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Amino Acid Sequence Determination of Human S100A12 (P6, Calgranulin C, CGRP, CAAF1) by Tandem Mass Spectrometry[1]

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S100A12 has been isolated from human neutrophils. The molecular weight and the amino acid sequence of S100A12 was determined by electrospray-mass spectrometry, tandem mass spectrometry and Edman microsequence analysis. Interestingly, a sequence comparison of S100A12 with all known human S100 proteins revealed that S100A12 is the most divergent of the S100 proteins.

The Ca^{2+}- (and Zn^{2+})- binding S100 proteins exert multiple intra- and extracellular functions and their altered gene expression is often associated with human diseases (1). As an example, S100A8 (MRP-8) and S100A9 (MRP-14), expressed in cells of myeloid origin, are linked to or useful indicators of inflammatory rheumatic diseases (2), cystic fibrosis (3) and different stages of HIV infections (4). Recently a protein of mol. wt. 6.5 kDa (P6) immunologically related to S100A8 and S100A9 was detected in human neutrophils (5). A partial amino acid sequence analysis revealed that P6, now renamed S100A12,[4] is a novel member of the S100 protein family. We purified S100A12 from human neutrophils and determined its protein sequence by tandem mass spectrometry and microsequence analysis.

MATERIALS AND METHODS

Purification of S100A12 from human neutrophils. Neutrophils were isolated from 450 ml of fresh buffy coats by dextran sedimentation and the cytosolic fraction was produced as described (5). This fraction was precipitated by adding ammonium sulfate to 29%. After centrifugation, the supernatant was adjusted to 2 mM $CaCl_2$ and applied to a phenylsepharose affinity column (Pharmacia, 1×5 cm; flow 0.5 ml/min). Proteins were eluted with 20 mM Tris-HCl, pH 7.5, and 5 mM EGTA. S100A12 was finally purified by reversed phase-HPLC using a Brownlee C_8-column (2.1 mm × 10 cm). Solvents were (A) 0.1 % trifluoroacetic acid and (B) 0.1 % trifluoroacetic acid in 80 % acetonitrile; a gradient of 40-70 % B in 45 min and a flow of 50 μl/min was applied. Homogenous S100A12 was eluted at 62 % B.

Gel electrophoresis. Reducing tricine SDS-PAGE (15 %) and non-reducing taurine SDS-PAGE (5-15 %) were performed as described (6). Isoelectric focusing was performed under native conditions (7). Carrier ampholytes, pH 3-10 and pH 4-6.5, were from Pharmacia. Proteins were stained with Coomassie blue.

Enzymatic cleavage and peptide separation. 50 μg of S100A12 was digested with trypsin (Boehringer-Mannheim).

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[1] The sequence of human S100A12 was submitted to the EMBL data library and is available under the accession number P80511.

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[4] Nomenclature: Human S100A12 (synonymes: P6, calgranulin c, calgranulin-related protein (CGRP), calcium-binding protein in amniotic fluid-1 (CAAF1)) and human S100A11 (S100C) are on human chromosome 1q21 (S100A12, Wicki et al., submitted; S100A11, Moog-Lutz et al., (1995) Int. J. Cancer 63, 297-303 and own unpublished results). For nomenclature of S100A1 up to S100A10 see Genomics 25, 638-643 (1995).

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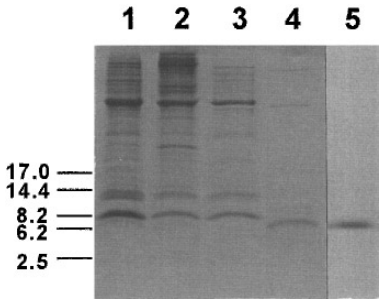

FIG. 1. Purification of S100A12 from human neutrophils: Tricine SDS–PAGE (15%) under reducing conditions. Lane 1: cytosolic fraction of neutrophils (100 μg of protein); lane 2: fraction precipitated with ammonium sulfate (100 μg); lane 3: flowthrough from phenylsepharose column (30 μg); lane 4: eluted proteins with 5 mM EGTA (10 μg); lane 5: purified S100A12 after HPLC purification (10 μg). Proteins were visualized by Coomassie blue.

S100A12 was dissolved in 50 μl cleavage buffer (100 mM Tris-HCl, 0.5 M urea, 20 mM methylamine, 10 % acetonitrile, pH 8.0). The enzyme/substrate ratio was 1:25 (wt/wt). The sample was incubated overnight at 37 °C, followed by immediate peptide separation on reverse-phase HPLC [Brownlee-C_8-column (2.1 mm $\times$ 10 cm); Applied Biosystem 130A].

25 μg of S100A12 was dissolved in 120 μl cleavage buffer (100 mM NH_4HCO_3, pH 8.1) and digested with endoproteinase Glu-C (Boehringer-Mannheim). The enzyme/substrate ratio was 1:20 (wt/wt). The sample was incubated for 48 h at 37 °C, followed by immediate peptide separation on reverse-phase HPLC.

Peptides were eluated with a gradient of 5-65% solvent B in 60 min and a flow rate of 100 μl/min. Fractions were collected manually, dried by speed-vac and redissolved in 5-10 μl solvent A. One μl of each peptide fraction was subjected to MS analysis.

Amino acid sequencing by mass spectrometry. Mass spectra were recorded on a TSQ-70 triple-stage-quadrupole instrument (Finnigan-MAT, San Jose, CA) equipped with a cesium-ion gun (Antek, Palo Alto, CA) (8,9).

The number and location of the acidic Glu and Asp were determined by additional methylation of the peptide fragments and the discrimination between Gln and Lys was achieved by acetylation (8,10,11).

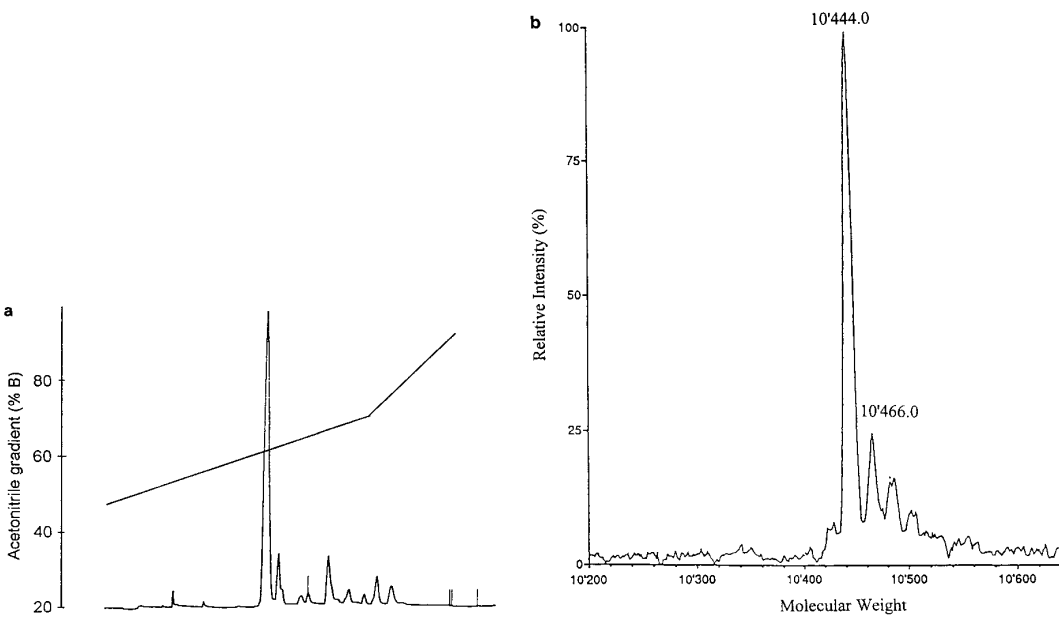

FIG. 2. HPLC separation of S100A12 (a); ESI-MS of S100A12 (b); smaller peaks correspond to $(Na)_n^+$-adducts of the protein.

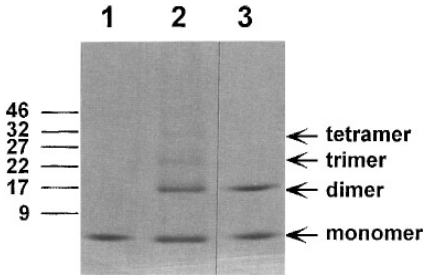

FIG. 3. Electrophoretic properties of human S100 proteins: SDS–PAGE (5–15%) was performed in a taurine buffer system under non-reducing conditions. Lane 1: S100A12; lane 2: S100A2; lane 3: S100A6. Five μg of each protein was applied. Proteins were visualized by Coomassie blue.

Leu and Ile were determined by Edman microsequencing with a pulse liquid-phase microsequencer, model 477A, from Applied Biosystems (Foster City, CA).

Molecular mass determination by electrospray ionization mass spectrometry (ESI-MS). The molecular mass of S100A12 was determined with a Sciex API III instrument, equipped with an ion spray source using a scan range form 500 to 2000 amu with 0.5 amu step size and a dwell time of 1.0 msec. The protein was desalted by reverse-phase HPLC using a BU-300 precolumn (2.1 mm $\times$ 3 cm, Brownlee), and diluted in 10 % acetonitrile, 0.1 % trifluoroacetic acid, followed by driven syringe injection (5 μl/min).

RESULTS AND DISCUSSION

In order to determine the sequence and biochemical properties, S100A12 was isolated from human neutrophils and purified by Ca^{2+}-dependent phenylsepharose chromatography and HPLC (Fig. 1).

The HPLC purification of S100A12 yielded a main peak eluting at 62 % B (Fig. 2a). The molecular mass of S100A12 was determined as 10′444 Da by ESI-MS (Fig. 2b).

To investigate if S100A12 is able to form dimers or higher polymers like several other S100 proteins (6), we subjected S100A12 to taurine SDS-PAGE under non-reducing conditions. S100A12 migrated as a monomer with an apparent molecular weight of 6 kDa (Fig. 3, lane 1). Under the same conditions S100A2 and S100A4 formed dimers, trimers and tetramers (Fig. 3, lane 2 and 3). It remains to be seen if S100A12 is active as a monomer or if it is able to form heteropolymers with S100A8 and/or S100A9. The isoelectric point (IEP) of S100A12 was 6.3 (calculated value: 6.24) under native conditions. S100A12 is a very basic protein when compared to e.g. S100A1 (IEP: 4.3), S100A4 (4.9) or S100A6 (5.3).

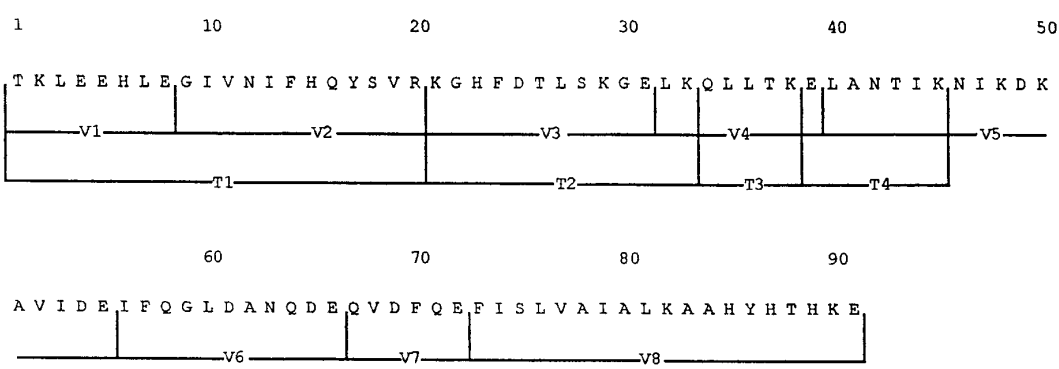

FIG. 4. Primary structure of human S100A12. T1-T4 represent peptides obtained by tryptic digestion and V1-V8 by digestion with endoproteinase Glu-C.

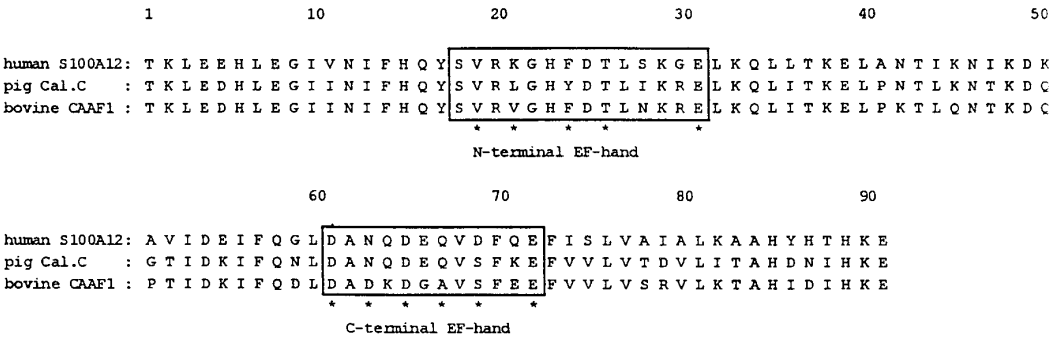

FIG. 5. Sequence comparison of S100A12, calgranulin c (pig) and CAAF1 (bovine). The N-terminal and C-terminal EF-hands are indicated by boxes. The amino acids binding Ca^{2+} are indicated by asterisks.

The primary structure of S100A12 (Mr 10′444) was determined by the combination of tandem mass spectrometry, automated Edman degradation and ESI-MS and showed no post translational modification. Automated Edman degradation was used for all peptides except peptide V7 to discriminate between Leu and Ile. The total fragmentation pattern obtained by the digests with two different proteolytic enzymes is summarized in Fig. 4.

Sequence comparison of S100A12 with the known corresponding proteins calgranulin c from pig granulocytes (12) and CAAF1 from bovine amniotic fluid (13) showed a 70 % and a 78 % identity, respectively (Fig. 5). The C-terminus, important for dimer formation (14) and Zn^{2+}-binding (12), differs in these 3 species in 11 amino acids in pig calgranulin c and 10 amino acids in CAAF1, while the N-terminus differs in 2 amino acids only, comparing the last and the first 20 amino acids of the respective proteins.

The sequence comparison with all known human S100 proteins shows that S100A12 is an unique member of the S100 protein family with a low identity compared to, e.g., 46 % for S100A9, 44 % for S100P and even less for the other proteins.

Nevertheless, S100A12 is a novel member of the S100 protein family, containing EF-hand structural motifs. Experiments are underway to examine the physiological role of S100A12 in human neutrophils.

APPENDIX

During preparation of this manuscript a calgranulin-related protein (CGRP) was purified from extracts of adult *Onchocerca volvulus*, a human tissue-dwelling parasite, and published by Marti et al. in Biochem. Biophys. Res. Commun. 221, 454-456 (1996). From these studies it was uncertain if CGRP is of human origin. The sequence of our S100A12 (isolated from human neutrophils), however, was completely identical to CGRP isolated from *O. volvulus* worm, demonstrating its human origin and the possible role of S100A12 in host-parasite interaction.

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