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Calreticulin functions as a molecular chaperone for the β-amyloid precursor protein☆

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Abstract

Processing of the β-amyloid precursor protein (APP) in the endoplasmic reticulum and the Golgi apparatus may be critical in generating the β-amyloid molecules linked to the pathogenesis of Alzheimer's disease. Since chaperone molecules such as calreticulin (Crt) have been shown to be important in the maturation of many glycoproteins, we investigated the interaction between Crt and APP. We show that APP binds transiently to Crt in a manner that is pH, divalent cation, and N-linked glycosylation-dependent. Both immature APP (containing only N-linked sugars) and mature APP (containing both N-linked and O-linked sugars) bind to Crt. Both proteins are part of a complex that appears to be large enough to accommodate other proteins as well. However, while most of the immature form is associated with the complexes, very little of the mature form is. The interaction between APP and Crt is likely to be of physiological significance with respect to APP maturation since Crt is involved in quality control of nascent glycoproteins in the secretory pathway. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: β-amyloid precursor protein; Alzheimer's disease; Chaperones; Calreticulin; Endoplasmic reticulum; β-amyloid

1. Introduction

The β-amyloid peptide (Aβ) is the major constituent of the amyloid plaques that characterize Alzheimer's disease. It is formed by proteolytic cleavage of a large, transmembrane glycoprotein, the β amyloid precursor protein (APP). Although it is highly expressed in brain and kidney, APP is detected in virtually all mammalian tissues [35]. It is produced as three major isoforms from a single alternatively spliced gene. A variety of post-translational modifications of APP have been characterized including N-linked glycosylation at one (residue 467) of two possible glycosylation sites, as well as O-linked glycosylation in the Golgi which results in a significant decrease in mobility during SDS-PAGE [29]. After glycosylation, APP may be cleaved in several ways. One cleavage occurs in a region on the N-terminal side of the membrane-spanning domain within the sequence that produces the β-amyloid peptide (Aβ). This cleavage results in a soluble secreted fragment, APP_s, and a 10 kDa non-amyloidogenic fragment, which remains in the membrane [9,39]. An alternate cleavage can occur at the N terminus of Aβ. This results in the production of an amyloidogenic fragment that can be further cleaved within the membrane to yield the secreted 39–43 amino acid peptide, Aβ [12,38]. Normally, the formation of Aβ begins after the full maturation of APP [11]. However, recent data suggest that Aβ can be generated in the ER or trans-Golgi network as well [14,48].

Aberrant processing of APP may result in either the overproduction of Aβ or an increased ratio of the 42 amino acid form of Aβ, $A\beta_{42}$, to the 40 amino acid form, $A\beta_{40}$. This shift in production to $A\beta_{42}$ (the more amyloidogenic

Abbreviations used: Aβ, β-amyloid peptide; AD, Alzheimer's disease; APP, β-amyloid precursor protein; CHAPS, 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate; Crt, calreticulin; DMEM, Dulbecco's Modified Eagle Medium; DMJ, deoxymannojirimycin; DTSSP, 3,3′-Dithio bis (sulfosuccinimidylpropionate); ECL, Enhanced Chemiluminescence; ER, endoplasmic reticulum; FBS, fetal bovine serum; HRP, horseradish peroxidase; kDa, kiloDaltons; MES, 2-(N-Morpholino) ethane sulfonic acid; NRS, normal rabbit serum; PBS, PBS; PMSF, phenymethylsulfonyl fluoride; PVDF, polyvinylidene fluoride.

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form) may be critical to the formation of senile plaques in the brains of Alzheimer's disease (AD) patients (for review, see 36). It is therefore of great importance to define those factors that control the maturation and processing of this protein. Calreticulin (Crt) is the major calcium binding protein found in smooth muscle sarcoplasmic reticulum and non-muscle ER [21,27,44]. Studies have indicated that Crt is a molecular chaperone that binds to many newly synthesized glycoproteins in the ER [15,24,26,28,31,46]. Along with other ER chaperones such as BiP and calnexin, Crt is thought to be involved in quality control of nascent glycoproteins in the secretory pathway.

Recent evidence indicates that Crt may play a role in the development of AD. Walker and co-workers [47] describe a general increase in Crt levels in AD brains with a decrease in the level of Crt in neurons. Taguchi and co-workers [42] also showed decrease in Crt expression in neurons from AD brains with no change in BiP. The mRNAs for both Crt and BiP were decreased.

In this paper we demonstrate that Crt and APP form a transient, detergent-stable complex that appears to require divalent cations and neutral pH. Our data support the possibility that a protein-protein interaction is involved although N-linked glycosylation is necessary for binding. The Crt/APP complex appears to be part of a larger protein complex that may include other molecular chaperones as well as Crt.

2. Materials and methods

2.1. Materials

Polyclonal (rabbit) anti-Crt antibody was from Affinity Bioreagents Inc. Antisera to APP included C8, a polyclonal antiserum to cytoplasmic C-terminal region of the APP [30] (from Dr. Dennis J. Selkoe from the Brigham and Women's Hospital at Harvard Medical School) and A4, a monoclonal antibody to the N-terminus of APP (Boehringer-Mannheim). Anti-rabbit IgG (H&L) conjugated to horseradish peroxide (HRP) was purchased from Rockland Inc. Protein A Sepharose™ CL-4B was from Pharmacia Biotech. 3,3′-Dithio bis (sulfosuccinimidylpropionate) (DTSSP) was obtained from Pierce. Zincov™ was from Calbiochem®. Enhanced Chemiluminescence (ECL™) Western blotting detection reagents were obtained from Amersham Life Science. Sequi-blot™ PVDF membrane was purchased from BioRad. HEK 293 cells transfected with APP_{695} that express APP at approximately ten times endogenous levels [37] were a generous gift from Dr. Dennis Selkoe. Geneticin® was from Gibco/BRL. EXPRE [35]S ^{35}S-Protein Labeling Mix (L-^{35}S-Met) was obtained from NEN. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and additional chemicals were from Sigma.

2.2. Cell culture and immunoprecipitation

HEK 293 cells transfected with APP_{695} [37] were grown in 6-well plates in DMEM containing 10% FBS, 0.2 mg/ml Geneticin® in a humidified atmosphere of 8% CO_2, at 37°C. The human neuroblastoma cell line, IMR-32, has been shown to express significant amounts of APP [25]. These cells were grown in MEM-α supplemented with 10% FBS. For harvesting, cells were washed three times with cold phosphate-buffered saline (PBS), pH 7.5, and then solubilized in 1 ml of lysis buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 8.0, 2% CHAPS, 0.25 μg/ml antipain, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 20 μM PMSF) at 4°C for 1 h with rotation. Protein concentration was determined using the bicinchonic acid method [40]. Lysates were precleared with protein A Sepharose™ for 1 h at 4°C with rotation and the cleared lysates were divided into two samples. Crt antiserum was added to one of these samples and normal rabbit serum (NRS) was added to the other. Both sera were used at a 1:500 dilution. The mixture was incubated overnight at 4°C with rotation. The following day, protein A Sepharose™ (50 μl packed beads per sample) was added to each sample for 2 h at 4°C. The beads were then pelleted by centrifugation for 10 s in a microcentrifuge, washed five times with the lysis buffer and twice with PBS. SDS-PAGE sample buffer was added to the immunoprecipitates and the samples were boiled for 5 min before being subjected to SDS-PAGE according to the method of Laemmli [19]. Gels were then transferred to a PVDF membrane and Western blotted with an antiserum to APP (C8 or A4) followed by an HRP-labeled secondary antibody and ECL™ Western blotting detection reagents.

To evaluate whether glycosylation was necessary for APP to interact with Crt, growth medium was removed from cells and replaced with medium containing 2 mM tunicamycin (an inhibitor of N-glycosylation), 1 mM castanospermine (a glucosidase inhibitor) or 1 mM DMJ (a mannosidase inhibitor) for 30 min prior to harvesting. To determine the dependence of APP/Crt binding upon divalent cations, either 5 mM EDTA, 5 mM EGTA, or 1 mM Zincov™ (a zinc chelating agent) was added to lysate samples immediately after harvesting. To determine the pH dependence of the APP/Crt interaction, cells were harvested in 0.1 M MES, 150 mM NaCl buffer, pH 5.5; 0.1 M MES, 150 mM NaCl buffer, pH 6.5; or 0.1 M Tris, 150 mM NaCl buffer, pH 7.5.

2.3. Reprecipitation and pulse/chase experiments

To confirm the identity of APP in the immunoprecipitates, cells were metabolically labeled with ^{35}S-Met at 10 μCi/ml for 4 h prior to harvest. They were then lysed and immunoprecipitated with anti-Crt as above. The resulting precipitates were resuspended in PBS containing 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton X-100 (immunomix) and then incubated overnight at 4°C with C8 (a polyclonal antibody to the C terminus of APP). This was

followed by precipitation using protein A IgG linked to Sepharose™. The beads were washed in immunomix and processed for SDS-PAGE as above. Gels were enhanced by soaking in 1 M sodium salicylate, 20% methanol, 1% glycerol, pH 7.2, for 1 h; dried; and the radiolabelled bands were detected by fluorography.

Metabolic labeling was also used to determine the time course of the APP/Crt interaction. Cells were labeled for 10 min with ^{35}S-Met and then chased for varying amounts of time before harvesting, lysing and immunoprecipitation with anti-Crt. Labeled, precipitated proteins were separated on SDS-PAG, which were then enhanced, dried, and analyzed using a Packard Instant Imager in order to quantify the amount of labeled APP co-immunoprecipitated with Crt. Identification of the APP band among the labeled proteins was made by reprecipitating a zero time-point sample with anti-APP as described above, and analyzing it on the gel along with samples from the other time points.

2.4. Cross-linking and ultracentrifugation

Cells were washed, suspended in PBS at 10^6 cells/ml, and then solubilized as above. DTSSP was added to 0.2 mg/ml and the cell lysate was incubated for 1 h at 4°C. The cross-linking reaction was quenched by adding 1M Tris, pH 7.5 buffer. For control experiments, cells were solubilized in 2% CHAPS without being cross-linked by DTSSP, or solubilized in immunomix with or without being cross-linked by DTSSP.

Cross-linked cell lysates were resolved on 5–20% continuous sucrose gradients in 2% CHAPS in PBS, pH 8.0; or in immunomix. 200 μl of each cell lysate were layered on the top of the gradient and centrifuged in a SW 50.1 rotor at 48,000 rpm for 6 h at 4°C. After centrifugation, the gradients were collected and the Crt and APP content of each fraction was analyzed by SDS-PAGE under non-reducing and reducing conditions followed by Western blotting. In experiments to determine the sedimentation coefficient of the gradients, proteins with known sedimentation coefficients were centrifuged under the same conditions and the concentration of protein in each fraction was measured. In this experiment, we used lysozyme (23.2 kDa, 2.4S), bovine serum albumin (BSA) (67 kDa, 6–7S), catalase (250 kDa, 11S), and thyroglobulin (685 kDa, 19–20S). To determine the density of each fraction, the density of corresponding fractions from a blank gradient was measured using a refractometer. This allowed us to ascertain the linearity of the gradient.

3. Results

Crt association with APP in HEK cells depends upon divalent cations, pH, and N-glycosylation. Crt has been shown to function as an ER chaperone, especially for glycoproteins [15,24,26,28,31,46]. We used HEK 293 cells

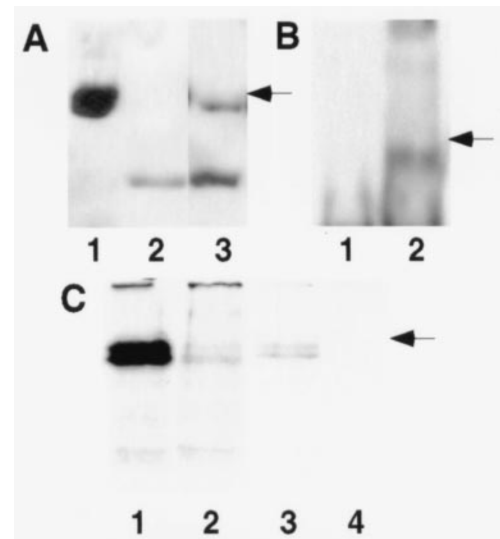

Fig. 1. APP co-precipitates with Crt in HEK 293 cells that over-express APP and in IMR-32 cells. HEK 293 cells that stably over-express APP_{695} [37] or human neuroblastoma IMR-32 cells were solubilized in lysis buffer and then immunoprecipitated using either NRS or an anti-Crt antibody. A) Western blot of anti-Crt immunoprecipitates from APP_{695}-transfected HEK 293 cells using a monoclonal antibody to the N-terminus of APP, A4. Lane 1) HEK 293-APP_{695} cell lysate; Lane 2) NRS immunoprecipitate; Lane 3) anti-Crt immunoprecipitate. B) Western blot of anti-Crt immunoprecipitates from IMR-32 cells using a polyclonal antibody to the C-terminus of APP, C8. Lane 1) NRS immunoprecipitate; Lane 2) anti-Crt immunoprecipitate. C) Cells were radiolabelled with ^{35}S-Met prior to solubilization and immunoprecipitation as above. Lane 1) anti-APP (C8) immunoprecipitate; Lane 2) anti-Crt immunoprecipitate; Lane 3) anti-Crt immunoprecipitate that was solubilized in immunomix and reprecipitated with C8; Lane 4) NRS immunoprecipitate that was solubilized in immunomix and reprecipitated with C8. Arrows indicate the 135 kDa molecular weight marker.

transfected with a cDNA expressing human APP_{695} at about 10 times the endogenous level [37] along with IMR-32 cells, which synthesize and secrete APP [25]. Cells were solubilized in a mild detergent, CHAPS, so that the protein-protein interactions were minimally perturbed. Figs. 1a and 1b show Western blots of proteins co-precipitating with Crt under these conditions. APP is present in precipitates from both cell types when anti-Crt (Fig. 1a, lane 3; Fig 1b, lane 2), but not normal rabbit serum (NRS) (Fig. 1a, lane 2; Fig. 1b, lane 1) is used as the precipitating antibody. The immunoreactive bands correspond in MW to one seen in the total cell lysate of each cell line. To ascertain with certainty that the band seen in Western blots was truly APP, APP_{695}-transfected HEK 293 cells were labeled for 4 h prior to harvesting and immunoprecipitation. This time period was chosen to ensure maximal labeling of APP since it has been shown that APP secretion peaks approximately 2h after labeling [2]. It can be seen in Fig. 1c, lane 2 that a number of proteins co-precipitate with Crt. If this precipitate is then subjected to immunoprecipitation with a polyclonal antibody to APP (C8) under conditions that disrupt the APP/Crt interaction (i.e. using SDS), APP alone is immunoprecipitated (lane 3). These results confirm the identity of APP in

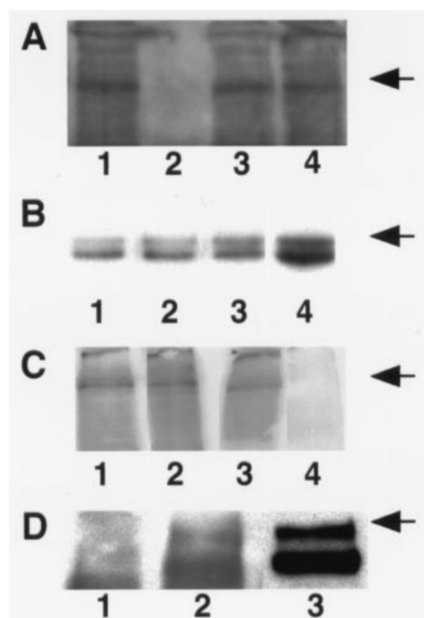

Fig. 2. Co-immunoprecipitation of APP with Crt is dependent upon divalent cations, glycosylation and pH. A) APP_{695}-transfected HEK 293 cells were solubilized in lysis buffer containing no additions (lane 1) 5 mM EDTA (lane 2), 5 mM EGTA (lane 3) or 1 mM Zincov™ (lane 4). Lysates were immunoprecipitated with an antiserum to Crt and the precipitates were separated by SDS-PAGE and analyzed by Western blotting with an antibody to APP, C8. EDTA was effective in disrupting the APP/Crt complex. B) Cells were treated with 5 mM EGTA (lane 1), 1 mM castanospermine (lane 2), 1 mM DMJ (lane 3) for 30 min prior to harvesting, immunoprecipitation with anti-Crt and Western blotting with a monoclonal anti-APP antibody (A4). None of the treatments effectively blocked the Crt/APP interaction. Lane 4 shows the cell lysate as a positive control for APP. C) Cells were treated with nothing (lane 1); DMSO (tunicamycin vehicle) (lane 2); 1 mM castanospermine (lane 3); or 2 mM tunicamycin (lane 4) for 30 min prior to metabolic labeling with ^{35}S-Met. Cells were lysed and immunoprecipitated above. Tunicamycin alone disrupts the APP/Crt complex and appears to abolish binding of other proteins to Crt as well. D) Cells were lysed in lysis buffers at pH 5.5 (lane 1), 6.5 (lane 2), and 7.5 (lane 3) (see Materials and Methods). They were then immunoprecipitated with anti-Crt and analyzed by Western blotting with C8. APP/Crt binding is abolished as the pH decreases. Arrows indicate the 135 kDa molecular weight marker.

the initial, anti-Crt precipitate. Two APP bands representing the mature (higher MW) and immature forms of APP [10, 29] are clearly visible in Fig. 1c. Using the A4 monoclonal antibody, the mature form is usually less well resolved than the immature form, and is frequently not seen at all (Figs. 1a, b). Co-precipitation of both forms of APP with Crt is consistently seen by re-precipitation and Western blotting with the polyclonal antibody, C8. We therefore believe that mature APP does interact with Crt in these cells.

It has been shown for several proteins that the chaperone function of Crt is mediated through a lectin-like interaction [13,31,41]. Since lectin interactions are Ca^{2+}-dependent, we first tested the Ca^{2+} dependence of the APP/Crt interaction by including chelators of divalent cations in the lysis buffer. Fig. 2a shows that the interaction was abolished when EDTA, a chelator of all divalent cations, was present (lane 2) but was unaffected by the specific Ca^{2+} chelator, EGTA (lane 3) or the specific Zn^{2+} chelator, Zincov™ (lane 4). The lack of Ca^{2+} dependence of the APP/Crt interaction suggests that APP and Crt may not interact through a lectin-like mechanism.

The lectin interactions of Crt have been shown to depend upon specific glucose trimming events in the ER [31,43]. We therefore tested the abilities of castanospermine (a glucosidase inhibitor), DMJ (a mannosidase inhibitor) and tunicamycin (an inhibitor of N-linked glycosylation) to inhibit APP/Crt binding. As shown in Fig. 2b, neither castanospermine nor DMJ affected the amount of APP in Western blots of anti-Crt immunoprecipitates. Using ^{35}S-Met labelled cells (Fig. 2c), tunicamycin (lane 4) but not castanospermine (lane 2) was able to inhibit the APP/Crt interaction. This suggests that while glycosylation is necessary for APP to bind to Crt, there is no dependence on specific oligosaccharide trimming events.

Secreted and integral membrane plasma membrane proteins such as APP are subjected to an increase in acidity as they pass through successive compartments in the secretory pathway. We tested the effect of altering the pH on the APP/Crt interaction. Fig. 2d shows that as the pH is decreased, less APP is immunoprecipitated with Crt. At pH 5.5, the interaction is abolished. This is consistent with the hypothesis that Crt acts as a molecular chaperone for APP in the ER and early Golgi.

The APP/Crt interaction is transient. A further characteristic of molecular chaperones is that the interaction between them and their substrate proteins is transient. The results of pulse/chase experiments shown in Fig. 3 indicate that this is the case for APP and Crt. Co-precipitation of labelled APP with Crt begins to increase at 5 min of chase, peaks at 10 min, and declines thereafter. It has been shown that APP secretion peaks at approximately 2h of chase [2]. These data are consistent with Crt acting as a chaperone for APP at an early point in its biosynthesis. However, it is possible that binding to another chaperone such as BiP occurs prior to Crt binding since peak occupancy of Crt with APP occurs at 10 min of chase.

APP and Crt are part of a high molecular weight protein complex. In order to determine the size of the complexes containing both APP and Crt, we separated the complexes using 5–20% sucrose gradients and collected the gradient in 18–22 fractions. We analyzed the amounts of APP and Crt in each fraction and compared the distributions of APP and Crt in untreated cells; in cells treated with the chemical cross-linker, DTSSP, after CHAPS solubilization; and in cells solubilized in immunomix. As shown in Fig. 4, in cells that were solubilized in the mild detergent, CHAPS, a significant portion of both APP and Crt occurred in the higher density fractions of the gradient. This was the case whether or not the cells were cross-linked. In contrast, the APP and Crt in immunomix-solubilized cells were predominantly in lower density fractions. This indicates that APP and Crt are associated with other proteins in a SDS-labile complex.

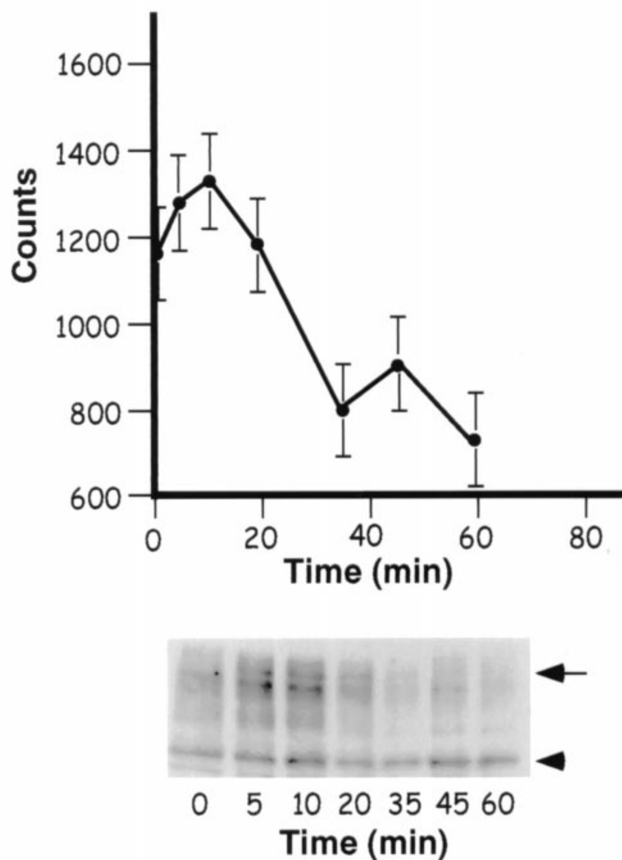

Fig. 3. The interaction between Crt and APP is transient in nature. APP-transfected HEK 293 cells were methionine starved, then labeled with ^{35}S-Met for 10 min. Labelled medium was replaced with unlabelled medium and the cells were chased for the indicated times at which point they were collected, solubilized in lysis buffer and immunoprecipitated with anti-Crt. The precipitates were analyzed by SDS-PAGE and fluorography. Quantitative analysis of bands was done using a Packard Instant Imager over 10 h. Labelled APP was maximal at 5 min of chase and declined thereafter. Arrow indicates the APP bands as determined by re-immunoprecipitation of a duplicate 0 time-point sample. Arrowhead indicates Crt.

Fractions from gradients of DTSSP-treated cells that contained both APP and Crt, were pooled and immunoprecipitated with anti-Crt. The precipitates were analyzed by SDS-PAGE under reducing conditions followed by Western blotting using the C8 antibody against APP (Fig. 5). APP was co-immunoprecipitated with Crt in fractions 7–12, which contained 1.347–1.357 g/ml sucrose. A standard curve (fraction number versus sedimentaion coefficient) using proteins of known sedimentation coefficients (thyroglobulin, MW = 685 kDa, S = 19–20; catalase, MW = 250 kDa, S = 11.3; BSA, MW = 67 kDa, S = 6–7; and lysozyme, MW = 23.2 kDa, S = 2.4) was generated. The APP/Crt-containing complexes sedimented between thyroglobulin and catalase with an approximate sedimentation coefficient between 13S and 15S. On a 5% SDS-PAG such as that shown in Fig. 6, the complex migrates significantly above the 210 kDa molecular weight marker. Taken together, these data suggest that the complex is large enough to accommodate more than one copy each of Crt (MW = 45 kDa) and APP (MW = 110 kDa.)

APP exists in two forms that differ largely in their various post-translational modifications, mainly O-linked glycosylation [37]. Both forms are detected in anti-Crt co-immunoprecipitations. We compared the protein interactions of the immature form of APP to those of the mature form in cells treated with DTSSP to determine the relative amounts of each that were complexed to other proteins. Fractions 4, 8 and 12 from cross-linked cells were analyzed by SDS-PAGE under non-reducing conditions, and Western blotted for APP using C8. Fig. 6 shows that virtually all of the immature form of APP is associated with other proteins in large complexes. In contrast much of the mature form of APP was not associated with these complexes. In fractions 4 and 10 from non-cross-linked cells, APP and Crt were not found in complexes that were stable to non-reducing SDS-PAGE conditions. This result indicates that mature APP, while present in the large complex, is not bound in a way that allows it to be cross-linked to other proteins.

4. Discussion

APP has become the subject of intensive investigation due to its endoproteolytic cleavage fragment, Aβ, which plays a critical role in the pathogenesis of Alzheimer's disease. There is evidence that a considerable quantity of Aβ is formed inside the cell in the ER, Golgi, and endosomal compartments [14]. Elucidation of the pathway and kinetics of APP trafficking is necessary for understanding how Aβ is formed, and the role of chaperones in this process. In this study, we address the interaction between APP and Crt. Crt has been shown to function as an ER chaperone, especially for glycoproteins [15,24,26,28,31,46] which include APP. We show here that APP can be co-immunoprecipitated with an antibody against Crt from cells lysed in a mild detergent. This interaction between APP and Crt exists whether or not the cross-linking agent DTSSP is present.

The precise nature of the interaction between Crt and APP is not clear. Crt binding appears to require glycosylation and, like its partial homolog calnexin, is often lectin-like in nature [26,28]. The lectin-mediated interactions of Crt are specific for the monoglucosylated, high mannose containing proteins [13,31,41,43]. However, there are documented examples of Crt binding via protein-protein interactions [1,26,17]. These include binding to other ER proteins such as BiP (Grp78), HSP90, Grp94, (stress proteins) [7,16], protein disulfide isomerase and ER57 (catalysts in disulfide bond formation) [3,6]. Crt also has specific binding sites for α-integrins [5,34], steroid hormone receptors [4,8] and various peptides [23] as well. It is thought that this ability to recognize specific peptide sequences accounts for the specificity of the protein interactions of Crt versus those

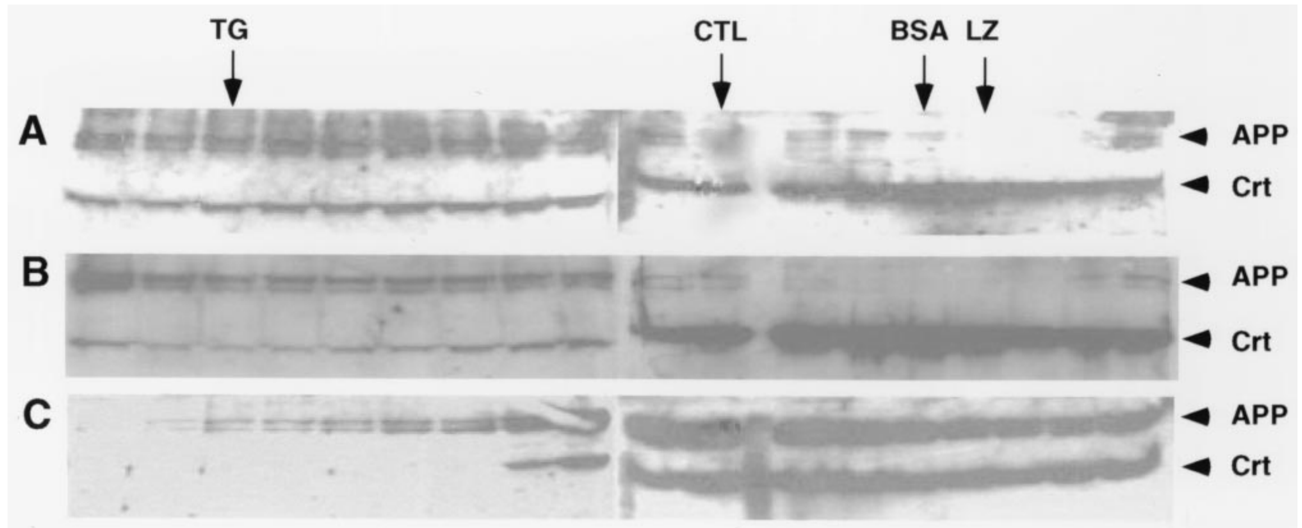

Fig. 4. APP and Crt occur in complexes that are separable on sucrose gradients. Cell lysates from DTSSP cross-linked and non-cross-linked HEK 293 cells were subjected to sucrose gradient centrifugation on 5–20% gradients. Samples from each of the 18 collected fractions were separated on an 8% SDS polyacrylamide gel under reducing conditions. The distribution of APP and CRT on each gradient was analyzed by Western blotting using antibodies against APP and CRT. A) Cross-linked, CHAPS-solubilized cells; B) Non-cross-linked, CHAPS-solubilized cells; C) Non-cross-linked, IMX solubilized cells. Both APP and Crt are shifted to the denser part of the gradient in cells solubilized under mild conditions (2% CHAPS). Arrowheads indicate the APP and Crt bands. Arrows indicate the location in the gradient of molecular weight markers. TG = thyroglobulin (685 kDa), CTL = catalase (250 kDa), BSA = BSA (67 kDa), and LZ = lysozyme (23.2 kDa).

of calnexin with which it shares a virtually identical carbohydrate specificity [45].

There is also evidence for Crt being involved in protein-protein interactions with newly synthesized proteins. Pipe and co-workers [32] found that castanospermine completely blocked the interaction between calnexin and factors V and VIII while the interaction of these proteins with Crt was only partially inhibited. It was recently found that the interaction of Crt with the gastrointestinal mucin, MUC 2, was inhibited by tunicamycin, but not by either castanospermine or DMJ [20]. Perforin, a constituent of lytic granules of cytotoxic T lymphocytes, has also been shown to bind to Crt via a protein-protein interaction [1]. In the case of APP, we see no evidence that inhibiting specific glucose or mannose trimming events interferes with its interaction with Crt. Neither does the interaction appear to require Ca^{2+}. Both of these results argue against a lectin-mediated interaction.

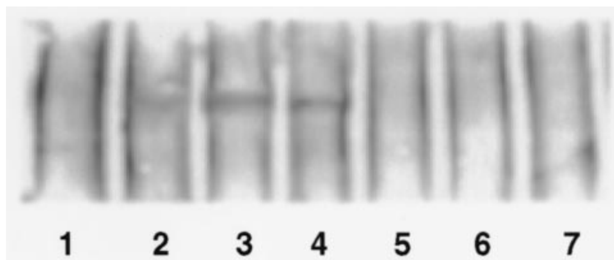

Fig. 5. Complexes containing both APP and Crt occur in discreet fractions. Cross-linked HEK 293 cells were solubilized and subjected to ultracentrifugation as above. Samples pooled in groups of 3 and these were immunoprecipitated with the antibody against Crt. Precipitates were assayed for APP by Western blotting after being subjected to 8% SDS-PAGE under reducing conditions. Complexes containing both APP and Crt occur in fractions 4–12, the peak occurring around fractions 7–9. This corresponds to an approximate molecular weight of 400 kDa. Lane 1–fractions 1–3, Lane 2–fractions 4–6, Lane 3–fractions 7–9, Lane 4–fractions 10–12, Lane 5–fractions 13–15, Lane 6–fractions 16–18, Lane 7–fractions 19–21.

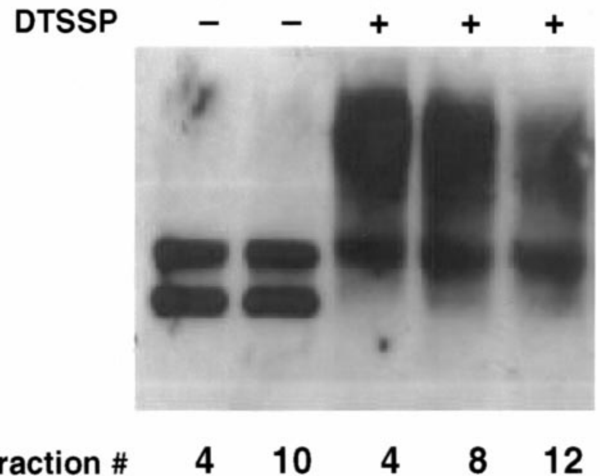

Fig. 6. Nearly all of the immature form of APP is associated with other proteins while most of the mature form is not. Cross-linked and non cross-linked HEK 293 cell lysates were subjected to ultracentrifugation as above. Fractions previously shown to contain both APP and Crt [4,8,10,12] were analzed by SDS-PAGE on a 5% gel under non-reducing conditions and subjected to Western blotting for APP. In cross-linked cells, the smaller, immature form of APP was not seen at around 120 kDa where it is seen in non-cross-linked cells. This indicates that it is likely to be associated with other proteins in large complexes. In contrast a band corresponding to the mature form of APP was seen not associated with other proteins. In non-cross-linked cells, neither the smaller nor the larger APP band was associated with other proteins.

Moreover, the presence of mature APP in co-immunoprecipitates suggests that for at least this form of APP, a lectin interaction is not involved. Since the removal of the terminal glucose of N-linked oligosaccharides occurs within either the ER, or at the latest, in the cis Golgi, mature APP molecules would not be expected to contain monoglucosylated high mannose sugars and should not be bound to the lectin site of Crt.

Tunicamycin treatment abolishes the binding of APP and Crt and appears to decrease the amounts of all proteins that co-immunoprecipitate with Crt. This suggests that N-glycosylation is indeed essential for Crt to bind to APP. It is conceivable that N-linked glycosylation promotes a conformational change essential for Crt binding. Alternately, an earlier permissive step may require N-linked glycosylation. Findings by Molinari and Helenius [22] reveal that glycoproteins lacking N-glycans near the amino terminus bind initially to the molecular chaperone, BiP, before binding to other chaperones such as Crt and calnexin. It has, in fact, been shown that APP, which lacks an amino terminal N-glycan, binds to BiP very early in its synthesis [50]. It is possible that when BiP binding is blocked with tunicamycin, subsequent Crt binding is abolished. Our pulse-chase data on the time course of APP binding to Crt does show a delay between the time of APP synthesis and maximal Crt occupancy by APP. These data are consistent with the hypothesis that APP binding to BiP is prior to and necessary for its binding to Crt.

We show that both the mature, O-glycosylated form of APP as well as the immature form interact with Crt. A similar finding has recently been reported by Goldgaber and co-workers [10]. We believe that the binding of mature APP to Crt in transfected HEK293 cells may be due to the fact that these cells overexpress APP approximately ten-fold [37] since we do not see mature APP bound to Crt in IMR-32 cells, nor in cultured cortical neurons (data not shown). Given that at pH 5.5, a pH level attained in the trans Golgi, very little APP can be co-immunoprecipitated with Crt, it is likely that in cells expressing physiological levels of APP, the APP/Crt interaction persists into the early cis Golgi but is disrupted by the decreased pH as it traverses the Golgi stacks. The mature APP is then transported to the plasma membrane while Crt is recycled to the ER via the KDEL receptor since these cells do not appear to express Crt on the surface [49]. Overexpressing cells may recycle mature APP back to the ER via the endosomal pathway and it is possible that this is the source of the mature APP bound to Crt. Our preliminary evidence indicates that mature APP can be found in the ER of the transfected cells.

Analysis of the size of Crt and APP-containing complexes using SDS-PAGE and sucrose rate-zonal centrifugation under conditions that do not disrupt the APP/Crt interaction revealed that some of the APP and Crt molecules are associated with other proteins in large complexes. Co-immunoprecipitation of cross-linked proteins with anti-Crt revealed that these complexes are quite large and may be over 400 kDa. Since the molecular weight of APP_{695} is about 110 kDa and that of Crt is about 45 kDa, it may be that there are more than one APP molecule and/or more than one Crt molecule per complex. It is likely, however, that in addition to Crt, other ER proteins such as BiP, Grp94, PDI, Erp57 and calnexin are present as well. Jethmalani and Henle [16] have demonstrated an interaction of Crt with Grp78 (BiP), Grp94 and HSP90 in heat stressed cells. The resulting complexes were determined by gel filtration chromatography to be from 400 kDa to 600 kDa in size. An interaction between Crt and BiP that is stable to chelators of divalent cations but sensitive to low pH has also been observed [7]. It has been shown that these chaperones may serve as a matrix that binds early folding and assembly intermediates and restricts their exit from the ER [43]. For example, Grp78 and Grp94 are involved in the maturation of thyroglobulin [18].

Although we found mature APP associated with Crt, we found that almost all of the immature form of APP in the cross-linked cells was associated with large complexes, while much of the mature form was not. This suggests there are fundamental differences in the nature of the Crt interaction with each of these forms.

In conclusion, we have found that in cells that express APP, the molecular chaperone, Crt, associates with APP in both its mature and immature forms. Both proteins participate in the formation of complexes that may contain other chaperones as well. This may have significant implications with respect to the pathogenesis of Alzheimer's disease since any insult that affects cellular levels of ATP, divalent cations or expression of stress proteins (including generation of oxidative radicals, excitotoxic injury, glucose or oxygen deprivation) could lead to a disruption of the complex. This could produce improperly folded APP resulting in an increase in $A\beta$. In this regard, Querforth and Selkoe [33], using the same cell line employed here, have shown that treatment of cells with a Ca^{2+} ionophore results in an increased secretion of $A\beta$.

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