Molecular Interactions of the Gβ Binding Domain of the Ste20p/PAK Family of Protein Kinases

AN ISOLATED BUT FULLY FUNCTIONAL Gβ BINDING DOMAIN FROM Ste20p IS ONLY PARTIALLY FOLDED AS SHOWN BY HETERONUCLEAR NMR SPECTROSCOPY*

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The transmission of the mating signal of the budding yeast Saccharomyces cerevisiae requires Ste20p, a member of the serine/threonine protein kinases of the Ste20p/PAK family, to link the Gβ subunit of the heterotrimeric G protein to the mitogen-activated protein kinase cascades. The binding site of Ste20p to the Gβ subunit was mapped to a consensus sequence of SSLφPL/VXφββ (X for any residue; φ for A, I, L, S or T; β for basic residues), which was shown to be a novel Gβ binding (GBB) motif present only in the noncatalytic C-terminal domains of the Ste20p/PAK family of protein kinases (Leeuw, T., Wu, C., Schrag, J. D., Whiteway, M., Thomas, D. Y., and Leberer, E. (1998) Nature 391, 191–195; Leberer, E., Dignard, D., Thomas, D. Y., and Leeuw, T. (2000) Biol. Chem. 381, 427–431). Here, we report the results of an NMR study on two Gβ binding peptides and the entire C-terminal domain derived from Ste20p. The NMR data show that the two peptide fragments are not uniquely structured in aqueous solution, but in the presence of 40% trifluoroethanol, the longer 37-residue peptide exhibited two well defined, but flexibly linked helical structure elements. Heteronuclear NMR data indicate that the fully functional 86-residue C-terminal domain of Ste20p is again unfolded in aqueous solution but has helical secondary structure preferences similar to those of the two peptide fragments. The NMR results on the two GBB peptides and the entire GBB domain all indicate that the two important binding residues, Serφ79 and Serφ880, are located at the junction between two helical segments. These experimental observations with the prototype GBB domain of a novel family of Gβ-controlled effectors may have important implications in understanding the molecular mechanisms of the signal transduction from the heterotrimeric G protein to the mitogen-activated protein kinase cascade.

The well ordered intracellular space of the cell is separated from the chaotic extracellular world by a lipid bilayer membrane. An important process for information transfer across the lipid bilayer is the G protein signal transduction system (1), out of which one of the most well studied is the activation of the mating process of the budding yeast Saccharomyces cerevisiae. In this process, small peptide pheromones produced by one type of cell are recognized by the opposite mating type, and this recognition initiates a series of events which includes cell cycle arrest, modulation of transcription, and changes in the cellular morphology (for review, see Ref. 2 and references therein). The transmission of the mating signal requires a protein Ste20p to link the Gβ subunit of the heterotrimeric G protein to the mitogen-activated protein kinase cascades (3).

Ste20p is a member of the Ste20/PAK family of serine/threonine protein kinases which have sequence similarity to protein kinase C and which are highly conserved from yeast to man and responsible for integrating multiple cellular signals (3, 4). In the yeast S. cerevisiae, Ste20p is involved in many important cellular processes, including pheromone signaling, morphological switching, vegetative growth, and polarized morphogenesis. Through binding interaction with the upstream Gβ subunit of the heterotrimeric G protein, Ste20p activates the downstream mitogen-activated protein kinase cascade and ultimately regulates the Ste12p transcription factor (5). Ste20p of the yeast S. cerevisiae is a 939-residue protein kinase, consisting of an N-terminal regulatory region over residues 1–62, a C-terminal kinase domain, and a noncatalytic domain at the extreme C terminus (Fig. 1). Several sites have been identified within residues 1–627 of Ste20p with binding activities for Ste5 (6), myosin I (7), Bem1p (8), and Cdc42 (9, 10). Recently, the binding site of Ste20p to the Gβ subunit was mapped to within the noncatalytic C-terminal domain of Ste20p and more specifically to a 14-residue fragment with a micromolar affinity (7, 11). Furthermore, the Gβ binding (GBB)1 sequence of Ste20p has been shown to represent a novel docking site for the heterotrimeric G protein, with a consensus sequence of SSLφPL/VXφββ (X for any residue; φ for A, I, L, S, or T; β for basic residues) present only in members of the Ste20p/PAK protein kinase family (7). In particular, the isolated C-terminal domain of Ste20p can completely restore the GBB function of the full-length Ste20p as shown by in vitro binding assays (7, 11). This observation indicates that the C-terminal domain of Ste20p is capable of binding Gβ independent of the other parts of Ste20p (7, 11).

Here, we report a NMR characterization of the conforma-

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1 The abbreviations used are: GBB, Gβ binding; Fmoc, N-(9-fluorenylmethoxycarbonyl); GST, glutathione S-transferase; TFE, trifluoroethanol; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence spectroscopy; DG, distance geometry; SA, simulated annealing; WASP, Wiscott-Aldrich syndrome protein; CPMG, Carr-Purcell-Meiboom-Gill.
A Bioactive and Partially Folded G\(\beta\) Binding Domain of Ste20p

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Purification**—A 14-residue peptide Ste20\(_{97-990}\) corresponding to residues 877–890 of Ste20p and a 37-residue peptide Ste20\(_{599-655}\) were chemically synthesized using standard Fmoc chemistry at the Sheldon Biotechnology Center of McGill University (Montreal, PQ). The synthetic peptides were purified by reversed phase high performance liquid chromatography and their identities verified by electrospray mass spectrometry.

**Expression and Purification of Ste20p**—A polymerase chain reaction was performed on a plasmid containing the Ste20p gene of the yeast *S. cerevisiae*, using 5'-CCCGGATCCGAGGAGCCGGAA-GAT-3' and 5'-CCGGAAATTCCTCCGTTGCTACT-3' as primers. The amplified DNA fragment encoding residues 854–939 (designated as Ste20\(_{854-939}\)) was cloned into the pET expression vector as a glutathione-S-transferase fusion protein using EcoRI and BamHII restriction sites. The sequence of the cloned DNA was confirmed by the automated DNA sequencing procedure. The cloned expression vector was transformed into the *Escherichia coli* strain BL21(DE3) to overexpress the GST-Ste20854 protein, which was subsequently purified using glutathione-Sepharose (Amersham Pharmacia Biotech). The cleavage of the GST-Ste20854 protein was performed directly on the glutathione-Sepharose column. The released Ste20859 protein was purified further on a fast performance liquid chromatography system using a Superdex G-75 column.

For NMR experiments, the Ste20854 protein was prepared in \(^{15}\)N- and \(^{15}\)N/\(^{13}\)C-labeled forms using a similar expression protocol except for growing *E. coli* cells in M9 medium instead of the 2YT medium. The \(^{15}\)N/\(^{13}\)C-SO\(_4\) salt was used for \(^{15}\)N labeling and \(^{15}\)N/\(^{13}\)C-glucose used for \(^{15}\)N/\(^{13}\)C labeling. The N-terminal 7 residues of the expressed Ste20854 protein samples were confirmed through sequencing and molecular masses verified by mass spectrometry. Deuterated dithiothreitol was included in the purified protein solution at a final concentration of 5 mM in order to prevent the formation of a disulfide bond.

**NMR Sample Preparation**—Samples for NMR experiments were prepared by dissolving the lyophilized peptides or the expressed protein in 40 mM imidazole buffer composed of 50 mM sodium phosphate at pH 7.2 and 0.2 mM EDTA. The pH values of the NMR samples were 6.8 for the two peptides and 5.5 for the entire domain because of a slight precipitation for the expressed protein at near neutral pH values. The deuterium lock signal for the NMR spectrometers was provided by the addition of 50 \(\mu\)l of D,O. Deuterated trifluoroethanol (TFE) was used to prepare NMR samples in the presence of 40% TFE.

**NMR Experiments and Sequence-assignments**—Proton NMR experiments were carried out on Bruker Avance-500 or Avance-800 spectrometers equipped with 5-mm triple resonance pulse field gradient accessories. Phase-sensitive detection by time-proportional phase incrementation was employed for both the two-dimensional Overhauser effect NOESY (12) and total correlation TOCSY (13) experiments. Mixing times of 150 and 200 ms were used for NOESY experiments and a rotation time of 280 ms for TOCSY experiments. A 1H cross-peak was also carried out using the XwinNMR spectrometer software (Bruker). A set of three-dimensional heteronuclear experiments, including HNCACB, CBCA(CO)NH, TOCSY-HSQC, and NOESY-HSQC (14 and references therein) were collected for the isotope-labeled Ste20854 protein at 288 K on the 800 MHz spectrometer. Water suppression was achieved using the WATERGATE method (15) with a 3:9:19 binomial selective pulse (16) incorporated into the last t2 periods of all of the three-dimensional pulse sequences as described previously (17). To improve the sensitivity and water suppression, a SEDUCE-1 (18) sequence with a field of 2.5 kHz for \(^{13}\)C was used to decouple the \(^{15}\)CO-\(^{13}\)C interactions in the t1 and t2 evolution periods. The NMR data were processed using NMRPipe/NMRDraw with 90°-shifted sine-square weighting functions in all three dimensions (19) and analyzed by the use of NMRView (20). All chemical shifts were indirectly referenced to that of 2,2-dimethyl-2-silapentane-5-sulfonic acid (21).

Sequence-specific assignments of the proton resonances for the two peptides were achieved through identification of spin systems in the TOCSY spectra combined with sequential NOE connectivities in the NOESY spectra (22). The amide \(^1\)H, \(^{15}\)N, \(^{13}\)C, and \(^{13}\)C\(^{\beta}\) resonances of Ste20854 were assigned using the three heteronuclear spectra, namely \(^{1}\)H HSQC, HNCA, and CBCA(CO)NH. This process provided assignments for the backbone resonances of most residues except for an acidic segment over residues 905–913 with a sequence of DND-DNDINE where the Asp and Asn residues occur repeatedly causing severe spectral overlappings.

\(^{15}\)N Relaxation and Measurements of Hydrodynamic Radius—\(^{15}\)N T1 and T2 relaxation times and \(^{1}\)H-\(^{15}\)N steady-state NOEs were determined on the 800 Hz spectrometer from a series of two-dimensional \(^{1}\)H-\(^{15}\)N correlation spectra as described by Farrow et al. (23). The \(^{1}\)H T1 values were measured from spectra recorded with relaxation delays of 40, 90, 140, 200, 280, 380, 500, 650, 830, 1,050, and 1,250 ms. \(^{1}\)H T2 values were determined with relaxation delays of 14.4, 28.8, 57.6, 86.4, 115.2, 144, 172.8, 216, 259.2, 316.8, 374.4, and 460.8 ms. \(^{1}\)H-\(^{15}\)N steady-state NOEs were obtained by recording spectra with and without \(^{1}\)H presaturation of duration 3 s plus a relaxation delay of 2 s. T1 and T2 relaxation times were determined from the 1H-15N HSQC spectra by nonlinear least squares fits of the cross-peak intensities in the T1 and T2 experiments, assuming a single exponential decay. \(^{1}\)H-\(^{15}\)N NOE values were obtained from the ratio of intensities of cross-peaks in the reference (no proton saturation) and NOE (with proton saturation) spectra. The presence of \(\mu\)-s scale conformational exchange processes was assessed by CPMG-based NMR experiments using procedures described previously (24–26). The intensities of the \(^{1}\)H-\(^{15}\)N HSQC spectra were followed as a function of the number of 180-degree \(^{15}\)N refocusing pulses (from 4 to 48) inserted within a constant period of 40 ms for the decay of the \(^{15}\)N transverse magnetizations (25). The HSQC intensities were converted to effective T2 values of the \(^{15}\)N nuclei as a function of the repetition rates (or effective B1 field strengths) of the CPMG pulse train, which varied from 100 to 1,200 \(\mu\)s for the different number of refocusing pulses used. The hydrodynamic radius was determined by

![Fig. 1. Schematic representation of the domain organization of Ste20p at the yeast *S. cerevisiae* and the Ste20p fragments employed in this study.](http://www.jbc.org/)

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**Fig. 1.** Schematic representation of the domain organization of Ste20p showing the N-terminal regulatory region (1–627), the kinase domain (628–853), and the C-terminal noncatalytic domain (854–939). The locations of the Cdc42 binding domain (CBD) and the GBB motif are also shown as labeled. Panel B, the amino acid sequence of a 14-residue fragment Ste20p\(_{97-990}\) containing the minimal GBB motif (underlined) as identified previously (11). Panel C, the amino acid sequence of a 37-residue extended fragment Ste20p\(_{599-655}\) containing the GBB motif. Panel D, schematic representation of the C-terminal noncatalytic domain (Ste20p\(_{939}\)). The minimal GBB motif is only conserved in the Ste20pPK family of protein kinases and is underlined in panels B and C.

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**Table:**

| CBD | Kinase | GBB |
|-----|--------|-----|
| 1   | 330    | 853 |
| 337 | 367    | 939 |

**Supplementary Information:**

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use of NMR pulsed field gradient measurements using the acetate ion as the internal reference (26).

Structure Calculations—NOE cross-peaks were collected from the NOESY spectra and were classified roughly as strong, medium, or weak, corresponding to distance upper bounds of 3, 4.0, and 5.0 Å. The obtained distance constraints were employed to calculate the solution structure of the 37-residue peptide by use of a standard protocol of distance geometry followed by simulated annealing, or DG/SA, implemented in the XPLOR software program (27). A set of 50 initial structures was generated by DG/SA, and 10 lowest-energy structures were selected for the final analysis and display. The protein structures are visualized and manipulated by use of the Sybyl molecular graphic program (Tripos Inc.) and the Insight II software package.

RESULTS

Conformational Properties of a 14-Residue GBB Peptide—Fig. 2 presents the conformational shifts of the 14-residue peptide Ste20877–890. Except for the C-terminal residue Leu890, the conformational shifts for all other residues are less than 0.05 ppm. This result, together with a poor chemical shift dispersion of the amide protons (−0.62 ppm) and the absences of medium or long range NOEs over the entire sequence (data not shown), indicates that the 14-residue peptide is flexible and may assume a predominantly random coil conformation in aqueous solution.

Secondary structure predictions using three different methods (28–30) all suggest that the C-terminal domain of Ste20p has helical structure propensities over most residues (not shown). It is well known that TFE can significantly stabilize helical conformations for peptides and proteins with intrinsic helical propensities (31–33). Therefore, the conformation of the 14-residue peptide was studied further in the presence of 40% TFE. As seen in Fig. 2, the introduction of 40% TFE significantly induces the conformational shifts of the CαH protons toward helical values. In agreement with this, strong sequential NH-NH NOEs were also observed over residues Val885 to Leu890 (spectra not shown). Very interestingly, even in the presence of TFE, the region around the two important binding residues, Ser879 and Ser880 (11), still remains predominantly flexible, as evident from the small CαH conformational shifts (Fig. 2) and the NOE patterns (data not shown).

Conformational Properties of the 37-Residue GBB Peptide—The 14-residue peptide Ste20877–890 has a relatively weak bind-
ing affinity for the Gβ protein (7, 11) and is flexible conformationally in aqueous solution as shown above. Therefore, we extended this 14-residue sequence to a 37-residue peptide Ste20_859–895 (Fig. 1C). Fig. 3a presents the CoH conformational shifts for this peptide. It appears that the longer peptide shows enhanced helical preferences for many residues even in aqueous solution, with helical conformational shifts for many residues larger than 0.05 ppm. However, the increase in the helical propensities is still not significant because only three nontail residues, Thr863, Thr872, and Val885, show conformational shifts larger than 0.1 ppm but less than 0.15 ppm. The analysis of the NOESY spectra for this peptide identified sequential NH-NH NOEs over many residues (data not shown), indicating the existence of helical conformations to some degree.

On the other hand, introduction of 40% TFE appears to stabilize the helical conformation of the longer peptide, Fig. 4.

**Fig. 4.** SDS-PAGE showing the purification steps for the recombinant Ste20_853–939 protein. Lane 1, molecular mass markers. Lane 2, GST-Ste20_853–939 protein after purification by glutathione-Sepharose column. Lane 3, mixture of GST-Ste20_859–939 and released GST proteins. Lane 4, released Ste20_859–939 protein in aqueous solution after thrombin cleavage.

**Fig. 5.** Panel a, an ¹H-¹⁵N HSQC spectrum of the entire C-terminal domain uniformly labeled with ¹⁵N and ¹³C at pH 5.5 and 15 °C with the assigned residues labeled. Panel b, differences between the Cα and Cβ conformational shifts over the sequence of the entire C-terminal domain. Black bars indicate the sequence corresponding to the 14-residue fragment Ste20_877–890, gray bars indicated the sequence corresponding to the 37-residue fragment Ste20_859–895.
Ste20853--939. Significantly, the deviations of the CaH chemical shifts increase significantly toward the helical direction (Fig. 3a). Consistent with this observation, a large number of medium range NOEs were identified as shown in Fig. 3b. In the presence of 40% TFE, strong sequential NH-NH NOEs span over nearly the entire sequence, from residue Ala862 to Glu876. A close inspection of the CaH conformational shifts (Fig. 3a) and the NOE pattern (Fig. 3b) indicates that in the presence of 40% TFE, two helical segments form in the 37-residue peptide, one from Ala862 to Glu876 and another from Ala862 to Leu890. Very strikingly, the binding region around residues Ser879 and Ser880 is still located at the junction between the two helical segments and may remain flexible even in the presence of 40% TFE, in agreement with the results for the 14-residue peptide.

Heteronuclear NMR Characterization of the Entire C-terminal Domain of Ste20p—The NMR data presented above showed that the 14- and 37-residue peptides containing the GBB consensus are unstructured in aqueous solution and only have helical secondary structure preferences. The lack of unique structures for the two peptides may be a result of missing long range interactions existing in the entire C-terminal domain of Ste20p. Therefore, the C-terminal domain over residues 853–939 of Ste20p was overexpressed and purified to homogeneity (Fig. 4) by affinity chromatography and gel filtration fast performance liquid chromatography (see “Experimental Procedures”). A quick inspection of the 1H-15N HSQC of Ste20853--939 showed that the chemical shift dispersions of both the amide 1H and 15N dimensions were still very poor, only 0.85 ppm for amide proton and 15 ppm for 15N, suggesting that the Ste20853--939 may also be unfolded in solution. Resonance assignments of unfolded/partially folded proteins are usually difficult because of chemical shift degeneracies and/or broadening of resonances resulting from the intermediate rates of conformational exchange at μs-ms time scales (34). Therefore, we used three-dimensional heteronuclear NMR experiments for resonance assignments, particularly by use of a combination of the HNCA-CB and CBCA-CO spectra. Some of the assignments were confirmed further by use of 15N-edited three-dimensional HSQC-TOSCY and HSQC-NOESY spectra. Using this procedure, most residues were assigned (Fig. 5a) with the exception of Ser879 and the Asp902-Asp-Asp-Asp-Asp-Glu920 region because of resonance overlaps. The missing of the Ser879 peak may result from chemical shift degeneracies and/or broadening of resonances.

Fig. 5b shows the difference between the conformational shifts of the Ca and Cβ carbons over the entire C-terminal domain, which have been shown to be a convenient method for secondary structure identification in peptides and proteins (35–37). Overall, the C-terminal domain of Ste20p has helical preferences over most residues (Fig. 5b). A detailed examination suggests that the C-terminal domain may consist of several helical segments, the first one centered around Leu865-Leu866, the second around Leu884-Val885, and the third around Thr915. It is extremely interesting to note again that in the entire C-terminal domain, the region containing the important binding residues Ser879-Ser880 is located at the junction between the first and the second helical segments as constantly observed in the 14- and 37-residue peptides. As seen in Fig. 5b, the chemical shift deviations suggest that the helical conformations are populated in the C-terminal domain but may still not be well formed. The analysis of the HSQC-NOESY spectrum indicates the absence of medium and long range NOEs for the entire C-terminal domain, confirming that the C-terminal domain indeed only has flexibly linked helical conformations and lacks tertiary packing interactions among the secondary structure elements.

We also introduced TFE into the Ste20853--939 sample for further enhancement of the helical structures. At low concentrations of TFE (5–10%), no significant changes were observed as judged by one-dimensional NMR spectra (not shown). Introduction of 40% TFE as done for the two shorter peptides led to significant broadening of the NMR peaks and prevented us from a complete NMR and NOE analysis. With a small portion of residues assigned, it appears that 40% TFE may induce increases in helical preferences for many residues, as judged from the assigned Ca chemical shifts. For example, the Ca conformational shift for Ile874 increases from 0.24 to 0.61 ppm, Lys892 from 0.26 to 0.46 ppm, Val893 from 0.41 to 0.98 ppm, Thr915 from 0.02 to 0.52 ppm, Ser924 from 0.45 to 1.03 ppm, and Lys925 from 0.9 to 1.28 ppm.

15N Relaxation Dynamics and Hydrodynamic Radius of the Entire GBB Domain—The 15N NMR relaxation data provide important insights into the conformational flexibility of protein backbones (38–39). Fig. 6 presents the backbone 15N NMR relaxation data including 1H-15N NOE intensities, T1 and T2 relaxation rates of the GBB domain. As seen in Fig. 6a, all of the 1H-15N heteronuclear NOE intensities are less than 0.42, and most are even less than 0.30, which are far smaller than the suggested value of 0.8 for a well folded proteins (38). This result clearly shows that the isolated C-terminal domain of Ste20p is conformationally flexible in solution. However, a closer examination of the heteronuclear NOE intensities indicates that several sequence segments may exhibit restricted motions to some degree, in agreement with the observation based on carbon chemical shifts (Fig. 5b) that these segments have helical secondary structure preferences. Interestingly, the region around residues Ser879 and Ser880 gives rise to relatively weak heteronuclear NOEs, indicating that this sequence seg-

![Fig. 6. Backbone 15N relaxation data of Ste20853--939 representing the entire C-terminal domain of Ste20p at pH 5.5 and 15 °C. Panel a, steady-state 1H-15N heteronuclear NOEs. Panel b, 15N T1 relaxation rates. Panel c, 15N T2 relaxation rates.](http://www.jbc.org/)

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ment is more flexible than those having helical conformation. A similar pattern can also be identified in the T2 relaxation times (Fig. 6c).

The motional properties of the GBB domain were assessed further by use of a CPMG-based experiment to detect the presence of \( \mu s \)-ms conformational exchange processes. However, no significant response of the \( ^{15}N \) T2 values to the CPMG pulse delays was observed up to the fastest CPMG repetition rate (1,200 s) for almost all residues of the GBB domain. In addition, the GBB domain exhibited relatively sharp \( ^{1}H \) HSQC peaks, with \( ^{15}N \) T2 mostly larger than 0.15 s or relaxation rates less than 6 s\(^{-1} \) even at a high magnetic field strength of 800 MHz (18.8 tesla) (Fig. 6c). These results indicate that the conformational exchange processes in this protein must either occur in the high \( \mu s \) time scale or involve other conformations with small chemical shift deviations or both. Furthermore, the hydrodynamic radius of the 86-residue GBB domain was determined to be 22.54 Å, larger than 18.6 Å for the folded state of a 90-residue SH3 domain but smaller than 27.5 Å for the unfolded state of the SH3 domain in the presence of 3.5 M GuHCl (26). This observation demonstrates that the GBB domain is not well folded and assumes a relatively expanded conformation which, however, is more compact than the unfolded state of proteins.

**Helical Structures of the GBB Domain**—NMR data on all three fragments indicate that the GBB domain is composed of several helical segments. To gain further insights into the functional interactions of the GBB domain, the helical structures of the 37-residue Ste20859–895, which carries the minimal GBB motif in the second half of the sequence, was determined using the extensive NOE data collected in the presence of 40% TFE (Fig. 3). For structure calculations, the assigned sequential and medium range NOEs were converted into 150 NOE distance constraints including 87 sequential and 63 medium range constraints. Fig. 7a is the lowest energy structure, showing two well defined helical segments linked by a flexible region. The N-terminal helix spans residues 5–16 and the C-terminal helix over residues 27–33. The junction between the two helices is poorly defined because of the lack of NOEs (Fig. 3b). The flexibility of the junction region is also reflected by the orientational differences between the two helical segments in different calculated structures, as highlighted in Fig. 7, b and c. Clearly, when one helix is well superimposed in the structure cluster, the other helix always shows an ensemble of orientations. Very importantly, these calculated structures emphasize the point that the two important binding residues, Ser879 and Ser880, are located in the flexible junction and another important residue Pro883 at the beginning of the second helix. These observations are completely consistent with the NMR conformational and \( ^{15}N \) relaxation data observed for the two peptide fragments and the entire GBB domain (Figs. 2, 3, and 6).

**DISCUSSION**

Functional studies and sequence analysis have suggested that the GBB consensus motif may represent an autonomous structural domain that is essential and sufficient for binding to the Gβ subunit of the heterotrimeric G protein (7, 11). In addition, residue Ser879 and especially the absolutely conserved residues Ser880 and Pro883 were shown to be important for the binding interaction (7, 11) as a triple mutant with these residues mutated to Ala abolished the functional binding (11). Our NMR results with two GBB peptides and the entire C-
Most strikingly, the N-terminal residues, Ser528 and Ser529, in the GBB motif in PAK1 also forms a short helical segment as part of the helical region. Interestingly, the C-terminal part of this PAK GBB motif corresponding to Ser879 and Ser880 in Ste20p are located in a loop region linking the GBB helix to the preceding helical region. Therefore, it appears that a short helix at the C-terminal part and the flexibility of the two conserved serines may be important for the functional binding to Gβ of GBB motifs in the Ste20/PAK family of protein kinases. On the other hand, in the yeast Ste20p, the noncatalytic C-terminal domain contains an additional 50 residues beyond the GBB motif, much larger than the 6 residues of human PAK1. Inclusion of these C-terminal residues in the form of the entire 86-residue C-terminal domain of Ste20p confers tight and specific binding of the GBB motif to Gβ (11). These observations taken together suggest that the additional parts in the C-terminal region of Ste20p may play roles in determining the affinity and specificity of the GBB-Gβ complex formation.

Our successful expression of the entire C-terminal domain of Ste20p made it possible to characterize the conformational and dynamic properties of this high affinity GBB domain by use of NMR spectroscopy. This is in itself significant because the expression of unstructured proteins still remains a difficult task. Even though this protein domain exhibits a narrowly dispersed 1H,15N HSQC spectrum, a total of 88% of the amide protons, backbone nitrogens, α and β carbons were assigned using heteronuclear NMR methods, enabling, for example, the use of 15N NMR relaxation data to pinpoint the conformational properties of this functionally active protein domain. Very interestingly, heteronuclear NMR data demonstrate clearly that even though the isolated C-terminal domain can completely restore the Gβ binding function of Ste20p independent of the rest of Ste20p, it is not compactly folded when isolated in solution, having only helical secondary structures similar to the conformational properties of the two GBB peptides, namely a 14-residue minimal fragment and a 37-residue extended peptide containing the GBB consensus.

At first sight, it is really surprising that such a highly active (for Gβ) peptide domain can be a flexible and partially folded protein. It has been shown that the two sets of binding residues for the Gβ domain are located on the opposite sides of the three-dimensional structure of the Gβ subunit of the heterotrimeric G protein with a distance larger than 40 Å (11). This observation implies that the GBB domain must bind to the Gβ subunit with a relatively extended conformation and with a large contact interface. It is possible that the entire C-terminal domain of Ste20p is structured in the full-length Ste20p kinase, as shown recently for the autoinhibited structure of human PAK1 (40). A presumably folded GBB structure in Ste20p may displace a subdomain of the kinase region and reorganize the kinase structure into an inactive conformation. Nevertheless, binding of the GBB domain to Gβ must dislodge the C-terminal domain of Ste20p from the kinase, potentially activating the kinase as required for function. A largely unfolded conformation of the Ste20p C-terminal region in the absence of association with the kinase, such as that determined here for the isolated C-terminal domain, may provide a facile mechanism for the formation of a tight binding and stable molecular complex with the β subunit of the heterotrimeric G protein. The NMR results reported here also fit into the recent hypothesis that many proteins and protein domains responsible for regulatory functions in cell cycle and signal transduction may be flexible in the free state and become uniquely structured only upon binding to their partners (41–46 and references therein). This binding-induced folding has been suggested to have some advantages for achieving desired binding specificity required for the integration of multiple response signals (46, 47), as also put forward for the multiple step formation of the N-WASP-Arp2/3 complex (47) and likely to be the case for Ste20p, a protein also responsible for integrating signals from multiple pathways.

In summary, we have studied two GBB peptides and the entire C-terminal domain of Ste20p by use of multidimensional NMR spectroscopy. The NMR results demonstrate that these protein fragments are all not uniquely folded in solution. The helical structures of a 37-residue GBB peptide were determined, showing that the C-terminal part of the GBB motif forms a short helix, whereas the conserved and functionally important residues are located at the flexible junction linking to a preceding helix. The present work represents the first detailed structural study on the GBB domain of Ste20p, the prototype and the largest of the novel family of Gβ binders, and may have important implications in understanding the molecular mechanisms of the signal transduction from the heterotrimeric G protein to the mitogen-activated protein kinase cascades.

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