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AMINO ACID SEQUENCE ANALYSIS OF HUMAN S100A7 (PSORIASIN) BY TANDEM MASS SPECTROMETRY †

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The Ca^{2+}-binding proteins regulate a number of cellular and extracellular activities and deregulations of S100 gene expression are associated with several human diseases. For example, S100A7 is upregulated in psoriatic skin, implicating a link with psoriasis, a chronic inflammatory dermatosis. We purified human S100A7 and determined its protein sequence by tandem mass spectrometry and Edman microsequence analysis. Interestingly, a sequence comparison of S100A7 with all known human S100 proteins showed that S100A7 is the most divergent of all S100 proteins.

The Ca^{2+}-binding S100 proteins regulate a number of intracellular, nuclear, and extracellular activities and deregulations of S100 gene expressions have been found to be linked to several human diseases (1-3).

Recently, the finding of the clustered organization of more than ten S100 genes on human chromosome 1q21 has led to the introduction of a new nomenclature for S100 proteins (4). One of these proteins, the S100A7 (previously referred to as psoriasin) was found to be highly upregulated in human psoriatic skin (5,6), suggesting a possible association of S100A7 with psoriasis, a chronic inflammatory dermatosis affecting ~2% of the population (7). We purified a cytosolic and a

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membrane-associated form of S100A7 from human psoriatic skin and determined the protein sequence by tandem mass spectrometry and Edman microsequence analysis.

MATERIALS AND METHODS

Purification

Psoriatic scales were homogenized in Tris buffer (50 mM Tris/HCl, 25 mM NaCl, 2.5 mM EDTA, 1 mM dithiothreitol, pH 7.5) and subsequently centrifuged at 100,000 *g*. The supernatant was used to purify cytosolic S100A7, using an identical purification procedure as described for epidermal fatty acid-binding protein (E-FABP) (8). In the last step of E-FABP purification, the single S100A7 peak eluted from the MonoQ column at 1.3 min was collected, concentrated, and filtrated through a Superose 12 column (Pharmacia), equilibrated with PBS containing 0.2 M NaCl. Purified cytosolic S100A7 eluted as a dimer with a mol. wt. of 21 kDa.

Membrane-associated S100A7 was extracted from the initial 100,000 *g* pellet. The cell membrane debris was washed 2 times with Tris buffer and subsequently treated for 1 h with 10 mM Tris-HCl, 2 mM EDTA, 10 mM monothioglycerol, 10 % glycerol, 0.8 M KCl, 1 mM PMSF, 10 mM aprotinin, and 10 mg/ml leupeptin, at pH 8.5. This homogenate was ultra-centrifuged, and the supernatant was dialyzed against 20 mM imidazole-acetic acid buffer at pH 6, and concentrated before injection onto a Resource S column (Pharmacia). The proteins were eluted from the column with a linear gradient of 0.06-0.5 M NaCl in imidazole-acidic acid buffer. The membrane-associated S100A7 fractions, eluted at 50-70 mM NaCl, detected by SDS-PAGE as a protein band at 12.5 kDa, were collected and concentrated. A purification step on a Resource S column was performed. Membrane-associated S100A7 was dialyzed against 10 mM sodium phosphate buffer at pH 6.8 and subjected to a Bio-Scale CHT2-I hydroxyapatite column (Bio-Rad) equilibrated with the same buffer. A linear gradient up to 500 mM phosphate was applied; S100A7 eluted at 14.5 mM phosphate. Final purification was achieved through a Superose 12 column as for cytosolic S100A7.

Reduction and derivatization of cysteine residues

Reduction with 10 mM dithiothreitol and reaction with 4-vinylpyridine were performed in 100 mM Tris-HCl, pH 7.4, 1 mM EGTA, and 6 M guanidine/HCl in the dark for 2 h at room temperature (9). The derivatized S100A7 was immediately separated from the other components by reversed phase-HPLC using a narrobore BU-300 pre-column (C_4, 2.1 mm x 3 cm, Brownlee). Solvents were (A) 0.1 % trifluoroacetic acid and (B) 80 % acetonitrile in 0.1 % trifluoroacetic acid. S100A7 was eluted with a gradient of 0-100 % B in 30 min and a flow of 200 µl/ min.

Enzymic cleavage and peptide separation

30 µg of S100A7 was used for each of the following reactions: enzymic digest with trypsin, chymotrypsin, and endoproteinase Asp-N (all enzymes purchased from Boehringer-Mannheim). S100A7 was dissolved in 50 µl cleavage buffer (50 mM NH_4HCO_3, pH 7.8, 1 mM EGTA, 10 % acetonitrile). For the Asp-N cleavage 1 mM EGTA was replaced by 2 mM $CaCl_2$ in the reaction buffer. For each reaction the enzyme/substrate ratio was 1:40 (wt/wt), the incubations were performed at 37° C for 4 h, followed by immediate peptide separation over reverse-phase HPLC. This separation was performed with an Applied Biosystems 130A solvent-delivery system on a Vydac C_8-column (1.0 mm x 25 cm). The peptides were eluted with a gradient of

5 -65 % solvent B in 60 min and a flow rate of 50 µl/min. Fractions were collected manually. The Asp-N digest was separated under the same conditions but 10 % of the eluent was split into the mass spectrometer (Sciex API III) and the molecular mass of the peptides was determined using a scan range from 500 to 2000 amu with 0.5 amu step size and a dwell time of 1.0 msec.

Amino acid sequencing by mass spectrometry
Partial analysis of the amino acid sequence of S100A7 by tandem mass spectrometry was performed as described previously (10,11) using a TSQ-70 triple quadrupole instrument (Finnigan-Mat) equipped with a 15 keV cesium-ion gun (Antek). The number and positions of acidic residues were determined by additional methylation of the fragments and the discrimination between Gln and Lys was achieved by acetylation (10,12). Leu and Ile were determined by amino acid analysis if present in the fragment only once, otherwise the peptide was sequenced additionally by Edman microsequence analysis.

Amino acid sequencing by Edman microsequence analysis
This was carried out using an Applied Biosystems model 477A pulse liquid-phase microsequencer.

Amino acid analysis
This was performed by gas-phase hydrolysis using 6 N HCl followed by conversion with dabsyl chloride. Separation and detection were performed with a Beckman 6300 amino acid analyzer.

Measurement of the molecular mass by electrospray ionization mass spectrometry (ESI-MS)
The molecular mass of both, 5 µg of cytosolic and membrane-associated S100A7, was obtained with a Sciex Api III instrument equipped with an ion spray source using a scan range from 500 to 2000 amu with 0.5 amu step size and a dwell time of 1.0 msec. The protein sample was desalted by reverse-phase HPLC using a BU-300 precolumn (2.1 mm x 3 cm, Brownlee) and diluted in 10 % acetonitrile, 0.1 % trifluoroacetic acid, followed by driven syringe injection (5 µl/min).

RESULTS AND DISCUSSION

Characterization of the cytosolic and membrane-associated forms of S100A7

The purified cytosolic and membrane-bound forms of S100A7 eluted from the gel filtration column as a dimer with an apparent molecular weight of 21 kDa. On SDS-PAGE (in the presence of reducing agent) both forms migrated as monomers with a slightly distinct electrophoretic mobility (Fig. 1). Cytosolic S100A7 (Fig. 1, lane 2) migrates more slowly than the membrane-associated S100A7 (Fig. 1, lane 3). Cytosolic and membrane-associated S100A7 proteins bound calcium on a $^{45}Ca^{2+}$ overlay blot (not shown). Electrospray mass spectrometry of both proteins, however, showed an almost identical molecular mass of 11'365 (+/- 0.7) Da for the cytosolic

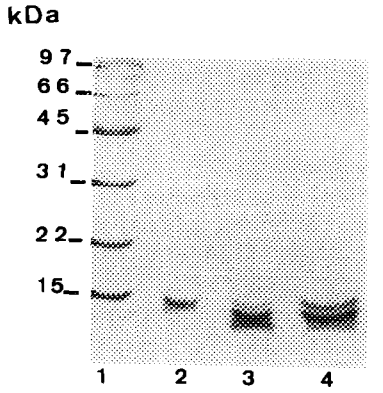

Fig. 1. SDS-PAGE (15 %) of cytosolic and membrane-associated S100A7 in the presence of reducing agent. Lane 1: low mol. wt. standard; lane 2: purified cytosolic S100A7 (1.5 μg of protein applied); lane 3: membrane-associated S100A7 (1.5 μg); lane 4: mixture of both (1.5 μg each). Proteins were stained with Coomassie blue.

and 11'368 (+/- 1.2) Da for the membrane-associated form. The apparent difference in mol. wt. observed in SDS-PAGE between the cytosolic (soluble) and the membrane-associated (insoluble) form could not be confirmed in ESI-MS analysis, even at less acidic mass spectrometry conditions (2 mM NH_4HCO_3, pH 6.5) and suggests that these two forms are conformationally different.

Primary structure of human S100A7

The purified protein was desalted by RP-HPLC and submitted to N-terminal sequence analysis. Because Edman degradation failed, we concluded that the protein is N-terminal blocked. The comparison of the calculated molecular mass deduced from the cDNA sequence (5) of 11'457 Da and the measured mass of 11'368 Da showed a difference of 89 Da. This mass difference can be explained by the loss of the N-terminal methionine and an additional acetylation of the first residue (Ser). This suggestion was confirmed by the endoproteinase Asp-N digest. The fragment A1 was identified as the N-terminal peptide (Fig. 2). Its molecular mass (1461 Da) was determined by both, ESI-MS and FAB triple quadrupole mass spectrometry, and was in agreement with the translated cDNA sequence replacing

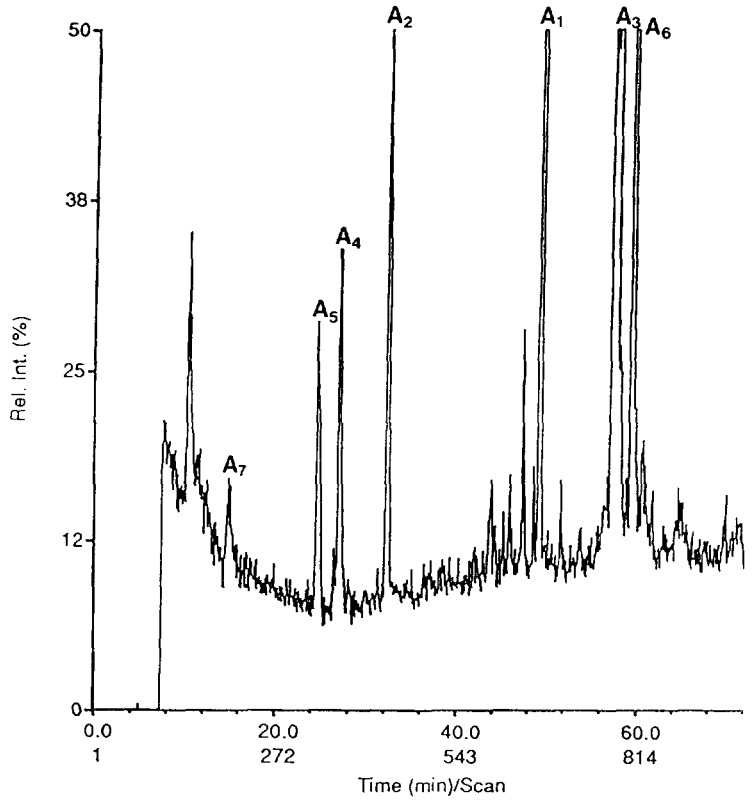

Fig. 2. LC-MS of an endoproteinase Asp-N digest of S100A7: HPLC separation of the digest followed by electrospray ionization mass spectrometry. The relative intensity represents the total ion current. Details of the procedure are described under 'Material and Methods'. Peaks A1-A7 represent peptides whose sequences were determined by tandem mass spectrometry. The nomenclature of the peptides is consistent with Fig. 3.

the N-terminal methionine (-131 Da) with an acetyl group (+42 Da). Its sequence was determined by tandem mass spectrometry. The three residues representing the mass of Leu or Ile were identified by amino acid analysis. The primary structure of the remaining part of the protein was determined by a combination of tandem mass spectrometry, automated Edman degradation and ESI-MS (Fig. 3). Automated Edman degradation also was used for peptides A3, A6, and C2, C6 to discriminate between Leu and Ile. The total fragmentation pattern obtained by the digests with the three different proteolytic enzymes is summarized in Fig. 3. Except for the N-terminal Met, the determined sequence of S100A7 is identical with the reported sequence deduced from the cDNA.

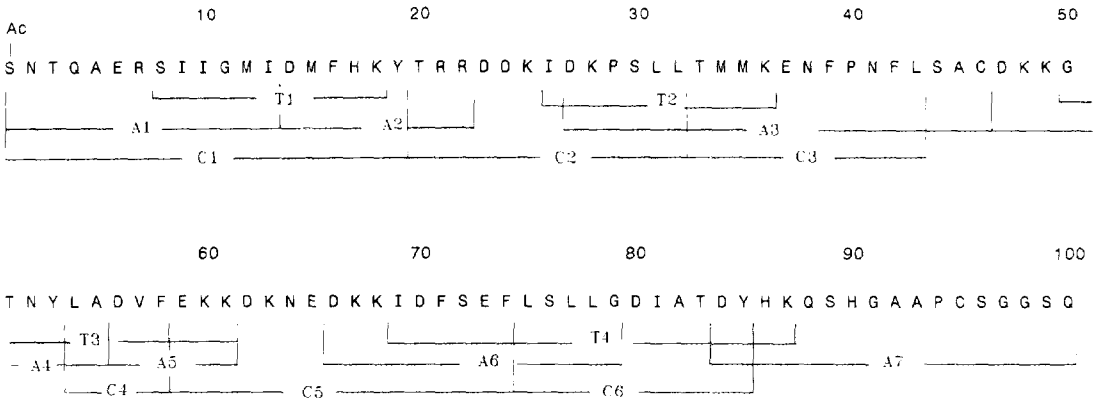

Fig. 3. Primary structure of human S100A7. T1-T4 represent peptides obtained by tryptic digestion, A1-A7 by endopeptidase Asp-N digestion, and C1-C6 by chymotryptic digestion.

A sequence comparison of S100A7 with all known human S100 proteins showed that S100A7 is the most divergent of all S100 proteins with, e.g., only 25 % identity (on the amino acid level) to S100B (Fig. 4). Nevertheless, S100A7 is a new member of the S100 protein family, containing EF-hand structural motifs. In addition, the S100A7 gene, like most other S100 genes, is clustered on chromosome 1q21.

Experiments are underway to examine the role of S100A7 in psoriasis and their

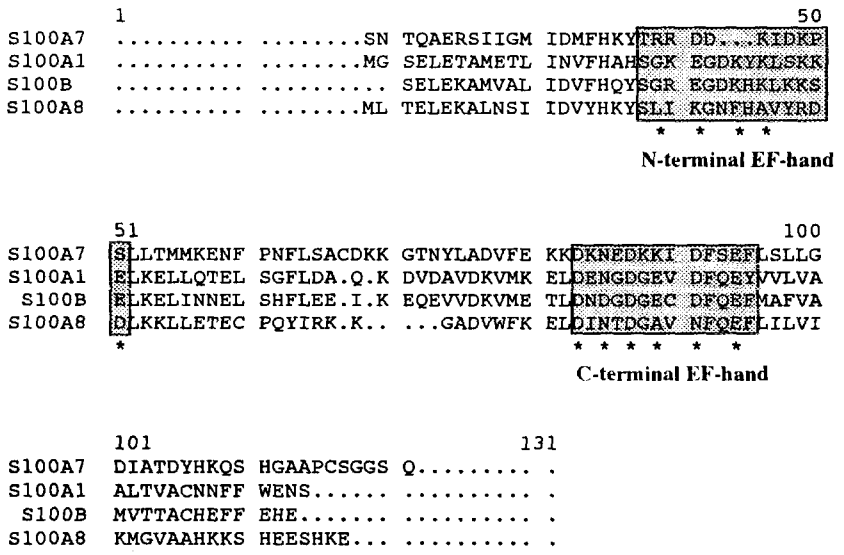

Fig. 4. Sequence comparison between S100A7 and selected members of the S100 family. The amino acid sequences have been aligned and the gaps have been introduced to maximum alignment. The N-terminal and C-terminal EF-hands are indicated by boxes. The amino acids coordinating Ca^{2+} are indicated by asterisks.

possible use in the diagnostic role of skin diseases with abnormal keratinocyte differentiation.

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