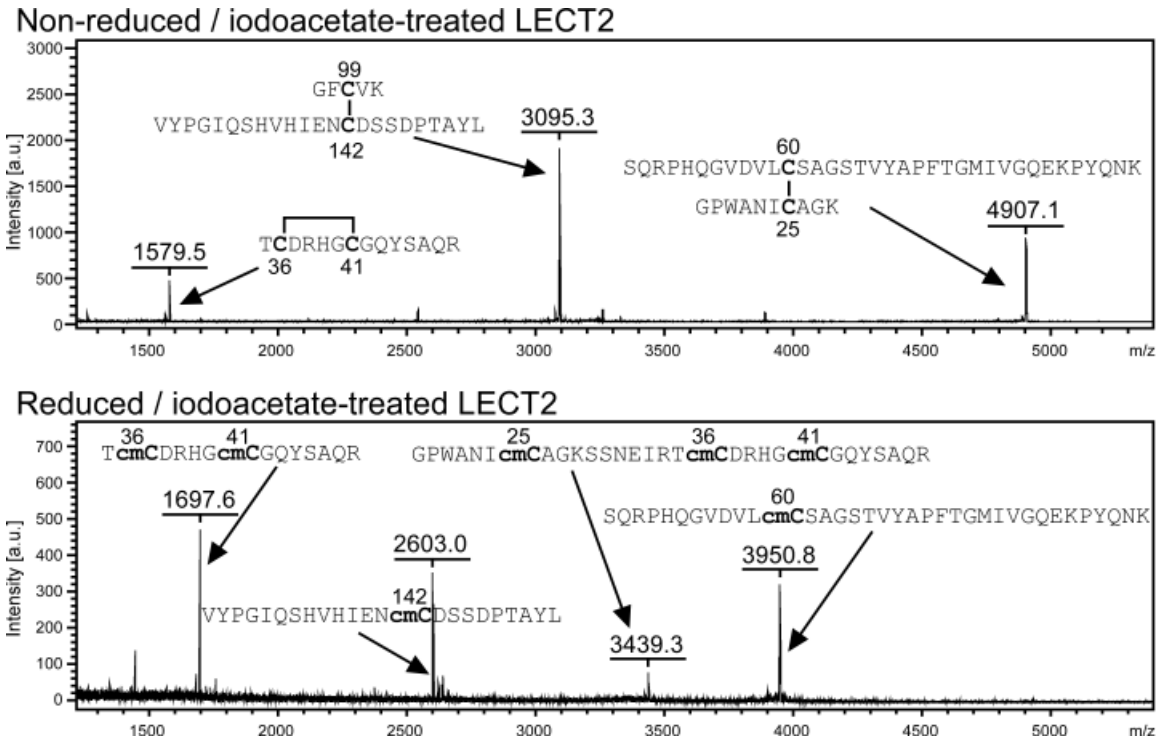

Figure 2. Representative MALDI-TOF mass spectra of trypsin digested human LECT2 peptides and treated with iodoacetate under non-reducing (upper) and reducing (lower) conditions. Bold C and cmC indicate disulfide cysteine and carboxymethylated cysteine residues, respectively.

To determine whether the three disulfide bonds identified in murine LECT2 are present generally in mammalian LECT2, we analyzed human LECT2 by the same procedures described above. Recombinant human LECT2 was treated with iodoacetate and analyzed by MALDI-TOF mass spectrometry. This analysis indicated that protein generally exists as a monomer and has three intramolecular disulfide bonds that are the same as those of murine LECT2 (data not shown). After trypsin digestion of human LECT2, we found that the resulting peptides gave three prominent peaks at *m/z* 1579.5, *m/z* 3095.3, and *m/z* 4907.1 (Figure 2). These peaks correspond to peptides with the disulfide linkages Cys36-Cys41, Cys99-Cys142, and Cys25-Cys60, respectively. Analysis of the reduced and carboxymethylated fragments produced four peaks at *m/z* 1697.6, *m/z* 2603.0, *m/z* 3439.3, and *m/z* 3950.8. These results indicated that human LECT2 has three intramolecular disulfide bonds, Cys25-Cys60, Cys36-Cys41, and Cys99-Cys142. This conclusion is consistent with that for murine LECT2.

4. Discussion

In this study, mass spectrometric analyses of murine and human LECT2 treated with iodoacetate showed that the proteins had no free cysteine residues. Moreover, mass spectrometric analysis of the products of digesting murine and human LECT2 with two different types of proteases indicated that the protein of both species had six cysteine residues involved in the formation of three disulfide bonds. These bonds were present between the first and fourth, second and third, and fifth and sixth cysteine residues, suggesting that assignment of these three disulfide bonds is likely to be conserved in many vertebrates. On the basis of these results, we suggest that the teleost LECT2, which lacks the two cysteine residues corresponding to the second and third cysteine residues of mammalian LECT2, may form two disulfide bonds between the first and second cysteine residues and the third and fourth cysteine residues. Likewise, in the chicken, which has two imperfect repeat units of Mim-1 with an extra cysteine residue between the fifth and sixth cysteine residues of mammalian LECT2 (Figure 1), we suggest that the latter two cysteine residues will not participate in a disulfide linkage.

Recently, the amino acid sequence of the C-terminal domain of LECT2 was categorized in the Pfam database as belonging to the peptidase M23 (PF01551) superfamily (*10*). However, we were unable to find any evidence of peptidase activity in a highly purified preparation of recombinant murine and human LECT2 (data not shown).

The disulfide bond between the second and third cysteine residues forms a tight intrachain loop in the N-terminus. Similarly, two cysteine residues separated by four amino acid residues have been reported in other proteins such as oxytocin and the insulin A-chain. As shown in Figure 1, the region around this disulfide bond has the common amino acid sequence $NX_2RXCDX_{2\sim4}GCG$ in most species, suggesting that the corresponding region in fish not only has the two

cysteine residues, but also forms a loop. This loop structure might be a key structural motif for LECT2 function.

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