A fast and simple method for probing the interaction of peptides and proteins with lipids and membrane-mimetics using GB1 fusion proteins and NMR spectroscopy

Lisa A. M. Sommer, Melanie A. Meier, and Sonja A. Dames*

Technische Universität München, Department of Chemistry, Biomolecular NMR Spectroscopy, Munich, Germany

Received 31 May 2012; Revised 11 July 2012; Accepted 12 July 2012

DOI: 10.1002/pro.2127

Published online 23 July 2012 proteinscience.org

Abstract: The expression of peptides and proteins as fusions to the B1 domain of streptococcal protein G (GB1) is very popular since GB1 often improves the solubility of the target protein and because the first purification step using IgG affinity chromatography is simple and efficient. However, the following protease digest is not always complete or can result in a digest of the target protein. In addition, a further purification step such as RP-HPLC has to be used to get rid of the GB1 tag and undigested fusion protein. Because the protease digest and the following purification step are not only time-consuming but generally also expensive, we tested if GB1 fusion proteins can directly be used for NMR interaction studies using lipids or membrane-mimetics. Based on NMR binding studies using only the GB1 part, this fusion tag does not significantly interact with different membrane-mimetics such as micelles, bicelles, or liposomes. Thus spectral changes observed using GB1-fusion proteins indicate lipid- and membrane interactions of the target protein. The method was initially established to probe membrane interactions of a large number of mutants of the FATC domain of the ser/thr kinase TOR. To demonstrate the usefulness of the approach, we show NMR binding data for the wild type protein and a leucine to alanine mutant.

Keywords: NMR spectroscopy; membrane-mimetic; protein–membrane interactions; protein–lipid interactions

tgczvations: DihepPC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPC, dodecylphosphocholine; GB1, B1 immunoglobulin binding domain of streptococcal protein G (56 residues); GB1-Xa, GB1 followed by a thrombin and a factor Xa recognition site (= LVPRGS-IEGR); (m)TOR, (mammalian) target of rapamycin; min, minute(s); SI, supplementary information; y1FATC, residues 2438–2470 of *S. cerevisiae* TOR1.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: German Research Foundation (DFG); Grant number: DA1195/3-1; Grant sponsor: TUM Graduate School.

*Correspondence to: Sonja A. Dames, Technische Universität München, Department of Chemistry, Biomolecular NMR Spectroscopy, Munich, Germany. E-mail: sonja.dames@tum.de

Introduction

Many signaling proteins can bind to specific regions of the plasma membrane or to membranes of different organelles in response to certain stimuli. Conditional membrane association can be mediated by posttranslational fatty acid modifications and/or a protein membrane anchoring region. Phagocytosis, for example, has been proposed to be regulated by local changes of the concentration of specific lipids such as for example different phosphoinositol-phosphate lipids (PIPs) as a function of time.^{1–3} This modulation in the local lipid composition regulates the assembly of specific protein complexes that contain for example small lipidated GTPases of the Rho family⁴ or different actin binding proteins that have domains that can interact with specifically composed membrane regions.^{5–8} Overall this highly ordered and well choreographed process results in the formation of a cup-like membrane structure and finally the formation of an internalized membrane vesicle.³ A better understanding of the membrane properties such as surface charge, curvature and acyl chain accessibility that are recognized by conditional membrane proteins usually involves interaction assays using a large number of differently composed membrane-mimetic particles. Moreover, one may want to find a mutant that does not anymore bind to these regions, which results in an even larger number of different binding reactions. Thus it would be desirable to have a fast and efficient method at hand to prepare the respective membrane-binding protein regions and to probe their ability to interact with different membrane-mimetics.

One aim of our group is to determine the structural basis of the localization of the ser/thr kinase target of rapamycin (TOR) to different cellular membranes and in the nucleus. TOR controls cell growth in response to nutrient availability and growth factor signals in all eukaryotes.^{9,10} TOR proteins are about 2500 residues long and share several functional domains.⁹ The small C-terminal FATC domain has been shown to influence the cellular stability of TOR through its redox state.¹¹ Subsequent NMR binding studies suggested that this domain contains further a redox-sensitive membrane anchor that consists of a hydrophobic bulb with a rim of charged residues.¹² To better understand the role of single residues for the interaction with membrane-mimetic particles a large number of mutants has to be tested. Usually, we express the FATC domain of yeast TOR1 (y1fatc) as fusion to the B1 domain of streptococcal protein G [GB1, Fig. 1(A)] using the vector GEV2.¹³ Following the first purification step by IgG affinity chromatography, factor Xa is used to cleave off the GB1 part. Finally, the free FATC domain is purified from the GB1 tag and undigested fusion protein by reversed phase HPLC.¹¹ Because this is fairly time consuming and considering the prices for commer-

cially available proteases also rather expensive, we tested if the fusion protein can be directly used for NMR-based interaction studies. As presented here the GB1 part shows no significant spectral changes in the presence of different membrane-mimetics. Thus the observed spectral differences for a specific GB1-fusion protein in the absence and presence of a particular membrane-mimetic correspond to the target protein. This strategy is generally applicable to test the membrane-binding properties of peptides and proteins. Moreover, in the case of peptides or small protein domains the one-step purification procedure can be sped up and improved making use of a published procedure involving incubation of the resuspended cells at elevated temperatures (80°C).¹⁴ This heat shock lyses the cells and results in the precipitation of most *E. coli* proteins.

Results and Discussion

The GB1 tag shows no significant spectral changes in the presence of membrane-mimetics

The ability of the GB1 fusion tag to interact with membranes was probed by recording ¹H-¹⁵N-HSQC spectra in the presence and absence of different membrane-mimetics. For this we used the cleaved off fusion tag from the purification of the free yeast TOR1 FATC domain, which consists of GB1 that is C-terminally followed by a linker region containing a thrombin (LVPRGS)¹³ and in this case additionally a factor Xa site (IEGR) [Fig. 1(A)]. This construct is referred to a GB1-Xa. Supporting Information Figures S1 shows the sequence and structure of GB1 (PDB-id 3gb1).¹⁵ Figure 1(B) shows a superposition of the ¹H-¹⁵N-HSQC spectra of ¹⁵N-GB1-Xa alone and in the presence of DPC or DihepPC micelles or DihepPC/DMPC bicelles or DMPC liposomes. Separate plots for each membrane-mimetic, in the case of DPC for different concentrations are given in Supporting Information Figures S2–S5. The majority of residues of GB1-XA show no significant spectral changes. Only in the presence of high concentrations of DPC and DihepPC micelles and DihepPC/DMPC bicelles, some residues show small local shifts, which presumably arise from unspecific interactions in the presence of high mM concentrations of the respective lipids. However, these shifts are so small that the overall spectral appearance is maintained [Fig. 1(b), Supporting Information Figs. S2–S5]. This was also not the case, when GB1-Xa is titrated stepwise with DPC in buffer (Supporting Information Figs. S2 and S7 in Ref. 16). Thus the presence of the GB1 fusion tag is not expected to disturb the detection of membrane-association of a linked target protein.

The GB1 fusion tag does not impair the detection of FATC membrane association

To test the usefulness of GB1 fusion proteins to probe protein–membrane interactions, we recorded

Figure 1. Schematic representation of the used GB1 fusion proteins and monitoring of protein membrane-mimetic interactions by NMR. (A) Built-up of the used GB1 fusion proteins. To the bottom right the sequence of the yeast TOR1 FATC domain (y1fatc) is shown. The disulfide bond and the membrane-anchoring region derived from earlier NMR studies¹² are indicated. (B) Superposition of the ^1H - ^{15}N HSQC spectra of GB1-Xa in the absence and presence of the indicated membrane-mimetics. (C) Superposition of the ^1H - ^{15}N HSQC spectra of GB1-Xa-y1FATC in the absence and presence of DPC micelles or DMPC liposomes. The indicated assignments for well-resolved peaks for the free y1FATC part were adapted from the published values (BMRB accession code 6228¹¹). (D-E) Superposition of the ^1H - ^{15}N HSQC spectra of y1FATC-L2459A and GB1-Xa-y1FATC-L2459A in the absence and presence of DPC micelles. (C and E) show additionally the spectrum of free GB1-Xa to better see the peaks that belong to the FATC domain and that shift upon addition of membrane-mimetics. For each plot the small region highlighted with a black rectangle has been zoomed and is shown to the upper left.

first spectra of the GB1-Xa-y1FATC fusion in the absence and presence of DPC micelles and DMPC liposomes [Fig. 1(C)]. In addition, the spectrum of the free GB1-Xa protein was superimposed. As can be seen, only peaks of the FATC part show major chemical shift changes. The observed spectral changes in the presence of DPC are overall similar as what has been observed for untagged y1FATC.¹² Using higher DPC concentrations (~ 150 mM) to drive the associa-

tion equilibrium to the bound state and for the oxidized form additionally higher temperatures (318 K) almost all peaks of the reduced and oxidized micelle-immersed state of y1FATC can be made visible.¹² This should similarly be possible using the GB1-fusion instead of the untagged protein. The resonances of the liposome-bound form are harder to be detected, because even SUVs are large¹⁷ compared to the rather small DPC micelles (~ 20 kDa).¹⁸ Thus

association with a liposome broadens the protein signals beyond detection. In this case higher lipid concentrations or higher temperatures will presumably not solve the problem. If the resonances of the liposome-bound state shall be made visible, one may consider the use of deuterated protein in combination with transverse relaxation optimized (TROSY) NMR methods.¹⁹

To directly compare the spectral changes obtained with the untagged protein and fused to GB1, we probed the interaction of a mutant of y1FATC in which L2459 was replaced by alanine, which is referred to as y1FATC-L2459A. Figure 1(D,E) shows superpositions of the ¹H-¹⁵N-spectra of y1FATC-L2459A alone or as fusion to GB1 in the absence and presence of DPC micelles, respectively. The observed spectral changes are similar to those observed for the wild type [Fig. 1(C)] indicating that replacing L2459 in the membrane anchor by alanine does not significantly impair micelle-association. For most residues, the chemical shifts of the backbone and side chain amides of GB1-tagged and untagged y1FATC-L2459A in the free and DPC-micelle bound states are very similar. Thus the presence of GB1 and the linker region appears not to affect the structure of the following FATC region.

The factor Xa (IEGR) and the thrombin site (LVPRGS) have a net charge of 0 and +1, respectively. However, the presence of more strongly charged protease sites, such as an enterokinase recognition site (DDDDK, net charge -3) may slightly lower the affinity for membrane-mimetics, especially if they contain negatively charged lipids such as phosphoinositides (PIPs) or phosphatidic acid (PA). We probed the interaction of the FATC domain of the TOR relative DNA-PKcs fused to either GB1-Xa or GB1-enterokinase with DPC and liposomes (data not shown). However, in this case we did not observe a difference in the association behavior.

Because of the linker region, GB1 and the attached target protein tumble rather independently. This and the small size of the GB1 tag allows the use of GB1 fusion proteins, at least for small to mid-size target proteins, to record NMR data for the chemical shift assignment and the structural characterization of the free and micelle-bound states. We tested this already successfully for the FATC domain of another phosphoinositol-kinase-related kinase (PIKK), namely DNA-PKcs. As soon as fully completed, the assignment will be deposited at the BMRB. Finally, besides micelles, bicelles, and liposomes, other membrane-mimetics such as for example protein-lipid-nanoparticles¹⁷ or such containing charged lipids^{7,8,12} may be tested for probing the interaction with conditional membrane-binding proteins fused to GB1. Finally, if ¹⁵N-labeling may be an issue, binding or nonbinding can be derived from a superposition of the amide region of a 1D ¹H experiment.

In summary, we presented an NMR-based approach to monitor protein- and peptide-lipid- and membrane-interactions using GB1 fusion proteins. The purification of the GB1 fusion protein is fast and cost-efficient since no protease digest followed by a further purification step has to be performed.

Materials and Methods

Plasmid cloning and protein expression and purification

Wild type *S. cerevisiae* TOR1 FATC (= y1FATC, residues 2438–2470) was cloned into the expression vector GEV2¹³ using the BamHI and XhoI sites and overexpressed in *E. coli* BL21 or Rosetta (DE3) (Novagen) as described.¹¹ Mutant versions of y1FATC were obtained by site-directed mutagenesis. The FATC domain without fusion tag was purified by IgG affinity chromatography, factor Xa digest, and reversed-phase HPLC purification as described.¹¹ GB1-Xa-FATC fusion proteins were purified as described in the following. Cells were harvested by centrifugation at 6000g and 4°C for 20 min. The cell pellet was resuspended in 50 mM Tris, 2 mM EDTA, 2 mM benzamidine, pH 7.5, thoroughly vortexed, and stored at -20°C. The next day the cell pellet was thawed and then heated for 5 min at 80°C in a water bath as described in the literature.¹⁴ Following incubation on ice for 10 min, the suspension was centrifuged at 20,000g and 4°C for 30 min. The supernatant containing the fusion protein was separated from the pellet by decanting and directly applied on an IgG sepharose column following the manufacturer's manual (GE Healthcare). Fractions containing based on SDS page GB1 fusion protein were pooled and lyophilized. The lyophilized protein was resuspended in 8–10 mL 50 mM Tris, 100 mM NaCl, pH 6.5 and concentrated to about 1–1.5 mL using an ultrafiltration spin column (Millipore, Amicon Ultra, MWCO 3000) and washed 2× with 13–15 mL 50 mM Tris, 100 mM NaCl, pH 6.5 (= NMR buffer). The pH of the resulting concentrated protein solution (ca. 0.5–2 mL) was checked and if necessary adjusted to 6.5. This protein stock was then used for the preparation of NMR samples.

NMR sample preparation

NMR samples contained ~50–150 μM ¹⁵N-labeled GB1-Xa or y1FATC or GB1-Xa-y1FATC fusion protein in 50 mM Tris buffer (pH 6.5, 95% H₂O/5% D₂O) with 100 mM NaCl, and 0.02% NaN₃. Dodecylphosphocholine (DPC) and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DihepPC) were purchased from Avanti polar lipids. The 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was obtained from Genzyme Pharmaceuticals and Affymetrix and deuterated DPC (d₃₈-DPC) from Cambridge Isotopes.

Generally, lipid stock solutions for the titrations were prepared as follows. A defined amount of lipid from a concentrated stock in chloroform was placed in a glass vial and dried under a stream of nitrogen

gas. The dried lipid was then dissolved in buffer or a protein sample. Only DihepPC was weighted and then directly dissolved in buffer. Micelles form above the critical micelle concentration (CMC), which is 1.1 mM for DPC and 1.4–1.8 mM for DihepPC.^{20,21}

Bicelles were prepared by first drying an appropriate amount of the long chain phospholipid in organic solvent (DMPC in chloroform) under a stream of nitrogen gas in a glass vial. The dried lipid was first resuspended in a small amount of the short chain lipid (DihepPC) in buffer (20 μ L), followed by stepwise addition of the rest. Following thorough vortexing of the bicelle mixture, the protein solution was added.

Liposomes were prepared by drying an appropriate amount of DMPC in chloroform under a stream of nitrogen gas. The resulting pellet was resuspended in buffer to obtain a 50 mM solution. To dissolve the pellet, it was exposed to seven cycles of freezing in liquid nitrogen, thawing by incubation in a water bath at 40°C and thorough vortexing. To induce the formation of small unilamellar vesicles (SUVs) from large uni- and multilamellar vesicles, the DMPC suspension was incubated in an ultra sonication bath for about 20–30 min. To remove the remaining large vesicles, the milky suspension was centrifuged in a tabletop centrifuge for 5 min at 14.8 K rpm. This resulted in a clear supernatant and a rather big fluffy white precipitate. For the preparation of a protein sample in the presence of liposomes, only the clear supernatant containing small unilamellar vesicles was used.

NMR spectroscopy

NMR spectra were acquired at 298 K on a Bruker DRX500 spectrometer equipped with a cryogenic probe. Data were processed with NMRPipe²² and analyzed using NMRView.²³ NMR samples in the presence of micelles contained either 50–150 mM DPC or 50 mM DihepPC. The lipid concentration in the bicelle sample was $\sim$ 45 mM DMPC and $\sim$ 225 mM DihepPC, corresponding to $q = 0.2$ and $c_L = 15\%$. The DMPC concentration in liposome sample was <30 mM. Note that the exact amount of DMPC left after centrifugation of the ultra sonicated liposome mixture is not known.

References

- Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443: 651–657.
- Yeung T, Ozdamar B, Paroutis P, Grinstein S (2006) Lipid metabolism and dynamics during phagocytosis. *Curr Opin Cell Biol* 18:429–437.
- Botelho RJ, Grinstein S (2011) Phagocytosis. *Curr Biol* 21:R533–538.
- Navarro-Lerida I, Sanchez-Perales S, Calvo M, Rentero C, Zheng Y, Enrich C, Del Pozo MA (2012) A palmitoylation switch mechanism regulates Rac1 function and membrane organization. *EMBO J* 31:534–551.
- Xian W, Janmey PA (2002) Dissecting the gelsolin-phosphoinositide interaction and engineering of a polyphosphoinositide-sensitive gelsolin C-terminal half protein. *J Mol Biol* 322:755–771.
- Kumar N, Zhao P, Tomar A, Galea CA, Khurana S (2004) Association of villin with phosphatidylinositol 4,5-bisphosphate regulates the actin cytoskeleton. *J Biol Chem* 279:3096–3110.
- Goult BT, Bouaouina M, Elliott PR, Bate N, Patel B, Gingras AR, Grossmann JG, Roberts GC, Calderwood DA, Critchley DR, Barsukov IL (2010) Structure of a double ubiquitin-like domain in the talin head: a role in integrin activation. *EMBO J* 29:1069–1080.
- Dames SA, Junemann A, Sass HJ, Schonichen A, Stopschinski BE, Grzesiek S, Faix J, Geyer M (2011) Structure, dynamics, lipid binding, and physiological relevance of the putative GTPase-binding domain of Dictyostelium formin C. *J Biol Chem* 286:36907–36920.
- Jacinto E, Hall MN (2003) Tor signalling in bugs, brain and brawn. *Nat Rev Mol Cell Biol* 4:117–126.
- Wullschlegel S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. *Cell* 124:471–484.
- Dames SA, Mulet JM, Rathgeb-Szabo K, Hall MN, Grzesiek S (2005) The solution structure of the FATC domain of the protein kinase target of rapamycin suggests a role for redox-dependent structural and cellular stability. *J Biol Chem* 280:20558–20564.
- Dames SA (2010) Structural basis for the association of the redox-sensitive target of rapamycin FATC domain with membrane-mimetic micelles. *J Biol Chem* 285: 7766–7775.
- Huth JR, Bewley CA, Jackson BM, Hinnebusch AG, Clore GM, Gronenborn AM (1997) Design of an expression system for detecting folded protein domains and mapping macromolecular interactions by NMR. *Protein Sci* 6:2359–2364.
- Koenig BW, Rogowski M, Louis JM (2003) A rapid method to attain isotope labeled small soluble peptides for NMR studies. *J Biomol NMR* 26:193–202.
- Gronenborn AM, Filpula DR, Essig NZ, Achari A, Whitlow M, Wingfield PT, Clore GM (1991) A novel, highly stable fold of the immunoglobulin binding domain of streptococcal protein G. *Science* 253:657–661.
- Rodriguez Camargo DC, Link NM, Dames SA (2012) The FKBP-rapamycin binding domain of human TOR undergoes strong conformational changes in the presence of membrane-mimetics with and without the regulator phosphatidic acid. *Biochemistry* 51:4909–4921.
- Warschawski DE, Arnold AA, Beaugrand M, Gravel A, Chartrand E, Marcotte I (2011) Choosing membrane mimetics for NMR structural studies of transmembrane proteins. *Biochim Biophys Acta* 1808:1957–1974.
- Tieleman DP, van der Spoel D, Berendsen HJC (2000) Molecular dynamics simulations of dodecylphosphocholine micelles at three different aggregate sizes: micellar structure and chain relaxation. *J Phys Chem B* 104:6380–6388.
- Sanders CR, Sonnichsen F (2006) Solution NMR of membrane proteins: practice and challenges. *Magn Reson Chem* 44:S24–S40.
- Stafford RE, Fanni T, Dennis EA (1989) Interfacial properties and critical micelle concentration of lysophospholipids. *Biochemistry* 28:5113–5120.
- Hauser H (2000) Short-chain phospholipids as detergents. *Biochim Biophys Acta* 1508:164–181.
- Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 6:277–293.
- Johnson BA (2004) Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Methods Mol Biol* 278:313–352.