Ubiquitinated annexin A2 is enriched in the cytoskeleton fraction

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Abstract Annexin A2 is a multifunctional protein and its cellular functions are regulated by post-translational modifications and ligand binding. When purified from porcine intestinal mucosa and transformed mouse Krebs II cells, SDS–PAGE revealed high-molecular-mass forms in addition to the 36 kDa protomer. These forms were identified as poly-/multi-ubiquitin conjugates of annexin A2, and ubiquitination represents a novel post-translational modification of this protein. Subcellular fractionation of mouse Krebs II cells revealed an enrichment of annexin A2-ubiquitin conjugates in the Triton X-100 resistant cytoskeleton fraction, suggesting that ubiquitinated annexin A2 may have a role associated with its function as an actin-binding protein.

Keywords: Annexin A2; Ubiquitin; Cytoskeleton; Post-translational modification

1. Introduction

Annexin A2 (anxA2) is a multifunctional Ca^{2+}-, lipid-, and actin-binding protein implicated in a number of intracellular functions such as signal transduction, membrane trafficking and mRNA transport [1,2], as well as the regulation of membrane/cytoskeleton contacts and extracellular functions [1,3]. The anxA2 protomer is cleaved by chymotrypsin into a 33 kDa C-terminal core domain, and a 3 kDa N-terminal domain which consists of 30 amino acids of which the first 14 residues constitute the binding site for its S100 protein binding partner, p11 [4]. Each of the two functional domains can be modified post-translationally in vivo and the modifications affect its binding of different ligands and thus its cellular functions. Acetylation at Ser1 is necessary for its binding of p11 [5], while proteolytic removal of the N-terminal domain abolishes binding to lipid rafts in smooth muscle [6]. Furthermore, anxA2 can be phosphorylated on Tyr23 [7], Ser11 and Ser25 (the primary phosphorylation site) [8,9]. It is *S*-glutathiolated on Cys8 in vivo [10], and in addition on Cys132 in vitro, which can be reversed by glutaredoxin [11]. These post-translational modifications affect the binding of phospholipids and F-actin [1,3,12]. The C-terminal core domain comprises the intracellular binding sites for Ca^{2+}, phospholipids and F-actin [1,12]. Calcium plays a major role in regulating the association of anxA2 with membranes and the cytoskeleton [1,3,13].

A novel post-translational modification of anxA2 is reported in the present study. Evidence is presented that high-molecular-mass forms of anxA2, as purified from porcine intestinal mucosa and mouse Krebs II cells, represent ubiquitin (Ub) conjugates which are enriched in a Triton X-100 insoluble particulate fraction, the cytoskeleton fraction [14,15].

2. Materials and methods

2.1. Purification of anxA2 from porcine intestinal mucosa and Krebs II cells

The heterotetrameric $anxA2_2p11_2$ complex was purified from porcine intestinal mucosa as described by Gerke and Weber [13], with minor modifications. No inhibitors of Ub-conjugating enzymes and Ub-isopeptidases were added during the procedure. Briefly, the Triton X-100 insoluble particulate fraction from porcine intestinal mucosa, obtained in the presence of 2 mM Ca^{2+}, was thoroughly washed before the release of anxA2 from membranes and the cytoskeleton by 15 mM EGTA in a defined buffered solution with protease inhibitors and 200 mM NaCl [13] (or as indicated in the figure legend). After high-speed centrifugation ($100\,000 \times g$, 60 min at 4 °C) of this EGTA extract, the supernatant was dialysed against buffer A containing 10 mM imidazole–HCl (pH 7.5) and 0.5 mM DTT before a second high-speed centrifugation ($100\,000 \times g$, 60 min at 4 °C). The dialysed supernatant was applied on a Whatmann DE52 column equilibrated with buffer A and the flow-through was subsequently applied on and eluted from a Whatmann CM52 column. Further purification was performed as described [13]. Subcellular fractionation of mouse Krebs II cells was performed essentially as described [2,15]. AnxA2 present in the cytoskeleton fraction [15] was released from the Triton X-100 insoluble material by a buffer containing 10 mM triethanolamine (pH 7.4), 250 mM sucrose, 10 mM EGTA, 130 mM KCl, and 2 mM $MgCl_2$. It was recovered in the supernatant after centrifugation ($100\,000 \times g$, 60 min at 4 °C). The salt concentration was subsequently lowered to 65 mM KCl by a 1:1 dilution with the same buffer without KCl and EGTA before application on the Whatmann DE52 column. AnxA2 present in the flow-through fraction was further purified as described for porcine anxA2 [13].

2.2. Immunoisolation

Rabbit anti-mouse IgG (5 μg/ml) was bound to Protein A–Sepharose CL-4B beads (Amersham Pharmacia Biotech) in NET buffer (10 mM Tris–HCl (pH 7.4), 150 mM KCl, and 1% (w/v) Triton X-100)

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Abbreviations: anxA2, annexin A2; CUE, coupling of ubiquitin conjugation to ER degradation; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; Ub, ubiquitin; UBA, ubiquitin-associated; UIM, ubiquitin-interacting motif

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during a 2 h incubation at 4 °C. The coated beads (200 μl) were washed three times in NET buffer by sedimentation (1200 g, 15 s) and further incubated with monoclonal antibodies (5 μg/ml) directed against Ub or anxA2 for 2 h at 4 °C. After three washes with NET buffer, 30 μg of purified anxA2 was added and incubated overnight at 4 °C. The Protein A–Sepharose beads with bound Ub or anxA2 antibodies were sedimented (1200 g, 15 s) and washed several times with NET buffer. The beads were finally resuspended in SDS sample buffer, heated for 3 min at 95 °C and the desorbed proteins were resolved by SDS–PAGE [16] and immunoblotted with either monoclonal anxA2 or Ub antibodies.

2.3. SDS–PAGE and Western blot analysis

SDS–PAGE was performed [16] in 10% (w/v) gels and the proteins were transferred onto 0.2 μm nitrocellulose membrane (Schleicher and Schüell) by overnight blotting performed at 120 V h [17]. AnxA2 and Ub were detected using monoclonal antibodies directed against anxA2 (Transduction Lab.) and Ub (Zymed), respectively, followed by anti-mouse horseradish peroxidase (HRP)-labelled secondary antibodies (BioRad). Binding was visualised using the enhanced chemiluminescence (ECL) method and ECL films.

2.4. Protein determination

Protein was measured by the Bradford dye-binding assay [18] using BSA as standard.

3. Results and discussion

3.1. High-molecular-mass forms of anxA2 in porcine intestinal mucosa

It was observed during purification of anxA2 from porcine intestinal mucosa that when 200 mM NaCl was used instead of 600 mM [13] during the preparation of the Triton X-100 insoluble particulate fraction and the subsequent EGTA extract (see Section 2), then several high-molecular-mass forms of anxA2 were identified by Western blot analyses using monoclonal anxA2 antibodies (Fig. 1C, lanes 2–3). On passage of the dialysed EGTA extract (100 000 × *g* supernatant) through a DE52 column, most of the anxA2 high-molecular-mass forms were retained on the column, whereas the 36 kDa form passed through (Fig. 1D, lanes 5–6). However, using a low concentration of NaCl (25 mM) resulted in an almost complete retention of anxA2 on the DE52 column (Fig. 1C and D, lanes 1 and 4) while the highest recovery of the 36 kDa form, with only trace amounts of high-molecular-mass forms (Fig. 1B, lane 6), was obtained in the presence of 600 mM NaCl. At this point, it should be noted that overexposure of Western blots reveals high-molecular-mass forms of anxA2 in all fractions (result not shown). However, it is clear that a salt concentration of 200 mM KCl promotes the release of these anxA2 forms as compared to 25 and 600 mM KCl and that they are largely retained on the DE52 column.

3.2. AnxA2 is enriched in the cytoskeleton fraction of mouse Krebs II cells

AnxA2 is enriched in the cytoskeleton fraction (Triton X-100 insoluble fraction) derived from Krebs II cells from which it is released (Fig. 2A and B, lane 2) by increasing the salt concentration to 130 mM KCl and the temperature to 20 °C [2,15]. In addition, 10 mM EGTA was added to promote the release of anxA2 associated with phospholipids in membranes and the cytoskeleton in a Ca^{2+}-dependent manner. Further purification of anxA2 derived from the cytoskeleton fraction by ion-exchange chromatography (Fig. 2B, lanes 3–5) revealed that several high-molecular-mass forms (~80–125 kDa) were adsorbed to the DE52 column (Fig. 2B, lane 5), as seen for anxA2 isolated from porcine intestinal mucosa (Fig. 1), while two anxA2 positive bands of 50–55 kDa were only partly retained on the DE52 column (Fig. 2B, lane 5). Some of the high-molecular-mass forms of anxA2 were recovered in the pellet on centrifugation (100 000 × *g*) of the EGTA extract (Fig. 2, lane 4).

3.3. The high-molecular-mass forms of anxA2 represent Ub conjugates

Ubiquitination of anxA2 was suspected, since the high-molecular-mass anxA2 positive bands appeared as a ladder. To verify that these forms of anxA2 purified from porcine intestinal mucosa (Fig. 3A, lane 1) or the transformed mouse Krebs II cells (Fig. 3A, lane 2) represent Ub conjugates, the

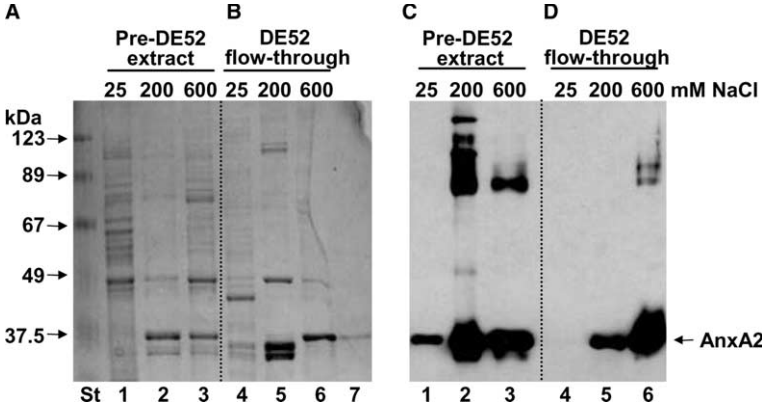

Fig. 1. High-molecular-mass forms of annexin A2. Dialysed EGTA extracts of the Triton X-100 insoluble particulate fraction from porcine intestinal mucosa were obtained using three different concentrations of NaCl in the extraction procedure (see Section 2), i.e., 25 mM (lanes 1 and 4), 200 mM (lanes 2 and 5) and 600 mM (lanes 3 and 6). 40 μg of protein present in the 100 000 × *g* supernatant before application to the DE52 column (A and C) and the corresponding flow-through from the DE52 column (B and D) was subjected to 10% (w/v) SDS–PAGE and Coomassie brilliant blue (A and B) or Western blot analysis (C and D). The membrane was probed with monoclonal anxA2 antibodies and HRP-conjugated goat anti-mouse secondary antibodies. Antibody binding was detected using the ECL method. AnxA2 standard (lane 7) and selected prestained molecular mass markers (St) are indicated.