FOR THE RECORD

Lysine 58-cleaved β2-microglobulin is not detectable by 2D electrophoresis in ex vivo amyloid fibrils of two patients affected by dialysis-related amyloidosis

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Abstract

The lysine 58 cleaved and truncated variant of β_2-microglobulin (ΔK58-β2m) is conformationally unstable and present in the circulation of a large percentage of patients on chronic hemodialysis, suggesting that it could play a role in the β2-microglobulin (β2m) amyloid fibrillogenesis associated with dialysis-related amyloidosis (DRA). However, it has yet to be detected in the amyloid deposits of such patients. Here, we extracted amyloid fibrils, without denaturation or additional purification, from different amyloidotic tissues of two unrelated individuals suffering from DRA, and characterized them by high-sensitivity bidimensional gel electrophoresis (2D-PAGE), immunoblotting, MALDI time-of-flight mass spectrometry, and protein sequencing. To confirm whether or not this species could be identified by our proteomic approaches, we mapped its location in 2D-PAGE, in mixtures of pure ΔK58-β2m, and extracts of amyloid fibrils from patients, to a discrete region of the gel distinct from other isoforms of β2m. Using this approach, the two known principal isoforms found in β2m amyloid were identified, namely, the full-length protein and the truncated species lacking six N-terminal amino acid residues (ΔN6-β2m). In contrast, we found no evidence for the presence of ΔK58-β2m.

Keywords: dialysis related amyloidosis; amyloid fibrils; proteolized variants of β2-microglobulin; proteomics

In 1985 the team led by Fumitake Gejyo discovered that β2-microglobulin (β2m) is the protein that generates amyloid fibrils in dialysis-related amyloidosis (DRA) (Gejyo et al. 1985). The pathogenesis of this amyloidosis, considered to be a prototypical man-made protein misfolding disease (Dobson 2006), is certainly related to the defective renal clearance of β2m in chronic kidney disease and its inefficient removal from the circulation by dialysis (Yamamoto and Gejyo 2005). The basis of the disease is most likely based on the elevated concentration of β2m, its misfolding propensity (Corazza et al. 2004), and its collagen-philia (Relini et al. 2006). While full-length β2m

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is an ubiquitous and major constituent and an important component of these amyloid fibrils, it has been demonstrated that a N-terminal truncated species lacking six residues (ΔN6-β2m) is present and that this species, with its high tendency to aggregate, could represent an influential element in the process of aggregation (Linke et al. 1987; Bellotti et al. 1998; Esposito et al. 2000). To date no other chemical species of β2m have been consistently reported in natural fibrils and products of mild oxidation and Asn deamidation that are detectable in natural fibrils have not attracted particular interest as a possible agent in β2m amyloidogenesis. However, another variant of β2m, ΔK58-β2m, has been discovered in the sera of ~40% of patients undergoing chronic hemodialysis (Corlin et al. 2005). ΔK58-β2m was discovered in 1987 by Nissen's group (Nissen et al. 1987) and it can be generated by incubating β2m with patient sera at 37°C. Activated C1s is the proteolytic enzyme capable of cleaving the protein at Lys58, giving rise to a new β2m species in which the two interrupted polypeptides corresponding to the sequences 1–58 and 59–99 are still covalently connected through the disulfide bridge at Cys 25–80. An additional cleavage, due to carboxypeptidase B-like activity, occurs in plasma and causes the removal of Lys58, yielding the N-terminal cleaved β2m species (Nissen et al. 1990). ΔK58-β2m so far has only been directly detected in blood samples from hemodialysis patients, but its conversion from parent β2m was also found to be accelerated by incubation with sera from patients suffering from autoimmune diseases, AIDS, and small-cell lung cancer (Plesner and Wiik 1979; Nissen et al. 1984; Bhalla et al. 1985). ΔK58-β2m represents conformer variants of β2m that may be important for the elucidation of the molecular basis of DRA amyloidosis. In vitro studies have demonstrated that in comparison with the intact protein, this species is highly destabilized and at 37°C, in the presence of 10% β2m fibrillar seeds, forms aggregates with amyloid characteristics in <1 h (Heegaard et al. 2005). In solution, ΔK58-β2m populates an intermediate state of folding (Mimmi et al. 2006) that specifically binds Congo red and displays a high propensity to aggregate (Heegaard et al. 2001). The role of ΔK58-β2m in amyloid deposition in vivo is, however, uncertain, since in natural amyloid fibrils this species has never been detected by classical amino acid sequencing or by the combination of two-dimensional (2D) gel electrophoresis and mass spectrometry (Stoppini et al. 2005). However, the effectiveness of this second approach in detecting ΔK58-β2m has never been ascertained. Prompted by these findings and our prior observations concerning potential loss of β2m fragments during typical amyloid extraction/denaturation procedures (Stoppini et al. 2000), we sought to establish whether or not proteomics approaches could identify the ΔK58-β2m variant by comparing β2m isoforms in ex vivo amyloid fibrils (i.e., fibrils obtained from patients) isolated in "native" fibrillar conformation from amyloidotic tissues of two patients with dialysis-related amyloidosis, in the absence and presence of added pure ΔK58-β2m.

Results and Discussion

Amyloid fibrils were isolated under nondenaturing conditions, without additional purification, from two different types of periarticular tissues derived from two unrelated individuals affected by dialysis-related amyloidosis. The morphology and dimensions of the fibrils were first compared by atomic force microscopy (AFM). The fibrils shown in Figure 1A were extracted from the shoulder capsule of a British patient, while the fibrils in Figure 1B were extracted from the amyloid material surrounding the femoral head of an Italian patient; both patients underwent hemodialysis, in the latter case for more than 20 yr. In both preparations the fibrils form bundles, in which adjacent fibrils are often intertwined or partially interconnected. Fibrils are more distinct in the sample extracted from the British patient, while a closer interconnection among fibrils is observed in the sample extracted from the Italian patient. However, the latter contains a larger amount of amorphous material, clearly visible in the background; in this case, fibrils embedded in amorphous material could result somewhat "glued" one to another. In contrast, amorphous material is nearly

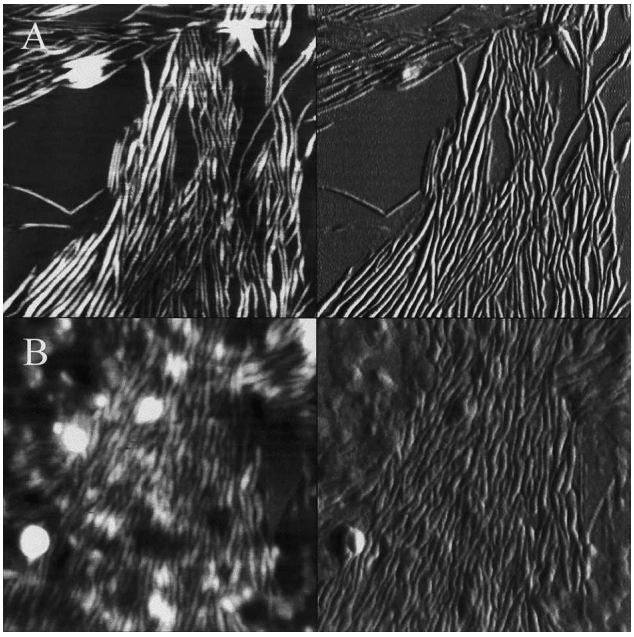

Figure 1. Tapping mode AFM images (*left*, height data; *right*, amplitude data) of natural amyloid fibrils extracted from the shoulder capsule of a British patient (*A*) and from the amyloid material surrounding the femoral head of an Italian patient (*B*). Scan size, 1.2 μm; Z range, 11 nm (*A*), 22 nm (*B*).

absent in the sample extracted from the British patient. Fibrils height, measured from the corresponding height in a cross-section of AFM images, is 2.2 ± 0.1 nm for the British patient and 2.3 ± 0.3 nm for the Italian patient; these values are comparable within experimental error. As the sample was dried for AFM imaging in air, the measured fibril heights are smaller than those expected to correspond to fully hydrated conditions.

Extracted material of both samples was directly analyzed in 2D-PAGE and immunoblotting with polyclonal anti-β2m antibodies. In Figure 2, A and B the immunoblots of amyloid fibrils obtained from the Italian and British patients are shown, respectively. In order to identify all of the spots corresponding to β2m, the blots were immunostained with polyclonal antiserum against human β2m. The samples showed marked electrophoretic heterogeneity with respect to molecular weight and especially for pI.

We have assigned the single spots detected by the antibody to the spots visualized in a correspondent 2D gel stained with Coomassie. In this way, we have identified several spots at ≅12 kDa and slightly lower molecular weight regions that are focused in a pH range from 4.0 to 6.2 that can be assigned to different isoforms of β2m. These spots were processed for the chemical characterization. The N-terminal sequence enabled the identification of the spots 1–6 as full-length β2m and spots 7–9 as the truncated form designated as ΔN6-β2m. These data are consistent with their respective predicted molecular masses of 11,731 and 11,007 Da. MALDI-TOF analysis of protein spots digested with protease Glu-C confirmed these assignments in further detail regarding the length and post-translational modifications. Results indicated that all spots terminate at Met 99 and that the main components are not cleaved at the C-terminal. Spot number 3 presents a partial deamidation of Asn42 and a partial oxidation of Met 99 to methionine sulphone. Spot numbers 4, 5, and 6 present different degrees of Asn17 and Asn42 deamidation, and Met99 partial oxidation. Spot numbers 7, 8, and 9 are characterized by different degrees of Asn17 and Asn42 deamidation, as well as Met99 oxidation.

There is a very close correspondence between spot patterns obtained from the fibrils of the Italian and the British patients. In order to identify a peptide possibly derived from ΔK58-β2m we searched all of the spots for the tryptic peptide corresponding to the sequence 59–75 with a N-terminal Asp residue and a molecular mass of 2203 Da (i.e., the N-terminal tryptic peptide from the B-chain of ΔK58-β2m), but no such peptide was detectable.

The pattern observed in our natural amyloid fibrils (Fig. 2) is remarkably consistent with that reported by Argiles and colleagues in 1995 in a study in which the natural β2m fibrils were analyzed by 2D-PAGE and immunoblotting (Argiles et al. 1995). In order to provide an immediate comparison of those data with our results, the 2D gel electrophoresis reported by Argiles is reproduced here with permission in Figure 3. In the case reported by Argiles, several species of β2m with heterogeneous isoelectric points were distributed along the axis of a common molecular weight

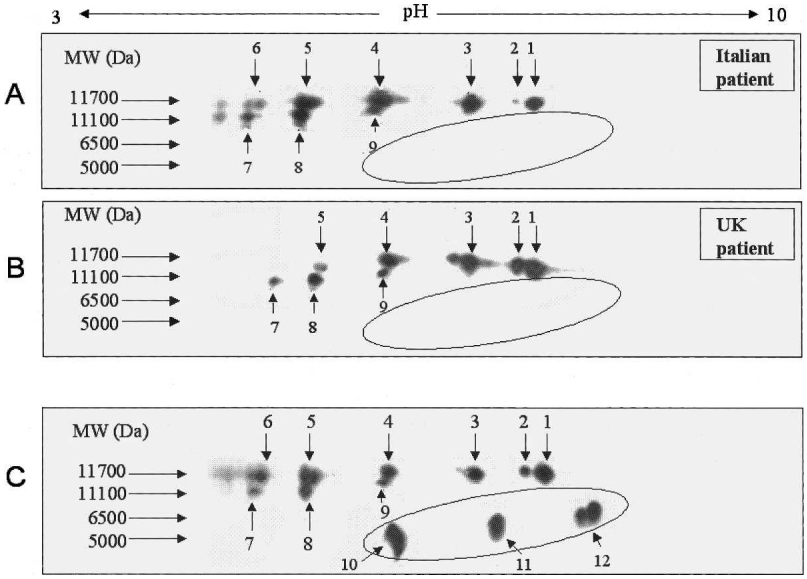

Figure 2. Western blot immunostained with anti β2m antibodies of bidimensional electrophoresis of the following material: (*A*) Fibrils extracted from the amyloid material surrounding the femoral head of the Italian patient; (*B*) fibrils extracted from the shoulder capsule of the British patient; (*C*) mixture 1:1 of amyloid fibrils of the Italian patient and ΔK58-β2m.

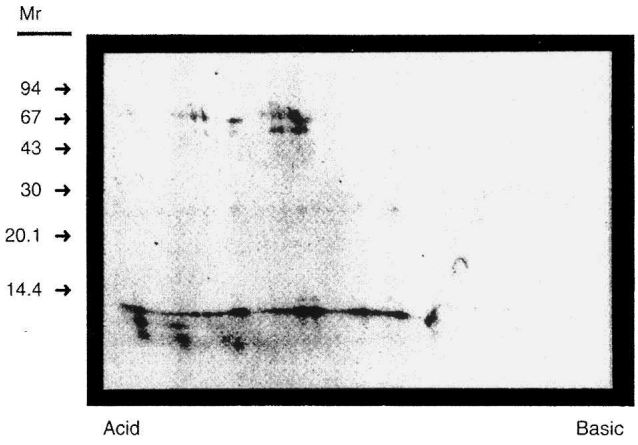

Figure 3. Reproduction with permission from Argiles et al. (1995). Immunoblotting with anti β2m antibodies of 2D-gel electrophoresis of natural amyloid fibrils from a DRA patient (the tissue source was not specified).

of ~12 kDa. These β2m species appear to correspond to spots obtained in our specimens and are indicated in Figure 2, A and B as spots 1–6 that correspond to the full-length β2m (Fig. 2). The spots of isoelectric point and slightly lower molecular mass (Fig. 3) most likely correspond to spots 7 and 8 in our sample (Fig. 2A,B) that we have assigned to ΔN6-β2m.

Interestingly, Argiles et al. (1995) did not report the presence of ΔN6-β2m nor ΔK58-β2m in their purified preparations. One explanation for the apparent absence of ΔN6-β2m, as we have previously suggested (Stoppini et al. 2000), could be related to the aqueous solvent used in the purification steps. In fact, purified ΔN6-β2m, in nondenaturing aqueous solutions, has a strong propensity to self aggregate and irreversibly precipitate (Esposito et al. 2000).

To validate that our approaches, reagents, and procedures could identify the ΔK58-β2m variant if present, we spiked increasing concentrations of the purified protein into amyloid fibrils of the Italian patient. Figure 2C shows that ΔK58-β2m is well visualized in Western blots of 2D-PAGE, migrating as three discrete spots (numbered 10, 11, and 12) that localize in a region of the gel distinct from the other isoforms. Indeed, the sensitivity of the procedure allowed detection of 1 ng of ΔK58-β2m. Notably, as mentioned above, the spots generated by ΔK58-β2m are completely absent in amyloid fibrils to which no pure protein was added (Figs. 2A,B), even after prolonged exposure of the blots.

The isoelectric point and molecular weight species of Figure 2C are consistent with the properties of a reduced ΔK58β2-m. In fact, the predicted pIs of peptides 1–57 (A-chain) and 59–99 (B-chain) are 6.3 and 5.13, respectively, while the molecular weights are 6531 and 5089 Da.

The corresponding spots in a 2D-gel analysis of reduced and S-alkylated ΔK58-β2m were stained by Coomassie blue, excised, and digested with trypsin. Two specific peptides of the A-chain (amino acids 1–57, peptide 20–41 $[M+H^+]$: 2555.87 Da) and of the B-chain (amino acids 59–99, peptide 1–23 $[M+H^+]$: 2999.28 Da) were used as indicators of the origins of the spots. Figure 4 shows different portions of the mass spectra of the four numbered spots excised from the Coomassie-stained gel. Despite differences caused by different pI-resolution, spots 2 and 9 of Figure 4 correspond to spots 12 and 10, respectively, of the immunoblot in Figure 2C. The two additional spots that were identified by mass spectrometry in Figure 4, i.e., spots 3 and 8, correspond to the intermediate material in Figure 2C represented by the splitting of spot 12 and the distinct intermediary spot 11. The exact correspondence between the spots in the two 2D experiments cannot be given more precisely, but the data unequivocally demonstrate that all material originates from ΔK58-β2m. Thus, the marked peaks were only found in spectra of the indicated spots. They were also found in the intermediary material (spots 3 and 8), and thus, both chains have a fraction of material with changed pI compatible with deamidation species as mentioned above for the β2m species found in amyloid extracts. The main result of these experiments is that we show that the ΔK58-β2m species are well resolved in a 2D gel and that no material corresponding to these spots appears in the amyloid fibril extracts from the two patients examined. The sensitivity of the 2D-PAGE immunoblotting method allows the detection of 1 ng of ΔK58-β2m loaded in the gel, i.e., a limit corresponding to about 1% of the amount of material loaded for the immunoblots (data not shown).

Conclusion

In this study we report new data regarding the protein composition of natural amyloid fibrils created by β2m in two different patients suffering from DRA. Particular attention was paid to the identification of the β2m species known as ΔK58-β2m, since this β2m conformer is conformationally unstable and putatively amyloidogenic in vitro (Heegaard et al. 2005), and because it was recently isolated and quantified in serum of patients submitted to hemodialysis (Corlin et al. 2005).

Our data demonstrate that, at least in the two patients studied here, ΔK58-β2m is not detectable in natural fibrils by a highly sensitive procedure such as immunoblotting with chemiluminescence visualization of 2D-PAGE separated extracted components. Through our analytical approach, the specimens are minimally manipulated, and therefore, we believe we can exclude artefacts concerned with the recovery of the putative ΔK58-β2m from the fibrils. The results of the analysis in which we have artificially created a mixture of fibrils and

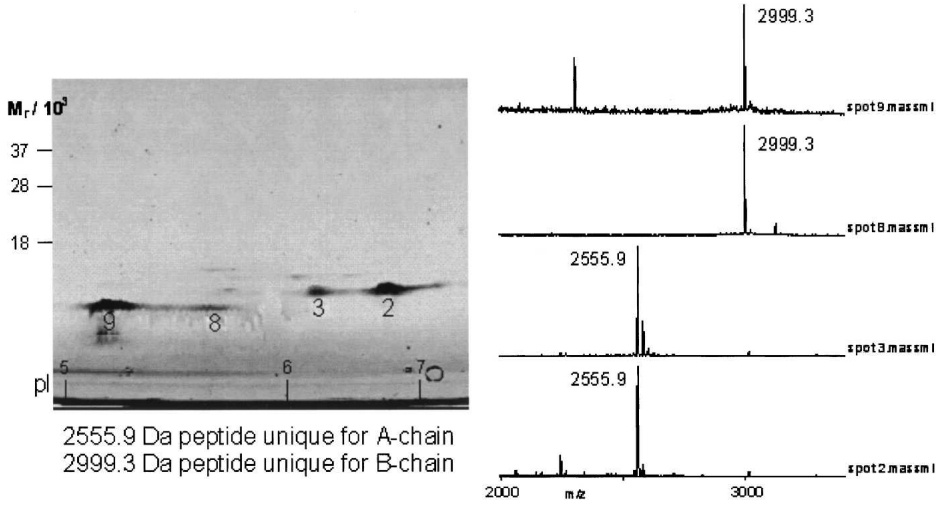

Figure 4. Spot identification by 2D-gel electrophoresis of purified ΔK58-β2m and MALDI-TOF MS analysis of tryptic spot digests. A portion of the 2D gel is shown with pI and molecular weight values indicated. The MS analyses of the numbered spots are shown in the mass spectra, focusing on the diagnostic peptides for the A-chain (amino acids 1–57) and the B-chain (amino acids 59–99). The A-chain peptide (2555.9 Da) is found only in the spectra of spots 2 and 3, and the B-chain peptide (2999.3 Da) is found only in the spectra of spots 8 and 9.

ΔK58-β2m (Fig. 2C) clearly demonstrate that this analytical procedure is highly suitable for this survey.

Even though the solubility of ΔK58-β2m (as in the case of the ΔN6-β2m material) is significantly reduced compared with full-length β2m (Mimmi et al. 2006), it is expected to be soluble at concentrations easily detectable in the techniques used here. Also, previous studies using extracts from other tissues containing β2m amyloid have shown that the pellet of material remaining after water extraction solely contains spots representing full-length β2m (Stoppini et al. 2005).

These findings highlight possible contradictions between the in vivo and in vitro data. The absence in natural fibrils of a highly unstable variant of β2m such as ΔK58-β2m is actually very surprising. This species is able to aggregate and generate fibrils at neutral pH and, if present in plasma, would become a predominant constituent of natural fibrils. In contrast, ΔN6-β2m, which is highly amyloidogenic in vitro, is not detectable in plasma (Stoppini et al. 2005), but it is a consistent component of natural fibrils. These findings support the general implication that the process of amyloid deposition in a target tissue requires that the fibrillogenic protein attains the amyloidogenic conformation at the right site, at the right time, and at the right concentration. In the case of ΔK58-β2m, it is conceivable that this species could populate a partially folded, potentially amyloidogenic conformation in serum, but in this fluid it cannot reach the critical concentration for aggregation and/or does not encounter relevant cofactors required for fibrillogenesis (Relini et al. 2006). Under such conditions, we can speculate that it may be more susceptible to degradation than to amyloid deposition. The identification of degradative pathways of amyloidogenic proteins that do not lead to fibril formation and deposition is of great interest and has implications for the interpretation of amyloid pathogenesis and will be important for consideration regarding new therapeutic strategies.

Materials and methods

Protein extraction from ex vivo fibrils

Amyloid fibrils were isolated from amyloidotic unfixed frozen (−80°C) femoral head tissue of an Italian patient and shoulder synovial tissue of an unrelated UK patient, with DRA, obtained after surgery with informed consent. Intense specific staining (Tennent 1999) of the amyloid deposits in tissue sections with polyclonal anti β2m antibodies confirmed that β2m was the major fibril protein. The fibrils were prepared independently by two researchers, by classical water extraction (Pras et al. 1968), according to previously described procedures (Tennent 1999; Relini et al. 2006) that differed by the use of ultracentrifugation (60,000*g* for 30 min; Beckman L8–704, Beckman Coulter S.p.A) and inclusion throughout of PMSF in the femoral head preparation (Relini et al. 2006). The presence of typical amyloid fibrils in the final isolates was confirmed by red-green dichroism/green-birefringence in cross-polarized light microscopy after staining with alkaline alcoholic Congo red and a characteristic fibrillar appearance in negative stain TEM. Fibril fractions containing the greatest abundance of amyloid, in each preparation, were pooled and lyophilized (Modulyo EF4, Edwards High Vacuum).

Atomic force microscopy

For AFM imaging, the amyloid fibril preparation obtained by the water extraction procedure was diluted 60 times; a 20-μL

aliquot of the diluted sample was deposited on freshly cleaved mica and dried under mild vacuum. Images were acquired in tapping mode in air using a Dimension 3000 microscope (Digital Instruments–Veeco), equipped with a 'G' scanning head (maximum scan size 100 μm) and driven by a Nanoscope IIIa controller. Single-beam uncoated silicon cantilevers (type OMCL-AC, Olympus, and RTESP, Veeco) were used. Fibril height was measured from the corresponding height in cross-section in the AFM images. The drive frequency was 300 kHz; the scan rate was between 0.3 and 0.5 Hz. Vertical displacements were calibrated measuring the depth of grating notches (180 nm) and the half-unit cell steps (1 nm) obtained by treating freshly cleaved mica with hydrofluoric acid. The horizontal displacements of the piezoelectric tubes were calibrated using a 3-μm pitch diffraction grating.

ΔK58-β2m preparation

ΔK58-β2m was derived from wild-type β2m purified from a pool of urine from nephropathy patients, as described previously (Nissen et al. 1987). By mass spectrometry, the molecular mass of the purified protein was in agreement with the theoretical value of 11,619 Da.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The first dimensional protein separation was performed using Immobiline Dry Strips, pH 3–10 (nonlinear gradient). The samples for Coomassie staining and blotting detection, respectively, were conditioned in a rehydration buffer: 8 M urea, 4% CHAPS, 65 mM DTE, 0.8% ampholine, and 0.002% bromophenol blue. Isoelectric focusing was performed on an IPGphor system (Amersham Biosciences). For the Coomassie staining, a standard concentration of 200 μg of protein was loaded, and for immunoblot, the gel was generally loaded with 10 μg of protein. The detection limit for ΔK58-β2m of the immunoblot was measured by carrying out the whole procedure using progressively diluted samples. After isoelectric focusing, proteins in the IPG strips were soaked for 15 min in SDS-PAGE equilibration buffer (50 mM Tris–HCl at pH 6.8, 6 M urea, 30% glycerol, 2% SDS) supplemented with 2% dithiothreitol. The SH groups were subsequently blocked with a solution containing 50 mM Tris–HCl (pH 6.8), 6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide, and a trace of bromophenol blue for 5 min. The second dimension separation was carried out in a vertical gradient slab gel with the Laemmli-SDS discontinuous system, using an acrylamide gel gradient of 9%–18% and 2% of cross-linking. Proteins were visualized using the Coomassie Brilliant blue G colloidal staining method according to previously described methods (Oakley et al. 1980; Hochstrasser et al. 1988).

The samples for 2D-PAGE mass spectrometry consisted of 30 μg of purified ΔK58-β2m, which was precipitated using a 2D cleanup kit from Bio-Rad. The precipitate was redissolved in 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer (pH 3–11) NL, 0.002% bromophenol blue. After rehydration of a 13-cm Immobiline DryStrip (pH 3–11) NL (Amersham Biosciences), the protein was focused using an IPGphor (Amersham Biosciences). After isoelectric focusing, the IPG strip was soaked 2 × 15 min in SDS-PAGE equilibration buffer (50 mM Tris-HCl at pH 8.8, 6 M urea, 2% SDS, 0.002% bromophenol blue) supplemented with 1% dithiothreitol and 2.5% iodoacetamide. Separation in the second dimension was carried out using a 10%–20% Criterion Tris-HCl gel (Bio-Rad) followed by visualization by colloidal Coomassie staining (Colloidal Blue Staining Kit, Invitrogen).

Immunoblotting

After 2D-PAGE, the proteins in the gel were transferred onto a PVDF membrane as previously described (Stoppini et al. 1997). The β2m isoforms were identified with an anti-human β2m polyclonal antibody (Dako) using a chemiluminescent procedure (ECL plus Western blotting detection reagent Amersham Bioscience).

MALDI/MS analyses and protein sequencing

The spots stained by Coomassie were manually excised from the gel, destained, and submitted to hydrolysis with endoproteinase Glu-C or proteomic grade trypsin (Sigma-Aldrich) according to the procedure previously carried out (Stoppini et al. 2005) or as detailed below. Peptide mixtures were analyzed by MALDI-TOF mass spectrometry using a Micromass spectrometer (Waters) or an Ultraflex MALDI-TOF instrument (Bruker-Daltonics). Samples were freeze-dried and then dissolved in 10 μL of 0.1% trifluoroacetic acid; 1 μL was mixed with 1 μL of a matrix solution of α-cyano-4-hydroxycinammic acid, 5 mg/mL in acetonitrile, 0.1% trifluoroacetic acid 7:3 (v:v), and the mixture was applied onto the metallic sample plate and air dried. Mass calibration was performed using a peptide standard mixture provided by the manufacturer. The instrument was operated in reflectron positive ion mode and spectra were acquired in the mass-to-charge ratio range (m/z) 800–3000. All mass values are reported as monoisotopic masses, and raw data were analyzed using the software MassLynx 4.0 (Micromass-Waters). In addition, the sequences of proteins electroeluted from 2D-PAGE were determined by adsorptive biphasic column technology using an HP G1000A protein sequencer (Hewlett-Packard) as previously detailed (Stoppini et al. 2005).

For the assignment of spots from 2D-gels as shown in Figure 4, the following procedure was applied: excised spots were washed 30 min with 40% v/v ethanol to remove most of the Coomassie stain and then shrunk using 100% acetonitrile followed by evaporation to dryness in a speedvac. The dried gel pieces were reconstituted in 50 mM NH_4HCO_3 with 12.5 ng/μL MS-grade trypsin (Stratagene) on ice for 45 min. Any remaining liquid not absorbed by the gel pieces was removed, and 20 μL 50 mM NH_4HCO_3 were added followed by incubation overnight at 37°C. A total of 10 μL supernatant were loaded on C18 stagetips (Proxeon Biosciences) equilibrated with 0.1% TFA and washed with 1% TFA. Peptides were eluted onto a MALDI target plate using an α-cyano-4-hydroxycinnamic acid solution (6.2 μg/μL, Agilent Technologies) and analyzed in positive ion-mode on the Ultraflex MALDI-TOF instrument controlled by FlexControl version 2.2, build 19. Recorded spectra were analyzed using the *MoverZ* freeware from Proteometrics.

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