Complex formation and submembranous localization of annexin 2 and S100A10 in live HepG2 cells

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Received 12 June 2001; accepted 12 June 2001

First published online 21 June 2001

Edited by Veli-Pekka Lehto

Abstract The Ca^{2+} and membrane binding protein annexin 2 can form a heterotetrameric complex with the S100A10 protein and this complex is thought to serve a bridging or scaffolding function in the membrane underlying cytoskeleton. To elucidate which of the subunits targets the complex to the subplasmalemmal region in live cells we employed YFP/CFP fusion proteins and live cell imaging in HepG2 cells. We show that monomeric annexin 2 is targeted to the plasma membrane whereas non-complexed S100A10 acquires a general cytosolic distribution. Co-expression of S100A10 together with annexin 2 and the resulting complex formation, however, lead to a recruitment of S100A10 to the plasma membrane thus identifying annexin 2 as the membrane targeting subunit. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Calcium; Live cell imaging; Membrane cytoskeleton; Membrane transport

1. Introduction

Annexins are a family of cytosolic Ca^{2+} binding proteins that interact with cellular membranes in a regulated and reversible manner. They have been implicated in a variety of membrane related events ranging from the stabilization of membrane structures to endocytic and exocytotic membrane traffic (for review see [1,2]). Central to the annexin action is their Ca^{2+} dependent binding to negatively charged phospholipids enriched in the cytosolic leaflet of cellular membranes. This property is contained in a conserved membrane binding module, the annexin core domain, which is built of four or eight tandemly repeated homology segments. The second principal building block of an annexin is the N-terminal domain which is unique in sequence and length. Though not being capable of mediating membrane binding themselves the N-terminal domains of different annexins have been shown to modulate the Ca^{2+} dependent membrane binding displayed by the annexin cores. Moreover N-terminal annexin sequences contain binding sites for specific protein ligands which can also affect canonical annexin properties. A number of such ligands has been identified using in vitro approaches, e.g. affinity chromatography on immobilized annexins, but the existence and functional significance of annexin–ligand complexes within cells has so far remained largely elusive (for review see [1,2]). The main exception is the complex formed between annexin 2 and the S100 protein S100A10 (p11). Annexin 2–S100A10 complexes can be isolated from cells and tissues and the two subunits of the complex often show overlapping intracellular distributions [3–5].

Complex formation between annexin 2 and S100A10 is mediated primarily through hydrophobic contacts. These involve aliphatic and aromatic amino acid residues located on one side of an amphiphatic α-helix comprising the N-terminal 14 residues of annexin 2 and a hydrophobic pocket formed by a S100A10 dimer [6–9]. When compared to monomeric annexin 2 the complex has a reduced Ca^{2+} requirement for binding to acidic phospholipids and is also capable of aggregating secretory granules at micromolar Ca^{2+} concentrations [10,11]. This latter activity is most likely due to the annexin 2–S100A10 complex forming symmetric cross-bridges between adjacent bilayers with two annexin 2 cores bound to two separate membranes and linked via a S100A10 dimer. Support for this view is severalfold. First, quick-freeze, deep-etch electron microscopy analysis of chromaffin and anterior pituitary secretory cells identified strands connecting secretory granule and plasma membrane which are likely to contain annexin 2–S100A10 [12,13]. Second, high resolution cryo-electron microscopic analysis of junctions formed by annexin 2–S100A10 between artificial liposomes or chromaffin granules revealed highly symmetric structures fitting in size with an annexin 2–S100A10 heterotetramer spanning the distance between two membrane surfaces [14]. Third, crystal structure analysis of the S100A10 dimer in a complex with a peptide covering the N-terminal annexin 2 sequence showed that two peptides reside on opposite sides of the S100A10 dimer which therefore could serve as a central unit connecting two membrane binding annexin 2 cores [9].

Within cells the annexin 2–S100A10 complex is located on both endosomal membranes and the plasma membrane and it is thought that the subplasmalemmal localization is required for a functioning of the complex in Ca^{2+} regulated exocytosis [12,13,15,16]. The Ca^{2+} dependent membrane binding of the annexin 2 core could mediate a direct interaction of the annexin 2–S100A10 complex with the plasma membrane although other modes of linking the complex to the submembranous region have been proposed. These include a tight association with the cortical cytoskeleton, possibly mediated through a binding of annexin 2 to F-actin or spectrin-like molecules [17] or an association with cholesterol-rich membrane domains [18,19]. Moreover it appears that in adrenergic

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Abbreviations: CFP, cyan fluorescent protein; YFP, yellow fluorescent protein

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PII: S0014-5793(01)02604-7

chromaffin cells a submembranously anchored S100A10 subunit participates in recruiting cytosolic annexin 2 to the cell cortex when cells are stimulated to secrete [15]. Such submembranous localization of non-complexed S100A10, however, is not observed in F9 mouse teratocarcinoma cells. In these cells which do not express annexin 2, S100A10 seems to be a cytosolic, non-membrane-bound protein [20].

To address this apparent controversy and to analyze the distribution of the subunits of the annexin 2–S100A10 complex in live cells we chose to record the distribution of fluorescent annexin 2 and S100A10 fusion proteins in HepG2 cells. These cells were selected because they express no or very little endogenous annexin 2 and S100A10 [21] thereby enabling us to specifically and separately analyze the ectopically expressed subunits of the complex. We show that annexin 2-cyan fluorescent protein (CFP) but not yellow fluorescent protein (YFP)-S100A10 assumes a plasma membrane localization in singly transfected HepG2 cells. When both fusion proteins are co-expressed they form complexes and are both localized to the plasma membrane indicating that the annexin 2 subunit is primarily responsible for anchoring the complex in the submembranous region.

2. Materials and methods

2.1. Expression constructs

Enhanced YFP, a bright yellow variant of GFP, was attached to the N-terminus of human S100A10 by cloning full-length S100A10 cDNA into the appropriately linearized pEYFP-C1 vector (Clontech, Heidelberg, Germany). Enhanced CFP, a bright cyan variant of GFP, was attached to the C-terminus of human annexin 2 by cloning annexin 2 cDNA into the appropriately linearized pECFP-N1 vector (Clontech, Heidelberg, Germany). To allow expression of a fusion protein the stop codon of the annexin cDNA was deleted. Moreover the cDNA used contained an A65E mutation installing the epitope for the H28 monoclonal anti-annexin 2 antibody [22] which was used for immunoprecipitation. Following cloning plasmids were amplified in *Escherichia coli* Top 10 F′ (Clontech, Heidelberg, Germany) and purified using the Jetstar 2.0 Plasmid kit (Genomed, Bad Oyenhausen, Germany).

2.2. Cell culture and transfections

HepG2 cells were maintained in RPMI medium with 10% fetal calf serum, glutamine and antibiotics in a 5% CO_2 incubator at 37°C. For transient transfections, cells were either grown on cover slips in 35 mm dishes or in 100 mm dishes and then transfected with Effectene (Qiagen, Hilden, Germany). 24 h after transfection cells were processed for fluorescence microscopy or immunoprecipitation.

2.3. Antibodies and immunoblotting

The mouse monoclonal antibody H28 directed against annexin 2 and the monoclonal antibody H21 directed against S100A10 have been described previously [5]. Living Colors™ A.v. Peptide antibody was purchased from Clontech (Heidelberg, Germany). Immunoblotting employed PVDF membrane (Millipore, Germany) and HRP-labelled secondary antibodies (Dako A/S, Denmark) which were visualized by ECL (Amersham, Germany).

2.4. Fluorescence microscopy

Living cells grown on cover slips were mounted in mowiol with 4% *n*-propyl-gallate as antifade agent and then inspected using a DM RXA fluorescence microscope (Leica, Wetzlar, Germany).

2.5. Immunoprecipitation

Cells were rinsed once with PBS and then lysed with IP-buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) containing a cocktail of protease inhibitors (1 mM PMSF, 4 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 0.5 μg/ml TPCK, 20 μM E64). During lysis dishes were maintained at constant agitation for 30 min on ice. Cells were then scraped from the dish with a rubber policeman and passed several times through a 22 gauge needle. The lysate was centrifuged for 10 min at $10\,000 \times g$ at 4°C and the supernatant was incubated in a pre-clearing step for 2 h at 4°C with Dynabeads M-450 (sheep anti-mouse IgG). The remaining non-bound lysate was then incubated over night at 4°C with Dynabeads coated with the respective monoclonal antibodies. Beads were washed three times with PBS containing 0.05% Triton X-100. Subsequently, adsorbed proteins were released by boiling in SDS–sample buffer and analyzed by immunoblotting.

3. Results and discussion

3.1. Annexin 2 and S100A10 assume different localizations when expressed individually in HepG2 cells

Previous Western blot analyses had revealed that hepatoblastoma HepG2 cells express no S100A10 and no or very little annexin 2 protein [21]. Hence, this cell line appeared to be a feasible model for studying the intracellular fate of ectopically expressed annexin 2 and S100A10 in the absence of the endogenous proteins. Annexin 2 and S100A10 were expressed as fluorescent protein chimeras to record their distribution in live cells. CFP and YFP were chosen to allow a simultaneous detection of both proteins in co-transfected cells. Selecting the site of fluorescent protein attachment deserved particular consideration. In the case of annexin 2, CFP was fused to the C-terminal end since a correctly acetylated

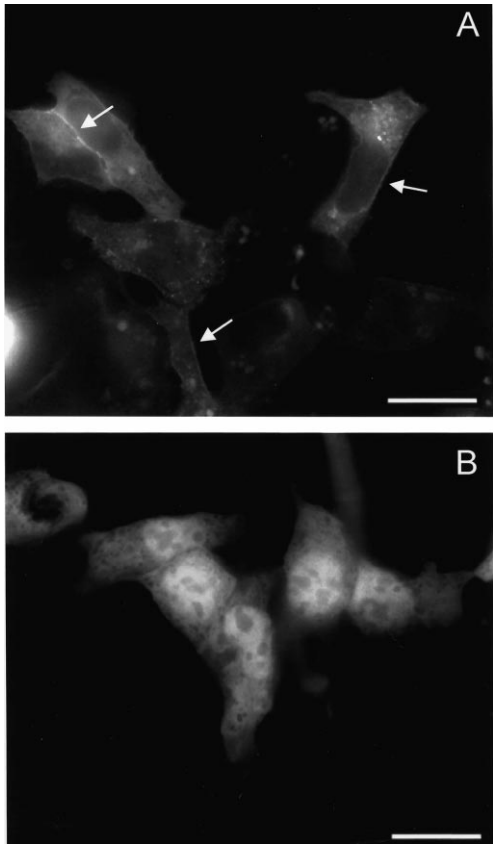

Fig. 1. Intracellular localization of annexin 2-CFP and YFP-S100A10 in living HepG2 cells. HepG2 cells were transfected with expression constructs encoding annexin 2-CFP (A) or YFP-S100A10 (B). Cells expressing annexin 2-CFP show plasma membrane and cytosolic staining, while the nucleus is devoid of label (A). S100A10 expression results in a diffuse cytosolic and nuclear fluorescence (B). Arrows indicate plasma membrane staining. Scale bar: 10 μm.

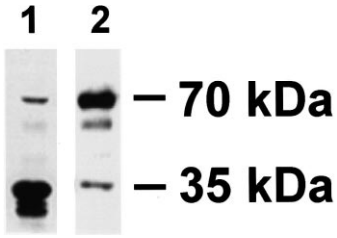

Fig. 2. Immunoprecipitation of annexin 2-CFP and YFP-S100A10 from HepG2 cell lysates. Cells were co-transfected with annexin 2-CFP and YFP-S100A10. Lysates from the transfected cells were subjected to immunoprecipitation with the monoclonal antibody H21 directed against S100A10 (lane 1) or with the monoclonal antibody H28 directed against annexin 2 (lane 2). Immunoblotting of the precipitates using polyclonal antibodies recognizing all GFP variants reveals the co-precipitation of both fusion proteins at 70 kDa (annexin 2-CFP) and at 35 kDa (YFP-S100A10).

N-terminal sequence has been shown to be required for efficient S100A10 binding in in vitro studies [6,7]. On the other hand, YFP was fused N-terminally to S100A10 since previous biochemical studies had identified residues in the C-terminal extension of S100A10 as being of crucial importance for annexin 2 binding [8].

Annexin 2-CFP assumes a general cytoplasmic distribution in transfected HepG2 cells (Fig. 1A). In addition, peripheral staining at the plasma membrane is observed. This distribution is reminiscent of what has been observed for endogenous annexin 2 in a number of cultured albeit fixed cells (for review see [2]) indicating that the CFP tag does not interfere with the binding of annexin 2 to its target membrane. As discussed before (for review see [2]) it appears likely that the cytosolic plus membrane distribution reflects the dynamic nature of the annexin 2-membrane interaction. Membrane binding of annexin 2 is both reversible and regulated, e.g. by Ca^{2+} and membrane cholesterol content [23], and the protein most likely shuttles between membrane and cytosol in a manner modulated by changes in intracellular Ca^{2+} concentration and membrane and/or cell cortex composition.

In contrast to annexin 2-GFP the ectopically expressed YFP-S100A10 protein shows a cytosolic and nuclear distribution with no enrichment at the plasma membrane (Fig. 1B). Such non-structured appearance is in line with what has been observed in non-differentiated mouse F9 cells. These cells do not express annexin 2 and the endogenous S100A10 is a cytosolic protein in the absence of annexin 2 [20,24]. However, this differs from the situation in adrenergic chromaffin cells where S100A10 is restricted to the subplasmalemmal region. Here the protein has been proposed to serve as a docking ligand for annexin 2 which translocates to this plasma membrane region upon nicotine stimulation [15]. Thus while the scenario might differ in certain secretory cells it appears to be the general rule that annexin 2 is a partly membrane-associated protein in the absence of S100A10 whereas the S100A10 protein is only found in the cytosol when no annexin 2 is present.

3.2. *Annexin 2 can recruit S100A10 to the subplasmalemmal region of HepG2 cells*

The experiments so far had been carried out with singly transfected HepG2 cells expressing either annexin 2 or S100A10. We next analyzed whether co-expression of both subunits of the complex can affect their subcellular localization. Therefore HepG2 cells were co-transfected with the annexin 2-CFP and YFP-S100A10 expression plasmids and the ability of the fusion proteins to form complexes with one another was first analyzed biochemically. Lysates of HepG2 cells transiently expressing the two fusion proteins (transfection rate approx. 50%) were subjected to immunoprecipitation using either annexin 2- or S100A10-specific antibodies. Immunoblot analyses of the immunoprecipitates using antibodies directed against either annexin 2 (not shown) or CFP/YFP reveal cross-precipitation indicating that complex formation had occurred (Fig. 2). All immunoprecipitations contained only sub-stoichiometric amounts of the subunit that was not directly bound by the antibody. Most likely this is due to the fact that the epitopes of the monoclonal antibodies used for immunoprecipitation partially overlap with the sites of complex formation (C. Thiel, E. Kube and V. Gerke, unpublished observation).

Given that the annexin 2-CFP and YFP-S100A10 fusion proteins are capable of forming complexes with one another we next monitored their intracellular localization by recording the fluorescence protein signals in co-transfected cells. Fig. 3A reveals that the distribution of annexin 2-CFP is indistinguishable of that observed in singly transfected cells. This shows that the intracellular membrane localization of annexin 2 is not significantly affected by formation of the annexin 2–S100A10 complex although a tight anchoring of annexin 2 in the cortical cytoskeleton appears to depend on complex formation [17]. However, in the case of YFP-S100A10 co-ex-

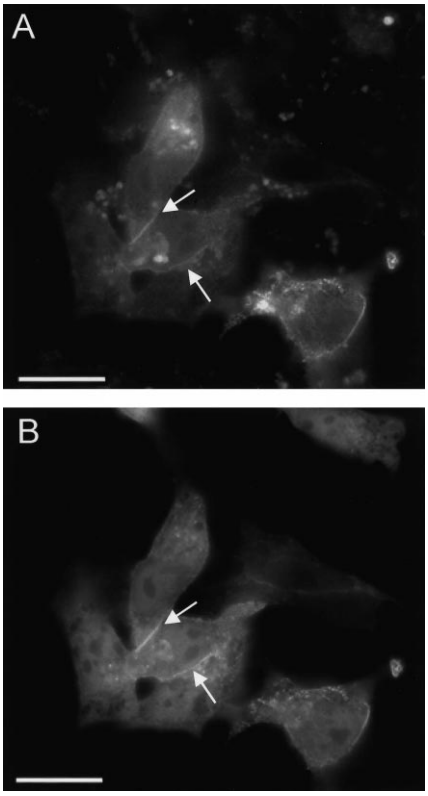

Fig. 3. Intracellular distribution of annexin 2-CFP and S100A10 in co-transfected live HepG2 cells. Cells were co-transfected with annexin 2-CFP (A) and YFP-S100A10 (B) and the intracellular distribution of both fusion proteins was analyzed 24 h following transfection. Note that both proteins co-localize at the plasma membrane in the co-transfected cells (arrows). Scale bar: 10 μm.

pression with annexin 2-CFP results in an altered intracellular distribution when compared to cells expressing YFP-S100A10 alone. The S100A10 fusion protein now localizes to both the cytoplasm and the plasma membrane thus showing a distribution overlapping with that of annexin 2-CFP (Fig. 3B). This indicates that in HepG2 cells complex formation between ectopically expressed annexin 2 and S100A10 leads to a recruitment of cytosolic S100A10 to the plasma membrane and that this localization is mediated through the annexin 2 subunit of the complex. Again this recruitment appears to differ from the scenario in adrenergic chromaffin cells where annexin 2 is translocated to the membrane only following cell stimulation and where S100A10 appears to represent the main membrane anchorage site [15]. Thus, different cells seem to regulate membrane attachment of the annexin 2–S100A10 complex in different ways with the mechanism described here for HepG2, i.e. membrane recruitment mediated through the membrane binding annexin 2 subunit, most likely representing the more general one.

3.3. Conclusions

By employing ectopic expression of fluorescent protein-tagged annexin 2 and S100A10 we could show that the two proteins assume different subcellular localizations when expressed individually. Complex formation obtained in co-transfected cells triggers a membrane recruitment of S100A10 which, in the absence of annexin 2, is solely cytosolic. Most likely, this membrane recruitment is mediated through a direct and Ca^{2+} regulated interaction of the annexin 2 subunit of the complex with membrane phospholipids. However, given the enrichment of annexin 2 at sites of high membrane cholesterol and/or F-actin attachment to membranes [18,19,23] other modes of interaction of annexin 2 with the membrane and/or cortical cytoskeleton possibly come into play as well. Once anchored in the plasmalemmal region the molecular architecture and multivalent properties of the annexin 2–S100A10 complex make it an attractive candidate for organizing in conjunction with the cortical cytoskeleton membrane domains and/or vesicular traffic in the cell cortex.

Acknowledgements: We thank Stefan Koch for help with cloning the fusion protein constructs. This work has been supported by a Grant from the Deutsche Forschungsgemeinschaft to V.G. (Ge 514/4-2).

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