Small Heat Shock Protein αB-Crystallin Is Part of Cell Cycle-dependent Golgi Reorganization*

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Rajendra K. Gangalum†, Matthew J. Schibler‡, and Suraj P. Bhat§$¶

From the †Jules Stein Eye Institute, ‡Research Institute, §Geffen School of Medicine and Molecular Biology Institute, University of California, Los Angeles, California 90095-7000

αB-Crystallin is a developmentally regulated small heat shock protein known for its binding to a variety of denatured polypeptides and suppression of protein aggregation in vitro. Elevated levels of αB-crystallin are known to be associated with a number of neurodegenerative pathologies such as Alzheimer disease and multiple sclerosis. Mutations in αB-crystallin gene have been linked to desmin-related cardiomyopathy and cataractogenesis. The physiological function of this protein, however, is unknown. Using discontinuous sucrose density gradient fractionation of post-nuclear supernatants, prepared from rat tissues and human glioblastoma cell line U373MG, we have identified discrete membrane-bound fractions of αB-crystallin, which co-sediment with the Golgi matrix protein, GM130. Confocal microscopy reveals co-localization of αB-crystallin with BODIPY TR ceramide and the Golgi matrix protein, GM130, in the perinuclear Golgi in human glioblastoma U373MG cells. Examination of synchronized cultures indicated that αB-crystallin follows disassembly of the Golgi at prometaphase and its reassembly at the completion of cytokinesis, suggesting that this small heat shock protein, with its chaperone-like activity, may have an important role in the Golgi reorganization during cell division.

αB-Crystallin is a member of the small heat shock family of proteins whose expression accompanies a number of developmental programs and pathologies in various tissues (1, 2). Mutations in αB-crystallin lead to cataractogenesis (3) and desmin-related cardiomyopathy (4). The appearance of αB-crystallin in a temporally and spatially controlled fashion during development has lead to speculations that this small heat shock protein may have important roles in the regulation of cellular physiology and growth (5–9). Quite remarkably, the elevated presence of αB-crystallin has been linked with a number of neurodegenerative disorders such as Alexander and Alzheimer diseases, dementia, and scrapie (1, 2). In multiple sclerosis the presence of αB-crystallin in astrocytes has been directly implicated in the pathological immune response (10).

Confluent cultures of the human glioblastoma cell line, U373MG, express appreciable amounts of αB-crystallin (about 2–4 μg/mg soluble protein) (11), thus presenting an excellent paradigm for investigating the physiological function of this protein. αB-Crystallin is predominantly a cytoplasmic protein, but it has also been shown to be present in the nucleus (7, 9). Considering that its chaperone-like activity has been demonstrated in vitro with a large number of substrates, under a variety of denaturing environments (12, 13), we wanted to ascertain whether αB-crystallin showed any preferential association with, or within, a cellular compartment in vivo. Rat tissues and the human glioblastoma cell line U373MG, not exposed to any specific physical or chemical stress, were used for these studies.

MATERIALS AND METHODS

Animals and Cell Culture—Experiments with animals were performed as per the guidelines of the Animal Research Committee, UCLA. Rats (Harlan Sprague-Dawley) were purchased from Charles River (Wilmington, MA). The human glioblastoma cell line U373MG (American Type Culture Collection, Manassas, VA) was maintained in complete MEM NEAA Earle’s salts medium (MEM NEAA) (Irvine Scientific, Santa Ana, CA) as per the supplier’s instructions. The cells were synchronized by a double thymidine block (14). For brefeldin A treatment the cells were synchronized and then released into MEM NEAA containing 5 μg/mL of brefeldin A (Fluka) for 1 h at 37 °C in the CO2 incubator (95% air, 5% CO2) before immunocytochemistry or cell fractionation.

Antibodies—An antiserum (anti-αB) against the conserved C terminus of αB-crystallin (cREEKPAVTAPKK, the small case “c” in the sequence is for cysteine; it was added for coupling to keyhole limpet hemocyanin; there is no cysteine in this sequence (15)) was raised commercially in rabbits (Sigma Genosys, The Woodlands, TX). This antiserum is as specific to αB-crystallin as the one made previously against the same peptide (15). Antibody GM130:FITC (anti-GM130) was a monoclonal antibody raised in mouse (BD Transduction Laboratories, Lexington, KY). For immunofluorescence, one of the following two secondary antibodies was used: 1) goat anti-rabbit TRITC or 2) goat anti-rabbit FITC (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For immunoblotting one of the following secondary antibodies was used: goat-anti-rabbit-horseradish peroxidase (Pierce) and goat-anti-mouse-horseradish peroxidase (Santa Cruz Biotechnology). Immunofluorescence—U373MG cells seeded on a microscope coverslip (Fisher Scientific) were fixed with methanol (–20 °C) for 6 min, washed, and incubated with the blocking buffer (5% normal goat serum, 1.0% bovine serum albumin, 0.20% Triton X-100 in phosphate-buffered saline) for 35 min. Subsequently, the cells were incubated with primary antibody (1:50 dilution) and then with the secondary antibody (1:200) following a standard protocol. The cells were stained in the dark with BODIPY TR ceramide (1:50,4-difluorothiophen-3-yl)hexyl acetoxysuccinimimidyl ester (BODIPY TR Ceramide) ( Molecular Probes Inc.) (16), and then counterstained with DAPI (4′,6-diamidino-2-phenylindole) and mounted on slides using Prolong anti-fade mounting medium (Molecular Probes Inc.) (17). For GM130 localization studies, instead of BODIPY, a secondary antibody (anti-GM130:FITC at 1:50 dilution) was used followed by DAPI. The pictures were taken using a Leica TCS-SP Multiphoton and Confocal Microscope with 100× oil immersion objective lenses (1.4). Z sections 0.5 μm thick at a 2–3-μm depth were taken and processed using Adobe Photoshop 6.0.

Cell Fractionation—The post-nuclear supernatants were fractionation.
Fig. 2. Characterization of the Golgi fraction by recentrifugation. The schematic on the top of each immunoblot represents the two gradients used (see “Materials and Methods”). Equal amounts of protein from six fractions (three from each gradient) were immunoblotted with anti-aB. The immunoblot (left panel) shows that the S2 fraction (the Golgi) contains an appreciable amount of aB-crystallin; upon recentrifugation of S2 (the immunoblot on the right), a significant amount can now be found in the SGF3 fraction (comparable with S3 in the first gradient and Fig. 1A). Fraction S1 is a very light whitish band seen in the top of the gradient.

Fig. 1. A, fractionation of post-nuclear supernatants of U373MG cells on a discontinuous sucrose gradient. Twenty-one fractions labeled 1–21, (about 100 μl each) were collected, and equal volumes (10 μl/fraction) were immunoblotted using anti-aB (top panel). The middle panel shows the same blot against anti-GM130. The brackets labeled S2 and S3 (top panel) indicate the position of the membrane fractions (see Fig. 2 for a schematic). Note that the lower membrane fraction, S3, contains both aB as well as the GM130. Asterisks in the middle panel indicate the presence of GM130 in the Golgi membrane fractions. A soluble preparation of purified human recombinant aB-crystallin (5 μg) was run under similar conditions for comparison (bottom panel). B, fractionation of post-natal day 10 rat heart post-nuclear supernatants (same experiment as in A, done with rat heart). Note S2 and S3 fractions (brackets); S2 has higher concentrations of aB-crystallin and GM130. Similar patterns were obtained using anti-GM130 with post-natal brain and liver extracts, which do not contain detectable aB-crystallin (data not shown). aB immunoblots do not show any other bands; the GM130 immunoblots show bands of a smaller size, which seem to be products of degradation (data not shown).

Existence of a Membrane-associated Fraction of aB-crystallin—Post-nuclear homogenates from U373MG cells were fractionated on a discontinuous sucrose gradient (shown schematically in Fig. 2, left panel). This gradient produced two easily visible membrane fractions (whitish bands), one near the bottom of the gradient at the interface of 1.3 and 0.86 M sucrose, fraction S3, and the other around the middle of the gradient at the interface of 0.86 and 0.50 M sucrose, fraction S2 (Fig. 1A, top panel). The band around the middle of the gradient (fraction S2, Fig. 1A, top panel) contains the Golgi (17). The fractions from this gradient were collected and immunoblotted for determining the distribution of aB-crystallin. Interestingly, while most of the aB-crystallin is seen in the top fractions of the gradient, a small but significant portion of aB-crystallin is seen associated with the membrane fraction S3 (Fig. 1A, top panel).

The Golgi membrane fraction, S2, also contains aB-crystallin, but the pattern of its presence is continuous with the rest of the soluble aB-crystallin in the top of the gradient (Fig. 1A, top panel). To gain insight into the identity of the two membrane fractions, S2 and S3, the same immunoblot was screened with anti-GM130 antibody for GM130, a well known Golgi matrix polypeptide (19) (Fig. 1A, middle panel). These data show that GM130 protein is concentrated in the same fractions (4–6) where aB-crystallin is found. GM130 is also present in fractions 13 and 14 (Fig. 1A, middle panel, asterisks) (although at much reduced levels), which includes the Golgi membrane fraction, S2 (Fig. 1A, top panel). Although aB-
crystallin pattern in the S2 fraction does not show a discrete separation from the soluble pool, the distinct pattern of the presence of GM130 (higher concentration of GM130 in fractions 13 and 14 in comparison with surrounding gradient fractions) clearly indicates that they contain the Golgi membranes. By the same token, the S3 fraction that contains a high concentration of GM130 protein must also contain Golgi membranes (see below).

The data obtained in Fig. 1A (top panel) is confirmed by the pattern of aB-crystallin and GM130 presence obtained with the fractionation of the post-nuclear supernatants prepared from post-natal day 10 rat heart (Fig. 1B). The pattern in Fig. 1B is very similar to that shown in Fig. 1A; there is however a clear concentration of aB-crystallin in two fractions (Fig. 1B, brackets), one nearer the bottom of the gradient, fraction S3 (similar to that in Fig. 1A), and the other in the middle, fraction S2 (the Golgi fraction). The Golgi fraction (S2) is much higher in concentration in comparison with the lower membrane (S3 fraction) as assessed by the relative presence of aB-crystallin and GM130 (Fig. 1B, compare anti-aB panel with anti-GM130 panel). The difference in the two patterns (between U373MG and rat heart) is probably related to higher lability of Golgi (S2) fraction that degrades to S3 fraction in this preparation of U373MG gradients. This possibility was addressed in another experiment with these cells when different membrane fractions (S1–S3) were collected directly, and the Golgi fraction (fraction S2) was refractionated on a second sucrose gradient (Fig. 2).

Fig. 2 shows that aB-crystallin is mostly in the fraction S2 in post-nuclear homogenates (Fig. 2, left panel). Upon refractionation of S2 (Fig. 2, right panel) most of the aB-crystallin is found in the fraction SGF3, the fraction comparable with S3 in the first gradient and Fig. 1A. Based on these data we conclude that the membrane fraction S3 (Fig. 1A) contains membranes and proteins derived from the Golgi fraction (S2, Fig. 1A). These data also confirm the lability of the Golgi membrane fraction (18). Extreme care is needed to keep the integrity of the Golgi fraction intact. The lability of the Golgi fraction isolated from the U373 MG cells (fraction S2) helped us identify Golgi as the origin of what fractionate as a discrete membrane fraction (S3) in heavier sucrose (Figs. 1 and 2). In summary the data presented in Fig. 1 indicate the presence of Golgi membrane-associated aB-crystallin in fractions S2 and S3.

aB-Crystallin Localizes to the Perinuclear Golgi—The astrocytoma cell line, U373MG, was synchronized with a double thymidine block (14), and the G1/S phase cells were immunostained with anti-aB. Prominent labeling of the Golgi apparatus resident in the perinuclear area is seen in these cells (Fig. 3A). The anti-aB staining co-localizes perfectly (Fig. 3A, Control-MeOH panel) with BODIPY TR ceramide (BODIPY), which preferentially stains the Golgi membrane. Fig. 3A (BFA-MeOH panel) the legend to Fig. 3A (only 17 fractions were collected). Note the disappearance of aB from Golgi membrane fractions 8–10 (arrows).

Fig. 3. A, presence of aB-crystallin in the Golgi. Two-photon and confocal laser scanning microscopy of U373MG cells immunostained with anti-aB (FITC, green) and BODIPY TR ceramide Golgi stain (red). Note the co-localization of aB-crystallin and BODIPY TR stain. The pictures shown are individual optical slices (confocal Z-sections 0.5 µm thick). This co-localization is sensitive to the exposure of cells to brefeldin A (20) (BFA-MeOH) (bottom panel). The control cells were exposed to the same amount of methanol as used in the preparation of brefeldin A. Staining of the Golgi by the anti-aB is lost upon preincubation of the antibody with recombinant human aB-crystallin (data not shown). The nuclei are stained with DAPI (blue). Bar = 10 µm. B, effect of brefeldin A on Golgi fractionation. Post-nuclear supernatants prepared from treated and untreated U373MG cells were fractionated (as described in the legend to Fig. 1) only 17 fractions were collected). Note the appearance of aB in the Golgi membrane fractions 8–10 (arrows).
of interest, therefore, to follow the presence of re-assembly at the completion of cell division, which allows its closely associated with its disassembly during mitosis and its disassembly during mitosis and its components to the appropriate site(s) in the daughter cells to template the biogenesis of the new Golgi. Future investigations should reveal the substrates for the αB-crystallin chaperone-like activity in the Golgi and during its mitotic transition.

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DISCUSSION

The chaperone-like function of αB-crystallin has been very well established in vitro, but the physiological function of this protein remains conjectural (12, 13). This protein is considered to be a cyttoplasmic soluble protein, yet it has been known for a very long time that α-crystallins associate with cellular membranes (22–24). In this paper we demonstrate that a discrete fraction of αB-crystallin is associated with the Golgi membrane complex (Figs. 1 and 2).

An interesting aspect of the Golgi apparatus in mammalian cells is that it exists as a stack of flattened membranes positioned in a perinuclear location in the cell. The data obtained by immunostaining and confocal microscopy (Fig. 3) clearly established that αB-crystallin associates with Golgi membranes. Although αB-crystallin is also known to be present in the nucleus (7, 9), this is the first time that this protein has been reported to be associated with a specific cyttoplasmic compartment such as the Golgi. The chaperone-like activity of αB-crystallin leads one to consider its role in well established functions of the Golgi complex as a major site of post-translational protein processing and sorting station for secreted proteins (25). In this connection the presence of αB-crystallin in the Golgi in astrocytes may be related to its implication in multiple sclerosis (10) and reactive gliosis and dementia (26).

A close examination of the mitotic panels in Fig. 4A reveals that staining obtained with either antibody (anti-αB or anti-GM130) is not amorphous but granular. It is particularly interesting that both bright red (αB-crystallin) as well as green grains (GM130) are seen around the newly formed nuclei (Fig. 4A, telophase). The presence of large granular co-localized αB-crystallin and GM130 is however unmistakable in the perinuclear area of the newly formed nuclei during cytokinesis (Fig. 4A, last panel, arrows). The timing of the αB-crystallin appearance in the nascent Golgi, immediately following cytokinesis, is notewor-thy and suggestive of a role in the biogenesis of the Golgi in the daughter cells. This speculation is supported by the fact that Golgi reorganization (or Golgi inheritance) is closely interlinked with mitosis checkpoints; inhibition of this process is known to inhibit mitosis (27). Interestingly, lens epithelial cell cultures derived from αB-crystallin null mice show an increase in the proportion of polyplloid cells (8, 28); this may be ascribed to the inability of the these cells to proceed unhindered through the cell cycle because of the absence of αB-crystallin.

The molecular mechanism of Golgi fragmentation and the molecular basis of its inheritance are a focus of intense and unresolved investigations (21, 29, 30). It is tempting to purport a role for αB-crystallin and other small heat shock proteins in Golgi inheritance by suggesting that the chaperone-like function of this protein protects and holds the Golgi components in the mitotic cytoplasm during cell division and then helps deliver these components to the appropriate site(s) in the daughter cells to template the biogenesis of the new Golgi. Future investigations should reveal the substrates for the αB-crystallin chaperone-like activity in the Golgi and during its mitotic transition.