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In vitro characterization of ligand-induced oligomerization of the *S. cerevisiae* G-protein coupled receptor, Ste2p

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ABSTRACT

Background: The *S. cerevisiae* α-factor receptor, Ste2p, is a G-protein coupled receptor that plays key roles in yeast signaling and mating. Oligomerization of Ste2p has previously been shown to be important for intracellular trafficking, receptor processing and endocytosis. However the role of ligand in receptor oligomerization remains enigmatic.
Methods: Using functional recombinant forms of purified Ste2p, atomic force microscopy, dynamic light scattering and chemical crosslinking are applied to investigate the role of ligand in Ste2p oligomerization.
Results: Atomic force microscopy images indicate a molecular height for recombinant Ste2p in the presence of α-factor nearly double that of Ste2p alone. This observation is supported by complementary dynamic light scattering measurements which indicate a ligand-induced increase in the polydispersity of the Ste2p hydrodynamic radius. Finally, chemical cross-linking of HEK293 plasma membranes presenting recombinant Ste2p indicates α-factor induced stabilization of the dimeric form and higher order oligomeric forms of the receptor upon SDS-PAGE analysis.
Conclusions: α-factor induces oligomerization of Ste2p *in vitro* and *in membrane*.
General significance: These results provide additional evidence of a possible role for ligand in mediation of Ste2p oligomerization *in vivo*.

1. Introduction

G-protein coupled receptors (GPCRs) comprise one of the largest known super-families of receptors with in excess of 4600 genes identified [1]. They represent a major class of pharmaceutical targets [2] and about 50% of all modern drugs and almost one-quarter of the top 200 best-selling drugs in the year 2000 modulated GPCR activity [3]. All GPCRs were initially assumed to exist and function as monomeric entities, and models of ligand binding and signal transduction and thus drug design efforts have been based on this hypothesis [3–6]. However, the more recent detection of oligomerized states for GPCRs has added a new dimension to rational drug design [3,7,8].

In living cells, the life cycle of GPCRs is divided into stages including biosynthesis and modification in the endoplasmic reticulum and Golgi, transportation to the plasma membrane, glycosylation, ligand-promoted activation and signal transduction and internalization events [5,9–11]. Several GPCRs including CCR5, Ste2p, C5a receptor, β_2-adrenergic receptor, oxytocin receptor, vasopressin V1a and V2 receptors, and serotonin 5-HT2C receptor have been shown to oligomerize in the endoplasmic reticulum during biosynthesis to enable processing [12–17]. Further studies have demonstrated that receptor intracellular trafficking and some pre-endocytotic signaling events are dependent upon, not only oligomerization, but specific homo- or hetero-oligomerization of constitutive or ligand activated receptors [3,4,10,18–21]. Among other things, these results indirectly suggest the possibility of a link between receptor activation and oligomerization, but any specific role for ligand in directly modulating the oligomeric state of plasma membrane localized GPCRs remains unclear [3–6,22–25].

Ste2p (α-factor receptor) is a GPCR present in the plasma membranes of the *S. cerevisiae* MATa mating haploid type. Activation of Ste2p by pheromone leads to well characterized G-protein mediated signal transduction events, growth arrest and mating [26–32]. Homo-oligomerization of Ste2p during transportation, processing and then again later during endocytosis has been detected *in vivo* by fluorescent resonance energy transfer and co-immunoprecipitation [13,19,33]. A GXXXG motif in transmembrane segment 1 has been proposed to mediate the homo-oligomerization interaction [34]. Interestingly, co-expression of functional and defective alleles of Ste2p was found to decrease signaling [35–37], while co-expression of constitutively

Abbreviations: AFM, atomic force microscopy; DLS, dynamic light scattering; DM, N-dodecyl-β-D-maltoside; GFP, green fluorescent protein; GPCR, G protein-Coupled receptor; PBS, phosphate buffered saline; Ste2p, α-factor receptor; SMCC, succinimidyl *trans*-4-(maleimidylmethyl) cyclohexane-1-carboxylate; 293E, human embryonic kidney cells stably expressing the 293Epstein Barr virus nuclear antigen

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active with normal Ste2p was found to suppress the constitutive signaling [37,38]. These results suggest that while mature Ste2p molecules are activated independently, following activation they function in a concerted fashion to promote G-protein activation [39,40], lending further indirect evidence to the possibility of a role for ligand in mediation of oligomerization.

Studies of receptor oligomerization have predominantly been carried out *in vivo* yielding important biologically relevant information. However *in vitro* analyses could lend additional important mechanistic insight into oligomerization-related processes including identification of specific factors contributing to oligomerization as well as characterization of associated changes in interactions and conformations. Such studies have generally not been possible due to the requirement for functional purified receptor to carry out biophysical analyses. Recently a recombinant form of ligand-activatible Ste2p was over-expressed and purified from human embryonic kidney 293 EBNA1 (293E) cell membranes [41,42]. Herein, this recombinant Ste2p has now been applied toward *in vitro* analysis of ligand-related receptor oligomerization. Specifically, α-factor-induced homo-oligomerization of Ste2p in detergent micelles has been detected by atomic force microscopy (AFM). This observation was further confirmed by dynamic light scattering (DLS) experiments in solution and by chemical cross-linking studies in cell membranes. In particular, a ligand-dependent stabilization of homo-dimeric and higher order homo-oligomeric forms of the recombinant receptor is reported.

2. Materials and methods

2.1. Cell culture and transient transfection

Samples were prepared following the method reported previously [26,42]. 293E cells were maintained in Low-Calcium Hybridoma Serum-Free medium supplemented with 1% bovine calf serum and 50 μg/mL G418 as previously described. Cells were maintained in agitated (110–130 rpm) shaking flasks under standard humidified conditions (37 °C and 5% CO_2). One day prior to transfection, cells were diluted to a density of 0.25×10^6/mL in growth medium, supplemented with 1% bovine calf serum and transfected 18 to 24 h later when the density reached approximately 0.5×10^6 cells/mL. Plasmid pTT-Fc-STE2-GFP or pTT-Ste2p-StrepTagII-(His)8 and polyethylenimine (PEI) were mixed together in medium at 1 μg and 2 μg DNA per mL of culture to be transfected, respectively. Upon the addition of PEI to the DNA, the mixture was vortexed, incubated for 15 min and added to the cells. Cells were harvested 72 to 100 h post-transfection by centrifugation at 1500 g for 10 min, followed by a wash with ice cold PBS and a subsequent centrifugation step at 1500 g for 15 min. The cell pellets were frozen at −80 °C.

2.2. Receptor solubilization and purification

Detail of plasmid constructs are described elsewhere [42]. Expressed Fc-Ste2p-GFP or Ste2p-StrepTagII-$(His)_8$ was extracted from the membrane fraction. The pelleted cells were thawed and re-suspended in extraction buffer (50 mM phosphate buffered saline (PBS) buffer, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM AEBSF) with gentle swirling on ice. The solution was homogenized at 1500 psi pressure using a French press and centrifuged at 3,000 g to remove debris. The remaining suspension was centrifuged at 100,000 g for 60 min. The final membrane pellet was washed and resuspended in detergent extraction buffer (50 mM PBS buffer, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM AEBSF, 1% N-dodecyl-β-D-maltoside (DM), 5 mM β-mercaptoethanol), stirred for 1 h and centrifuged at 10,000 g. The solubilized Fc-Ste2p-GFP samples were then applied to protein-A Sepharose, washed with 10 column volumes of 100 mM Tris buffer pH 7.0 containing 100 mM NaCl, 0.05% DM, 5 mM $MgCl_2$, 20% glycerol (v/v) and eluted with 50 mM, pH 3.0, glycine buffer containing 100 mM NaCl, 0.05% DM, 5 mM $MgCl_2$, 20% glycerol (v/v) and immediately neutralized with 20% (v/v) 1 M Tris–HCl pH ~7.0. The solubilized Ste2p-StrepTagII-$(His)_8$ fractions were applied to Talon™ affinity resin. The Talon™ affinity column was washed by 10 column volumes buffer A (50 mM PBS, pH 8.0, 100 mM NaCl, 0.05% DM, 5 mM $MgCl_2$, 20% glycerol (v/v)) containing 50 mM imidazole and eluted with buffer A containing 250 mM imidazole.

2.3. Atomic force microscopy

The buffer system supporting purified Fc-Ste2p-GFP or Ste2p-StrepTagII-$(His)_8$ samples from membrane fractions, prepared as described above, were exchanged by ultrafiltration (MW cut off 10 kDa) into distilled water containing 0.05% DM to remove the bulk of salt from the sample. Samples were subsequently filtered through a 0.1 μm membrane filter and diluted in milliQ-water containing 0.05% DM to final concentrations of 0.1 mg/mL (0.9 μM Fc-Step-GFP and ~1.8 μM Ste2p-StrepTagII-$(His)_8$) with or without 1 μM final concentration α-factor (Zymo research) and then adsorbed to freshly cleaved, poly-L-lysine-coated (Sigma, MW 4000) mica. After a 10 min incubation at room temperature, the mica slide was rinsed carefully with milliQ-water and dried at 4 °C in a refrigerator for 5 min. Samples were imaged using a PicoSPM atomic force microscope (Molecular Imaging, Tempe, AZ, USA) using contact mode in air. The deflection images are shown. The AFM probes were silicon cantilevers from Mikromasch (force constant, 0.65 N/m).

2.4. Dynamic light scattering

DLS experiments were performed on a DynaPro-MS800 instrument (Protein Solutions Inc., Charlottesville, VA, USA) that monitors the scattered light at 90° from the incident light beam (wavelength of laser diode is 824.9 nm). The intensity fluctuation of the scattered light was recorded by single-photon-counting electronics. Assuming Brownian motion and a hard sphere model for the particles, the apparent diffusion coefficient (D) was converted to a hydrodynamic radius (R_h) using the Stokes–Einstein relation: $R_h=\kappa_B T/6\pi\eta_o D$, where κ_B is the Boltzmann constant, T is the absolute temperature, and η_o is the viscosity of the solvent. At least 20 measurements each of 10 s duration were collected for each sample. All samples were derived from the same protein preparation. Purified solutions of Fc-Ste2p-GFP and Ste2p-StrepTagII-$(His)_8$ were concentrated to a final concentration of 0.2 mg/ml by ultrafiltration (100 kDa MWCO) with washing into 50 mM PBS, pH 8.0, 100 mM NaCl, 0.01% DM, 5 mM $MgCl_2$, 20% glycerol (v/v) in the presence or absence of 100 μM α-factor and were subsequently filtered through 20 nm filters (Whatman, Brentford, UK). Extreme care was taken to reduce contamination of samples by dust. DynaILS software provided by the manufacturer to calculate membrane protein hydrodynamic radius of particles was used in analyzing the results. Solutions of bovine serum albumin (typical hydrodynamic radius 3.0 nm) were used as standards. All measurements were carried out at 10 °C.

2.5. Chemical cross-linking

Membranes from 293E cells expressing Fc-Ste2p-GFP by transient transfection were prepared as described above. Membranes resuspended in PBS buffer (150 mM NaCl, 50 mM phosphate buffer, pH 7.4) were split into two equal volumes; to which either α-factor to final concentration of 10 μM or an equal volume PBS buffer was added. After stirring for 30 min at 4 °C, each of these samples were split into two equal volumes and hetero-bifunctional crosslink reagent succinimidyl *trans*-4-(maleimidylmethyl) cyclohexane-1-carboxylate (SMCC) (PIERCE) was added to one of each sample type to a final concentration of 1 mM, and then the samples further stirred for 60 min at 4 °C. The reaction was terminated by the addition of 10 mM β-ME in Tris buffer (10 mM pH 7.0). Fc-Ste2p-GFP was enriched by

protein A sepharose column chromatography, concentrated and applied to 6% SDS-PAGE and further analyzed by Western blot using anti-GFP-HRP as the primary antibody.

3. Results

3.1. Observation of agonist-induced recombinant Ste2p oligomerization by atomic force microscopy

Fc-Ste2p-GFP and Ste2p-StrepTagII-$(His)_8$ were individually over-expressed and purified from membranes of 293E cells. Testing for ligand binding functionality demonstrated that approximately 80% of the purified receptors are in a biological active form as reported previously [42]. In the case of purified Fc-Ste2p-GFP, although quantitative binding assays have not been completed, a 10 nm blue shift of the fluorescent emission spectrum maximum arising from a purified Fc-Ste2p-GFP/fluorescent-modified α-factor complex (463 nm maximum) is observed compared to that observed for fluorescent-modified α-factor alone (473 nm maximum) [42]. This shift is indicative of the ligand either remaining bound to the receptor or re-binding to native receptor following the neutralization step after the low pH elution, and supports conservation of a native Fc-Ste2p-GFP receptor conformation after purification. The purified receptors were washed with distilled water containing 0.05% DM and characterized by AFM in the presence and absence of α-factor. After purification, any aggregated receptor was removed by passing the samples through a 100 nm membrane filter immediately prior to adsorbing the sample onto a poly-L-lysine coated mica surface.

Typical AFM images obtained in these experiments are shown in Fig. 1. In these experiments the resolution of the horizontal dimension is limited by tip convolution such that the overall observed sizes of particles on the mica surface are significantly larger than theoretically expected, and thus interpretation must be limited primarily to comparison of heights, which remain very precise. For Fc-Ste2p-GFP it was found that in the presence of approximately equimolar α-factor, the particle dimension was approximately twice that observed in the absence of agonist (Fig. 1A). Statistical calculations indicated an average of 2.8 nm molecular height in the absence of α-factor and two distinct species with 2.9 nm and 5.2 nm molecular heights in the presence of α-factor (Fig. 1B and C). Similar results were observed for Ste2p-StrepTagII-$(His)_8$ fusion protein (Fig. 2), where binding of α-factor increased molecular heights on average from 1.8 nm to 3.6 nm. The existence of two different species in the presence of agonist likely represents a mixture of unoccupied and α-factor occupied/oligomerized Ste2p on the mica surface. The smaller height (unoccupied) population likely represents the presence of some inactive recombinant purified receptor in the sample as well as some minor dissociation of ligand from native state Ste2p during sample preparation. In the case of Ste2p-StrepTagII-$(His)_8$, the smaller height species might also arise from the presence of a slight excess of receptor in the sample. As well, by way of a control for detergent concentration effects during filtration, a highly concentrated detergent-only sample was applied to the poly-Lys mica slide, which when imaged by AFM showed no significant molecules associating with the slide (data not shown). Thus either any detergent micelles don't adhere, or are 'scraped off or destroyed' by the passing probe and thus do not factor into the receptor samples. Overall, these results indicate a fusion-tag independent, α-factor induced Ste2p oligomerization event *in vitro*.

To confirm the identity of the particles being observed on the mica surface, the receptor samples were imaged following incubation with a mouse monoclonal antibody that recognizes the C-terminal GFP fusion. Initially, antibody was imaged alone (Fig. 3A) yielding much smaller particle sizes than Fc-Ste2p-GFP or Ste2p-StrepTagII-$(His)_8$ in molecular height and both horizontal dimensions. The images arising from samples of Fc-Ste2p-GFP mixed with anti-GFP antibody showed

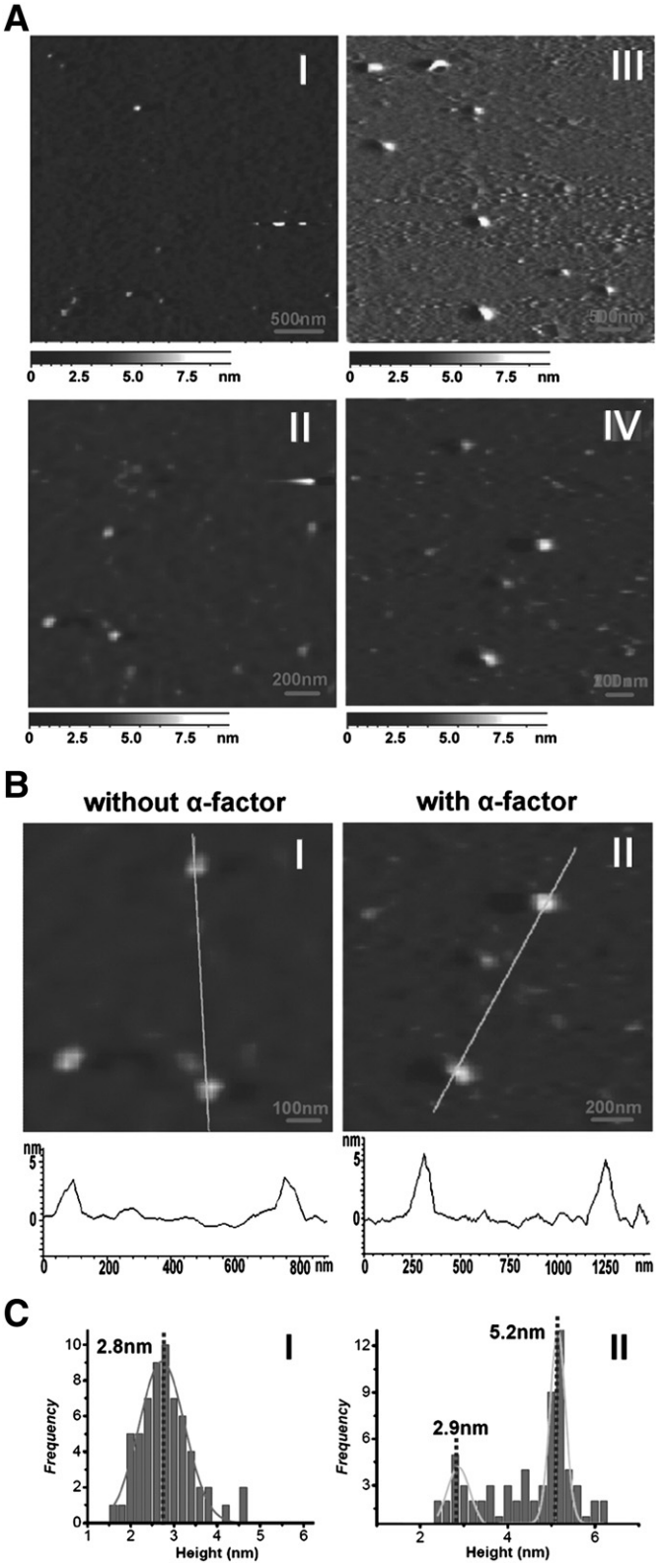

Fig. 1. AFM images of Fc-Ste2p-GFP in the presence and absence of agonist. (A) Left panel: in the absence of α-factor, low (I) and high (II) magnification images of Ste2p receptor; right panel: in the presence of α-factor, low (III) and high (IV) magnification images of Ste2p receptor. (B) the molecular height of Ste2p molecules in the absence (left panel) or presence (right panel) of α-factor. (C) the frequency distribution of molecular height of a total of 62 and 68 particles respectively, indicating heights of 2.8 nm on average in the absence of α-factor (I) and 2.9 nm and 5.2 nm on average in the presence of α-factor (II).

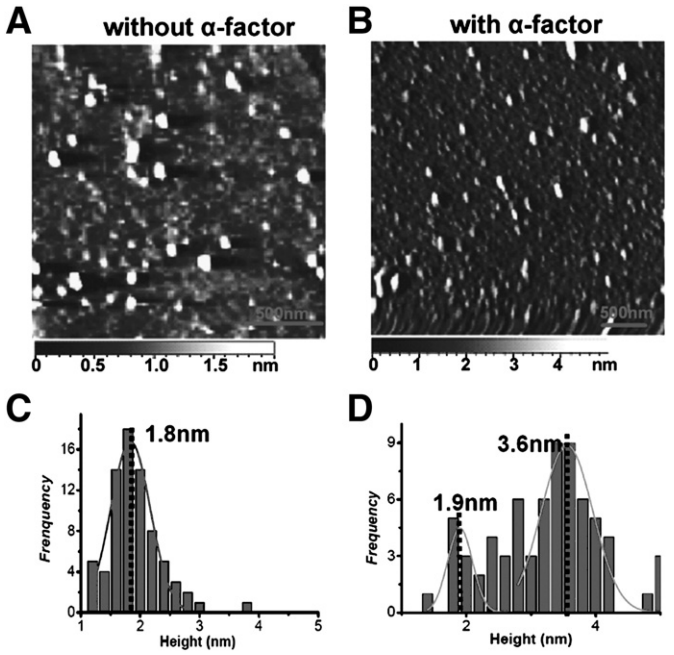

Fig. 2. AFM images of Ste2p-StrepTagII-$(His)_8$ in the presence and absence of agonist. (A) images of Ste2p-StrepTagII-$(His)_8$ receptor in the absence of α-factor; (B) images of Ste2p-$(His)_8$ receptor in the presence of α-factor; (C) the frequency distributions of molecular height for a total of 77 particles in the absence of α-factor indicating 1.8 nm on average; (D) the frequency distribution of molecular height for a total of 71 particles in the presence of α-factor indicating 1.9 nm and 3.6 nm on average.

some small particles designated free anti-GFP antibody and larger particles with heights of ~2.9 nm on average assumed to be complexes of Fc-Ste2p-GFP with anti-GFP antibody (Fig. 3B). An excess of anti-GFP antibody was used in the system to ensure the bulk of Ste2p was bound to antibody. In the presence of α-factor, the particle heights (and overall dimensions) were significantly increased up to 7.0 nm on average (Fig. 3C and D). This is significantly larger than the 5.2 nm average observed for activated Fc-Ste2p-GFP alone, thus further confirming antibody-binding and the agonist-induced oligomerization of Fc-Ste2p-GFP.

3.2. Dynamic light scattering determination of agonist-dependent recombinant Ste2p oligomerization

Dynamic light scattering measurements allow determinations of changes in protein size in solution. Fig. 4 shows the autocorrelation functions measured for Ste2p in the absence (Fig. 4A) and presence (Fig. 4C) of α-factor. The size distributions calculated from the autocorrelation functions (Rh values) are shown in Fig. 4B and D respectively. Purified Fc-Ste2p-GFP in the absence of α-factor yielded an average Rh of 6.7 + 0.4 nm, and in the presence of 100 μM α-factor yielded an average Rh of 8.4 + 0.3 nm, representing a ~1.7 nm increase. The wider size distribution observed in the presence of α-factor indicates an increase in polydispersity of particle sizes and likely represents averaging of coexisting low molecular weight oligomer and higher-level ligand-bound oligomeric forms. A small amount of large molecular weight species (>30 nm) was also observed (data not shown) and attributed to very small quantities of non-specifically aggregated receptor. By way of control, 100 μM α-factor was analyzed, yielding only a ~0.2 nm size distribution (data not shown). While this clearly excludes any significant contribution to the receptor/ligand complex Rh value by α-factor alone at 100 μM, the value of 0.2 nm should not be considered an accurate measure of the radius of α-factor in solution. Due to the small molecular weight of the peptide (~1600 Da) much higher concentrations would have to be analyzed to obtain an accurate radius. In contrast, Ste2p-StrepTagII-$(His)_8$ was found to be much more unstable in DM solution, and prone to faster aggregation with very high size distributions (>100 nm particles) regardless of the presence of α-factor (data not shown). While the ratio of aggregated to un-aggregated molecules was still relatively low, the DLS assay is much more sensitive to the higher size molecules thereby masking any remaining lower weight signals making further DLS analysis with Ste2p-StrepTagII-$(His)_8$ unfeasible. The difference in apparent non-specific aggregation of Ste2p-StrepTagII-$(His)_8$ between AFM and DLS experiments likely arises due to intrinsic differences between the two techniques (solid phase versus solution analyses) and is further compounded by the necessary use of higher receptor concentrations in the DLS work. Comparisons of the Fc-Ste2p-GFP results to observations of structural changes upon light induction of the GPCR rhodopsin [43], emphasize that conformational changes alone could not possibly introduce a 1.7 nm Rh increase. Thus the only possibility is Ste2p oligomerization. Lacking a reliable model for Ste2p oligomerization, we simply conclude that the DLS results further support the AFM data demonstrating ligand-induced Ste2p oligomerization.

3.3. Agonist-induced recombinant Ste2p oligomerization observed by crosslinking in membranes

Toward relating the observed ligand-dependent oligomerization results to a slightly more biologically relevant situation, crosslinking

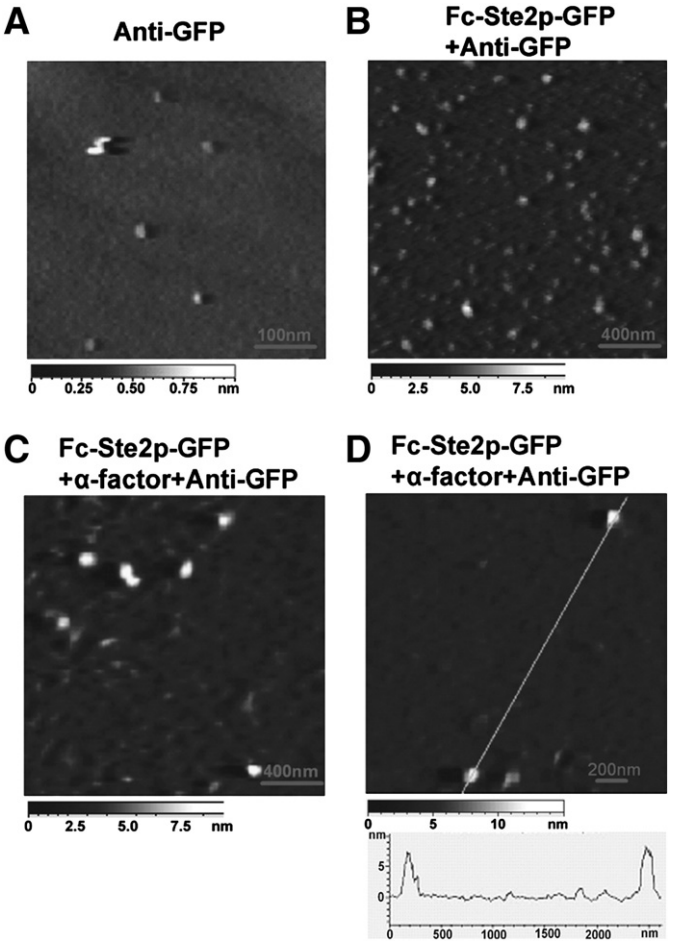

Fig. 3. AFM images of complexes between Fc-Ste2p-GFP and anti-GFP antibody. (A) Images of Anti-GFP antibody only. (B) Images of Fc-Ste2p-GFP incubated with antibody. (C) Images of Fc-Ste2p-GFP incubated with antibody and α-factor. (D) The molecular height of complexes between Fc-Ste2p-GFP and anti-GFP in the presence of α-factor.

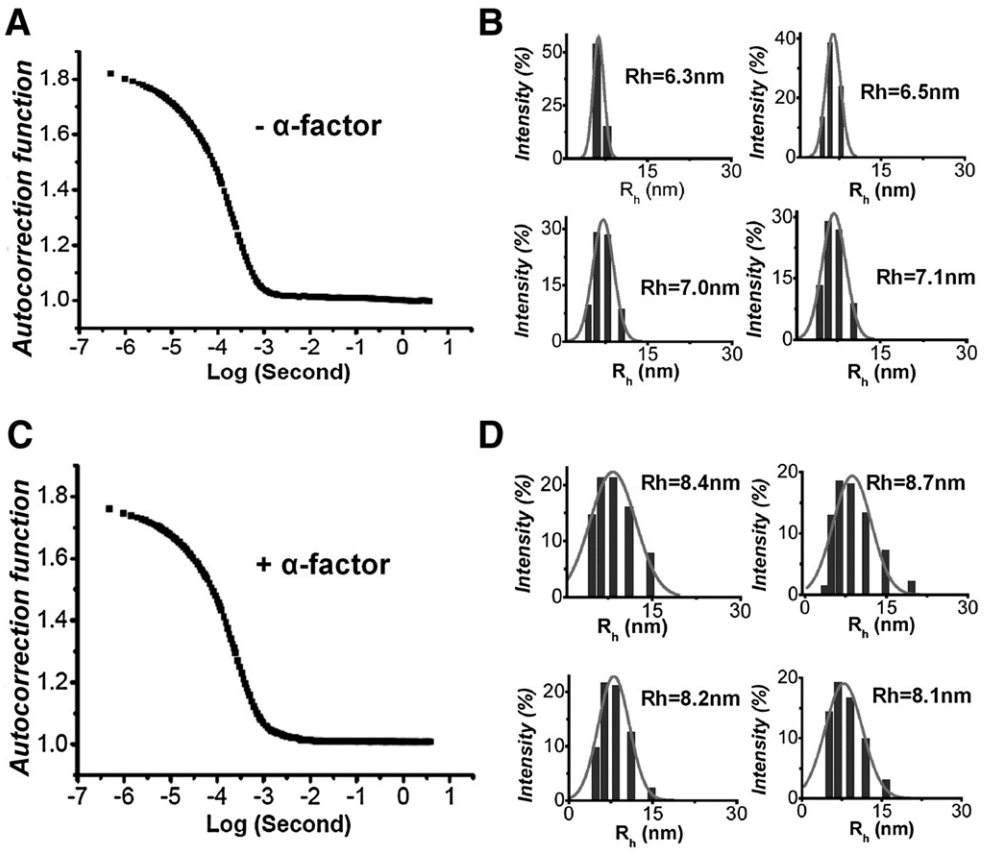

Fig. 4. Agonist induced Ste2p oligomerization probed by DLS. Experimental autocorrelation functions for Fc-Ste2p-GFP without (A) and with (C) α-factor. The size distribution of 4 separate experiments in the absence (B) or presence (D) of α-factor respectively.

analyses of receptor oligomerization were carried out in transfected 293E membranes presenting Fc-Ste2p-GFP. SMCC is a commonly used hetero-bifunctional chemical cross-linker that reacts both with amine and thiol groups [44]. Following the crosslinking step in the presence or absence of ligand, the receptors were detergent solubilized, purified and analyzed by 6% SDS-PAGE and Western blot using anti-GFP antibody. As shown in Fig. 5, two discrete bands are observed at approximately 100 kDa and 200 kDa respectively when crosslinked in the absence of α-factor as expected based on the theoretical MW of Fc-Ste2p-GFP. These same two bands were also observed in uncrosslinked samples in both the presence and absence of ligand. The upper band represents a well documented SDS-resistant dimeric form of the receptor that is commonly observed by SDS-PAGE [33]. The slightly broader aspect of the crosslinked bands compared to the uncrosslinked bands is likely attributable to heterogeneous modification of the Fc-Ste2p-GFP molecules by different numbers of SMCC molecules [42]. Crosslinking in the presence of α-factor increased the number of observed crosslinked species to three, including a weaker ~100 kDa band, a more concentrated ~200 kDa band and a new band at ~300 kDa (Fig. 5), representing monomeric, dimeric and trimeric forms of Fc-Ste2p-GFP. Overall, these results indicate that α-factor stabilizes the dimeric form of Ste2p and induces formation of higher-order recombinant Ste2p oligomers, including at least a trimeric form. However, the existence of even higher molecular weight oligomers cannot be excluded, as they may simply not be isolatable in sufficient quantities to be visualized, due to the statistical probabilities associated with the cross-linking event.

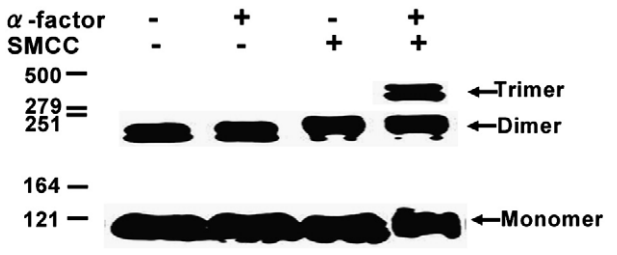

Fig. 5. SMCC cross-linking of Ste2p in 293E membranes. Visualization of the products of cross-linking Fc-Ste2p-GFP with SMCC in the absence and presence of α-factor. 6% SDS-PAGE and western blot with anti-GFP as the primary antibody were used in the determination.

4. Discussion

This work further extends earlier resonance energy transfer [13,19,34,37,39,40] and co-immunoprecipitation [33] based studies in which Ste2p was found to be oligomerized in both the presence and absence of agonist. While these earlier studies raised the possibility, they ultimately left ambiguous a specific role for ligand in oligomerization.

Herein it is demonstrated that ligand is sufficient to induce recombinant receptor oligomerization *in vitro*. The recombinant receptors were fusion proteins and two alternate fusion tagged forms of the receptor were tested. The observation of similar AFM effects with different tags essentially dismisses any possibility of the observed effects being mediated by the fusion tags. Another issue of potential concern is related to the biological relevance or specificity of the aggregation. Toward this we have demonstrated by crosslinking that ligand induces oligomerization in membranes. This 'in membrane' experiment essentially eliminates the possibility of any 'non-specific aggregation' arising from the relative instability of membrane

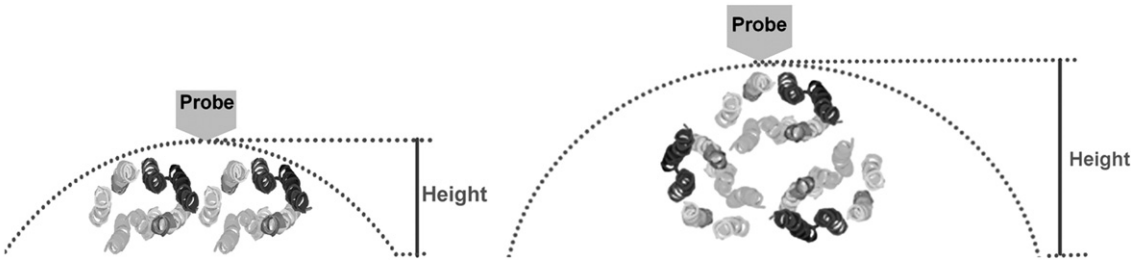

Fig. 6. Cartoon representations of Ste2p oligomerization events observed in AFM experiments. The expected relative heights for monomer, dimer and trimer forms of oligomerized Ste2p are modeled based on the three dimensional structure of rhodopsin [45]. An approximate two fold increase in height upon trimerization is highlighted. The models assume the GPCR is lying on its side on the mica surface.

protein in detergent solutions. Further to this, comparison to the crosslinking results obtained in the absence of ligand emphasize that these 'in membrane' crosslinking results are not simply the product of molecularly crowded over-expressed receptors. However, while 293E membranes may control for non-specific aggregation, they do not represent a full biologically relevant system and as such, as with most *in vitro* studies, we present the observed results only as additional indirect evidence of a possible role for ligand in mediating the oligomerization of Ste2p.

The width of bovine rhodopsin (measured perpendicular to the direction of the helices) is estimated to be approximately 2.5 nm based on the crystal structure [45]. As such the ~2–3 nm heights of single particles observed for Fc-Ste2p-GFP and Ste2p-StrepTaggII-$(His)_8$ by AFM in the absence of α-factor correlate with the crystal structure assuming the purified receptor is oriented to be lying on its side on the mica slide and taking into consideration the relative sizes of fusion tags. However this height observation cannot distinguish between monomeric or dimeric oligomerization (Fig. 6). Thus these results cannot confirm or preclude earlier data that suggests Ste2p exists in a dimeric form in the absence of agonist [13,19,33,34,37,40]. Similarly, cross-linking data shows both monomeric and dimeric bands in the absence of α-factor where the dimeric band has been previously attributed to an SDS-resistance dimer of Ste2p [42]. As such the work herein does not yield any new information about the specific oligomeric nature of the receptor prior to ligand exposure. Rather these studies provide insight into the dynamic changes that occur in oligomerization upon the introduction of ligand. Addition of α-factor, is shown herein to increase Ste2p particle heights supporting induction of oligomerization to higher levels. Further consideration of models (e.g. Fig. 6) indicates this oligomerization must be at least trimeric in nature to produce the observed AFM height increases. This coincides with the observed increases in both DLS determined Rh values and the population of dimeric and trimeric species isolated in membrane crosslinking experiments. This ligand-dependent oligomerization of Ste2p is similar to photobleaching induced rhodopsin higher-order oligomerization reported previously [46].

Based on consideration of additional reports on the oligomeric state of rhodopsin in detergent solution and membranes [46–48], dimerization of Ste2p [13,19,33,34,37,40] and the data presented herein based on AFM, DLS and crosslinking, we hypothesize that the native conformation of Ste2p is possibly dimeric, with α-factor stabilizing the dimeric form and further inducing higher order oligomerization. Further speculation related to the functional significance of ligand-induced Ste2p oligomerization suggests two possibilities: 1) the functional unit for interaction with the heterotrimeric G-protein and transduction of signaling is a higher order oligomer [39,40,49]; 2) the higher-order oligomerized activated receptor stabilizes interactions with opposite membrane/opposite membrane localized receptors for initiation of membrane fusion processes [26,50–52]. Characterization of the functional significance and structural aspects of this ligand related oligomeric recombinant receptor state will be essential for a complete understanding of the functionality of Ste2p and GPCRs in general.

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