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SEQUENCING ANALYSIS OF MUTANT ALLELE cdc28-srm OF PROTEIN KINASE CDC28 AND MOLECULAR DYNAMICS STUDY OF GLYCINE-RICH LOOP IN WILD-TYPE AND MUTANT ALLELE G16S OF CDK2 AS MODEL

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Анализ нуклеотидной последовательности мутантного аллеля *cdc*28-*srm* протеинкиназы дрожжей CDC28 и молекулярно-динамическое исследование последствий замены аминокислоты G16S на модельной системе протеинкиназы человека CDK2

Центральная роль, которую циклин-зависимые киназы играют на протяжении всего цикла деления клетки, а также большая вероятность присутствия генетических изменений CDK или дерегуляции CDK ингибиторами в раковых клетках, делают протеинкиназу CDC28 дрожжей *Saccharomyces cerevisiae* чрезвычайно привлекательной моделью для изучения механизма регуляции CDK. Ранее было обнаружено, что определенные генные мутации, включающие *cdc28-srm*, влияют на прохождение клеточного цикла, поддержание различных генетических структур и повышают чувствительность клеток к ионизирующему излучению. Мутация *cdc28-srm* не является температурно-чувствительной и отличается от известных *cdc28-ts*, так как имеет фенотипические проявления при 30 °C. Анализ нуклеотидной последовательности *cdc28-srm* выявил единичное замещение аминокислоты G16S. Глицин20 является третьим глицином в консервативной последовательности GxGxxG в G-богатой петле, находящейся напротив активационной T-петли. Несмотря на важность и консервативность G-петли, ее роль остается до сих пор неясной. Кристаллическая структура человеческой CDK2 служит моделью каталитического остова других CDK, включая CDK28. Нами проанализированы молекулярно-динамические (МД) траектории комплекса CDK2/ATP наносекундной длительности. МД-исследования замещения CDK2-G16S (CDK28-G20S) показали конформационные отличия конечной структуры CDK2, проявляющейся в отдалении G-петли от ATP и при формировании иной конформационной структуры T-петли.

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Sequencing Analysis of Mutant Allele *cdc*28-*srm* of Protein Kinase CDC28 and Molecular Dynamics Study of Glycine-Rich Loop in Wild-Type and Mutant Allele G16S of CDK2 as Model

The central role that cyclin-dependent kinases play in the timing of cell division and the high incidence of genetic alteration of CDKs or deregulation of CDK inhibitors in a number of cancers make CDC28 of the yeast *Saccharomyces cerevisiae* very attractive model for studies of mechanisms of CDK regulation. Earlier it was found that certain gene mutations including *cdc28-srm* affect cell cycle progression, maintenance of different genetic structures and increase cell sensitivity to ionizing radiation. A *cdc28-srm* mutation is not temperature-sensitive mutation and differs from the known *cdc28-ts* mutations because it has the evident phenotypic manifestations at 30 °C. Sequencing analysis of *cdc28-srm* revealed a single nucleotide substitution G20S. This is a third glycine in a conserved sequence GxGxxG in the G-rich loop positioned opposite the activation T-loop. Despite its demonstrated importance, the role of the G-loop has remained unclear. The crystal structure of the human CDK2 has served as a model for the catalytic core of other CDKs, including CDC28. Nanoseconds long molecular dynamics (MD) trajectories of the CDK2/ATP complex were analyzed. The MD simulations of substitution CDK2-G16S (CDC28-G20S) show conformational changes of CDK2 structure resulting in the moving of the G-loop away from ATP and a new rearrangement of amino acids in the T-loop.

The investigation has been performed at the Veksler and Baldin Laboratory of High Energies, JINR.

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INTRODUCTION

The cyclin-dependent kinases (CDKs) are a subfamily of serine/threonine-specific protein kinases. The enzymes catalyze a transfer of the gamma-phosphate of adenosine triphosphate (ATP) to a protein substrate. CDKs are crucial regulators of the timing and coordination of eukaryotic cell cycle events. Transient activation of these kinases at specific cell stages is thought to trigger the principal transitions of the cell cycle, including DNA replication and entry into mitosis. In yeast, both transitions are controlled by single CDK (CDC28 in *Saccharomyces cerevisiae* [3]). In human cells, cell cycle events are governed by several CDKs. CDK4-cyclin D is necessary for passage through G1, CDK2-cyclin E is necessary for the transition from G1 to S phase, CDK2-cyclin A is necessary for progression through S, and CDC2-cyclin B is necessary for the transition from G2 to M phase. Two of these proteins CDK2 and CDC2/CDK1 have long been studied extensively. Both are closely related to yeast CDC28/CDK1 (CDK2 62% and CDC2 60% identity in amino-acid sequence).

The central role that cyclin-dependent kinases play in the timing of cell division and the high incidence of genetic alteration of CDKs or deregulation of CDK inhibitors in a number of cancers make CDC28 very attractive as a model for studies of mechanisms of CDK regulation. We have found that certain gene mutations in *Saccharomyces cerevisiae* including *cdc28-srm* affect cell cycle progression and maintenance of different genetic structures [1]. The *cdc28-srm* was accompanied by decreased mitotic stability of excess natural chromosomes and recombinant circular plasmids containing centromeres as well as decreased rates of spontaneous mitochondrial rho$^-$ mutations [1], increased cell sensitivity to ionizing radiation [2] and conferred checkpoint defects [4]. A *cdc28-srm* mutation is not temperature-sensitive mutation and differs from known *cdc28-ts* mutations because it has the evident phenotypic manifestations at 30 °C.

Crystallographic studies of several eukaryotic protein kinases have shown that they all share the same fold and tertiary structure. The crystal structure of the human CDK2 [5, 6] has served as a model for the catalytic core of other CDKs, including CDC28. The CDK2 structure is bilobed with N-terminus that is primarily beta-sheet and C-terminus that is primarily alpha-helix. ATP binds in a cleft between the two lobes. CDKs are inactive as monomers. Cell cycle-dependent oscillations in CDK activity are induced by complex mechanisms that include binding to positive regulatory subunits (cyclins) and phosphorylation at positive (pT160 in T-loop) and negative (pT14, pY15 in G- loop) regulatory sites.

The aim of the present work is to determine the nature of *cdc28-srm* mutation and to simulate the structure of the mutant allele through the experimental and molecular dynamics simulation studies.

1. MATERIALS AND METHODS

1.1. Preparing of Chromosomal DNA. Preparing of chromosomal DNA from yeast cells *cdc28-srm* was done by the standard technique [7].

1.2. Analysis of Nucleotide Sequence of Mutant Allele *cdc*28-*srm*. A set of coloured fluorescent terminators (ABIPRISM Dye Terminator Kit with AmpliTaq, Perkin–Elmer) and ORF specific primers were employed for determining the nucleotide sequence of the mutant allele *cdc28-srm* in an ABIPRISM 3700 sequencer according to standard procedure.

MD Simulations of Conformational Changes of Proteins. For the MD simulations the SANDER modules of the program package AMBER 8.0 [8] and of the modified version of AMBER 7.0 for a special-purpose computer MDGRAPE-2 [9] were used. The starting geometries for the simulations were prepared using X-ray structures from the Prookhaven Protein Data Bank (http://www.pdb.org). The all-atom force field [10] was used in the MD simulations. A system was solvated with TIP3P molecules [11] generated in a spherical (non-periodic) water bath. The system temperature was kept constant by the Berendsen algorithm with coupling time of 0.2 ps [12]. Only bond lengths involving hydrogen atoms were constrained using the SHAKE method [13]. The integration time step in the MD simulations was 1 fs. The simulation procedures were the same in all calculations [14]. Firstly, for each system a potential energy minimization was performed on an initial state. Next, the MD simulation was performed on the energy-minimized states. The temperatures of the considered systems were gradually heated to 300 K and then kept at 300 K the next 1 million time steps [15, 16]. The trajectories at 300 K for 2 ns were compared and studied in detail. The result of simulations and images of simulated proteins were analyzed by RasMol [17] and MOLMOL [18] packages.

2. RESULTS

2.1. *cdc*28-*srm* is a Substitution of Glycine with Serine in Position 20. Sequencing analysis of *cdc28-srm* revealed a single nucleotide substitution of glycine with serine in position 20 (G20S). This is a third glycine in a conserved sequence GxGxxG in G-rich loop. Glycine is the smallest amino acid, but serine is larger. Both amino acids belong to a neutral polar class. They are well solvable in water because their polar R-groups may form hydrogen bonds with

water (for glycine and serine polar R-groups – H and HO-CH2, respectively). It is worth noting that the observation of a key mutation in a protein extended structure is important and it allows understanding the mechanism of the protein kinase regulation activity. In our case we have obtained a mutation in a highly conservative sequence in G-rich loop. Despite its demonstrated importance, the role of G-loop has remained unclear. We will simulate the consequence of the conserved glycine substitution in the G-rich loop on the structure of the protein kinase. We use the human CDK2 as a model. Substitution of G20S in yeast CDC28 corresponds to G16S in human CDK2.

At the beginning we analyze inactive complex CDK2/ATP. Analysis of the CDK2/ATP binary complex [5] indicates that ATP interacts with several residues lining the cleft between the two lobes. The adenine base is positioned in a hydrophobic pocket between the beta-sheet of the small lobe and the L7 loop between beta-5 and alpha-2. The ATP phosphates are held in position by ionic and hydrogen-bonding interactions with several residues, including K33, D145, and the backbone amides of the G-rich loop between beta-1 and beta-2.

2.2. The CDK2/ATP Structural Conformations. We simulated the CDK2-G16/ATP of the wild-type structure and compared the conformational changes with CDK2-S16/ATP structure. The calculation results of the wild-type CDK2-G16/ATP and CDK2-S16/ATP structural conformations are shown in Figs. 1

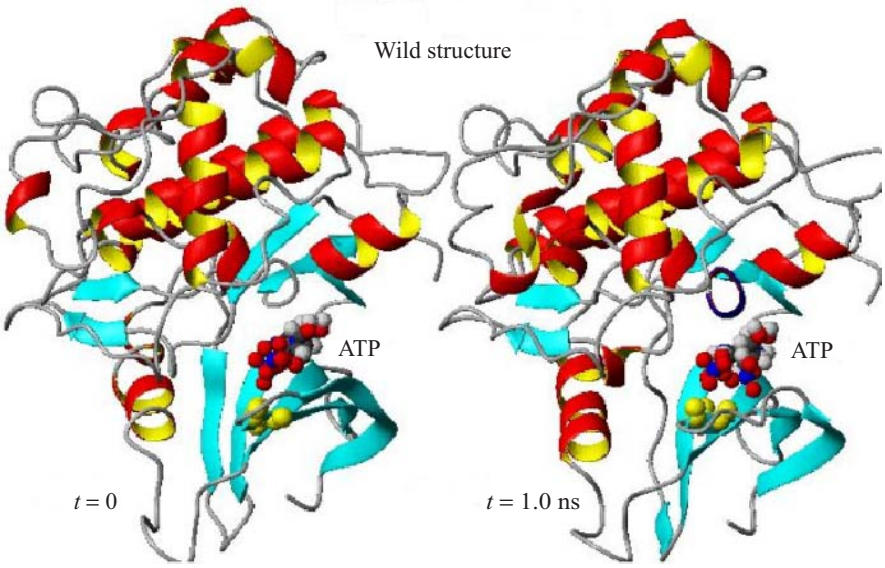

Fig. 1. The initail and final (1-ns state) structures of the CDK2/ATP of the wild-type complex. The ATP molecule and residue 16 of the G-loop are drawn by balls

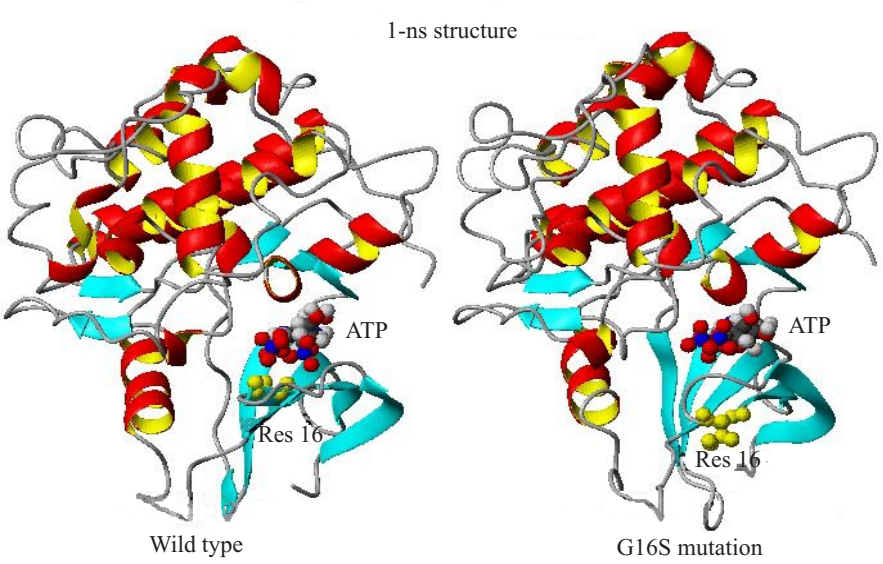

Fig. 2. The comparison of the final (1-ns state) structures are shown (left — wild type, right — S16 protein). The ATP molecule and the residue 16 of the G-loop are drawn by balls

and 2. The left snapshot displays the initial states, the right snapshot – the final 1-ns states, respectively. For CDK2-G16 and CDK2-S16 structures we have also generated a number of the animation movies to display the real-time dynamical motions. We concentrate on the positional changes between the ATP, residue 16 and T-loop (the latter covers a left bottom alpha-helix shown in Fig. 1).

If to make the comparison of the pictures of the initial and final states of wild-type CDK2-G16/ATP structure, it is easy to see that there is not a visually large difference between these two structures. Moreover, the position of the amino-acid residue 16 does not change within 1 million MD time steps in comparison to that at the initial configuration. So, for the wild-type protein we found that it keeps its conformation stable relatively to the original state.

Regarding the CDK2-S16 variant (Fig. 2), a completely different picture of the dynamics and conformational changes has seen. First of all, the amino-acid residue 16 moves dramatically far from the ATP location site. In comparison to the wild structure, the distance between the position S16 and ATP, as is seen from the snapshots, increases of about 2–2.5 times at average. At the same time, such movement results on a relative shift of the T160 residue and the whole T- and G-loops positions.

On the one hand, it is obvious that the increase of the ATP-S16 distance influences on the picture of the hydrogen bonds formation involving the ATP and G-loop. On the other hand, the mutation induced CDK2' conformational change results in an interhelical protein movement, covering a phosphorylation point (viz. T160).

2.3. The G16S Mutation Induced Corruption of a Hydrogen Bond Network. In Fig. 3 we display the T160, ATP and S16 positions (an «activation triangle») for the final (1-ns) state to estimate (though indirectly) a possibility

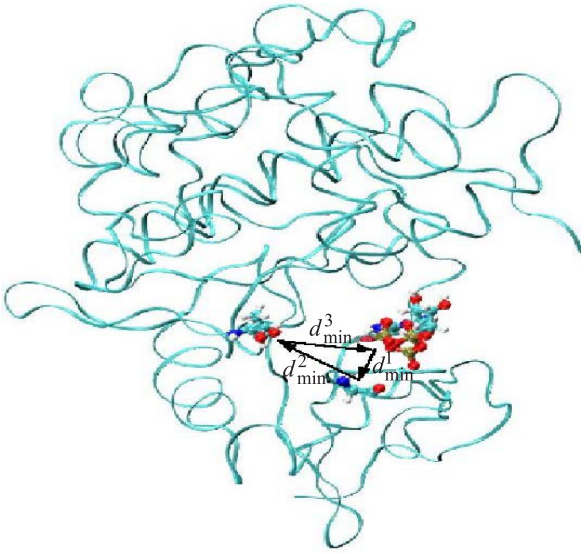

Fig. 3. The positions of the T160, ATP and res16 (an «activation triangle») are shown. The ATP molecule, residues T160 and 16 are drawn by balls

of the hydrogen bond formation in the ATP and G-loop region. We note that in general a hydrogen bond is regarded as being effective when the distance between the hydrogen atom of the proton donor and the proton acceptor is less than 2.6–2.4 Å. For the above «activation triangle» the calculated values of the distances between the ATP-res16 and ATP-T160 are shown in Figs. 4 and 5, respectively. From Fig. 4 we observe that the ATP-res16 distance for the wild-type and mutant structures has a completely different behavior. It is easily seen from Fig. 4 that the ATP-res16 distance for the wild-type structure lies within ~ 2.5 Å during the all 1-ns dynamical changes. At the same time, for the mutation structure, ATP-res16 distance lies within of 7.5 Å. That is the G16S mutation causes the increase of the ATP-res16 distance of about three times large than that of the wild-type structure. Thus, the mutation induced conformational changes in the CDK2 structure are obviously corrupt the all hydrogen bond network in the

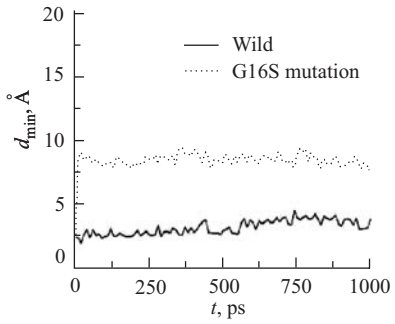

Fig. 4. The time dependences of the ATP-res16 distance are shown for the CDK2-G16/ATP (solid line) and CDK2-S16/ATP (dotted line) in accordance to the «activation triangle»

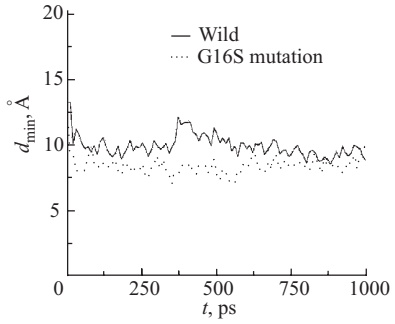

Fig. 5. The time dependences of the ATP-res16 distance are shown for the CDK2-G16/ATP (solid line) and CDK2-S16/ATP (dotted line) in accordance to the «activation triangle»

ATP-res16 binding site. In Fig. 5 we present the time-dependent dynamics for the ATP-T160 distance. We see that the ATP-T160 distance for the wild-type protein is comparably shorter than that for the CDK2-S16/ATP variant. The behavior of the ATP-T160 distance agrees with the results shown in the previous figure.

2.4. Rotational Changes of the Amino-Acid Residues Around of Phosphorylated Regulatory Site T160. The CDK2/ATP' dynamical peculiarities around of the posphorylation site (T160) we have analyzed in detail, displaying by the snapshots and animation movies all amino-acid positions in the T-loop. From these data we have caught a rotation of two neighboring amino acids of T160 (viz. residues 159 and 161) that possesses different behavior. The residue 159 (Y159) is tyrosine with a non-charged polar R-group, and the residue 161 (H161) is histydine with a polar charged R-group. A summary of these observations is presented through the following two sets of snapshots (Figs. 6 and 7). From Fig. 6 (left snapshot — initial state) we see that two carbon rings of residues Y159 and H161 are occupy a well-separated positions. The carbon rings during their

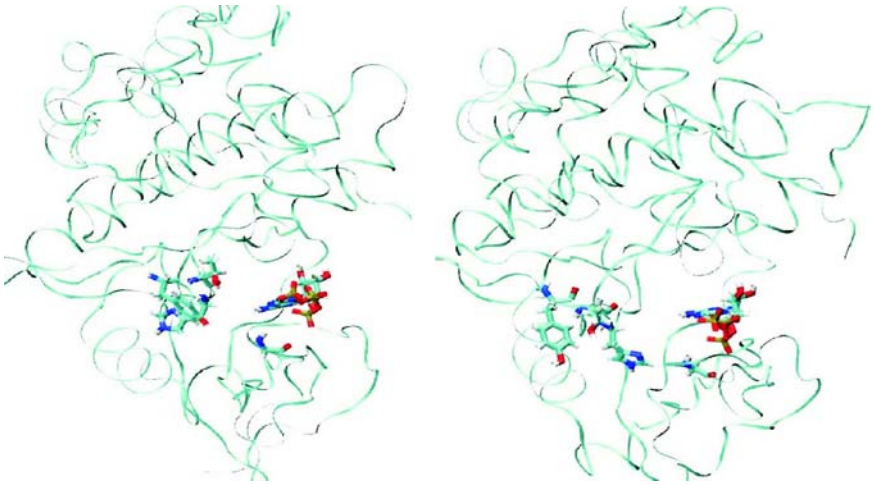

Fig. 6. The initial (left) and final (right) configurations of the wild-type protein. The snapshots display the orientational changes of two neighboring amino acids around phosphorylation site (viz. the residue T160). It is seen that the Y159 and H161 exchange their relative orientations with respect to residue T160

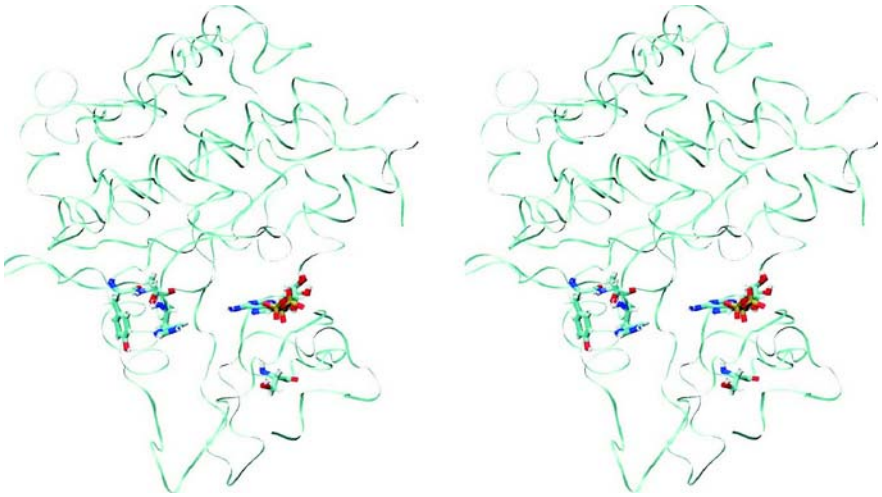

Fig. 7. The initial (left) and final (right) configurations of the CDK2-S16 protein. The snapshots display the orientional changes of two neighboring amino acids (Y159 and H161) around a phosphorylation site (T160). The positions of the ATP molecule and the res16 are also drawn

dynamical motions abruptly exchange (at around of 0.5 ns from the start) their orientation positions. The rings have begun to «screen» the amino-acid residue T160. Such «screening» does not change until the end of the dynamics (right snapshot — final 1-ns state). The overlapping of the carbon rings, on the contrary, does not manifest itself for the S16 variant (Fig. 7). During the all simulation period no «screening» of the site T160 has to be seen.

Thus, the mutation induced changes for the protein structure affect the overlapping of the carbon rings at a key phosphorylation point T160. The described-above «screening» phenomena we connect with the positional changes of the activation T-loop induced by the mutation at the residue 16.

From the described-above «activation triangle» we have estimated the T160-res16 distance (see Fig. 8) for the wild-type CDK2/ATP, CDK2-S16/ATP and

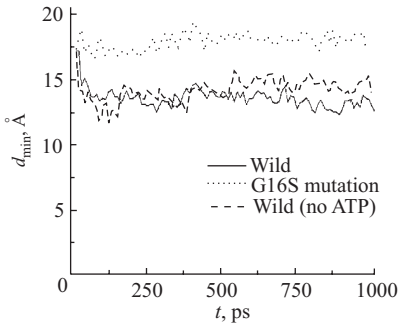

Fig. 8. The time dependences of the T160-res16 distance are shown for the CDK2-G16/ATP wild type (solid line), a wild-type CDK2 (dashed line) and CDK2-S16/ATP (dotted line) in accordance to the «activation triangle»

wild-type monomeric (without ATP) CDK2 proteins. It is seen from Fig. 8 that the T160-res16 distance in the mutant CDK2-S16/ATP structure is significantly larger than that of the wild-type CDK2-G16/ATP. At the same time, the T160-res16 distance for the wild-type CDK2/ATP and wild-type monomeric CDK2 structures follows almost the same law. This fact probably means that the «screening» phenomena do not origin from the presence of ATP molecule or T160-ATP exchanges.

DISCUSSION

The G-loop enables protein kinase to adopt a wide range of backbone conformations. The significance of this domain is demonstrated by the fact that substitution of the glycine residues in the G-loop, particularly the first and the second glycine (GxGxxG) with either alanine or serine results in a dramatic

decrease in cAPK activity. The functional importance of the G-loop has been described in detail for cAPK [19–22], but its importance for CDK regulation has not yet been discussed. It is believed that the G-loop catalytic function – that is, correct ATP binding and alignment – is the same as its function in cAPK, but it exhibits a new inhibitory function for CDK [23]. We have obtained the mutation *cdc28-srm* that posseses a pleotropic manifestation in yeast cells. Analyses have shown that this mutation is localized in the glycine-rich loop (G-loop) and is a substitution of the third glycine with serine.

The crystal structure of inactive monomeric CDK2 showed that the T-loop (residues 147–153) would block access of substrates to the active site and that ATP would bind with the wrong geometry for efficient catalysis. Binding to cyclin A simultaneously moves the T-loop of CDK2 away from the substrate binding cleft and repositions of the G-loop (residues 11–18), so that they can interact properly with the ATP phosphates. This complex has a low but detectable activity. Less dramatic changes occur in the structure of the CDK2/cyclin A complex following activation by phosphorylation of T160 [24]. The phosphorylation of T160 might relieve a proposed steric interference between the substrate and the unphosphorylated T-loop and help to organize an acidic patch containing E162, E208 and D235 by repositioning E162, a T-loop residue. The serine of the peptide substrate is hydrogen bonded to the ATP gamma-phosphate oxygen, to the catalytic aspartate D127, and to the conserved lysine K129 [25].

MD simulation analysis shows that in CDK2-S16/ATP the distance between ATP and the residue 16 in the G-loop dramatically increased. The shift is equal 5 Å. It is known that the G-loop was changed during the early stage of inactive (CDK2/ATP), partly active (CDK2/cyclin A/ATP), and fully active (pT160-CDK2/cyclin A/ATP) CDK2 simulations in comparison with its conformation as found in the crystal structures [23]. The G-loop moves away from the ATP phosphate moiety binding site after the interaction of CDK2 with cyclin A and again after CDK2/cyclin A/ATP complex phosphorylation at the T160 site. The shift of the G-loop is equal to 3.5 Å (CDK2/cyclin A/ATP) and 8.6 Å (pT160-CDK2/cyclin A/ATP) in comparison with the G-loop position found in the CDK2/ATP system. It is interesting that we observe this shift in the mutant allele CDK2-G16S/ATP as well.

We find unexpected consequence of substitution G16S – new orientation of two neighboring amino acids of T160 in the T-loop. It may influence on the interactions of the protein kinase with cyclins. In further studies we intent to examine some other complexes of the CDK2, including cyclin A and substrate.

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