Crystal Structure of the Homolog of the Oncoprotein Gankyrin, an Interactor of Rb and CDK4/6*

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The oncoprotein gankyrin plays a central role in tumorigenesis and cell proliferation. Gankyrin interacts with the retinoblastoma tumor suppressor (Rb) and cyclin-dependent kinase 4/6 (CDK4/6), increases phosphorylation at specific residues of Rb by CDK4/6 in vivo, and promotes tumorigenesis. The phosphorylation of Rb by CDK4/6 leads to the deregulation of the cell cycle during G1/S transition. Although how phosphorylation occurs on Rb has been studied extensively, the mechanism of site-specific phosphorylation of Rb remains unclear due to a lack of information on the structural arrangement of Rb and CDK4/6. Here, we have determined and refined to 2.3-A resolution the crystal structure of a gankyrin homolog, the non-ATPase subunit 6 (Nas6p) of the proteasome from yeast. The crystal structure reveals that Nas6p contains seven ankyrin repeats. The number of the repeats is different from that predicted from the primary structure. Nas6p also possesses an unusual curved structure with two acidic regions at the N- and C-terminal regions separated by one basic region, suggesting that it has at least two functional surfaces. The tertiary structure of Nas6p, together with the previous biochemical studies, indicates that the CDK4/6 and Rb binding surfaces of gankyrin are located at the N- and C-terminal regions, respectively, and face the same side of gankyrin. These observations suggest that gankyrin brings Rb and CDK4/6 together through gankyrin-Rb and gankyrin-CDK4/6 interactions and determines the relative positioning of the substrate (Rb) and the enzyme (CDK4/6). Our findings provide mechanistic insight into site-specific phosphorylation of Rb caused by CDK4/6.

In this regulatory pathway one of the key factors is the retinoblastoma tumor-suppressor protein (Rb)1 (1). Rb binds to the transcription factor E2F, which is required for the expression of genes involved in the G1/S transition and inhibits its transactivation function. The hyperphosphorylation of Rb by cyclin-dependent kinases 4 and 6 (CDK4/6) leads to the release of E2F from the Rb:CDK4/6 complex and degradation of Rb by the proteasome via a ubiquitin-dependent pathway. The release of E2F triggers activation of a number of genes required for G1/S transition and tumorigenesis. Therefore, to better understand the regulatory mechanism of the cell cycle and tumorigenesis, regulation of site-specific phosphorylation of Rb by CDK4/6 must be clarified.

Rb phosphorylation by CDK4/6 is regulated mainly by proteins containing ankyrin repeats, gankyrin (gann ankyrin: gann means cancer in Japanese) for positive regulation and INK4 (CDK4/6 inhibitor) for negative regulation (2, 3) in addition to cyclin for activating CDK4/6 (4). Cyclin interacts on one side of the CDK catalytic cleft, inducing large conformational changes in its active center and activating the kinase by broadening the entrance of the catalytic cleft (5). Rb contains at least 16 consensus sequences for CDK phosphorylation, and the significance of these residues has been demonstrated (6). However, the mechanism of Rb phosphorylation at specific residues by CDK4/6 remains unclear. Recent studies reveal that gankyrin also interacts with CDK4/6 and enhances CDK4/6 phosphorylation activity toward Rb by competing with INK4 (3). In sharp contrast to cyclin, gankyrin interacts with Rb in addition to CDK4/6 (3) and increases Rb phosphorylation at specific residues in vivo (2). These studies indicate that gankyrin has different or additional roles from cyclin for activating CDK and determining site specificity for Rb.

Although the mechanism of Rb phosphorylation at specific residues by CDK4/6 in this pathway is still unclear, the tertiary structure of gankyrin will provide insight into the mechanism of site-specific phosphorylation of Rb for the following reasons. The interaction of gankyrin with the substrate (Rb) and enzyme (CDK4/6) is simultaneously involved in the determination of the site-specific phosphorylation of Rb (2, 3) and likely adjusts the relative position of those molecules to facilitate Rb phosphorylation. Additionally, the mechanism of negative regulation has been clarified by analysis of the tertiary structure of the CDK4/6-INK4 complex (7, 8). INK4 binding to CDK4/6 is well known to induce conformational changes in the ATP-binding site of CDK4/6, to interfere with ATP binding, and to the ATP binding site of CDK4/6.
inactivate CDK4/6 phosphorylation activity (7, 8). Therefore, the tertiary structure of gankyrin provides a mechanism for site-specific phosphorylation of Rb by CDK4/6.

Here, we present the tertiary structure of the non-ATPase subunit 6 (Nas6p) and gankyrin homolog refined to a resolution of 2.3 Å. Our results reveal that Nas6p has a concave structure

| Table I
| Summary of data collection and refinement statistics |

| Source | PF BL18B | PF BL6A | PF BL18B |
|—— | ———— | ———— | ———— |
| Heavy atom concentration | 1 mM | 1 mM | 1 mM |
| Soaking time | 30 h | 6 h | 2.5 |
| Resolution (Å) | 2.3 | 2.8 | 2.5 |
| Unique reflections | 11,582 | 6,576 | 8,563 |
| Completeness (%) | 99.0 | 99.9 | 95.9 |
| Rmerge (%) | 8.5 | 10.0 | 6.5 |
| Rsites (%) | 13.3 | 12.4 | |
| Rfree (%) | 0.66 | 0.77 | |

Refinement statistics

| Resolution (Å) | 30.0–2.3 |
| Bond length (Å) | 0.006 |
| Bond angle (Å) | 1.30 |

* Rmerge = Σ |Fobs| - |Fcalc| / Σ |Fobs| summed over all observations and reflections.

* Rfree was calculated with 5% of the data omitted from refinement.

** FIG. 1: Sequence alignment of Nas6p and gankyrin. ** Accession numbers of the amino acid sequences are P50086 (Nas6p) and O75832 (gankyrin). The α helices and β strands are indicated as helices and arrows, respectively, and ANK1–7 repeats are indicated by blue bars. Residues 1–27 and 200–228 in Nas6p are newly identified in this study as ANK1 and -7, respectively. Residues 1–138 in gankyrin (cyan box) are required for interaction with CDK4/6. Residues 178–182 in gankyrin (LXCE motif (green bar)) are required for interaction with Rb (2, 3). The figure was generated by Espript (22). Identical or similar residues are defined as G; A, V, I, L; M; F, Y, W; P; C; S, T; N, Q; D, E; and H, K, R.
with seven ankyrin repeats. The CDK4/6 and Rb binding surfaces of Nas6p are located at N- and C-terminal regions, respectively, and are facing on the same side, suggesting that gankyrin promotes the correct placement of Rb and CDK4/6. These structural results provide a gankyrin-mediated mechanism of site-specific phosphorylation of Rb by CDK4/6.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Crystallization—Yeast Nas6p was expressed in Escherichia coli and purified and crystallized as described elsewhere (9).

Structure Determination—All diffraction data were collected at the beamlines BL6A and BL18B of the Photon Factory, Tsukuba, Japan. The structure was determined by the MIR method at 3.0-Å resolution and refined to 2.3 Å with data from a native crystal (Table I). The native and derivative data were indexed and processed with DPS (10). Two platinum sites and one gold site were found and refined using SOLVE (11). The experimental map at 3.0 Å produced by SOLVE was improved by solvent flattening and extended to 2.7 Å with SOLomon incorporated in CCP4 (12). After solvent flattening and phase extension, the map was good enough to trace the secondary structure elements using O (13). The map was further improved by combining the experimental phases with the calculated phases obtained from initial fitting. After the first refinement round, the R-factor was 27.4%, and Rfree was 32.3%.

The model was refined with CNS (14) using a maximum likelihood target that included amplitude and phase probability distributions. Phase-combined, σA-weighted electron density maps were used throughout to guide further model building and to determine the location of water molecules. The current refined model at 2.3-Å resolution

Fig. 2. Tertiary structure of Nas6p. A, stereo view of a section of the final σA-weighted 2F- - Fc map at 2.3-Å resolution, contoured at 1.0 σ. B, ribbon representation of the tertiary structure of Nas6p. Each ankyrin repeat is indicated by ANK1–7. The figures were generated by Bobscript (23) and Molscript (24). C, topological diagram of the secondary structure elements of the Nas6p protein. ANK1–7 indicates the seven ankyrin repeats. α helices and β strands are indicated by circles and arrows, respectively. Residue numbers are indicated at the start and end of each secondary structural element. D, structure-based sequence alignment of the seven ankyrin repeats of Nas6p. Positions of α helices and β strands are indicated above the sequences. Red letters indicate the conserved PLH motif except for AED in ANK7. Green letters indicate other identical or similar residues between each ankyrin repeat.

Fig. 3. Curved structure of ankyrin repeats in Nas6p. A, superposition of ANK1–2 domains of Nas6p (red) and the ANK1–2 of Bcl-3 (cyan). Equivalent Ca atoms of helical segments were used for least squares fit calculation. Note that Nas6p structure is more concave compared with that of Bcl-3 (PDB code: 1K1A). B and C, representative secondary structural elements forming more concave structures. Residues involved in the inter-ankyrin repeat interactions between ANK1 and -2 (B) and between ANK3 and -4 (C) are shown. α2 helices are the same secondary structural elements as shown in Fig. 2, C and D.
RESULTS AND DISCUSSION

Overall Structure of Gankyrin Homolog Nas6p—Gankyrin was isolated as an oncoprotein in hepatocellular carcinoma (2).

Gankyrin was also isolated as a p28 subunit of the human 19S proteasome (16). Nas6p, which is similar to gankyrin, is also a subunit of the yeast 19S proteasome (16) and shares high sequence similarity with gankyrin. Sequence comparison studies showed that gankyrin possesses 35% identity and 52% similarity with Nas6p (Fig. 1). These structural and functional observations have indicated that Nas6p is a yeast homolog of gankyrin.

The crystal structure of yeast Nas6p composed of 228 residues was solved by the MIR method at 2.7-Å resolution and was refined to 2.3-Å resolution (Fig. 2A). The tertiary structure of Nas6p is an elongated structure with overall dimensions 74 Å × 36 Å × 33 Å (Fig. 2B). Previous primary structure analysis suggested that Nas6p and gankyrin have five or six ankyrin repeats with flexible N- and C-terminal segments (2, 16). However, the predicted number of ankyrin repeats of Nas6p is different from its crystal structure, which consists of seven ankyrin repeats (ANK1 to ANK7 in Fig. 2C).

Each ankyrin repeat is formed by ~30 amino acid residues with a β-hairpin and helix-loop-helix motif (the only exception is ANK1, which lacks the β strand β1) (17). Many conserved residues have been found between the ankyrin repeats (Fig. 2D). Hydrophobic interactions caused by conserved residues Leu-6, Ala-9, and Leu-21 occur within and between the repeats. A motif, PLH is conserved among the ankyrin repeats and is positioned at the start of the α1 helix. However, in ANK7, this PLH motif is absent; also, the helix α1 corresponding to this region is shorter compared with the equivalent helix in other ankyrin repeats. ANK2–6 possess hydrophobic faces on either side formed by preceding and successive repeats, whereas ANK1 and -7 are highly exposed to the solvent and contain a high proportion of charged residues.

An Unusual Curved Structure of Ankyrin Repeats in Nas6p—To obtain further insight into the tertiary structure of Nas6p, we compared Nas6p to another ankyrin repeat protein. Among the tertiary structures of known ankyrin repeat proteins, the oncoprotein Bcl-3 also contains seven ankyrin repeats (18). Bcl-3 is a member of the 1B family of proteins that can enhance NFκB-dependent transcription. The primary structure of Bcl-3 has 29% identity and 43% similarity with that of Nas6p and 29% identity and 55% similarity with that of gankyrin. Although both proteins, Nas6p and Bcl-3, have the same number of ankyrin repeats, the overall tertiary structures of these proteins are quite different (Fig. 3A). Superposition of the Nas6p (red in Fig. 3A) and Bcl-3 (cyan in Fig. 3A) structures reveals that Nas6p has a more concave structure than does Bcl-3.

The seven ankyrin repeats in Nas6p possess uniform backbone conformations, as expected from the relatively high degree of sequence similarity between the motifs (Fig. 2D) except for ANK7. ANK1 lacks the β strand β1 and possesses a shorter linker region that connects ANK2 compared with other repeats (ANK2–7). In ANK2, -3, and -5, the linker chain is relatively longer compared with that found in ANK1, -4, and -6. This insertion of a longer chain causes more bending, and thus, the overall shape of Nas6p resembles a concave structure (called “banana-shaped” structure).

The helix α2 in ANK1 is not parallel to its equivalent helix in ANK2 (Fig. 3B). Region 27–30 forms a sharp turn toward the α2 region of ankyrin repeats, and thus, the side chains of Leu-29 and Leu-30 undergo hydrophobic interactions with residues in the linker region of ANK2. In addition, more bulky side chain residues (Phe, Lys, Gln, Glu, and His) are found in helix α2 of these two repeats (ANK1 and -2). These arrangements might be the reason for the wider separation of the C-terminal ends of these two helices.

Similarly, helix α2 in ANK3 is also not parallel to its equivalent segment in ANK4 (Fig. 3C). A wide separation between these two segments at the C-terminal end is probably due to Arg-94 and Pro-95. The side chain of Arg-94 has strong electrostatic interactions with the backbone carbonyl oxygen atoms of Met-59, Glu-60, and Val-62, which are positioned in the linker region of ANK2. These electrostatic interactions favor inclination against α2 of ANK4 by pulling the C-terminal end of helix α2 of ANK3 segment toward ANK2. In addition to these electrostatic interactions, the presence of bulky side chains in these helices also favors this type of arrangement; otherwise severe clashes would occur if these two segments are in parallel.

Also, the axes of equivalent helices α2 in ANK5 and -6 are slightly widened at C-terminal ends from each other, probably due to the presence of a bulky side chain Tyr-197 in ANK6. The equivalent residue in other repeats possesses a small hydrophobic residue, except in ANK2 and -3 (Lys-58 and Arg-94), where they contribute to similar structural arrangements in these regions. Another interesting feature is that the linker region between ANK2 and -3 is longer, whereas that between ANK4 and -5 is shorter than those found in other repeats. Thus, the above structural arrangements are key factors that contribute to the overall tertiary structure of Nas6p, which looks more bent and in a concave-like structure.
**FIG. 5.** Prediction of gankyrin interaction with Rb and CDK4/6 based on the tertiary structure of Nas6p and biochemical and mutational studies. A. sequence alignment of the first four ankyrin repeats of INK4d, Nas6p, and gankyrin. Amino acids involved in hydrogen bond and nonpolar interactions between INK4d and CDK4/6 are shown in h (red box) and n (cyan box), respectively (7, 8). B. Co atom superposition of ANK1–4 of Nas6p and ANK1–4 of INK4 in INK4/CDK4/6 complex (PDB code: 1BI8). Red, green, and yellow indicate Nas6p, INK4 and CDK4/6, respectively. C, comparison of the electrostatic surface charge of Nas6p with INK4. Negative, neutral, and positive charges are shown in red, white, and blue, respectively. The orientation of this figure is a 90° rotation about the vertical axis to B. Interacting surfaces between INK4 and CDK4/6
**Features of the Molecular Surface of Nas6p—Electrostatic surface potential analysis of Nas6p showed that the regions of ANK1–4 and ANK6–7 have negatively charged surfaces (Fig. 4A). In addition, the central region contains mainly basic residues, like a stepping-stone (Nas6p: Lys-117, Lys-118, Arg-134, Lys-136, Lys-138, Arg-146, Lys-154, Lys-164, and Lys-172). These residues seem to be a boundary that divides the function of the N- and C-terminal regions.**

**Comparison of Nas6p and INK4 Tertiary Structures—**The tertiary structure of Nas6p reveals a long and concave structure with two acidic and one basic region. Because gankyrin is a human homolog of Nas6p, gankyrin might also possess similar structural features. Intriguingly, the tertiary structure of gankyrin is similar to that of Nas6p. In addition, the amino acid distributions on the molecular surface, including the N-terminal, boundary, and C-terminal regions, are also conserved through evolution between Nas6p and gankyrin (Figs. 1 and 4B). Therefore, we have attempted to predict the tertiary structure of the CDK4/6-gankyrin complex based on the tertiary structures of Nas6p and the CDK4/6-INK4 complex.

Recent biochemical studies show that the N-terminal region of gankyrin (corresponding to ANK1–4) is responsible for CDK4/6 binding (3). To understand the characterization of the gankyrin-CDK4/6 interaction, we have superimposed ANK1–4 of Nas6p and ANK1–4 of INK4 (Fig. 5A) (7, 8). The superposition reveals that the folds of ANK1–4 of both proteins are quite similar (Fig. 5B). Thus, gankyrin and INK4 would bind to CDK4/6 in a similar manner. The crystal structures of CDK4/6-INK4 complexes showed that ANK2 and -3 are mainly involved in intermolecular interactions. ANK2 and -3 in gankyrin, therefore, might be good candidates for interactions with CDK4/6.

Unique features of INK4 are, compared with other ankyrin repeats in this family of inhibitory factors, a shorter helix α1 of ANK2 and a long loop that connects helices α1 and α2. These regions of INK4 alter the tertiary structure of the ATP-binding site of CDK4/6, interfering with ATP binding and inhibiting the phosphorylation activity of CDK4/6 (7, 8). However, ANK2 of Nas6p does not have these features. This suggests that structural deviation of the ANK2 in gankyrin from INK4 might be important for the interaction between gankyrin and CDK4/6 without CDK inactivation.

**Comparison of Nas6p and INK4 Molecular Surface—**The surface charges of the interacting surface (ANK1–4) of both Nas6p and INK4 with CDK4/6 are quite different; Nas6p is acidic, and INK4 is hydrophobic (Fig. 5C, yellow circle). Gankyrin is also acidic, as the residues exposed on the surface as well as the distribution of charged amino acids in this region are well conserved with Nas6p (Fig. 1, cyan box, and Fig. 4B). Interestingly, the surface charge of the CDK4/6-interacting surface of INK4 is weakly basic, whereas the surface charge of the predicted CDK4/6-interacting surface of gankyrin is acidic. Thus, the interaction between gankyrin and CDK4/6 might be stronger than the interaction between INK4 and CDK4/6. These findings agree well with previous biochemical studies showing that gankyrin counteracts the inhibition of CDK4/6 by INK4 (3). Based on these observations, we propose a model that gankyrin and INK4d bind to the same surface of CDK4/6 through an almost similar binding fashion but compete with different surface charge. The elucidation of the structure of the CDK4/6-gankyrin complex and biochemical and mutational studies are necessary to further understand how gankyrin interacts with CDK4/6 by competing with INK4.

**Model for Interaction of Gankyrin with Rb and CDK4/6—**In the proposed structure of the gankyrin-CDK4/6 complex (Fig. 5B), the C-terminal region of gankyrin extends and bends toward the active center of CDK4/6. This free C-terminal region might interact with some target molecules and recruit them to CDK4/6 as substrates. The C-terminal region of gankyrin contains an LXCXE motif, which is responsible for the binding of Rb in vivo (2). Recent biochemical studies indicate that the LXCXE motif region of gankyrin is sufficient for Rb binding in vivo (3). It has also been shown that the binding of CDK4/6 and Rb with gankyrin is independent (3). Taken together, gankyrin is physically associated with CDK4/6 and Rb at its N- and C-terminal regions, respectively (Fig. 5D). The LXCXE peptide binding to Rb is an extended β-strand-like conformation (19), whereas the LXCXE motif in Nas6p and gankyrin is an α helical conformation. These results suggest three possibilities for gankyrin-Rb interaction through the LXCXE motif; they are (i) conformation of the LXCXE motif in a peptide and in a protein are different, (ii) the LXCXE motif of gankyrin is induced to fit to Rb and subsequently alters its structure in the β strand-like conformation, or (iii) gankyrin binds Rb through other surfaces.

The dual independent functional roles of gankyrin are most likely due to structural features. Therefore, our proposed complex structure model of CDK4/6 binding to ANK1–4 and of Rb binding to ANK6 and ANK7 of gankyrin would logically fit well with both biochemical and mutational studies in vitro (3), and previous studies reveal that overexpression of gankyrin induces Rb phosphorylation in vivo (2).

The region of Rb whose structure is solved contains at least two consensus sequences for CDK phosphorylation (19). In this proposed complex structure model the residue in Rb (Thr-373) that is phosphorylated depending on gankyrin overexpression (2) is close to the active center of CDK4/6 (Fig. 5D, orange circle). In contrast the residue in Rb (Ser-780) that is not phosphorylated by the dependence of gankyrin overexpression (2) is located in the opposite side of the active center of CDK4/6 (Fig. 5D, cyan circle). This model indicates that gankyrin bridges Cdk4/6 and Rb and makes the active center of Cdk4/6 and the phosphorylation site of Rb face each other.

Rb also interacts with E2F. Previous studies show that E2F and the LXCXE binding motifs are located in different regions of Rb (19–21) and that, using another surface for INK4 interaction, CDK4/6 might also interact with cyclin D (5). Taken together, these studies along with our proposed model suggest that gankyrin bridges Rb-E2F and CDK4/6-cyclin D complexes in a large regulatory complex (Fig. 5E). These issues will be clarified by the elucidation of the crystal structure of the CDK4/6-Rbgankyrin complex, the CDK4/6-cyclin D-Rb-E2F-gankyrin complex, and so on.

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