In vitro aggregation of the regulated secretory protein chromogranin A

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Aggregation chaperones, consisting of secretory proteins that contain a hexa-histidine epitope tag, enhance the calcium-induced aggregation of regulated secretory proteins and their sorting to secretory granules. The goal of this study was to gain a better understanding of this unusual aggregation mechanism. Hexa-histidine-epitope-tagged secreted alkaline phosphatase, an aggregation chaperone, enhanced the *in vitro* aggregation of chromogranin A in the presence of calcium, but not in the presence of magnesium or other divalent cations. As an exception, chromogranin was completely aggregated by zinc, even in the absence of the aggregation chaperone. In addition, fluorescence spectroscopy of the aggregation reaction mixture showed an increase in fluorescence intensity consistent with the formation of protein aggregates. The calcium-induced aggregation of chromogranin A was completely inhibited by 0.2% Triton X-100, suggesting that it involves hydrophobic interactions. In contrast, the detergent did not affect chaperone-enhanced aggregation, suggesting that this aggregation does not depend on hydrophobic interactions. EDTA-treated chaperone did not enhance chromogranin A aggregation, indicating that divalent cations are necessary for chaperone action. Although the structure of the aggregation chaperone was not important, the size of the chaperone was. Thus the free His-hexapeptide could not substitute for the aggregation chaperone. Based on these results, we propose that the hexa-histidine tag, in the context of a polypeptide, acts as a divalent cation-dependent nucleation site for chromogranin A aggregation.

Key words: aggregation chaperone, calcium, hexa-histidine, His epitope tag, secreted alkaline phosphatase.

INTRODUCTION

Regulated secretory proteins, including peptide hormones, chromogranins, processing enzymes and digestive enzymes, are stored in secretory granules of endocrine cells, exocrine cells and neurons [1]. The sorting and storage of regulated secretory proteins in secretory granules depends on the storage conditions. These conditions (i.e. the ionic milieu of the *trans*-Golgi network and secretory granules) are similar in endocrine cells, exocrine cells and neurons and include a moderately acidic pH (5.0–6.5) and a high (millimolar) calcium concentration. Consistent with their similar storage conditions, many regulated secretory proteins exhibit similar biochemical properties that distinguish them from constitutive secretory proteins, which are not stored in secretory granules. As an example, regulated secretory proteins, but not constitutive secretory proteins, bind to other regulated secretory proteins *in vitro* [2,3]. Perhaps the best-characterized common property of regulated secretory proteins is their ability to aggregate from solutions of millimolar concentrations of calcium and moderately acidic pH, i.e. under the conditions of the distal secretory pathway (e.g. [4–7]). This aggregation distinguishes most regulated secretory proteins from constitutive secretory proteins, which do not aggregate under these conditions.

The interaction of different regulated secretory proteins with one another, while excluding constitutive secretory proteins, has been proposed to play a role in the sorting and storage of regulated secretory proteins in secretory granules [8,9]. This sorting mechanism may involve binding of a regulated secretory protein to a sorting receptor, which is also a regulated secretory protein [10], the use of a regulated secretory protein as a helper protein for sorting of another regulated secretory protein [11] or the aggregation of regulated secretory proteins [12–14]. Thus the common theme amongst multiple sorting mechanisms appears to be that sorting involves specific interactions with proteins that are destined for the regulated secretory pathway and secretory granule storage [15].

Given the above considerations, it came as a surprise that sorting of the regulated secretory protein chromogranin A (CgA) is enhanced by the co-expression of a constitutive secretory protein [16]. The constitutive secretory protein secreted alkaline phosphatase (SEAP) was modified by addition of a hexa-histidine epitope tag which neither altered the secretory behaviour of SEAP nor induced aggregation of the constitutive secretory protein [16]. Instead, the hexa-histidine-modified protein dramatically enhanced the aggregation of regulated secretory proteins. Based on these properties, this class of hexa-histidine-tagged proteins was termed aggregation chaperones [16].

An artificial system has been designed to mimic the regulated secretion of biologically active peptides [17]. In this approach, a therapeutic protein is cleaved from an aggregating fusion protein to allow drug-induced control of protein secretion. As an alternative, aggregation chaperones may be useful in directing peptide precursors to the regulated secretory pathway where they would be available for proteolytic processing, amidation and stimulated secretion. The latter system does not require modification of the peptide precursors and can be used for protein production in endocrine tumour cell lines that do not exhibit optimal sorting of regulated secretory proteins. Practical use of this system requires a better understanding of aggregation chaperones and how this type of aggregation differs from the physiological aggregation of regulated secretory proteins. In this report, we have further characterized the unusual properties of aggregation chaperones. It is shown that the chaperone does not

Abbreviations used: CgA, chromogranin A; SEAP, secreted alkaline phosphatase; SEAP-HIS, hexa-histidine-epitope-tagged SEAP.

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act by hydrophobic interaction and that the structure of the reporter protein does not determine chaperone function. Instead, divalent cations bound to the aggregation chaperone are critical for its function.

EXPERIMENTAL

Plasmids and cell lines

pcDNA3-SEAP-HIS was prepared and SEAP-HIS (hexahistidine-epitope-tagged SEAP) was stably expressed in GH4C1 cells, as described previously [16].

Purification of SEAP-HIS

This was accomplished using a previously developed protocol [16]. Briefly, Krebs–Ringer–Hepes secretion medium from GH4C1 cells that stably express SEAP-HIS was collected and concentrated by centrifugation on a 30 kDa-cut-off membrane filter (Centricon-30; Amicon/Millipore, Bedford, MA, U.S.A.). The concentrated sample was mixed with lysis buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl and 1 mM imidazole] and re-centrifuged. The retentate was mixed with Ni^{2+}–agarose that had been equilibrated in lysis buffer and incubated for 1 h on a rotary shaker. The Ni^{2+}–agarose gel was packed in a mini column, which was washed with wash buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl and 20 mM imidazole]. The column was then eluted with elution buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl and 250 mM imidazole] and the fractions with the highest SEAP activity were pooled and concentrated by filtration. The retentate was diluted in 10 mM Hepes, pH 7.4, and again concentrated by filtration to remove the imidazole-containing column buffer. The final product was stored frozen. A negative control sample was similarly prepared from GH4C1 cells that express SEAP lacking the 6 × His epitope tag.

In vitro aggregation assay

CgA was purified from bovine parathyroid glands as described in [18]. Each sample contained 3 or 5 μg of purified bovine CgA in 30 or 50 μl of 50 mM Hepes or 50 mM Mes (pH 5.5–7.4), with or without 0.2 % Triton X-100 and with or without 0.6 or 1 μg of purified SEAP-HIS. In some experiments, a His hexapeptide was substituted for SEAP-HIS in the aggregation assay. $MgCl_2$, $ZnCl_2$, $NiCl_2$ or $CaCl_2$ were added as noted in the text. NaCl was added to samples lacking divalent cations to maintain a constant salt concentration. The samples were incubated for 1 h at room temperature followed by centrifugation at 16000 ***g*** for 30 min. The supernatants and resuspended pellets were analysed by SDS/PAGE on 10 % gels, followed by immunoblotting using a polyclonal antiserum to bovine CgA (diluted 1:20000), as described in [13].

EDTA treatment of SEAP-HIS

To remove divalent cations, SEAP-HIS was treated with EDTA (25 mM) for 30 min at 4 °C and then washed twice by centrifugation in 2 ml centrifuge filters (Centricon-30). Pilot experiments had shown that SEAP was quantitatively recovered in the retentate. The final retentate was further concentrated in a lyophilizer and then resuspended in EDTA-free 10 mM Hepes buffer (pH 7.4) and used for aggregation assays.

Alkaline phosphatase assay

When pretreated SEAP-HIS or the native SEAP-HIS was used for the aggregation experiments, the SEAP activity in aggregation supernatants was analysed by Phosphalight alkaline phosphatase assay (Perkin Elmer Tropix, Bedford, MA, U.S.A.), as described in [19].

Fluorescence spectroscopy

Steady-state fluorescence measurements were made using an Aminco Bowman Series 2 Luminescence Spectrometer. Excitation and emission bandpasses were set at 4 nm and the scan rate was 1 nm/s. An excitation wavelength of 280 nm was used and emission spectra were collected from 275 to 400 nm. All fluorescence measurements were performed at 20 °C using a 0.5 ml cuvette with 1 cm path length. Each sample contained 7 μg of bovine CgA, the appropriate salt (15 mM) and SEAP-HIS (0.1 μg) as indicated. The buffer was 7.5 mM Tris/HCl (pH 7.5). Fluorescence measurements were taken within 5 min of mixing the samples.

RESULTS

Effect of Triton X-100 on aggregation of CgA

Most regulated secretory proteins, including CgA, exhibit calcium-induced aggregation [4,20]. The aggregation efficiency is generally low (30–40 % aggregation [13]) but it can be improved by the addition of so-called aggregation chaperones to the reaction [16]. Thus 6 × His-tagged secretory proteins, including SEAP-HIS, can mediate 100 % calcium-induced aggregation of CgA even at the physiological pH of 7.4 [16]. Calcium-induced aggregation of chromogranins appears to depend on ionic interactions, as it is inhibited by high salt concentrations [4]. In addition, the divalent cations may cause charge neutralization, leading to hydrophobic interactions of the protein molecules. To test this possibility, CgA was subjected to calcium-induced aggregation in the presence or absence of the aggregation chaperone SEAP-HIS and the non-ionic detergent Triton X-100 (0.2 %; Figure 1). In the absence of detergent or SEAP-HIS, CgA exhibited only moderate aggregation with a small increase attributed to calcium (Figure 1, lanes 1 and 2). Triton X-100 prevented this low-level aggregation, suggesting a dependence on hydrophobic interactions (Figure 1, lanes 5 and 6). In contrast, the SEAP-HIS-enhanced aggregation was not affected by the

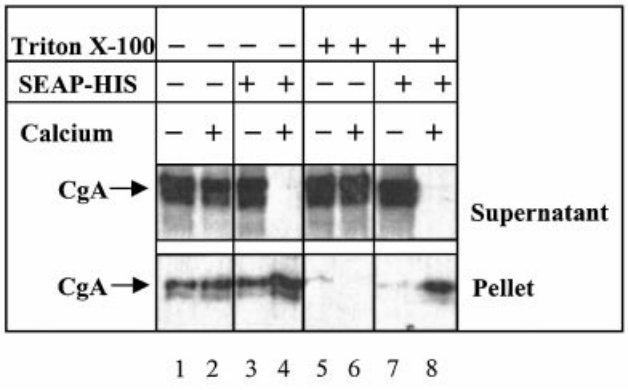

Figure 1 Calcium-induced aggregation of CgA

CgA (5 μg) was incubated in the absence or presence of 0.2% Triton X-100 for 1 h at room temperature. The samples contained 50 mM Hepes, pH 7.4, and 15 mM $CaCl_2$ (+Calcium) or 15 mM NaCl (−Calcium). At the end of the incubation, the samples were centrifuged for 30 min at 16000 ***g*** and the supernatants and pellets were analysed by immunoblotting using antiserum to CgA. The arrows point to the positions of intact bovine CgA. Similar results were obtained in three other experiments.

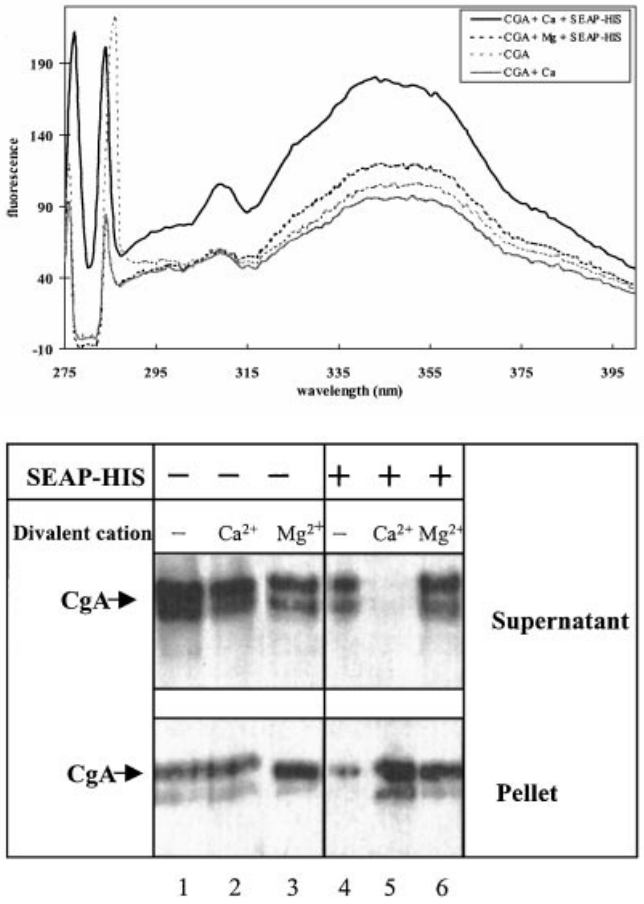

Figure 2 Fluorescence spectroscopy of CgA aggregation and the effect of magnesium

Top panel: fluorescence spectroscopy of CgA aggregation. CgA (7 μg) was incubated in 7.5 mM Tris/HCl (pH 7.5) with 0.1 μg of SEAP-HIS, 15 mM $CaCl_2$ or 15 mM $MgCl_2$, as indicated. Bottom panel: effect of magnesium on aggregation. CgA (5 μg) was incubated with or without 1 μg of SEAP-HIS for 1 h at room temperature. The samples contained 50 mM Hepes (pH 7.4) and 15 mM $CaCl_2$ (Ca^{2+}), $MgCl_2$ (Mg^{2+}) or no divalent cation (—). At the end of incubation the samples were centrifuged for 30 min at 16000 ***g*** and the supernatants and pellets were analysed by immunoblotting using antisera to CgA. The data are from a single experiment performed with duplicate samples.

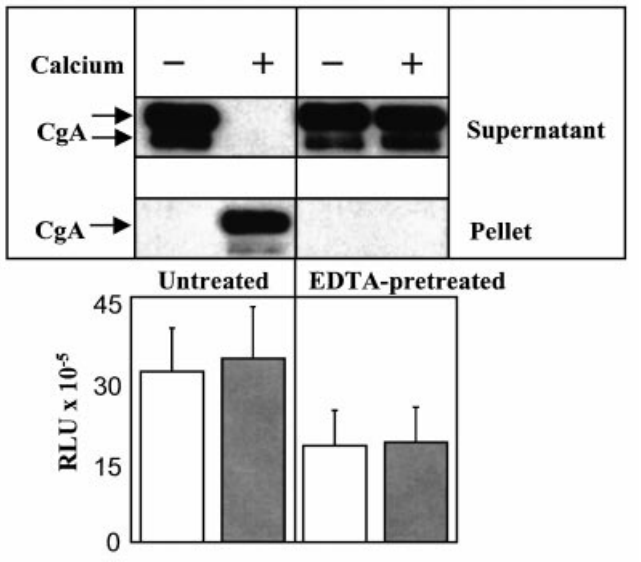

Figure 3 Aggregation with EDTA-treated SEAP-HIS

CgA (5 μg) was incubated in the presence of SEAP-HIS that had been pretreated with EDTA or not, as described in the Experimental section. The samples contained 50 mM Hepes (pH 7.4) and 0.2% Triton X-100, with or without 15 mM $CaCl_2$. At the end of incubation the samples were centrifuged for 30 min at 16000 ***g*** and the supernatants and pellets were analysed by CgA immunoblotting (top panel). SEAP activity in the supernatants was measured by alkaline phosphatase assay and presented as means ± S.E.M. ($n = 8$) (bottom panel); RLU, relative light units.

detergent, suggesting that aggregation chaperones do not act through hydrophobic interactions (Figure 1, lanes 4 and 8).

Effect of magnesium on aggregation of CgA

Calcium has been studied extensively in aggregation experiments, since it is the most abundant divalent cation in most secretory granules that store CgA and other regulated secretory proteins [4,21–25]. To test if chaperone-enhanced aggregation is specific for calcium, the aggregation reaction was performed in the presence of magnesium, another divalent cation that is found in significant amounts in living cells. Figure 2 (top panel) shows the fluorescence spectrum of CgA incubated in the presence or absence of SEAP-HIS and 15 mM $CaCl_2$ or $MgCl_2$. The increased fluorescence intensity detected in samples containing both SEAP-HIS and Ca^{2+} are consistent with tryptophan residue(s) shifting into a more hydrophobic environment, as would occur during the formation of chromogranin aggregates. Smaller increases in fluorescence were observed in the absence of SEAP-HIS or in the presence of SEAP-HIS and Mg^{2+} (Figure 2, top panel). In a control experiment, we confirmed that SEAP-HIS did not significantly enhance the aggregation of CgA in the presence of Mg^{2+} (Figure 2, bottom panel). In addition, no aggregation was seen when Mg^{2+} was used in the presence of 0.2 Triton X-100 (results not shown). Hence, magnesium cannot effectively substitute for calcium in the aggregation chaperone reaction. The effect of calcium is consistent with the dominant concentration of this cation in secretory granules.

Effect of divalent cations on the function of SEAP-HIS

Calcium and magnesium are the most abundant divalent cations in most cell types. However, less-abundant cations could affect the aggregation reaction if they are bound to or donated by the aggregation chaperones. Indeed, SEAP binds Zn^{2+} and Mg^{2+} at the active site [26] while His epitope tags have a relatively high affinity for divalent metal ions [27]. To test if chaperone-bound divalent cations play a role in the function of aggregation chaperones, SEAP-HIS was treated with EDTA and then used for the aggregation assay. As expected, there was some loss in the enzymic activity of SEAP-HIS (Figure 3), presumably due to the loss of Mg^{2+} and Zn^{2+} from the active site of the enzyme [26]. While the loss of enzymic activity of SEAP was only partial, the chelator caused complete loss of aggregation chaperone activity (Figure 3). The loss of chaperone activity was not merely due to the partial inactivation of the enzyme, since inactivation by heat denaturation did not eliminate the chaperone activity (results not shown). Together these results indicate that chaperone-bound divalent cations are necessary for its activity, whereas the three-dimensional structure of the chaperone is less important.

Several secretory proteins aggregate in the presence of Zn^{2+} (R. K. Jain, S. G. Venkatesh, C. Geetha, D. J. Cowley and S.-U.

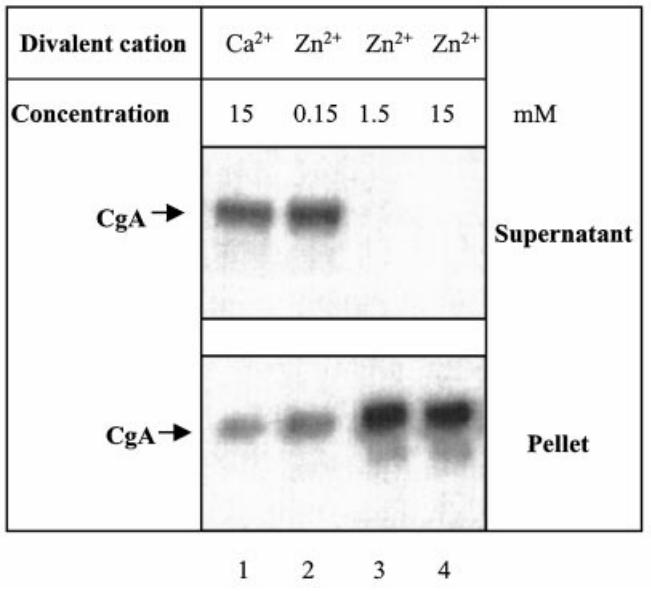

Figure 4 Effect of zinc on CgA aggregation

CgA (5 μg) was incubated in the presence of $CaCl_2$ or $ZnCl_2$ at the concentrations indicated. The samples were centrifuged and the supernatant and pellet fractions analysed by immunoblotting.

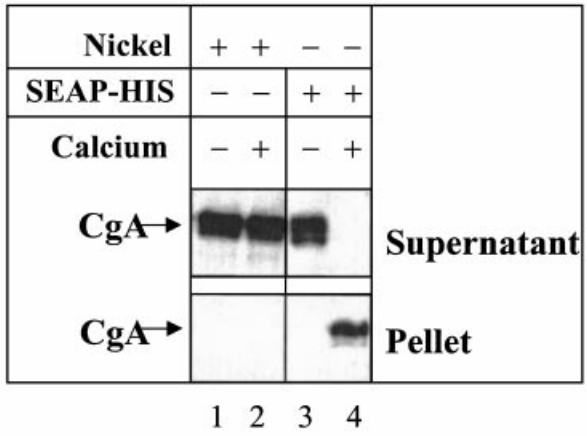

Figure 5 Effect of nickel on CgA aggregation

CgA (5 μg) was incubated in the presence of 1 mM $NiCl_2$ or 1 μg of SEAP-HIS with or without 15 mM $CaCl_2$. The samples were centrifuged and the supernatant and pellet fractions analysed by immunoblotting. The experiment was repeated with similar results.

Gorr, unpublished work). SEAP binds Zn^{2+} and this divalent cation could act as the precipitating agent in the aggregation chaperone reactions. To test this, the aggregation of CgA was tested in the presence of Zn^{2+} with no SEAP-HIS added. Figure 4 shows that 15 mM Zn^{2+} caused complete aggregation of CgA in the absence of SEAP-HIS. However, 0.15 mM Zn^{2+} did not enhance the aggregation of CgA in the absence of SEAP-HIS (Figure 4). Since the concentration of SEAP-HIS in the standard aggregation reactions is only 0.4 μM, it is unlikely that SEAP-HIS acts as an aggregation chaperone by donating Zn^{2+} from the active site of alkaline phosphatase or the His tag.

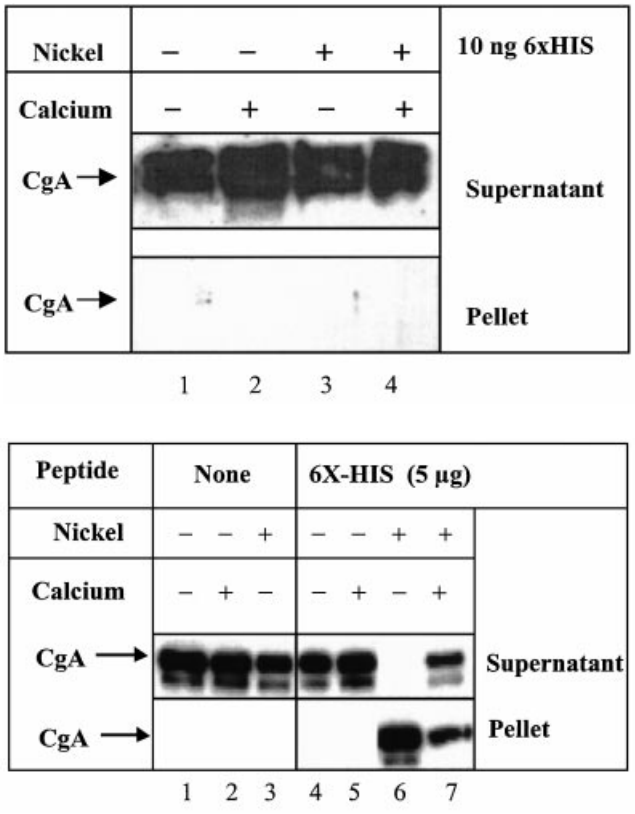

Figure 6 6 × His-peptide-induced aggregation of CgA

Top panel: CgA (3 μg) was incubated with 10 ng of 6 × His peptide (molar equivalent of SEAP-HIS) and 15 mM $NiCl_2$ and/or $CaCl_2$ as indicated. The samples were centrifuged and the supernatant and pellet fractions analysed by immunoblotting. The experiment was repeated with similar results. Bottom panel: CgA (5 μg) was incubated with or without 5 μg of 6 × His peptide in 50 mM Hepes, pH 7.4, and 0.2% Triton X-100 with or without 15 mM calcium and 1 mM nickel. The sample in lane 3 did not contain 15 mM NaCl. The samples were then centrifuged for 30 min at 16000 ***g*** and the supernatants and pellets analysed by immunoblotting using antiserum to CgA. The results from a representative experiment are shown. The data in each lane were reproduced in 2–5 experiments.

Three different His-tagged proteins, SEAP, 7B2 and guanylate cyclase, have been used as aggregation chaperones in this and a previous study [16]. The proteins were purified in different laboratories, but in each case Ni^{2+} columns were used for purification [16,28,29]. To test if Ni^{2+} from the columns could account for the aggregation properties of column-purified SEAP-HIS, calcium-induced aggregation of CgA was performed in the presence of 1 mM $NiCl_2$. Figure 5 shows that Ni^{2+} did not induce aggregation of CgA in either the presence or absence of calcium. Thus Ni^{2+} cannot substitute for SEAP-HIS to enhance the aggregation of CgA. As expected, SEAP-HIS caused complete aggregation of CgA in the presence of calcium (Figure 5). Column buffer alone or a control sample consisting of SEAP-containing medium passed through the Ni^{2+}column (both subjected to filtration) did not enhance the calcium-induced aggregation of CgA (results not shown).

Effect of the 6 × His epitope tag on the aggregation of CgA

The 6 × His epitope tag is common to the three aggregation chaperones tested in this and our previous study [16]. To test directly whether the 6 × His epitope tag was sufficient for

enhanced aggregation of CgA, a hexa-histidine peptide was used in aggregation experiments. When the peptide (840 Da) was added at a concentration of 0.4 μM, corresponding to the concentration of SEAP-HIS in previous experiments, it did not enhance the calcium-induced aggregation of CgA (Figure 6, top panel, lanes 1 and 2). Ni^{2+} (1 mM) had no effect on the aggregation of CgA at this peptide concentration (Figure 6, top panel, lanes 3 and 4).

The hexa-histidine peptide was then tested at a 500-fold higher concentration (200 μM), corresponding to 1 μg of peptide/μg of CgA. The peptide did not enhance aggregation of CgA in the presence of NaCl or $CaCl_2$, but caused complete aggregation of CgA in the presence of 1 mM $NiCl_2$ (Figure 6, bottom panel, lanes 1–6). This Ni^{2+}-induced aggregation was inhibited by 15 mM Ca^{2+}, but not by 15 mM Na^+ (Figure 6, bottom panel, lanes 6 and 7), suggesting that the divalent cations compete for binding sites on the hexa-histidine peptide. These results indicate that a hexa-histidine peptide is most effective as an aggregation chaperone when the peptide is part of a fusion protein, although it does not appear that the conformation of the fusion protein is important for its function.

DISCUSSION

Peptide precursors are sorted to secretory granules where they undergo proteolytic processing and amidation prior to release of the biologically active peptides by stimulated secretion [1,30]. An artificial system that uses regulated release of therapeutic peptides from fusion protein constructs to mimic regulated secretion was recently described in non-endocrine cells [17]. An alternative approach to modifying the regulated secretory protein is to increase the efficiency of sorting to take advantage of the processing and secretory machinery in endocrine cells. This is particularly important for protein expression in endocrine tumour cell lines, which often exhibit poor sorting efficiency. Calcium-induced aggregation of regulated secretory proteins has long been thought to play a role in sorting and storage of these proteins in secretory granules [8]. Recently we determined that aggregation chaperones, hexa-histidine-tagged secretory proteins that enhance calcium-induced aggregation, also enhance sorting of regulated secretory proteins to secretory granules [16]. The present study addresses the mechanism of action of the aggregation chaperone.

Unlike calcium-induced aggregation of CgA, the aggregation chaperone-induced aggregation does not appear to involve hydrophobic interactions. Similarly, while CgA aggregates at low pH in the absence of calcium, chaperone-enhanced aggregation is dependent on calcium. These findings suggest an aggregation mechanism that involves ionic interaction of chromogranin molecules, mediated by calcium. It is not clear whether SEAP-HIS acts as a calcium donor to the aggregation reaction or if the chaperone provides the appropriate milieu for enhanced aggregation.

Divalent cations play an important role in aggregation chaperone function. Removal of chaperone-bound divalent cations with the chelator EDTA resulted in a complete loss of chaperone function. Nickel, a constituent of the columns used for purification of His-tagged proteins, did not restore function of the EDTA-treated aggregation chaperone. The finding that function was not restored by the addition of various other divalent cations suggests that chelation caused an irreversible conformational change in the chaperone. This change is independent of the structural changes associated with heat denaturation of the protein.

The structure of the aggregation chaperone does not appear to be critical for function. However, the finding that free 6 × His peptide does not act as a chaperone in the presence of calcium suggests that the size of the chaperone matters. A possible explanation for these findings is that the larger aggregation chaperones serve as a nucleation site for aggregation of CgA, while the hexapeptide is not of sufficient size to induce nucleation.

The identification of aggregation chaperones suggests that calcium-induced aggregation is not a passive process but, rather, it can be regulated without modifying the aggregating protein. We previously found that CgA is sorted more efficiently to the regulated secretory pathway in the presence of SEAP-HIS than with SEAP alone [16]. This finding suggests that aggregation chaperones can function in living cells and that these artificial constructs can be used to enhance intracellular processes.

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