Biochimica et Biophysica Acta 1783 (2008) 964–973

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Structural model of a complex between the heterotrimeric G protein, Gsα, and tubulin

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ARTICLE INFO

Article history:
Received 27 March 2007
Received in revised form 11 February 2008
Accepted 13 February 2008
Available online 4 March 2008

Keywords:
G protein
Microtubule
Crystal structure
GTPase
Cytoskeleton

ABSTRACT

A number of studies have demonstrated interplay between the cytoskeleton and G protein signaling. Many of these studies have determined a specific interaction between tubulin, the building block of microtubules, and G proteins. The α subunits of some heterotrimeric G proteins, including Gsα, have been shown to interact strongly with tubulin. Binding of Gα to tubulin results in increased dynamicity of microtubules due to activation of GTPase of tubulin. Tubulin also activates Gsα via a direct transfer of GTP between these molecules. Structural insight into the interaction between tubulin and Gsα was required, and was determined, in this report, through biochemical and molecular docking techniques. Solid phase peptide arrays suggested that a portion of the amino terminus, α2–β4 (the region between switch II and switch III) and α3–β5 (just distal to the switch III region) domains of Gsα are important for interaction with tubulin. Molecular docking studies revealed the best-fit models based on the biochemical data, showing an interface between the two molecules that includes the adenylyl cyclase/Gβγ interaction regions of Gsα and the exchangeable nucleotide-binding site of tubulin. These structural models explain the ability of tubulin to facilitate GTP exchange on Gα and the ability of Gα to activate tubulin GTPase.

1. Introduction

Microtubules, an essential component of the cytoskeleton, are composed of tubulin dimers where each dimer consists of an α and β monomer. One GTP binds to the nonexchangeable site (N-site) on α-tubulin and another GTP binds at the exchangeable site (E-site) on β-tubulin. GTP hydrolysis occurs at the E-site when another dimer binds to the growing microtubule at the positive end [1,2]. Over the past few years, significant progress has been made in the structural determination of the tubulin dimer. The domains on tubulin where drugs such as taxanes, colchine and vinblastine bind have been revealed. Much less information exists on where the microtubule associated proteins (MAPs) bind tubulin. However, many of the sites have been proposed to be on the C-terminus of tubulin. As the structure/function of tubulin dimers and microtubules is deciphered, the interfaces between a number of interacting molecules and tubulin will be revealed [2].

G proteins are heterotrimeric structures composed of α, β, and γ subunits. Upon agonist binding to membrane receptors, the Gα subunit is activated by the exchange of GDP for GTP leading to the extracellular message being passed to the intracellular side [3]. Activated Gα interacts with effector proteins and allows Gβγ to interact with effectors as well. Recently, it has become apparent that Gα and Gβγ proteins interact with a vast array of other cellular proteins that can affect the G protein activation/deactivation cycle [4,5]. Although distinct in structure (and other properties) from other G protein regulators, tubulin has long been known to interact with certain G proteins [6,7]. Of the Gα family of proteins, an inhibitory G protein α subunit of adenylyl cyclase (Giα1) and the stimulatory G protein α subunit of adenylyl cyclase (Gsα) bind with a high affinity to tubulin while other Gα subunits (e.g., the α subunit of the retinal G protein transducin; Gtα) show no measurable tubulin binding [7].

Tubulin–Gα interaction has been shown to induce changes in the GTP/GDP binding and kinetics in both Gα and tubulin. Gα proteins binding to tubulin activate the GTPase activity of tubulin, destabilizing the microtubules [8]. Conversely, Gα proteins can be activated in a receptor-independent mechanism in which a direct transfer of GTP (transactivation) from the E-site on tubulin to the Gα subunit occurs [9]. In the case of Gsα, this receptor-independent activation of Gsα subunits increases the coupling of Gsα to adenylyl cyclase [10]. Elucidation of the binding sites between Gα subunits and tubulin dimers should provide insight into this complex and novel interaction.

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Abbreviations: Giα, inhibitory G protein α subunit of adenylyl cyclase; Gsα, stimulatory G protein α subunit of adenylyl cyclase; Gtα, α subunit of the retinal G protein transducin; MAPs, microtubule-associated proteins; N-site, nonexchangeable site; SPR, surface plasmon resonance

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doi:10.1016/j.bbamcr.2008.02.017

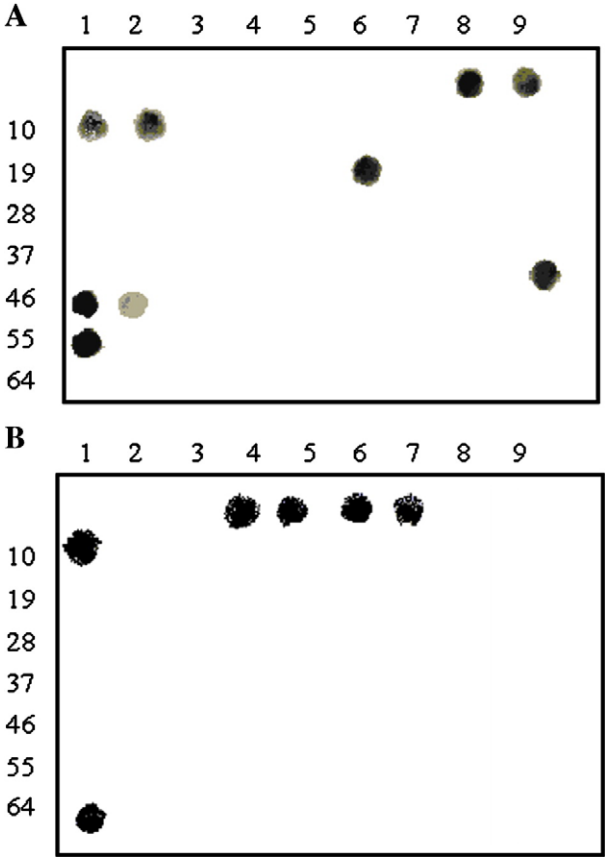

Fig. 1. Binding of tubulin to the Gsα-peptide SPOT Membrane. The SPOT membrane included 73 spots where the numbered spots correspond to the peptides (12 amino acids) which have been covalently attached to the cellulose membrane. Each peptide corresponds to a portion of the amino acid sequence of Gsα, which is divided into overlapping peptide (7 amino acid overlap to each peptide, total 73 spots). Membranes with overlapping sequences of Gsα were incubated with 150 nM tubulin-GDP (A) or tubulin-GTP (B). Quadruplicate experiments under the same conditions generated an identical pattern of tubulin binding.

Crystallographic studies provide a definitive method to determine protein–protein structures. However, tubulin has been difficult to study by crystallographic approaches [11–13]. Absent the ability to form Gα-tubulin crystals, another approach to determine protein–protein structures is through molecular docking programs with verification through biochemical assays.

In this study, we combine biochemical and molecular docking techniques to propose a model of Gsα and tubulin interaction. We have covalently attached overlapping peptides of the primary amino acid Gsα sequence to a membrane and determined tubulin binding to specific spots on the membrane [14], which provided potential high affinity sites important for tubulin–Gsα interaction. Further exploration and confirmation of these studies were made by comparing tubulin binding of these Gsα peptides to the sequence homologous peptides from Gtα, a G protein known to not bind tubulin despite significant structural similarities to Gsα [7]. To complement these studies, protein–protein docking algorithms were used to generate and refine a model for the articulating facets of these molecules [15,16].

This approach of combining biochemical and computational methods has allowed us to propose how tubulin and Gsα interact. This report reveals the first structural models of the Gsα–tubulin complex and suggests that tubulin interacts with Gsα predominantly in the GTPase domain, more precisely with regions essential to adenylyl cyclase activation (α2–β4 and α3–β5) [17]. This model also suggests that Gsα binds to tubulin such that it surrounds the nucleotide-binding site of β-tubulin, in a region of tubulin normally involved in docking other tubulin molecules during microtubule polymerization. These structures reveal how tubulin might transactivate Gsα and how Gsα can activate tubulin GTPase. This allows further understanding of the interface between heterotrimeric G protein signaling and the cytoskeleton.

2. Materials and methods

2.1. Protein preparations

Ovine brain tubulin was prepared as previously described [18]. Briefly, the brains were obtained at a local slaughterhouse from freshly-killed animals and were placed on ice upon removal from animals. Microtubule associated proteins were removed by phosphocellulose chromatography. Purity of the prepared tubulin, as determined by SDS gel electrophoresis, was always greater than 95%. Nucleotide replacement on tubulin was performed as before [19], using charcoal to strip bound nucleotide. Protein concentrations were determined by Coomassie Blue binding (BioRad Protein Assay) with Bovine Serum Albumin as a standard [20].

2.2. SPOT membranes

Peptides were synthesized on to a cellulose membrane with PEG spacer (8×12 cm^2) (AIMS Scientific Products, Braunschweig, Germany) via the C-terminal amino acid in sequential spots by the use of a SPOT synthesis kit (SIGMA genosys, St. Louis, MO) [14,21]. The peptides corresponded to the amino acid sequence of Gsα (accession number P04895, homo sapiens, Gsα long form), The primary sequence was divided into overlapping peptides for a total of 73 spots (12 amino acids in length with 7 amino acid overlap between sequential peptide). For the first SPOT membrane, the Gsα membrane, Spot 1 corresponded to amino acids 1–12 and Spot 2 corresponded to residues 6–17 in the primary amino acid sequence, etc.

Another SPOT membrane, the Gsα–Gtα membrane, was created based on the results of the Gsα membrane. For this SPOT membrane, we compared Gsα-peptides that bound tubulin to Gtα-peptides, because Gtα does not bind tubulin. The sequence of the Gsα-peptides that were found to bind tubulin in Fig. 1 (with an approximate 5 amino acid extension added to the N-terminal and C-terminal) was used as well as the corresponding amino acid sequence of Gtα. For instance, the Gsα sequence of spots 4–11 in Fig. 2 was taken, the 5 amino acids from the Gsα sequence toward the N-terminal

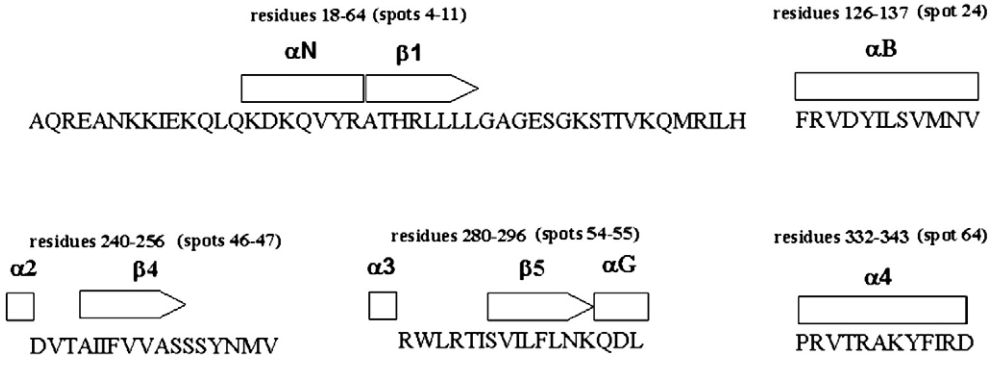

Fig. 2. Residues of Gsα that interact with tubulin according to SPOT membranes shown in Fig. 1. The specific domains, α-helices (α) or β-sheets (β), as well as their corresponding number or letter are indicated as described previously [17].

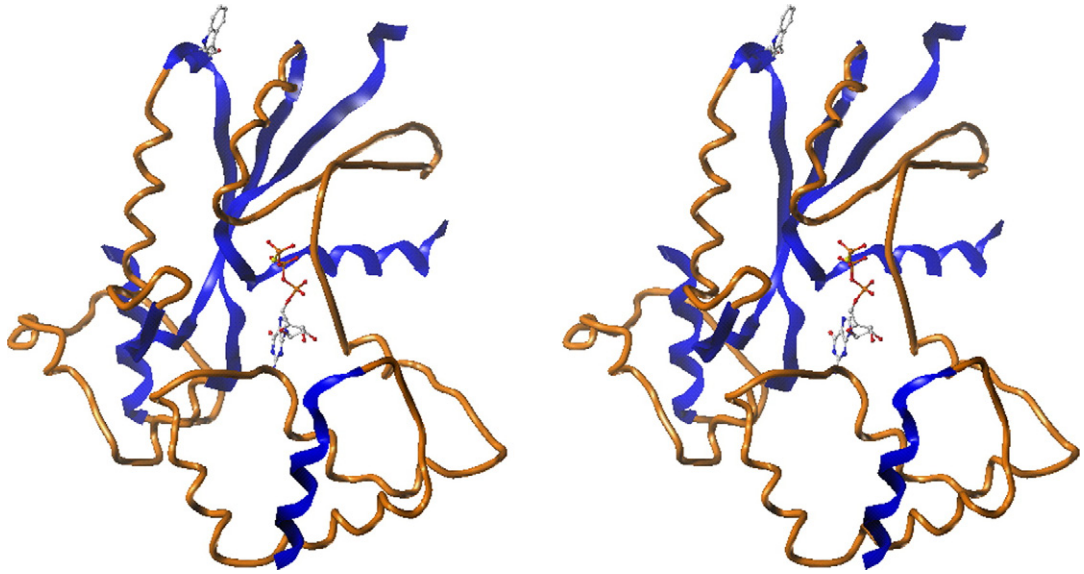

Fig. 3. Tubulin binding regions on the tertiary structure of Gsα. Stereo structure of Gsα–GTPγS in a line ribbon form (structure from ref. [17]) with the regions showing binding to β-tubulin. The side chain of TRP281 and GTPγS are shown in the ball and stick configuration with the color scheme by atom type. The structure of Gsα–GTPγS [17] is missing the following residues determined to be important from the results shown in Figs. 1 and 2: ALA18–LYS34 and ASN66–PHE68.

as well as C-terminal were added to both ends of the sequence, and then the sequence was divided into overlapping 15 amino acid length peptides. These peptides were divided into overlapping peptides (15 amino acids in length with 10 amino acid overlap between sequential peptide, 70 total spots). Spot 1 corresponded to the 1st 15 amino acids from Gsα that bound tubulin in Fig. 1, spot 2 corresponded to sequence aligned amino acids from Gtα, spot 3 corresponded to a peptide shifted 5 amino acids toward the C-terminal end of that sequence from Gsα and spot 4 corresponded to the sequence aligned amino acids from Gtα, etc. Certain regions of Gsα lacked corresponding regions in Gtα. For these regions of Gtα, we substituted the corresponding amino acids of Gsα into the Gtα-peptides.

For the Gsα membrane studies, the membranes were blocked with TBS-containing 0.1% Tween-20 (TBS-T) with 2.5% milk for 1 h, washed with TBS-T and incubated overnight at 4 °C with 150 nM tubulin in RIPA buffer (10 mM Tris–Cl, pH 7.4, 1% Titron-X-100, 1% Sodium Deoxycholate, 1% SDS and 500 mM NaCl). Next, the membranes were washed 3× with RIPA buffer and incubated with anti α-tubulin antibody (Sigma, St. Louis, MO), followed by the horseradish peroxidase conjugated secondary antibody (1 h each at room temperature in the RIPA buffer containing 1% milk) and developed with enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham Biosciences). For the Gsα–Gtα membrane studies, TBS-T was substituted for the RIPA buffer through the above methods. The RIPA buffer was used for the Gsα membrane studies to provide stringent conditions as means to avoid non-specific binding, whereas, TBS-T alone was used with Gsα–Gtα membrane studies to verify that the strong detergent did not interfere with the results seen in the presence of the RIPA buffer.

For stripping the membranes, we modified a previously described procedure [14]. Following the tubulin binding experiments, the membrane was always stripped and re-probed with ECL to verify that residual protein–antibody complexes were stripped before reuse. An example of one of the control experiments is shown, which demonstrates the absence of residual tubulin–antibody binding to the membrane following stripping (Figure S1). Also, controls were also done to verify that there is no non-specific binding of the primary or secondary antibody to the membranes and that no residual tubulin remained bound to the peptides following the stripping procedure. For these controls, the membranes were incubated with primary and secondary antibody following

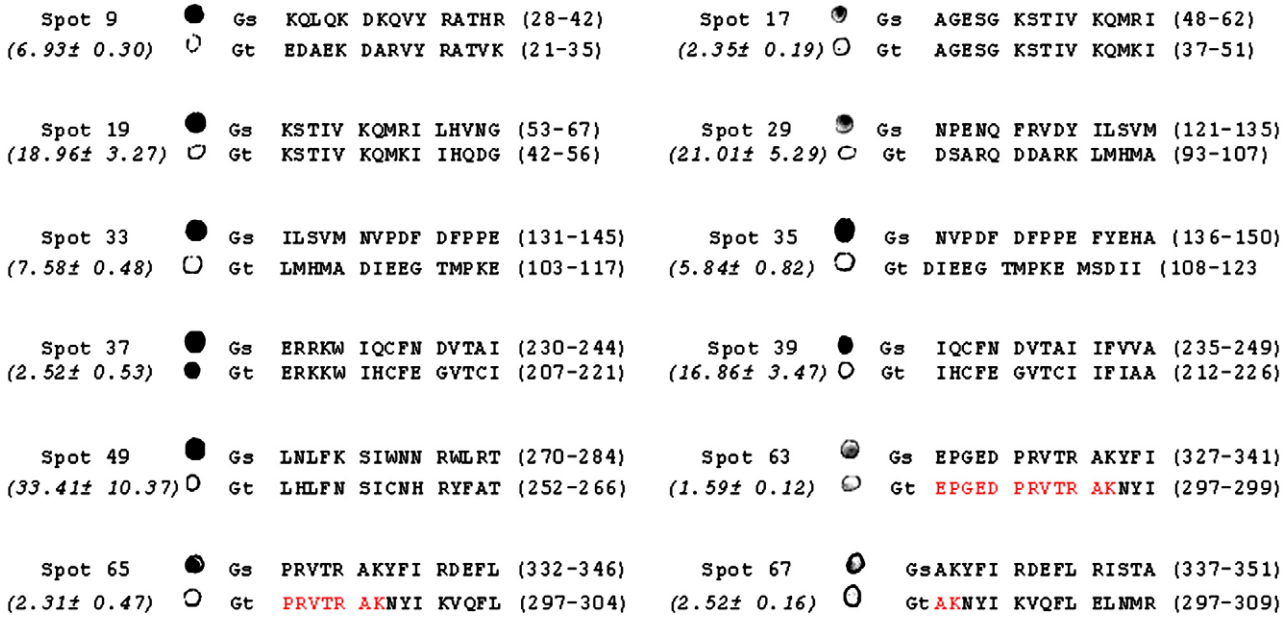

Fig. 4. Binding of tubulin to Gsα-peptides as compared to Gtα-peptides. Peptides from Gsα showing tubulin binding were compared to the corresponding Gtα peptide. The numbers shown in parenthesis represent the ratio between densitometric determinations of tubulin binding to Gsα vs, Gtα on the spot membrane ($n=3\pm$SEM). The membrane was incubated with 150 nM tubulin (as in Fig. 1). Note that spot 63, 65, and 67, the residues in red in the Gtα peptides are those of Gsα, as these amino acids are missing from Gtα (see Materials and methods for full explanation).

by development with ECL and the result consistently showed that there was no non-specific binding of either primary or secondary antibody to the membrane or any remaining tubulin bound to the peptides after stripping (Figure S2). In summary, these control experiments verified the absence of non-specific binding of the primary antibody, secondary antibody or tubulin with the peptides after stripping the membranes. To further control for non-specific binding, tubulin was incubated with equimolar Gsα (150 nM) at 37 °C for 1 h to form protein complexes and next, the complexes were incubated with the SPOT membrane (Gsα membrane) overnight followed by incubation with the antibodies

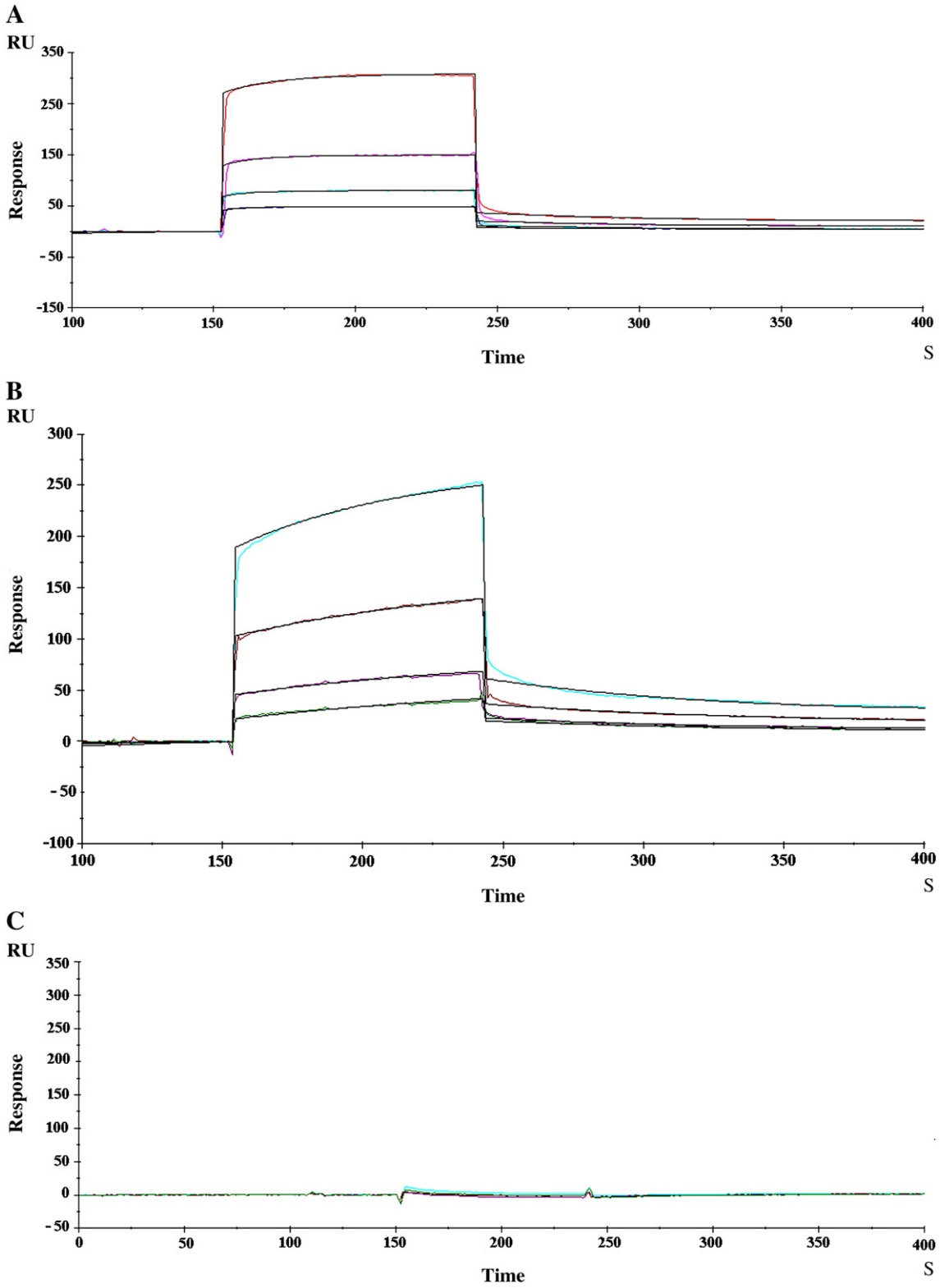

Fig. 5. Sensorgrams representing the surface plasmon resonance analysis of the interaction between immobilized tubulin and the peptides corresponding to Gsα (A), Giα (B) and Gtα (C) in spot 49 of Fig. 4 (peptide 3 and Gi or Gt analogs). (A) Binding of the Gsα peptide to tubulin with $k_a = 171\ M^{-1}s^{-1}$, $k_d = 8.07 \times 10^{-3}\ s^{-1}$ and $K_D = 4.07 \times 10^{-5}$ M. (B) Binding of the Giα peptide to tubulin with $k_a = 1 \times 10^3\ M^{-1}s^{-1}$, $k_d = 9.61 \times 10^{-3}\ s^{-1}$ and $K_D = 1.11 \times 10^{-5}$ M. (C) Binding of Gtα peptide to tubulin. The binding signals obtained for Gtα-peptide were too weak to provide reliable kinetic fits. The concentration series used were 100, 50, 25 and 12.5 μM. Curves were fit using a 1:1 binding model with drifting baseline (*black*).

and detection with ECL as above Figure S3). This prior incubation with Gsα prevented tubulin from binding to the immobilized peptides on the Gsα membrane. Note that, in order to ensure the continued validity of the membrane, control experiments were interspersed, temporally, with those testing tubulin binding.

2.3. Molecular modeling

Because the crystal structure of Gsα was determined as a dimer [22], one of the Gsα molecules in the dimer was deleted along with all of its corresponding ligands (the PDB file is 1AZT). In the remaining Gsα molecule, the Mg^{2+} and PO_4^{4-} molecules were deleted and GTPγS was retained. For the structure of the tubulin dimer [23], the α-tubulin subunit was removed along with its corresponding nucleotide and taxol (the PDB file is 1TUB). For the remaining β subunit, GDP was retained and taxol was removed.

The docking algorithm, ZDOCK2.3, http://zlab.bu.edu/zlab/protein.shtml0 [15] was used first for the unbound protein–protein docking where 2000 predictions were generated using β-tubulin as receptor and Gsα as ligand. ZDOCK was downloaded to a Linux system. Parameters were added to uniCHARM for GTPγS (GSP, as named in the original Gsα PDB). The parameters used were from the uniCHARM file for GNP and the sulfur in GTPγS was used from the sulfur in CYS. ZDOCK uses a fast Fourier Transform algorithm. The protein–protein interface is evaluated by shape complementarity, desolvation energy, and electrostatics. A number of protein docking algorithms are available, and each have strengths and weaknesses. ZDOCK has been ranked in the top 3 protein–protein docking programs at the CAPRI competition [24] for accurately predicting protein–protein structures.

The 2000 complexes generated from ZDOCK were then submitted to ClusPro, http://nrc.bu.edu/cluster/ [16]. Since ZDOCK has been shown to give more accurate protein–protein docking results than the ClusPro docking program [15], ClusPro was just used to cluster the 2000 protein–protein complexes generated by ZDOCK, and to identify the highest ranked representative complex. ClusPro calculates pair-wise RMSD values to find neighbour complexes within 9 Å of another complex. These complexes were clustered and the top 30 clusters were returned for further evaluation. To minimize the side chain clashes, the ranked complexes in the clusters were subjected to a minimization using CHARMM [16]. These clusters were then ranked according to population in each cluster. The parameters for ClusPro were set in the advanced options section for filtering and clustering, with a radius of 9 Å, the electrostatic hits at 1500, and a return cluster output of 30. The representative complex for each particular cluster is the complex that is most centrally located in the array of complexes.

Further characterization of the top five complexes was performed by energy minimization of the ZDOCK/ClusPro-derived complexes. Each of the top 5 complexes was further examined for an additional 5000 cycles with the SANDER package within AMBER7 and a minimization energy score was determined. The buried surface area (BSA) of each complex was determined within the GRASP program, as to determine which complex has the largest contact area. Visualization of protein structures was performed via GRASP [25] or SYBYL 6.9 (Tripos, Inc, St. Louis, Mo) software.

2.4. Binding analysis by surface plasmon resonance (SPR)

Quantitative analysis of peptide–tubulin interactions were performed on a BIAcore 1000 system (Pharmacia Biosensor AB, Uppsala, Sweden). Tubulin was immobilized in HBS-P buffer pH 7.4 (0.01 M Hepes, 0.15 M NaCl, with 0.005% (v/v) surfactant P20) at a flow rate of 10 μL/min on sensor chip CM5 according to the manufacturer's instructions. Different concentrations of synthetic peptides (12.5, 25, 50 and 100 μM in HBS-P buffer) were injected onto the flow cell at a flow rate of 30 μL/min. Curves from buffer blank injections and the reference flow cell (no ligand) were subtracted from all binding curves to correct for non-specific binding and buffer shifts created during injection. The surface was regenerated by injection of 1 M NaCl, 0.5% Triton X-100 in HBS-P buffer. Curves were global fitted to the 1:1 binding with a drifting baseline reaction model using the BIA evaluation 4.1 program (Pharmacia Biosensor AB).

3. Results

To determine the specific domains on Gsα that bind to tubulin, we used the SPOT technique [14], in which peptides corresponding to the primary Gsα sequence were covalently attached to a cellulose-based membrane. The Gsα membrane was probed with tubulin in the GDP or GTP bound stages (Figs. 1A and B, respectively). Tubulin–GDP interacted with 9 peptides and tubulin–GTP interacted with 6 peptides. Control experiments described in the methods section indicated no non-specific binding of the primary or secondary antibodies or residual tubulin binding to the membrane following stripping (see Methods).

Tubulin binds with a high affinity ($K_D \cong 130$ nM) to Gsα and Giα, but does not bind to the photoreceptor G protein, Gtα [7]. Fig. 2 shows the amino acids of the Gsα-peptides that exhibited binding to tubulin. Certain domains that interacted with tubulin, the α2–β4 and α3–β5 regions, are known to be important in the interaction with adenylyl cyclase as well as with Gβγ [17,26]. Furthermore, in the α3–β5 region, there is a tryptophan in Gsα (W281) and Giα1 that corresponds to a tyrosine in Gtα. This residue is located on the protein surface where solvent-exposed hydrophobic residues often contribute to protein–protein interactions (Figs. 2 and 3).

The structure of Gα proteins includes two domains: a GTPase domain and an α-helical domain [3]. Data from peptide binding (SPOT studies) in Figs. 1A and B suggest that the primary tubulin binding sites on Gsα are localized primarily to the GTPase domain (Fig. 3). The GTPase domain of Gsα includes the switch regions [3]: switch I (αF–β2); switch II (β3–α2–β4), and switch III (β4–α3), which are important for adenylyl cyclase activation and are structurally altered upon exchange of GDP for GTP [17,27]. Two domains of Gsα that bound tubulin on the SPOT membrane, α2–β4 (spots 46–47, residues 240–256) and α3–β5 (spots 54–55, residues 280–296), are included in these regions.

By examining the structure of the Gsα (Fig. 3) for which peptides are external and freely available to bind other proteins, it is apparent that some of the peptides are buried, and would not be accessible to bind other proteins. Specifically, peptides 4–11 (see Fig. 2) have very limited regions exposed to the surface. However, the first few peptides are not fully visualized on the crystal structure, so this may not apply to this whole stretch of amino acids (the residues not visualized include ALA18–LYS34 and ASN66–PHE68). The C-terminal of peptide 11 is external, and could be involved in protein–protein interactions. Spot 24 is likely a false positive, as it is not near our predicted binding interface. SPOTS 46–47, 54–55, and 64 (see Fig. 2) all have regions that are solvent-exposed, as seen on the Gsα structure shown in Fig. 3.

Table 1
Gsα and β-tubulin residues in the protein–protein interface

Complex (#)[a]	Gsα[b]	β-tubulin[b]
1 (17)	3/15	10/21
2 (16)	6/16	11/25
3 (15)	0/10	14/24
4 (15)	9/21	8/27
5 (14)	3/17	12/20
6 (14)	6/11	15/28
7 (13)	6/12	13/22
8 (13)	4/16	12/24
9 (12)	3/19	0/27
10 (11)	9/11	10/19
11 (11)	3/26	0/27
12 (11)	7/12	0/16
13 (11)	8/20	16/29
14 (11)	3/10	9/17
15 (11)	0/20	4/19
16 (10)	3/11	1/14
17 (10)	3/10	10/17
18 (10)	2/23	16/32
19 (9)	2/13	10/15
20 (9)	3/25	19/30
21 (9)	3/16	12/25
22 (9)	5/6	0/15
23 (9)	4/21	10/17
24 (9)	9/17	0/25
25 (8)	5/7	7/15
26 (8)	3/15	0/23
27 (8)	7/14	0/13
28 (8)	5/19	0/19
29 (8)	3/19	0/23
30 (8)	1/18	14/26

[a]The number in parentheses indicates the number of ZDOCK-generated complexes that made up that particular cluster as determined by ClusPro. [b]The numerator corresponds to number of residues in Gsα (middle column) or β-tubulin (right column) within 5Å of the other protein that were detected from the SPOT membrane data (middle column) or predicted from a hypothetical β-tubulin interface (right column, see text for full explanation), respectively. The denominator corresponds to the total number of residues within the interface, as determined from that ZDOCK-generated complex. The residues that were less than 5Å from another residue on the other protein were considered to be in the protein interface.

Importantly, these are all regions displaying flexibility depending on the nucleotide state of Gsα.

To test further the binding of tubulin to the Gsα-peptides, we compared the binding of Gsα-peptides to Gtα-peptides, using a separate peptide array membrane. Gtα does not bind tubulin despite significant sequence and structural similarities to Gsα. Peptides from Gsα that display enhanced binding as compared to the corresponding Gtα peptide are shown (Fig. 4). As before, this membrane was probed with tubulin in both the GDP and GTP stages. Since we were probing for binding with antibodies, control experiments were performed which demonstrated no non-specific binding of the primary or secondary antibodies or residual tubulin binding to the membrane following stripping (see Materials and methods). Distinct regions in the amino terminus and the switch II and III regions of Gsα bind tubulin while corresponding regions of Gtα do not, providing leads into differences between Gtα and Gsα which allow the latter to bind tubulin (see Fig. 4). These data also reconfirmed the binding shown with the first SPOT membrane (Fig. 1) even though the size of the peptides were slightly longer in this membrane (15 vs. 12 amino acids).

To further confirm the results from Fig. 4, the real time interaction of two of these peptides (spot 49, the Gsα peptide LNLFK SIWNN RWLRT and the Gtα peptide LHLFN SICNH RYFAT) with tubulin was determined using Surface Plasmon Resonance (SPR). Tubulin was immobilized on CM5 sensor chips and the binding of various concentrations of injected peptide was monitored (Fig. 5). Gsα peptide bound to immobilized tubulin ($K_D = 4.07 \times 10^{-5}$ M) in a concentration dependent manner (Fig. 5A, see Figure legend for all binding parameters obtained). In contrast, the corresponding Gtα peptide does not bind to tubulin (Fig. 5C). Based on the previous finding that Gsα and Giα bind to tubulin with similar affinity but not Gtα, we also tested the binding of a corresponding Giα peptide (MKLFD SICNN KWFTD) to tubulin. We found that a corresponding Giα peptide also binds to tubulin ($K_D = 1.11 \times 10^{-5}$ M) (Fig. 5B) with a similar affinity as the Gsα peptide. These results confirm the specificity of this Gsα domain in tubulin binding.

To further analyze the interaction between Gsα and tubulin, two protein–protein docking programs (ZDOCK [15] and ClusPro [16]) were used to generate and analyze protein complexes. These docking algorithms do not include possible conformational changes induced

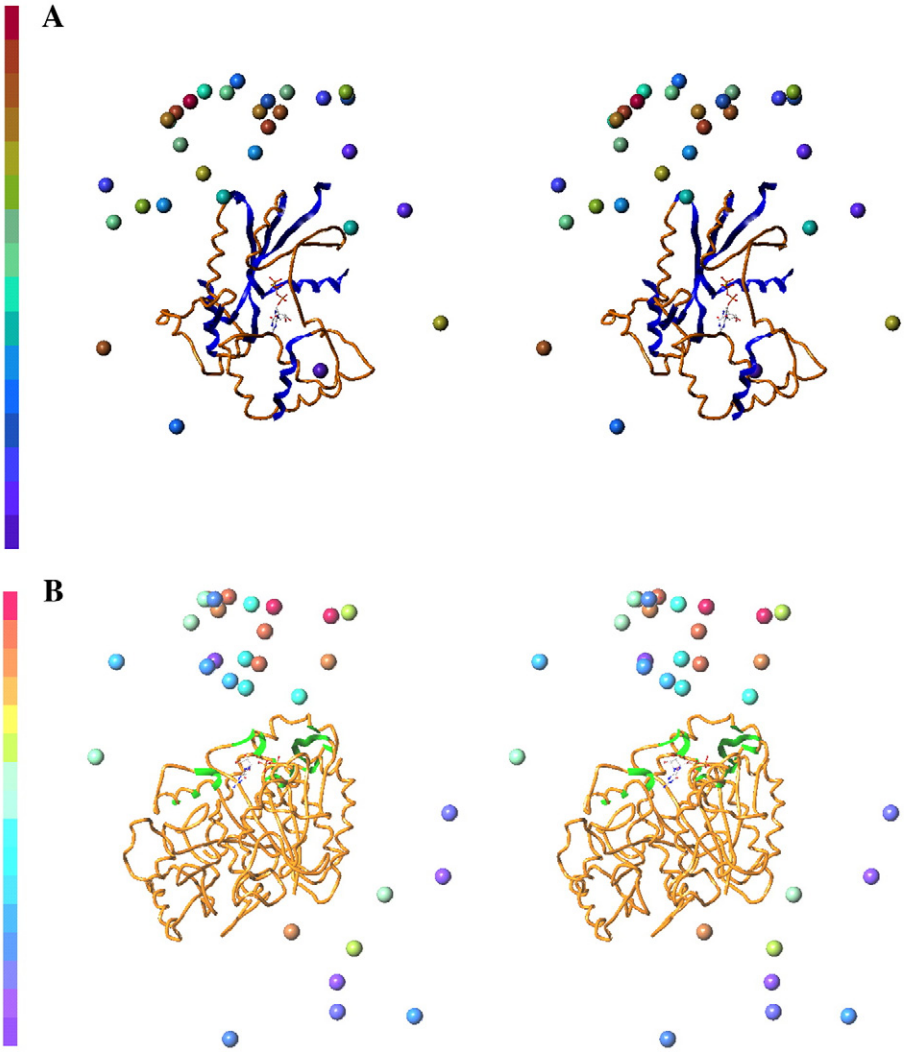

Fig. 6. Relative orientation of β-tubulin and Gsα for the top 30 complexes, calculated by ZDOCK and ClusPro. (A) Stereo representation of the orientation of β-tubulin relative to a fixed Gsα–GTPγS, in which the backbone is rendered as a tube, for the top 30 complexes. Gsα–GTPγS is shown as described in Fig. 3 with the regions of Gsα–GTPγS showing binding to tubulin highlighted. A circle at its geometric center represents β-tubulin for each of the 30 complexes. A color bar on the figure corresponds to the ranking of the complexes from 1 to 30, with red indicating highest ranked complex, blue indicating lowest ranked complex. (B) Stereo representation of the orientation of Gsα–GTPγS relative to a fixed β-tubulin, in which the backbone is rendered as a tube, for the top 30 complexes. A circle at its geometric center represents the Gsα molecules for each of the 30 complexes. A color bar on the figure corresponds to the ranking of the complexes from 1 to 30, with red indicating highest ranked complex, blue indicating lowest ranked complex. The highlighted residues in green on β-tubulin represent the predicted interface with Gsα, as described in the text.

Table 2
Minimization energies, buried surface area (BSA), and interacting regions between the different domains of β-tubulin and Gsα for complexes 1–5[a]

Complex	Energy	BSA(Å2)	β-tubulin	Gsα
1	−11,515	3326	H1(11)	α3–β5(280–283)
			B2–H2(69–74)	α3–β5(280–281)
			B3–H3(98–105)	α3–β5(278–283), α4–β6(351–356)
			B4–H4(142)	α4–β6(356)
			H5(179–185)	α4–β6(354–356)
			H11–H12(404–411)	α4–β6(348–355), α3(277)
2	−12,207	3954	H1(11)	α3–β5(283), α4–β6(356)
			B2–H2(71–77)	α3–β5(283–284), α4–β6(354–358)
			B3–H3(93–113)	α5(386–391), α3–β5(280–285, N-term(38)
			B4–H4(142–143)	α3–β5(280)
			B5–H5(178–185)	α3–β5(280–281)
			H11–H12(407–411)	α2(235–239), α3–β5(281)
3	−11,475	3552	H1(11–15)	C-term(391)
			B3–H3(95–110)	α3(283), β6(354–358), C-term(389)
			B4–H4(142–143)	C-term(389)
			B5–H5(176–185)	β6(358–360), C-term(385–389)
			H7(224)	C-term(388)
			H11–H12(404–411)	α4–β6(352–358)
4	−11,079	3397	H1(11–25)	α2(235–240), N-term(35–38)
			B2–H2(71–83)	α2(236–239), β5(207–211), α2(220–236), N-term(35–42)
			H7(220–232)	C-term(389–391), α3–β5(280–284), α4–β6(355–356), α2(239–240), N-term(38)
			B7–H9(278)	C-term(391)
5	−10,936	3132	H1(11–15)	αG–α4(309–317), α4(336)
			B2–H2(71–76)	αG–α4(307–310)
			B3–H3(96–105)	αG–α4(304–307), α4(331–332)
			B5–H5(175–180)	αG–α4(320–325)
			H7(224)	αG–α4(318–320)
			H11–H12(407)	αG–α4(329–331)

[a]Domain interactions between the two proteins were determined by identifying residues that were less than 4Å from another residue on the other protein and were considered to be potential protein contacts. Based on these residue-residue contacts, the domains that defined these residues are shown. In parentheses, the residue number or the range of residues that contributed to the residue-residue contacts are indicated. For β-tubulin, α-helices are listed as H1–H12 and β-sheets are listed as B1–B10 [23].

by the interactions of the two proteins; however, these programs have been successful in predicting near-native structural complexes for other systems [15,16,28]. Using the structures of Gsα–GTPγS [22] and β-tubulin–GDP [23], we generated 2000 potential complexes by ZDOCK and clustered these complexes with ClusPro. ClusPro created and ranked 30 clusters with the cluster ranking based on the number of similar complexes in each cluster. The complex that is most centrally located in each cluster is used to represent that cluster [16]. In the docking programs, only the structure of the β-tubulin subunit was used, based on the assumption that Gα proteins interact only with this subunit because this subunit contains the exchangeable nucleotide that is likely involved in the transactivation mechanism [7–9].

After obtaining the final 30 complexes, we analyzed the number of amino acids on Gsα that were in the interface of this protein–protein interaction and were also predicted from the SPOT membrane (Table 1). Twenty-eight of the 30 complexes included some residues on Gsα that were in the protein interface that were also determined by the peptide array technique. Fourteen of the 30 complexes had over thirty percent of the residues in the interface predicted by the direct binding results of the SPOT membrane.

In Fig. 6, the relative position of β-tubulin (for the top thirty complexes) to a fixed Gsα (represented as a whole molecule) is shown. Most of the potential β-tubulins cluster near the GTPase domain of Gsα. This indicates a favorable orientation of β-tubulin predicted by the docking programs to be located in the GTPase domain of Gsα. Further, the regions predicted by the SPOT membrane are highlighted on Gsα in Fig. 6 indicating many of the predicted interfaces of the top 30 complexes are in this region.

The interactions that occur between tubulin dimers within microtubules (called interdimer interactions) have been well defined and occur at several residues surrounding the nucleotide on β-tubulin (27 amino acids have been defined) [23]. We hypothesized that Gsα binds close to the nucleotide-binding site on β-tubulin and in a similar region to which the α-tubulin of another tubulin dimer would bind. Modeling of the top thirty complexes (Fig. 6B) with the relative position of Gsα, represented by circles, to a fixed β-tubulin, indicates that a majority of these docked Gsα molecules are located around the exchangeable nucleotide-binding site of β-tubulin. This is consistent with the above hypothesis. We also analyzed whether these hypothesized residues are in the interface of the top 30 complexes, and found that most of the complexes did indeed contain these β-tubulin residues in the interface (Fig. 6B. and Table 1). For 18 of the 30 complexes, over 40% of the residues on β-tubulin in the interface were predicted by the above assumption. Of the top 10 complexes, only complex 9 did not contain any of these residues.

The top five complexes from the ZDOCK/ClusPro analysis were further analyzed by energy minimization cycles (5000) and the buried surface area (BSA) of each of these complexes was then determined (Table 2). The energy values indicate that complex 2 has the lowest energy, followed by both complexes 1 and 3 being slightly higher in

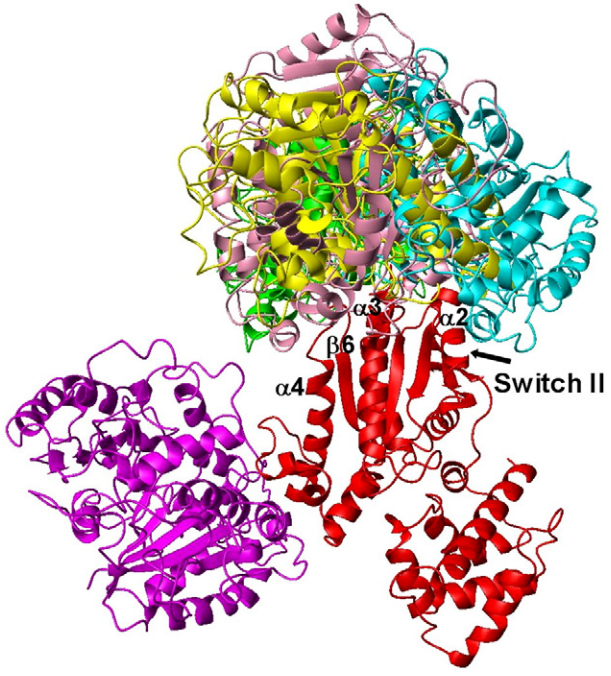

Fig. 7. Representations of Complex 1-5 for Gsα–β-tubulin Interaction. Both proteins are presented in the α carbon form, where Gsα is in red and fixed in orientation to the position of the β-tubulin molecules for each of the top 5 complexes. β-tubulin molecules for the different complexes are in: pink (complex 1), yellow (complex 2), green (complex 3), cyan (complex 4) and purple (complex 5).

energy than 2, with 4 and 5 showing the highest energies of the five complexes. Complex 2 also has the most buried surface area at the interface, perhaps suggesting a better interaction of the two molecules.

Fig. 7 shows an α-carbon alignment of the top five complexes, with a fixed Gsα and the relative position of corresponding β-tubulin for the top 5 complexes. It is apparent that the top four complexes are similar in the location of the binding surface between β-tubulin and Gsα. Complex 5 is in a different location on the Gsα, and is higher in energy than the other four complexes, and is therefore, presumably, a less favorable complex. Complex 4 is significantly higher in energy than complexes 1–3, but has a number of similar regions of interactions with the top three complexes (Table 2). At this time, it would be difficult to determine which of these three is the most plausible complex. However, it is noteworthy that the top complexes have a number of common contact regions between Gsα and β-tubulin, in particular, in the regions of α3–β5 and α4–β6 loops of Gsα (Table 2).

4. Discussion

In this report, we construct the initial working model of a complex between tubulin and Gsα using a peptide binding array, data from chimeric G proteins [28] and molecular docking approaches. Definitive determination of structures is always best undertaken with more precise methods (i.e., X-ray crystallography). However, this protein–protein interaction will be more difficult to determine by these methods considering tubulin has thus far defied attempts at crystallization The approach taken in this study, with the consideration of earlier data, has provided a working model.

Protein–protein interactions occur as three-dimensional structures. The SPOT membrane technique has limitations in that linear peptides are used to determine binding domains, which will likely produce some false leads. As seen in Fig. 3, the likely protein interface is through regions near the switch II and III regions. Around these domains, there appears to be a continuous protein binding interface on Gsα. The identified binding site on the α-helical domain of Gsα as well as some the peptides that extend into the interior of the protein are not likely to be genuine tubulin binding sites on Gsα. Further, as seen in Fig. 1, tubulin-GDP and tubulin-GDP show limited overlap in their binding to the peptide array. To date, however, there are no data supporting major structural changes between the GDP and GTP bound states of tubulin. Hence, we do not suggest different mechanisms of binding to Gsα dependent on the nucleotide bound state of tubulin. Likely, the differences are a reflection of the limitations of the SPOT membrane technique.

The majority of docking algorithms, including the programs used here, do not address the possibility of conformational changes induced by the formation of these protein–protein interactions. This adds difficulty in the interpretation of these data because many protein–protein interactions induce structural change in the proteins from their original, unbound conformation. The programs employed in this study, however, have been quite successful in predicting structures that are close to the near-native conformation [29]. By utilizing these two approaches, we have generated a reasonable working model of how these two proteins interact.

As seen in Table 1, most predicted complexes contained at least a few residues on Gsα predicted from the binding of tubulin to Gsα-peptides. Although the Gsα-peptide membrane predicted many residues that were not found in the Gsα interface of the complexes, the reason might be that only selected residues in the peptides from the SPOT membrane may be important in this interaction. Also, the crystallographic structure of Gsα was missing a portion of the proximal amino terminal, a region predicted by the SPOT membrane to be important for tubulin binding [22]. Additionally, many of the residues selected by the SPOT membrane approach may be important when the proteins (either Gsα or β-tubulin) are in a particular nucleotide state or even in a transition state during their transformation from GDP to GTP. Further, our definition of being included in the interface was quite stringent (≤5Å). By comparing the highlighted Gsα-peptide membrane-predicted domains in Gsα (Fig. 3) vs. the relative orientation of the docked Gsα to β-tubulin (Fig. 6), it is apparent that both methods had a predilection to the GTPase domain, in particular, the amino terminal and the switch II and switch III regions of Gsα.

Prior to this study, chimeric proteins comprised of Gsα, Giα1 and Gtα were used to define the domains on Gα proteins that are important in the interaction with tubulin. One study [30] demonstrated that the amino terminal portion of Gsα (residues 1–63 which includes the N-terminus of Gsα which corresponds to residues 1–40 as well as residues 41–62 which correspond to the Ras-like domain) may be important for tubulin to activate adenylyl cyclase. The data in Figs. 1–3 indicate that the amino terminus plays a role in the binding to tubulin. For the top five complexes, complex 4 (Table 2) includes a significant portion of the amino terminus of Gsα in the interface, and complex 2 includes only a small portion of the amino terminus of Gsα in the interface. The simplest reason for seeing less amino terminal involvement than expected in the top five complexes is that the structure of Gsα used in the molecular docking studies was missing portions of the amino terminus. This absent region of the amino terminus included the following residues predicted by the SPOT technique to be important: ALA18–LYS34 and ASN66–PHE68. Therefore, a significant portion of the amino terminus of the Gsα was not available for analysis by the protein–protein docking programs.

In a previous study [28], it was demonstrated that one of the major Giα1-tubulin interacting domains was between residues 237–270 of Giα1 (which corresponds to residues 253–293 in Gsα, regions β4–α3–β5). However, this was not the only region involved in binding

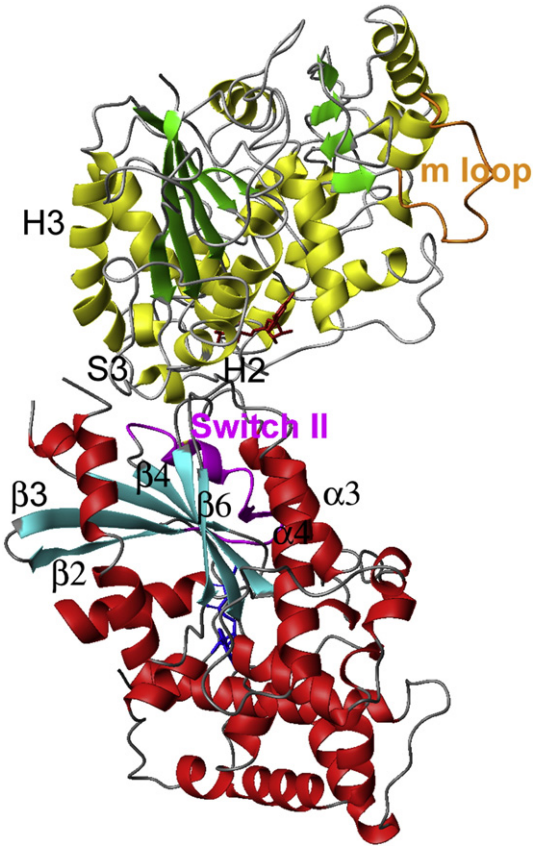

Fig. 8. A Ribbon Representation of Complex 2. Gsα is displayed in ribbon/tube form in red and cyan with the Switch II region highlighted in pink. The β-tubulin is displayed in ribbon/tube form in yellow and green, with the m loop highlighted in orange. Important interacting domains on Gsα and β-tubulin are labelled. Location of GTP bound to both Gsα and tubulin is indicated.

of the two molecules [28]. This is consistent with data presented in Figs. 1–3. Since we assume that Gsα and Giα1 complex with tubulin at a similar interface, regions α3–β5 of the Gα protein family appear important for interaction with tubulin. As seen in Table 2, the α3–β5 region of Gsα is included in the interface of 4 of the 5 top complexes indicating that the predicted models agree with the data from both SPOT membrane and Gtα–Giα1 chimeras [28]. Further, the switch II region (α2–β4) is in the interface of complex 4 (and complex 2), which is another domain predicted from the binding data (Figs. 1–3) and studies with chimeric Gα proteins [28].

Previous studies on G protein–tubulin interaction showed that the certain G protein heterotrimers could form a complex with tubulin [31]. The deduced structure of Giα1β1γ2 [32] suggests that most of the switch II region (including the following domains, α2–β4) as well as the amino terminus of Giα1 are involved in the interface with the $\beta_1\gamma_2$ subunits [26]. This interface on the Gα subunit has many similarities to our proposed interface on Gsα to tubulin, which could be interpreted to mean that Gα and tubulin could not bind contemporaneously to Gβγ. Nonetheless, previous data indicated that the Gαβγ heterotrimer binds tubulin, yet this binding surface on the heterotrimer is not known [31]. Of interest, the effects of either Gβγ or Gα alone on tubulin (promotion of microtubule polymerization by Gβγ or tubulin GTPase activation by Gα) are not observed with intact heterotrimers [33,34]. This suggests that the active interface between Gsα and tubulin is at least partially occluded when Gβγ is binding to Gsα, consistent with observation of Ford et al [26]. Thus, the most likely site for the binding of a Gαβγ heterotrimer is at a region of Gβ or Gγ which does not interact with Gα. Several such interactions have been described (See ref. [35] for review), however the complexity of how Gα and Gβγ subunits interact with other proteins, and the possibility that heterotrimers have additional interactive facets is just starting to be understood [36]. It is possible that the acetylated domain of Gγ is involved in binding to tubulin, as prenylation deficient Gγ2 mutants or the farnesylated Gγ1 (as opposed to the geranylgeranylated Gγ2) fail to interact with tubulin [37]. Interestingly, prenylated domains of small G proteins have also been implicated in tubulin binding [38].

Comparison of the interacting surfaces of Gsα and β-tubulin for the top five complexes in Table 2 indicate similar interactions. However, in Fig. 8, we show complex 2, because this model has the lowest energy as seen in Table 2. In this model, the switch II region (α2–β4) and the amino terminal of Gsα both contain contacts to the H1 and H2 regions of β-tubulin (Fig. 8). Interestingly, the amino terminal of Gsα (seen directly under the switch II region, Fig. 8) extends directly into the nucleotide-binding pocket of β-tubulin. This is consistent with the role of this region in the activation of tubulin GTPase by Gα [8].

Several isotypes of tubulin are known to exist [39]. It is not clear whether any single isotype preferentially binds Gα. Nonetheless, both the current microtubule-based tubulin structure [11] as well as that inferred from tubulin–stathmin complexes [13] does not distinguish dimer isotypes. At this point, we also are unable to make such a distinction.

In recent years, it has become apparent that tubulin interacts with many different proteins [2]. Structural information on these protein interactions has been limited except in a few cases [2], [13]. Many of these proteins, in particular Tau proteins, appear to interact with tubulin through the acidic C-terminal of tubulin [2]. The model presented in this report suggests a binding site for Gα at the plus end of a microtubule which is quite different from that of MAPs. As seen in Figs. 6–8, the nucleotide-binding pocket and the surrounding residues of β-tubulin comprise a majority of the Gsα interacting surface. Further, the Gsα molecule may completely encase the nucleotide-binding pocket of β-tubulin. Gsα appears to be the first protein identified to associate with β-tubulin at the GTP binding site.

The interaction between Gα proteins and tubulin can result in a novel mode of Gα activation [7–9]via the direct transfer of GTP from tubulin to the Gα protein in exchange for GDP. This mechanism is not unique to G proteins, as a similar direct transfer of NADH has been demonstrated between alpha-glycerol phosphate dehydrogenase and lactate dehydrogenase [40]. Recently, the structure of a complex between two GTP binding proteins, FtsY and Ffh was determined [41,42]. These two proteins form a complex when both are GTP bound and dissociate when both GTP molecules are hydrolyzed. The crystal structure of this complex showed that a catalytic core is formed and contains both GTP molecules. Due to the transfer of GTP that has been observed between Gα proteins and tubulin [9], we suggest that the Gsα–tubulin complex bears similarity to the FtsY–Ffh complex in that the two-nucleotide-binding sites must be in close proximity. The predicted complexes seen in Figs. 6–8 interface near the nucleotide-binding sites of β-tubulin and Gsα.

The structural interaction between tubulin and Gsα proposed here has implications for the function of each protein. The domains on Gsα (α2–β4 and α3–β5) that are essential to the binding and activation of adenylyl cyclase [17] are also important for the interaction with tubulin. These observations indicate how formation of a complex may alter the interaction of Gsα with adenylyl cyclase [7–10]. Further, these observations provide a structural basis to the finding that Gsα interacts with tubulin at the exchangeable site on β-tubulin activating the GTPase of tubulin and increasing dynamics of microtubules [8]. Gsα appears to surround the exposed GTP on the β-tubulin, which includes the region of the GTP cap of microtubules. Substitution of this region with the identical region from transducin yields a protein that blocks interaction between tubulin and Gsα. This results in decreased cellular microtubule dynamics [8] and diminished ability of cells to form microtubule-based extensions [30]. The proposed structural model thus provides a testable hypothesis that will inform new experiments to further probe the functional features of this system; a system that may serve as the interface between G protein signalling and cellular structure and trafficking.

Gsα facilitates GTP hydrolysis on tubulin, which leads to microtubule depolymerization by increased GTPase activation on tubulin [8]. This is consistent with the structural models presented in this report. The experimental and theoretical analyses in this report provide the first proposed structural model for the Gsα–tubulin complex. This study will be pivotal in guiding future studies of G protein–tubulin interaction and may prove germane to discerning the action of hormones and neurotransmitters in modifying the cytoskeleton and the relationship between cellular signalling molecules and cytoskeletal proteins.

Acknowledgements

We thank the Protein Research Laboratory at the University of Illinois at Chicago and Dr. Bao-Shiang Lee for help with the SPOT membrane. We thank Dr. Tomoyoshi Kobayashi and Rahul Dave′ for their generous help with surface plasmon resonance experiments. This work was supported by the National Institutes of Mental Health Grant MH 39595 and DA 02568 (to M. M. R.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.02.017.

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