

STRUCTURE NOTE

X-ray Structure of Human Gankyrin, the Product of a Gene Linked to Hepatocellular Carcinoma

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Introduction. Gankyrin, a newly described oncoprotein, is identical to the p28 subunit of the 26S proteasome. The protein is derived from an oncogene linked to hepatocellular carcinoma (HCC) and thus represents a potential target for drug therapy against liver cancer. As the name indicates, gankyrin contains an ankyrin repeat stack (6 repeats) with a 38-amino-acid *N*-terminal domain [Fig. 1(a)], and the first letter “g” stands for “gann,” which means cancer in Japanese. Similar to other ankyrin-repeat proteins, gankyrin mediates protein-protein interactions with diverse regulatory proteins, including the retinoblastoma tumor suppressor (Rb), the cyclin-dependent protein kinase 4 (CDK4), melanoma antigen A4 (MAGE-A4), and the S6 ATPase of the 26S proteasome.^{1–3}

Rb, the first described human tumor suppressor,⁴ plays an important role in regulating the cell cycle. The inactivation of Rb is thought to be involved in the majority of human malignancies.⁵ X-ray structures of complexes of the pocket domain of Rb with a peptide derived from the papillomavirus E7 protein⁶ and with a fragment of SV40 large T antigen,⁷ which contain the LXCXE Rb-binding motif have been determined. Interestingly, the sequence of gankyrin also contains a potentially Rb-binding LXCXE motif.⁸ Recently, gankyrin was shown to be involved in the destabilization of Rb,¹ and its involvement in the Rb pathway was demonstrated using both *in vivo* and *in vitro* approaches.⁹ The effect of gankyrin on Rb phosphorylation is exerted through CDK4, which was reported to form a gankyrin-CDK4-cyclin D2 ternary complex similar to the previously established p16^{INKA4}-CDK4-cyclin D2 complex. Therefore, gankyrin appears to compete with the tumor suppressor p16^{INKA4} for CDK4 binding. However, the binding of gankyrin to CDK4 does not affect CDK4 activity, indicating that the binding modes of p16^{INKA4} and gankyrin to CDK4 might be different. Furthermore, gankyrin uses different surfaces for Rb and CDK4 binding, i.e., the first three or four ankyrin repeats are involved in CDK4 binding, whereas the fifth repeat contains the LXCXE motif implicated Rb binding. MAGE-A4 is a mem-

ber of the MAGE (melanoma antigen) family, a large group of proteins that contain a well-conserved ~200-amino acid region known as the MAGE-homology domain.^{10,11} A recent report demonstrated that MAGE-A4 binds to gankyrin and suppresses its oncogenic activity.³ Finally, gankyrin specifically binds to the C-terminus of the proteasomal S6 ATPase of the 26S proteasome.¹² GST pull-down analyses using full-length gankyrin and various gankyrin deletion mutants demonstrated that a full-length, presumably correctly folded, gankyrin is essential for interaction with the S6 ATPase.²

In addition to biochemical studies of gankyrin and its molecular interactions, the crystallization of human gankyrin and its yeast homolog have been recently reported.^{13,14} Here, we report the X-ray structure of human gankyrin to 2.8 Å resolution, which was determined within the Protein Structure Factory¹⁵ structural genomics program.

Materials and methods. A cDNA fragment corresponding to the open reading frame of gankyrin (GenBank AAH11960) was amplified by PCR from a cDNA clone of the German Resource Center (RZPD, <http://www.rzpd.de>), under the identifier MPMGp800G03570, using primers Pr1388 and pQE276, of sequences 5'-GAGGATC-CGAGGGGTGTGTGTCTAACCT-3' and 5'-GGCAAC-CGAGCGTTCTGAAC-3', respectively. The PCR product was cloned into the vector pQTEV (GenBank accession no.

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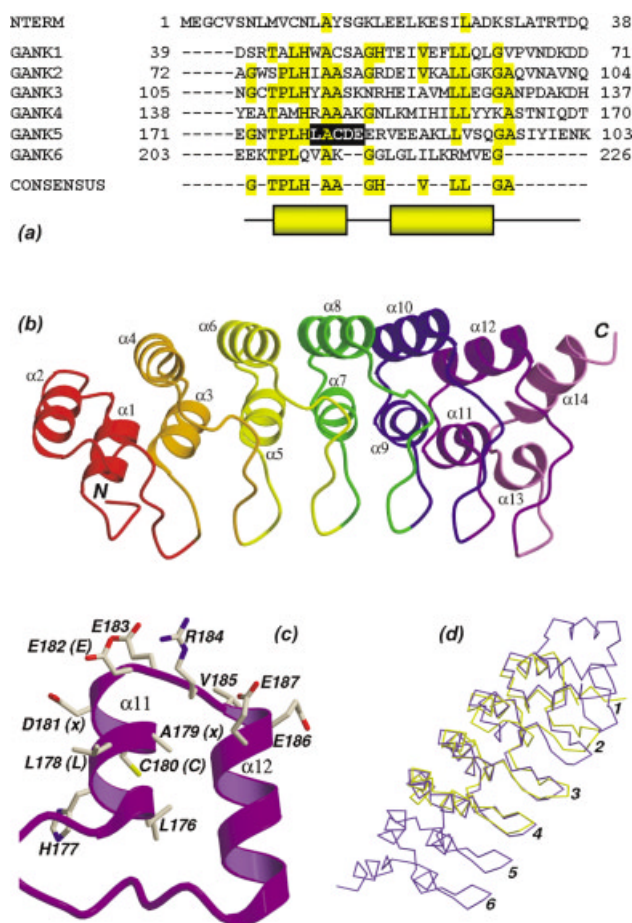


Fig. 1. (a) Sequence of human gankyrin. The six ankyrin repeats are aligned with the ankyrin consensus sequence.³⁰ In the fifth ankyrin repeat, the potentially Rb-interacting LXCXE sequence motif is highlighted. Below the sequence, the position of the α -helices are indicated. (b) Ribbon diagram of human gankyrin. The N-terminal domain is colored red, and the six ankyrin repeats are colored individually. (c) Structure of the fifth ankyrin repeat. Side chains of the Rb-binding motif are shown explicitly. The residues corresponding to the Rb-binding motif (LXCXE) are indicated within the brackets. (d) Superposition of the structures of gankyrin and p16^{INK4A} (bound to CDK6, PDB entry 1B17). The ankyrin repeats are numbered.

AY243506) using restriction enzymes *Bam*HI and *Not*I. The resulting plasmid was introduced into *Escherichia coli* SCS1 cells carrying the Rosetta plasmid (Novagen). The resulting clone used for protein expression has the PSF clone ID 108465 and is available from the RZPD under the ID PSFEp250A062.

Clone 108465 was grown to an OD₆₀₀ of 1.5 in 4 L of SB medium [12 g/L Bacto-tryptone, 24 g/L yeast extract, 0.4% (v/v) glycerol, 17 mM KH₂O₄, 72 mM K₂HPO₄], supplemented with 20 μ g/mL thiamine, 100 μ g/mL ampicillin, and 34 μ g/mL chloramphenicol in shaker flasks. Protein expression was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 4 h at 37°C. All further steps were performed between 4 and 8°C. Cells were pelleted by centrifugation and resuspended in a threefold volume of 20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 10 mM imidazole, 5 mM 2-mercaptoethanol, 1 mM PMSF, a protease inhibitor

cocktail tablet (EDTA-free, Roche) and 500 units Benzamide (Merck, Darmstadt). Cells were lysed by sonification, followed by centrifugation (23,000 G, 45 min) and filtration through a 0.22- μ m syringe filter. The filtrate was applied to a 1.6 mL Ni POROS20 column (Applied Biosystems) in four batches. After washing with 30 column volumes of 20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 10 mM imidazole, the protein was eluted using 250 mM imidazole. The eluted protein was supplemented with 2 mM dithiothreitol and 1 mM EDTA. The His-tag was removed with 1.2 μ g TEV protease overnight. After fourfold dilution with 20 mM Tris-HCl, pH 7.4, the protein was bound to a 1.6 mL HQ-POROS20 column (Applied Biosystems) and eluted with a NaCl gradient. The protein was further purified on a Superose 12 16/50 column (Amersham Biosciences), equilibrated in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, followed by concentration with Biomax concentrators (Millipore, 10-kDa cutoff) to a final concentration of 18.3 mg/ml in 600 μ L. The purified and concentrated protein was checked for monodispersity by dynamic light scattering (Laser Scatter201, RiNA GmbH, Germany).

Crystals of gankyrin were obtained by the sitting-drop method using a 96-well Greiner plate at 20°C from drops containing 400 nL of protein (18.3 mg/mL) plus 400 nL of reservoir solution (3.5 M Na formate, pH 7.0) equilibrated against 75 μ L of reservoir solution. All pipetting steps were done using semiautomated dispensing systems.¹⁹ Single crystals suitable for diffraction experiments, belonging to space group P4₁2₁2 with unit cell dimensions of $a = 116.4$ Å and $c = 74.4$ Å and one molecule in the asymmetric unit (76% solvent content), grew within 3 days. Native diffraction data were collected to 2.8 Å resolution from a single crystal on a MAR345 imaging plate detector at the BESSY (Berlin) beamline PSF-ID14.2. Before data collection, the crystals were cryoprotected using mother liquor containing 10% glycerol and flash-cooled directly in the liquid nitrogen stream. The data were processed using the program DENZO and scaled with SCALEPACK.²⁰ The data collection statistics are shown in Table I.

The crystal structure was determined by molecular replacement using the program MOLREP.²¹ The search model was prepared using SWISS-MODEL, an automated protein modelling server²² using the “first approach mode” (only amino acid sequence is required to build a model). The search model of gankyrin was based upon the template structures of Protein Data Bank entries 1N1I, 1N0R, 1MJ0, and 1N0Q1, which were selected automatically by the server. The best solution obtained from MOLREP yielded a correlation coefficient of 0.474 (0.340 for the second best) for one molecule in the asymmetric unit. A few cycles of refinement with the program REFMAC²³ resulted in free R falling to 0.458 from 0.571, and the figure of merit increasing to 0.548 from 0.295, which provided a strong indication that the solution was correct. The phases were subjected to prime-and-switch phasing using RESOLVE²⁴ to remove model bias. Further phase improvement was achieved using the free-atom refinement method in ARP/wARP.²⁵ TLS parameters were determined and TLS restrained refinement²⁶ was per-

TABLE I. Data Collection and Refinement Statistics

| Data collection | |
|--|--------------|
| Data set | Native |
| Wavelength (Å) | 1.0000 |
| Resolution (Å) | 2.8 |
| Total observations | 169,487 |
| Unique observations | 12,978 |
| Completeness (%) ^a | 99.8 (99.3) |
| R_{sym}^a | 0.072 (0.62) |
| Average $I/\sigma(I)^a$ | 35.4 (4.0) |
| Refinement | |
| Resolution range (Å) | 20–2.8 |
| No. of reflections | 12,144 |
| No. reflections in R_{free} set | 632 |
| R factor ^b | 0.187 |
| Free R factor ^c | 0.233 |
| r.m.s.d. bond lengths (Å) | 0.011 |
| r.m.s.d. bond angles (°) | 1.281 |
| No. of nonhydrogen atoms | 1680 |
| Average B factor (Å ²) | 28.9 |

^aStatistics for the highest resolution bin (2.9–2.8 Å) are given in parentheses.

^b $R = \sum_h ||F_o(h) - k|F_c(h)|| / \sum_h |F_o(h)|$.

^cFree R factor was calculated using a 5% randomly selected subset of the total number of reflections.

formed. The final refinement statistics are shown in Table I for a model consisting of 223 residues and 53 water molecules. The three N-terminal residues cannot be seen in the electron density. The atomic coordinates and structure factors are available from the Protein Data Bank under accession code 1QYM. The figures were prepared using Molscript,²⁷ Bobscript,²⁸ and Raster3D.²⁹

Results. The structure of gankyrin consists of five complete ankyrin repeats, one incomplete ankyrin repeat at the C-terminus and the N-terminal domain [Fig. 1(b)]. Each ankyrin repeat forms an L-shaped structural unit consisting of a β -turn, followed by two antiparallel α -helices and a long loop leading to the turn of the next repeat. The last ankyrin repeat lacks the long loop. Pairs of α -helices from adjacent ankyrin repeats pack in parallel forming four-helix bundles with a left-handed twist. Each repeat is slightly twisted counterclockwise and forms spiral-like steps. The overall fold of the N-terminal domain resembles an ankyrin-repeat structure even though the level of sequence identity with the consensus sequence is much lower [see Fig. 1(a)]. In the crystal, the N-terminal surfaces of neighboring molecules interact with each other and form a spiral stack upwards and complete a bow or core-like structure with a large solvent accessible surface (not shown).

The fifth ankyrin repeat (GANK5) of gankyrin contains the retinoblastoma-binding motif, LXCXE, which is important for inactivation of Rb. The structure of the GANK5 and the Rb-binding motif together with neighboring residues are shown in Fig. 1(c). Interestingly, the Rb-binding motif ¹⁷⁸LACDE¹⁸² of gankyrin is located within an α -helix (α 11). In contrast, in the X-ray structures of complexes of the pocket domain of Rb with a peptide derived from the papilloma virus E7 protein⁶ and with a fragment of SV40 large T antigen,⁷ which contain the LXCXE Rb-binding motif, it adopts a β -sheet conformation. The side chains of

L178, D181, and E182 and the backbone oxygen atom of C180 are solvent-exposed, whereas the side chains of A179 and C180 are buried within the molecule [see Fig. 1(c)]. Therefore, L178, D181, and E182 might play important roles in Rb binding, which is consistent with previous mutagenesis studies. Gankyrin interaction with Rb is absent in an E182A point mutant, and only a weak interaction remains with the gankyrin L178A point mutant.¹ Peptides with substitution of one of the three conserved residues of the LXCXE motif (L178A, C180A, and E182A) lost their ability to disrupt the binding of gankyrin to Rb.⁹

Previously, truncation mutagenesis studies on gankyrin suggested that the first three or four ankyrin repeats are involved in CDK4 binding. Moreover, gankyrin competes with p16^{INK4A} for CDK4 binding.⁹ The NMR structure of p16^{INK4A} has been reported,¹⁶ but the mechanism of CDK4 inhibition by this molecule has remained elusive because of the difficulty in obtaining structural information about CDK4. However, the possible nature of interactions between p16^{INK4A} and CDK4 were analyzed in detail through a large number of mutants,¹⁶ and it was concluded that residues H66, D84, R124, E26, and D82 are important for binding to CDK4. The X-ray structure of the CDK6-p16^{INK4A} binary complex^{17,18} shows a continuous binding interface which buries a large surface area. The second and third ankyrin repeats of p16^{INK4A} contribute most of the interactions, whereas its first and fourth repeats engage in fewer interactions. A least-squares superposition of the C α positions of corresponding ankyrin repeats from gankyrin and p16^{INK4A} [Fig. 1(d)] yields an rms displacement of 1.03 Å. However, major structural differences are obvious, mainly at the second ankyrin repeat of gankyrin. Gankyrin and p16^{INK4A} exhibit very low sequence similarity, and the CDK4-binding residues of p16^{INK4A} are not conserved in the gankyrin sequence, suggesting that the mode of binding to CDK4 and the biological activity may be quite different between the two proteins.

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NOTE ADDED IN PRESS

Since submission of this article, a structure of gankyrin in a different crystal form³¹ and the crystal structure of Nas6p, the yeast homolog of gankyrin³² have been published.

REFERENCES

- Higashitsuji H, Itoh K, Nagao T, Dawson S, Nonoguchi K, Kido T, Mayer RJ, Arai S, Fujita J. Reduced stability of retinoblastoma protein by gankyrin, an oncogenic ankyrin-repeat protein overexpressed in hepatomas. *Nat Med* 2000;6:96–99.
- Dawson S, Apcher S, Mee M, Higashitsuji H, Baker R, Uhle S, Dubiel W, Fujita J, Mayer RJ. Gankyrin is an ankyrin-repeat oncoprotein that interacts with CDK4 kinase and the S6 ATPase of the 26 S proteasome. *J Biol Chem* 2002;277:10893–10902.
- Nagao T, Higashitsuji H, Nonoguchi K, Sakurai T, Dawson S, Mayer RJ, Itoh K, Fujita J. MAGE-A4 interacts with the liver oncoprotein gankyrin and suppresses its tumorigenic activity. *J Biol Chem* 278;2003:10668–10674.

4. Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, Dryja TP. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 1986;323:643–646.
5. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell* 1995;81:323–330.
6. Lee JO, Russo AA, Pavletich NP. Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. *Nature* 1998;391:859–865.
7. Kim HY, Ahn BY, Cho Y. Structural basis for the inactivation of retinoblastoma tumor suppressor by SV40 large T antigen. *EMBO J* 2001;20:295–304.
8. Nevins JR, Leone G, DeGregori J, Jakoi L. Role of the Rb/E2F pathway in cell growth control. *J Cell Physiol* 1997;173:233–236.
9. Li J, Tsai MD. Novel insights into the INK4-CDK4/6-Rb pathway: counter action of gankyrin against INK4 proteins regulates the CDK4-mediated phosphorylation of Rb. *Biochemistry* 2002;41:3977–3983.
10. Barker PA, Salehi A. The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease. *J Neurosci Res* 2002;67:705–712.
11. Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T, Lucas S. An overview of the MAGE gene family with the identification of all human members of the family. *Cancer Res* 2001;61:5544–5551.
12. Dawson S, Hastings R, Takayanagi K, Reynolds S, Low P, Billett M, Mayer RJ. The 26S-proteasome: Regulation and substrate recognition. *Mol Biol Rep* 24;1997:39–44.
13. Krzywda S, Brzozowski AM, Al-Safty R, Welchman R, Mee M, Dawson S, Fujita J, Higashitsuji H, Mayer RJ, Wilkinson AJ. Crystallization of gankyrin, an oncoprotein that interacts with CDK4 and the S6b (rpt3) ATPase of the 19S regulator of the 26S proteasome. *Acta Crystallogr D* 2003;59:1294–1295.
14. Adachi N, Padmanabhan B, Kataoka K, Kijima K, Yamaki M, Horikoshi M. Purification, crystallization and preliminary X-ray diffraction analysis of yeast regulatory particle non-ATPase subunit 6 (Nas6p). *Acta Crystallogr D* 2002;58:859–860.
15. Heinemann U, Büsow K, Mueller U, Umbach P. Facilities and methods for the high-throughput crystal structural analysis of human proteins. *Acc Chem Res* 2003;36:157–163.
16. Byeon IJ, Li J, Ericson K, Selby TL, Tevelev A, Kim HJ, O'Maille P, Tsai MD. Tumor suppressor p16^{INK4A}: Determination of solution structure and analyses of its interaction with cyclin-dependent kinase 4. *Mol Cell* 1998;1:421–431.
17. Russo AA, Tong L, Lee JO, Jeffrey PD, Pavletich NP. Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumour suppressor p16^{INK4a}. *Nature* 1998;395:237–243.
18. Jeffrey PD, Tong L, Pavletich NP. Structural basis of inhibition of CDK-cyclin complexes by INK4 inhibitors. *Genes Dev* 2000;14:3115–3125.
19. Mueller U, Nyarsik L, Horn M, Rauth H, Przewieslik T, Saenger W, Lehrach H, Eickhoff H. Development of a technology for automation and miniaturisation of protein crystallisation. *J Biotechnol* 2001;85:7–14.
20. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 1997;276:307–326.
21. Vagin A, Teplyakov A. An approach to multi-copy search in molecular replacement. *Acta Crystallogr D* 2000;56:1622–1624.
22. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res* 2003;31:3381–3385.
23. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D* 1997;53:240–255.
24. Terwilliger TC. Map-likelihood phasing. *Acta Crystallogr D* 2001;57:1763–1775.
25. Perrakis A, Harkiolaki M, Wilson KS, Lamzin VS. ARP/wARP and molecular replacement. *Acta Crystallogr D* 2001;57:1445–1450.
26. Winn MD, Isupov MN, Murshudov GN. Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr D* 2001;57:122–133.
27. Kraulis P. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr* 1991;24:946–950.
28. Esnouf RM. Further additions to MolScript version 1.4, including reading and contouring of electron-density maps. *Acta Crystallogr D* 1999;55:938–940.
29. Merritt EA, Murphy MEP. Raster3d version 2.0—a program for photorealistic molecular graphics. *Acta Crystallogr D* 1994;50:869–873.
30. Michaely P, Tomchick DR, Machius M, Anderson RGW. Crystal structure of a 12 ANK repeat stack from human ankyrinR. *EMBO J* 2002;21:6387–6396.
31. Krzywda S, Brzozowski AM, Higashitsuji H, Fujita J, Welchman R, Dawson S, Mayer RJ, Wilkinson AJ. The crystal structure of gankyrin, an oncoprotein found in complexes with cyclin-dependent kinase 4, a 19 S proteasomal regulator, and the tumor suppressors Rb and p53. *J Biol Chem* 2004;279:1541–1545.
32. Padmanabhan B, Adachi N, Kataoka K, Horikoshi M. Crystal structure of the homolog of the oncoprotein gankyrin, an interactor of Rb and CDK4/6. *J Biol Chem* 2004;279:1546–1552.