RESEARCH COMMUNICATION

Lack of association of G-protein β_2 - and γ_2 -subunit N-terminal fragments provides evidence against the coiled-coil model of subunit- $\beta\gamma$ assembly

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The association between peptides from bovine G-protein β_2 - and γ_2 -subunits was studied by CD spectroscopy and cross-linking. Both peptides had ~ 25 % stable α -helical structure at 25 °C, but there was no increase on mixing subunits as expected for coiled-

coil formation. Also, disulphide cross-linking gave more $\beta_2\beta_2$ homodimer than $\beta_2\gamma_2$ heterodimer. These data do not support the proposed N-terminal coiled-coil model of $\beta\gamma$ -subunit association.

INTRODUCTION

Heterotrimeric G-proteins play a major role in signal transduction in nervous, endocrine, cardiovascular and other mammalian and eukaryotic systems. The structures of the α -subunit of transducin and of the inhibitory G-protein, G_{i1} , have recently been solved [1,2]. In contrast, the structures of the β - and γ -subunits are much less well understood. The β - and γ -subunits form a tight complex, which can not be separated without denaturation. There is specificity in the interactions of the five subtypes of β -subunit which will bind to the ten γ -subunit subtypes [3–6], and there are specific γ - and α -subunit interactions [3]. It is now recognized that $\beta\gamma$ -subunits as well as α -subunits are important in regulating effectors [7]. Functions mediated by $\beta\gamma$ -subunits include: targeting α -subunits to membranes [8], enhancing coupling of α -subunits to receptors [9], activation of ion channels [10,11], inhibition of Type I adenylate cyclase and potentiation of α -subunit activation of Type II adenylate cyclase [12], activation of phospholipase C [13,14], and modulation of receptor function by targeting and activation of G-protein-coupled receptor kinases [15]. A better understanding of the structure of the β - and γ -subunits and their complex will be important in elucidating the molecular mechanisms of their specificity and functions.

Based on its sequence, the β -subunit appears to be composed of two domains: a putative N-terminal coiled-coil region of approx. 30 amino acids [16], followed by seven repeating units of roughly 40 amino acids each, known as the WD-40 (or GH-WD) repeat [17]. Several lines of evidence have suggested that the coiled-coil region of β -subunit interacts with a similar coiled-coil region in the N-terminus of the γ -subunit [16,18,19]. In particular, deletion of the putative coiled-coil region of β -subunit and mutations that disrupt the heptad repeat in β -subunit, which is characteristic of a coiled-coil, abolish interaction with γ -subunit [19]. However, more recently, regions of β - and γ -subunit distinct from the N-terminal regions were found to determine specificity of subunit-subunit interactions [5,6].

We wished to test the hypothesis that the proposed coiled-coil

regions of β - and γ -subunits do bind tightly to one another. Since these interactions have been modelled on the X-ray structure of a leucine zipper transcription factor [19], we used methods developed for that system to study β -/ γ -subunit interactions. Association of synthetic peptides from leucine zipper transcription factors [20–22] was demonstrated by CD and by spontaneous covalent cross-linking of cysteine residues in their structure. We report here that the putative coiled-coil regions of the G-protein β_2 - and γ_2 -subunits show little evidence for a tight association, raising questions about the postulated N-terminal coiled-coil interaction.

EXPERIMENTAL

β_2 - and γ_2 -monomer peptides were prepared by treating peptide stock solutions with 3 mM (final concn.) dithiothreitol (DTT) for 25 min at room temperature, followed by separation on a Rainin Dynamax C₄ reverse-phase HPLC column. The column was eluted with 0.1 % trifluoroacetic acid and a 30 min linear gradient of 25–45 % acetonitrile. Fractions from the single peaks were dried in a Speed-Vac instrument. To monitor dimerization, they were dissolved in the dimerization buffer (50 mM Tris buffer, pH 8.0) at 100 μ M each of β_2 and γ_2 peptide, and then incubated at room temperature for 0–33 h. Samples were taken at the indicated times and separated by the same reverse-phase HPLC method used for preparation of monomers, but without any DTT. The A_{220} was measured and data were analysed with Rainin DYNAMAX software, and peaks were integrated to calculate peak areas in μ V.

RESULTS AND DISCUSSION

The N-terminal regions of G-protein β - and γ -subunits are predicted to be α -helical and to form a coiled-coil as a mechanism of β - and γ -subunit association [16,19]. Formation of a coiled-coil enhances the α -helical character of peptides from the leucine

Abbreviations used: DTT, dithiothreitol; TFE, trifluoroethanol.

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Coil position	<i>g</i>	<i>abc</i>	<i>defg</i>	<i>abc</i>	<i>defg</i>	<i>abc</i>	<i>de</i>
β_2 peptide	E	LEQLRAE	AEALRNQ	IRDARKA	CGDST		
γ_2 peptide	S	IAQARKL	VEQLKME	ANIDRIK	VSKCA		

Figure 1 Sequences of β_2 and γ_2 peptides

The sequences of peptides prepared from the bovine G-protein β_2 (residues 3–29) and γ_2 (residues 8–34) are shown aligned with their predicted coiled-coil positions. For disulphide-cross-linking studies, alanine-33 in the native γ_2 sequence was changed to cysteine, as indicated by *italics*.

Figure 2 CD spectra and melting curves for G-protein β_2 and γ_2 peptides

(a) CD spectra were determined as described in the Experimental section with 100 μ M of either β_2 (—) or γ_2 (---) peptide in 50 mM NaCl/10 mM sodium phosphate buffer, pH 7.0. Samples were maintained at 25 °C. The vertical dotted line indicates 222 nm, the wavelength at which α -helix predominates in the spectrum. A buffer blank (not shown) showed minimal signal at 222 nm. (b) The ellipticity of 100 μ M of either β_2 (lower trace) or γ_2 (upper trace) peptide at 222 nm was measured as in (a), and the temperature was scanned from 5 to 75 °C at 50 °C/h. The vertical dotted line indicates 25 °C, the temperature at which spectra in (a) were obtained.

zipper transcription factors GCN4 and fos and jun [20,21]. Thus we examined CD spectra for 27-amino acid peptides from the G-protein β - and γ -subunits. Figure 1 illustrates the peptide sequences used and the predicted alignment of the coiled-coils [16]. The peptides contained $3\frac{1}{2}$ turns of the predicted coiled-coils, and include cysteine residues, which are predicted to be directly adjacent to one another in a coiled-coil complex based on the model of Garritsen et al. [19]. CD spectra of the β_2 and γ_2 peptides showed evidence of partial α -helical character, with molar ellipticities at 222 nm and 25 °C of approx. 16000 and 11000 respectively (Figure 2a). Temperature scans showed that the β_2 peptide had a low-stability α -helical component with a melting temperature of about 20–25 °C, but also showed evidence of a high-stability α -helical component which retained significant

Figure 3 Spectra of mixtures of β_2 and γ_2 peptides are not concentration-dependent

CD spectra of equimolar mixtures of β_2 and γ_2 peptides were determined at 25, 50 and 100 μ M concentrations. Absolute ellipticities are shown in (a) and molar ellipticities (calculated by using the indicated concentrations) are shown in (b).

negative ellipticity at 222 nm at 75 °C (Figure 2b). In contrast, the γ_2 peptide appeared to show only the high-stability component. This stable component does not appear to be due to disulphide cross-links, as incubation with 2 mM DTT before and during collection of spectra did not decrease the apparent α -helix content (results not shown), and the γ_2 subunit, which shows a greater proportion of apparently stable α -helix, does not form dimers to any appreciable extent (see below).

Surprisingly, the CD data did not reveal any evidence of interaction between the peptides. There is no concentration-dependence of the CD spectrum of a mixture from 25 to 100 μ M peptide (Figure 3). Also, the CD spectrum of the mixture of the two peptides is identical with the sum of the spectra of the two peptides measured individually (results not shown). Finally, the melting temperature (Figure 2a, and result not shown) of the low-stability component of the β_2 -subunit spectrum is unchanged (22 °C for β_2 alone, 23 °C for the sum of spectra of β_2 and γ_2 , and 22 °C for a mixture of β_2 and γ_2).

Peptide synthesis and MS

Peptides corresponding to the N-terminal regions of bovine β_2 (residues 3–29) and γ_2 (residues 8–34) (Figure 1) subunits were synthesized by the University of Michigan Protein and Carbohydrate Structure Core Facility, using Fmoc (fluorenylmethoxycarbonyl) chemistry. Alanine-33 in the γ_2 sequence was replaced by cysteine to permit cross-linking studies. The identity and purity of the peptides was determined by reverse-phase

Figure 4 TFE enhances α -helix formation in both the β_2 and the γ_2 peptides

CD spectra of the β_2 and γ_2 peptides were measured at 25 °C as described in Figure 2 legend. TFE was added to the samples at the indicated concentrations (0–15%) and spectra were repeated. Both peptides can be induced to form additional α -helix upon addition of TFE, as indicated by the increase in negative ellipticity at 222 nm and positive ellipticity at 190 nm.

HPLC and electrospray MS. MS was done by the University of Michigan Protein and Carbohydrate Structure Core Facility, using a Vestec single-quadrupole mass spectrometer and electrospray interface.

CD measurements

Determination of CD spectra and melting curves was done in a J710 Jasco Spectropolarimeter in a 1 mm cell with continuous nitrogen purging. Temperature scans were measured from low to high temperatures with a scan rate of 50 °C/h. Peptides were freshly dissolved in buffer containing 50 mM NaCl and 10 mM sodium phosphate, pH 7.0, before the scans. Masses were determined by weighing, based on molecular masses of 3157 Da for β_2 and 3192 Da for γ_2 . For some studies, 10 mM DTT was added to prevent disulphide cross-linking during data collection. Controls with DTT alone showed no CD signal.

Disulphide cross-linking studies

The fact that the mixture of β_2 and γ_2 peptides does not show an increase in negative ellipticity at 222 nm could result from a large baseline α -helical character or some inability of the β_2 and γ_2 peptides to form α -helix. To test these possibilities, we added trifluoroethanol (TFE), which induced a significant increase in both negative ellipticity at 222 nm and positive ellipticity at 190 nm, indicating formation of substantial α -helix (Figure 4).

Figure 5 Time course of disulphide dimer formation

Monomeric β_2 and γ_2 peptides were mixed in 50 mM Tris buffer at a concentration of 100 μ M each. A sample was taken immediately for HPLC analysis; then the reaction mixture was incubated at room temperature for 0–33 h. Samples were taken at the indicated times and analysed by HPLC as described in the Experimental section. The peak area of the monomers (a) and the homo- and hetero-dimers (b) was determined by integration and is expressed in arbitrary units.

At 20% TFE at 5 °C both β_2 and γ_2 peptides had molar ellipticities of $-44\,000$ degrees $\cdot$ cm² $\cdot$ mol⁻¹ at 222 nm, indicating $\sim 100\%$ α -helical character (results not shown).

It is possible that the β_2 and γ_2 peptides form a complex without any significant change in α -helical character. Consequently, we used another technique to detect interactions between peptides, which exploits the ability of cysteine-containing peptides to form disulphide-linked heterodimers [21]. Both peptides contain a cysteine residue near the C-terminus. The cysteine in β_2 is in the *a* position of the predicted coiled-coil and the cysteine in the γ_2 is in the *d* position, which should be directly adjacent to one another in a coiled-coil complex [21,22]. The equivalent residues have been cross-linked in transducin β - γ complexes by using oxidative conditions [23]. If there was a specific interaction between β and γ peptides, one would expect the proportions of heterodimer to homodimers to be greater than the 2:1:1 ratio resulting from random dimerization. Monomers were prepared as described in the Experimental section. Homo- and hetero-dimers were produced by incubation of β_2 and γ_2 alone or together in 50 mM Tris buffer, pH 8, at room temperature. HPLC retention times (min) were β (10.8–11.3), γ (14.8–15.4), $\beta\beta$ (14.2–14.8), $\gamma\gamma$ (20.4–21.0) and $\beta\gamma$ (18.2). All dimers could be converted back into the appropriate monomers by incubation with DTT (results not shown). The kinetics of dimerization were determined by incubation in Tris buffer, pH 8.0, at room temperature for the indicated periods of time (Figure 5). The total amount of peptide recovered in the five peaks (i.e. the sum of the peak areas) remained constant to 24 h, but declined slightly at 33 h. The β_2 peptide rapidly forms homodimers, but the γ_2 peptide does not dimerize very much under identical conditions. In the mixture, the formation of $\beta_2\gamma_2$ occurred, but to

a lesser extent than that of the $\beta_2\beta_2$ homodimer. If a strong association of β_2 and γ_2 occurred, one would expect predominantly the $\beta_2\gamma_2$ heterodimer to be formed, which was not the case. Since the d position of a coiled-coil has enhanced disulphide stability [24], it is rather surprising that the β_2 peptide, with the cysteine in position a , has a greater propensity for dimerization than the γ_2 peptide, which has its cysteine in position d .

The lack of enhancement of α -helix content on mixing β_2 and γ_2 peptides and the relatively low melting temperature for the β -subunit peptide contrasts with the clear enhancement of α -helical content for several leucine zipper type coiled-coil peptides [21]. It is possible that the regions picked for synthesis either were incorrect or were too small to form the coiled-coils. This does not appear likely, as the regions chosen encompass the more stable N-terminal ends of the regions used in the modelling studies in Garritsen et al. [19]. Molecular-dynamics simulations in our laboratory confirmed that the N-terminal end of these peptides should form a more stable association than the C-terminal ends (results not shown). Another difference between the expression studies with intact subunits and the peptide studies reported here is our use of β_2 sequence rather than β_1 . It is possible that the relative contributions of the N- [19] and C-terminus [6] of the β -subunit to association with γ -subunits differs between β_2 and β_1 ; however, this does not seem likely, given the high degree of sequence conservation.

There are several pieces of evidence supporting the model of a coiled-coil interaction between the N-terminus of β - with γ -subunits in bovine transducin. Deletion of the N-terminal 34 residues of the β_1 -subunit blocks its association with a soluble form of γ_2 -subunit in a co-expression system [19]. In the same work, Garritsen et al. [19] found that mutations predicted to disrupt a coiled-coil structure (E10K and an insertion of alanine at position 10) block association in the same system. Cysteine-25 in β_1 and -36 or -37 in γ_1 can be chemically cross-linked in the native transducin $\beta\gamma$ structure [23], indicating a proximity of these regions.

In contrast, proposals regarding the role of the N-terminus of γ -subunit are entirely based on modelling [16,19]. Indeed, Bubis and Khorana [23] predicted that the region on the C-terminal side of Cysteine-37 and γ_1 would bind to the residues on the N-terminal side of cysteine-25 in β_1 . More recently, Spring and Neer [5] found that amino acids 33–46 in γ_2 determined the specificity of $\beta\gamma$ -subunit interactions, whereas Rahmatullah et al. [3] showed that amino acids 1–15 of γ_2 determine the specificity for α -subunits. These data, in combination with our studies on synthetic peptides derived from the N-termini of β_2 and γ_2 ,

support a model in which the N-terminus of β -subunit interacts with a middle region of γ -subunit, whereas the N-terminus of γ -subunit interacts with the α -subunit.

The dimerization time-course experiment in which significant $\beta_2\beta_2$ complex is formed is intriguing in light of our evidence for clustering and low mobility of bovine brain $G\beta\gamma$ -subunits in cell membranes [25,26]. This raises the intriguing speculation that the N-terminus of β -subunits might mediate clustering of the $G\beta\gamma$ -subunits as well as association of β - with γ -subunits.

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