Journal of Biomolecular NMR **27:** 185–186, 2003.
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Letter to the Editor: Backbone assignments of Grb2 complexed with ligand peptides for SH3 and SH2 domains

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Received 10 March 2003; Accepted 8 May 2003

Key words: deuterated protein, Grb2, multidomain, NMR, resonance assignments

Biological context

Grb2 is a SH2/SH3 containing adapter protein with molecular weight of 25 KDa. Grb2 comprises three src homology (SH) domains, N-terminal SH3, central SH2 and C-terminal SH3. SH2 and SH3 are prototypes of the structural and functional domains involved in the intracellular signalling proteins which recognize and bind to phosphotyrosine containing sequences and proline rich sequences in target proteins, respectively (Schlessinger, 1994). Through its SH3 domains, Grb2 constitutively binds to proline rich sequences in the C-terminal tail of Sos, a guanine nucleotide exchange factor for Ras. When a growth factor receptor is activated upon binding of a ligand, tyrosine residues on the receptor or substrate proteins such as IRS or Shc are phosphorylated. Grb2 binds to the target sequences containing a phosphotyrosine residue through its SH2 domain and relocates Sos in the vicinity of the membrane from the cytosol, enabling Sos to interact with Ras. Thus, the inactive GDP bound form of Ras is activated to the GTP bound form. The signal transduction process mediated through Grb2 is recognized as a major pathway for cell proliferation.

The structure of Grb2 was determined by X-ray crystallography in 1995 (Maignan et al., 1995), where the two SH3 domains are in close contact with each other so that Grb2 forms a compact structure. A question arises whether this compact structure is retained in solution or is due to crystal packing force in the crystal state. This prompted us to study the structural property of Grb2 in solution by NMR spectroscopy. We report here the NMR backbone assignments of Grb2. These assignments provide a basis for further structural and dynamic studies of Grb2.

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Methods and experiments

The mutant Grb2 (residues 1-217) where Cys32 and Cys198 were replaced by Ser32 and Ala198 (We referred this mutant as Grb2 in the followings.) were cloned into a pET-3a expression vector. Transformed Escherichia coli BL21 (DE3) pLysS cells were grown at 25 °C in Luria broth (LB) to obtain unlabeled Grb2. Uniformly ^{15}N- labeled protein sample was prepared by growing transformed E.coli on M9 minimal medium containing 1 g/l $^{15}NH_4Cl$ as a sole nitrogen source. A uniformly ^{15}N/^{13}C-labeled and deuterated protein sample was prepared by growing the cells in the presence of 95% D_2O, after the method of protein deuteration described by Venters et al. (1992). Selective ^{15}N labeled proteins at Val, Met and Lys residues were prepared by growing the cell in amino acid broth containing 50 mg/1 l α-^{15}N labeled Val, Met or Lys, respectively. A uniformly ^{15}N labeled protein with selectively ^{13}C labeled carbonyl groups of proline residues was prepared by growing the cell in M9 broth containing $^{15}HN_4Cl$ and 50 mg/1 l 1-^{13}C-Pro. The proteins were purified as described previously (Yuzawa et al., 2001).

The buffer was changed to NMR sample buffer containing 20 mM phosphate buffer (pH 7.2), 0.05% (w/v) sodium azide and 90% H_2O/10% D_2O using an Amicon concentrator. The Grb2-ligand complex formation was monitored by observing chemical shift changes in ^{1}H-^{15}N HMQC spectra of Grb2 upon

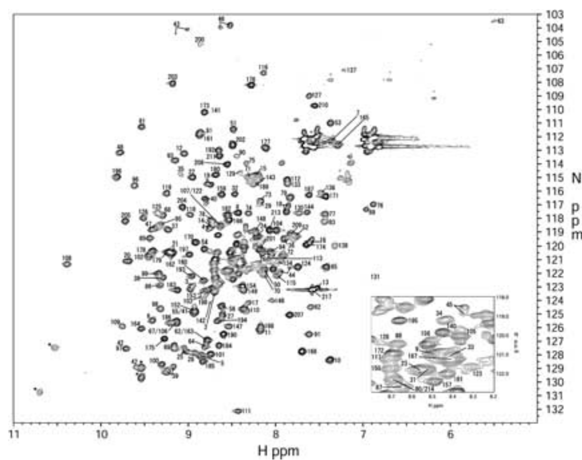

Figure 1. ^{1}H-^{15}N HSQC spectrum of ^{2}H/^{13}C/^{15}N labeled Grb2 complexed with the EGF receptor-derived peptide and the Sos derived peptide in 90% H_2O/10% D_2O solution at pH 7.2 and 25 °C. Sequential assignments are indicated with residue number. The Trp indole ring NHcross peaks are labeled with ∗. Double peaks are observed due to conformational heterogeneity for some residues.

addition of aliquots of the Sos-peptide (VPPPVP-PRRR) derived from human Sos1 and the EGFR-peptide (EpYINSQV) derived from the human EGF receptor. These peptides were purchased from Kurabo Industries Ltd. and used without further purification. The NMR sample solution contained 1.2 mM of Grb2, 1.2 mol equiv. of the EGFR-peptide and 5 mole equiv. of the Sos-peptide which was placed in a volume restricted microcell (Shigemi, Hachiouji, Japan) so that each binding site on SH3 and SH2 was saturated by the ligand. NMR data were acquired on Varian Unity Plus 600 MHz and Varian Unity Inova 500 MHz spectrometers with a triple-resonance pulsed field gradient 5 mM probe. The NMR data were processed using VNMR (Varian Instruments, Pala Alto, CA), NMRPipe (Delaglio et al., 1995) and Felix 95.0 (Molecular Simulations, San Diego, CA). The ^1H_N, ^{15}N and ^{13}C backbone resonances of Grb2 were assigned from an array of deuterium decoupled triple resonance experiments including 3D-HNCA, 3D-HN(CO)CA, 3D-HN(CA)CB, 3D-HN(COCA)CB, 3D-HN(CA)CO and 3D-HNCO recorded using uniformly labeled ^{2}H/^{13}C/^{15}N-labeled Grb2 (Yamazaki et al., 1992; Matsuo et al., 1996). In order to confirm the backbone resonance assignments, ^{1}H-^{15}N HSQC spectra were recorded for specifically ^{15}N- labeled proteins at Val, Met and Lys residues (Tate et al., 1992). For the assignments of the main chain amide groups between Pro-X sequences (X means any type of amino acid residue.), we recorded the HNCO spectrum of uniformly ^{15}N- labeled Grb2 containing proline residues with selectively ^{13}C-labeled carbonyl carbons.

Extent of assignments and data deposition

For the sequential assignments of the backbone ^1H_N, ^{13}C and ^{15}N resonances, a series of heteronuclear deuterium decoupled triple resonance experiments was made including 3D-HNCA, 3D-HN(CO)CA, 3D-HN(CA)CB and 3D-HN(COCA)CB. Ambiguities due to overlap of Cα and/or Cβ resonances were removed using the triple resonance, deuterium decoupled 3D-HNCO and 3D-HN(CA)CO spectra. The backbone resonance assignments were further confirmed by the 2D ^{1}H-^{15}N HSQC spectra recorded on the protein samples with selectively ^{15}N labeled amino acid residues; Met, His, Lys and Val. In order to confirm the assignment for the resonance of residues succeeding to proline residues, 3D-HNCO spectrum were recorded for the uniformly labeled ^{15}N protein sample labeled with $^{13}C'$ labeled proline residue.

The backbone sequential assignments have been made. Total 189 out of 205 possible backbone resonance (217 residues *minus* 11 proline residues and the amino terminal residue) were observed in the ^{1}H-^{15}N HSQC spectrum, and were unambiguously assigned. The several backbone resonances were not observed possibly due to exchange broadening. About 80% of the aliphatic side chain carbon resonances were assigned. Assignments of the side chain protons and aromatic ring carbons have not been made. A list of chemical shifts for deuterated protein have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under accession number 5693.

Acknowledgements

We are grateful to Mr Masashi Yokochi of CREST/JST for helpful discussions.

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