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

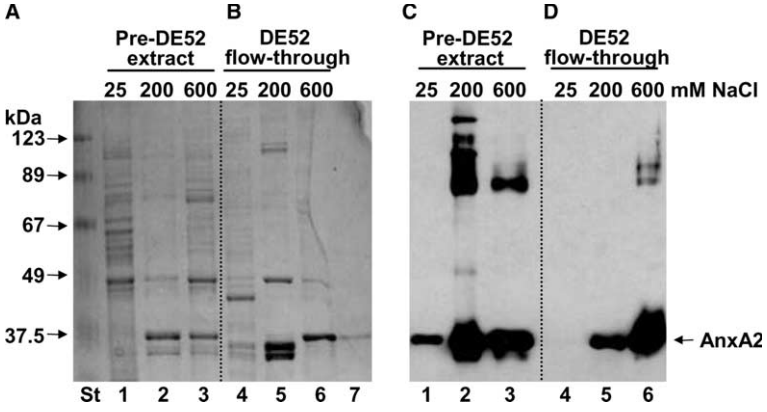

Fig. 1. High-molecular-mass forms of annexin A2. Dialysed EGTA extracts of the Triton X-100 insoluble particulate fraction from porcine intestinal mucosa were obtained using three different concentrations of NaCl in the extraction procedure (see Section 2), i.e., 25 mM (lanes 1 and 4), 200 mM (lanes 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.

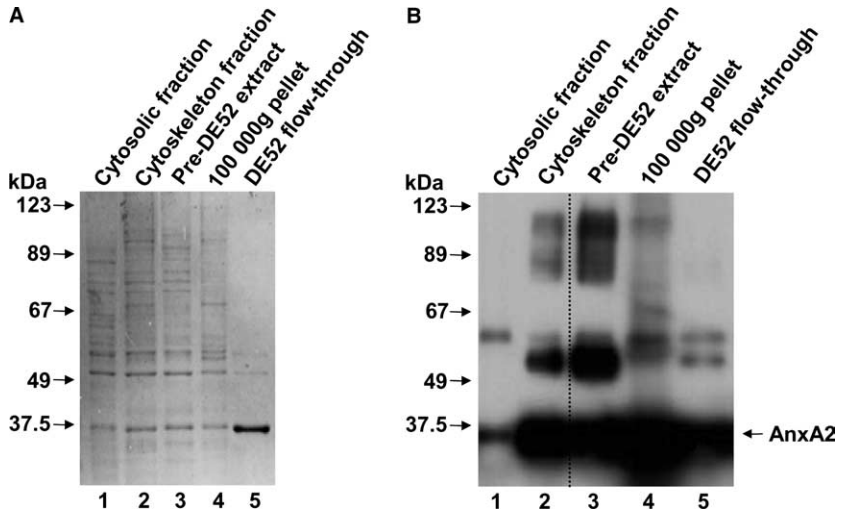

Fig. 2. Enrichment of annexin A2 and its high-molecular-mass forms in the cytoskeleton fraction of Krebs II cells. Subcellular fractionation of mouse Krebs II cells was performed as described in Section 2. 20 μg of total protein in the cytosolic and cytoskeleton fractions was subjected to 10% (w/v) SDS–PAGE and Coomassie brilliant blue (A) or Western blot (B) analysis with monoclonal anxA2 antibodies and HRP-conjugated goat anti-mouse secondary antibodies. Antibody binding was detected using the ECL method. The analysed fractions were the cytosolic (lane 1) and cytoskeleton fractions (lane 2), as well as anxA2 preparations derived from the cytoskeleton fraction by further purification (lanes 3–5): $100\,000 \times g$ supernatant (lane 3), $100\,000 \times g$ pellet (lane 4) and the DE52 flow-through (lane 5). Selected prestained molecular mass markers are indicated.

preparations were subjected to immunoisolation with Ub (Fig. 3B) or anxA2 (Fig. 3C) antibodies. Subsequent SDS–PAGE and Western blot analysis with anxA2 (Fig. 3B) and Ub (Fig. 3C) antibodies, respectively, demonstrated the presence of several high-molecular-mass forms of Ub conjugates of anxA2 (Ub-anxA2), in the range of ~80–125 kDa (Figs. 3B and 2C). Interestingly, these forms appeared as a ladder start-

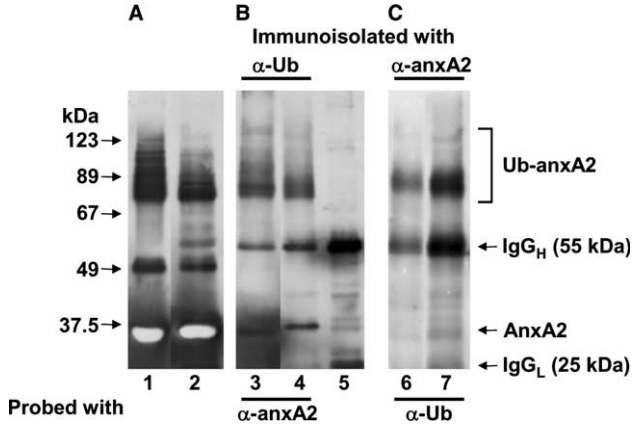

Fig. 3. Identification of high-molecular-mass forms of annexin A2 as ubiquitin conjugates. 30 μg of the anxA2 preparations purified from porcine intestinal mucosa (in the presence of 600 mM KCl) (lanes 1 and 3) or mouse Krebs II cells (lanes 2 and 4) was subjected to (10%, w/v) SDS–PAGE and Western blot analysis with monoclonal anxA2 antibodies before (A) and after (B) immunoisolation by Ub antibodies. 30 μg of the anxA2 preparations was likewise subjected to immunoisolation by anxA2 antibodies, subjected to 10% (w/v) SDS–PAGE and Western blot analysis with monoclonal Ub antibodies (C). Primary and secondary antibodies characterised by heavy and light chain IgG subunits of ~55 and ~25 kDa, respectively, were recovered and represent additional internal molecular mass markers (B, lane 5). Antibody binding was detected using the ECL method and HRP-conjugated goat anti-mouse secondary antibodies. Ub-anxA2 conjugates are indicated by a square bracket. Selected prestained molecular mass markers are indicated.

ing at ~80 kDa, both when purified from porcine intestinal mucosa and mouse Krebs II cells. This pattern was found to be consistent in several preparations of purified anxA2. Bands of 36 kDa are detected on the blot probed with anxA2 antibodies (Fig. 3B), that most likely result from interaction of a non-ubiquitinated form of anxA2 and a Ub-anxA2 species. Note that the 50 kDa anxA2 positive band (Fig. 3A, lanes 1 and 2) is not immunoprecipitated under these conditions and its nature remains to be identified. Interestingly, a ~47 kDa anxA2 form which is resistant to SDS-treatment, heat denaturation and 2-mercaptoethanol reduction has previously been reported [19].

3.4. Functional significance of anxA2 ubiquitination

Since anxA2 contains an α-helical overall structure, we searched for Ub-binding domains of α-helical secondary structure such as coupling of Ub conjugation to ER degradation (CUE), Ub-associated (UBA) and Ub-interacting motif (UIM) domains. The region 176GRRAEDGSVID (key residues underlined) spanning the fifth helix of domain II appears to contain a conserved UIM with Gly176 as a weak hydrophobic residue [20]. UIM-containing proteins both bind polyUb-chains and promote ubiquitination [20]. Interestingly, UIM-dependent polyubiquitination does not lead to degradation of the modified protein. No UBA and CUE domains [21] were found and the ubiquitination site(s) in anxA2 remains to be identified. It should also be noted that anxA2 contains a species conserved sequence 36RDALNIETA analogous to the so-called "destruction box" linked to intracellular proteolysis [22].

After having shown that anxA2 in vivo is a target protein for ubiquitination, the question was whether this post-translational modification represents a targeting signal for degradation by the Ub-proteasome system. To address this possibility, [^{35}S] methionine labelled anxA2 was synthesised in a coupled in vitro transcription/translation system, and the stability of newly synthesised protein was followed by

further incubation in an enriched rabbit reticulocyte lysate system as described [23]. However, neither ubiquitination nor degradation of anxA2 during a 3-h chase was observed (data not shown), presumably due to the lack of a substrate specific Ub ligase or necessary ancillary proteins and/or other post-translational modifications in this assay system. Judged by the consistent pattern of anxA2-Ub conjugates in the cellular extracts, it is concluded that polyUb forms of anxA2 are relatively stable. Interestingly, it has been found that the turnover of cytoskeleton-bound anxA2 in the Triton-X 100 insoluble fraction ($t_{1/2}$ = 40–50 h) in human lungs [14] is three to four times slower than for the soluble anxA2 ($t_{1/2}$ = 15 h). Thus, while degradation by proteasomes is a process requiring a polyUb-chain with a minimum of four Ub molecules in the chain linked in a Lys48 branching configuration [24], other Lys residues in Ub have been reported to support the formation of polyUb-chains, branching at Lys29 and Lys63 [25,26]. Ub-chains in these configurations target the protein to non-proteasome-dependent cellular processes such as DNA repair, regulation of gene expression, apoptosis, and subnuclear trafficking [26]. AnxA2 is involved in endocytosis [1] and ubiquitination, in particular monoubiquitination, has also been implicated in the internalisation of several plasma membrane proteins destined to the endosomal/lysosomal pathway [26].

The cellular functions of Ub are truly ubiquitous, since ubiquitination regulates protein localisation, activity and binding partners by a number of different methods [26]. The finding of an enrichment of polyUb anxA2 in the cytoskeleton fraction suggests a function in a non-proteasomal process. AnxA2 is an actin-binding protein and is associated with lipid rafts [1,3]. Considering that the Triton X-100 insoluble fraction contains both cytoskeletal elements and certain lipids associated with rafts and caveolae, it is possible that polyubiquitination may be involved in the dynamic regulation of the actin cortical cytoskeleton network as shown for some established regulatory proteins. Thus, the identification in *Saccharomyces cerevisiae* of actin (Act1p) and some of its regulators (Rho3p, Sac6p, and Rvs167p) as membrane-associated substrates for ubiquitination suggests a role of Ub as a direct regulator of the actin cytoskeleton [27]. The possibility should therefore be considered that the binding of anxA2 to the actin cytoskeleton makes it a substrate for specific Ub ligases associated with the cytoskeleton network.

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