14-3-3 Protein C-terminal Stretch Occupies Ligand Binding Groove and Is Displaced by Phosphopeptide Binding**

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14-3-3 proteins are important regulators of numerous cellular signaling circuits. They bind to phosphorylated protein ligands and regulate their functions by a number of different mechanisms. The C-terminal part of the 14-3-3 protein is known to be involved in the regulation of 14-3-3 binding properties. The structure of this region is unknown; however, a possible location of the C-terminal stretch within the ligand binding groove of the 14-3-3 protein has been suggested. To fully understand the role of the C-terminal stretch in the regulation of the 14-3-3 protein binding properties, we investigated the physical location of the C-terminal stretch and its changes upon the ligand binding. For this purpose, we have used Förster resonance energy transfer (FRET) measurements and molecular dynamics simulation. FRET measurements between Trp^242 located at the end of the C-terminal stretch and a dansyl group attached at two different cysteine residues (Cys^256 or Cys^189) indicated that in the absence of the ligand, the C-terminal stretch occupies the ligand binding groove of 14-3-3 protein. Our data also showed that phosphopeptide binding displaces the C-terminal stretch from the ligand binding groove. Intramolecular distances calculated from FRET measurements fit well with distances obtained from molecular dynamics simulation of full-length 14-3-3^ζ protein.

The 14-3-3 protein family represents one of the most important group among proteins recognizing phosphorylated targets (1–4). Two canonical 14-3-3 binding motifs have been defined, RXpXpXp and RXpY/XpXpXp (5, 6), where pS denotes phosphorylated serine. Many of the 14-3-3 protein binding partners identified so far contain one of these motifs. Through the functional modulation of a wide range of binding partners, 14-3-3 proteins are involved in many biologically important processes, including cell cycle regulation, metabolism control, apoptosis, and control of gene transcription (1–4).

All 14-3-3 proteins form very stable homo- and heterodimers with characteristic cup-like shape and a large, 40-A-wide, deep central channel (5–10). Each monomer consists of nine antiparallel a-helices and an amphipathic groove where the phosphorylated segment of the binding partner is bound. The primary structure of 14-3-3 proteins is highly conserved, with completely conserved residues forming either the dimer interface or the walls of the ligand binding groove. Maximal sequence diversity occurs within the flexible C-terminal stretch, which has been shown to be involved in the regulation of binding properties of 14-3-3 proteins (11–16). In addition, the C-terminal stretch of two vertebrate 14-3-3 isotypes (ζ and ζ) contains a casein kinase Iα phosphorylation site at position 232 (17). Phosphorylation of 14-3-3 proteins has been suggested to be an important regulatory mechanism of individual isozymes, and it has been shown that in vivo phosphorylation of the C-terminal phosphorylation site inhibits the interaction between 14-3-3ζ and Raf-1 kinase (17, 18). The structure of the C terminus is unknown, because this region is disordered in all available 14-3-3 protein crystal structures (5–10). Liu et al. (7) suggest that, in the absence of the ligand, the 14-3-3 protein C-terminal stretch could occupy the ligand binding groove and thus has to be pushed away during the ligand binding. Moreover, Truong et al. (14) show that removal of the C terminus increases the binding affinity of 14-3-3ζ protein for several tested ligands and propose that the C terminus might function as an autoinhibitor by suppressing unspecific interactions between 14-3-3 protein and inappropriate ligands. In addition, we have recently shown that phosphopeptide binding changes the conformation and increases the flexibility of the 14-3-3 protein C-terminal stretch (16).

To fully understand the role of the C-terminal stretch in the regulation of 14-3-3 protein binding properties, we have attempted to provide evidence for the location of the missing C-terminal part of the 14-3-3 protein molecule. Förster resonance energy transfer (FRET) measurements between Trp^242 (located within the C-terminal stretch) and a dansyl group (attached at two different cysteine residues) indicate that, in the absence of the ligand, the C-terminal stretch occupies the ligand binding groove of the 14-3-3 protein. Our data also showed that phosphopeptide binding displaces the C-terminal stretch from the ligand binding groove. Intramolecular distances calculated from FRET measurements fit well with distances obtained from molecular dynamics simulation of full-length 14-3-3^ζ protein.

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Experimental Procedures

Expression and Purification of 14-3-3 Proteins—All 14-3-3 protein mutants were generated using the QuickChange kit (Stratagene). The 14-3-3 proteins were expressed and purified as described by Obisola et al. (16).

Labeling of 14-3-3 Protein Mutants by 1,5-IAEDANS—Human 14-3-3 protein possesses three cysteine residues (Cys25, Cys94, and Cys189). To prepare proteins suitable for FRET measurements, we constructed mutants containing a single cysteine residue (either at position 25, 94, or 189, respectively) and a single Trp residue located at the end of the C-terminal stretch at position 242. Covalent modification of the 14-3-3Δc cysteine protein containing the single Trp242 and single Cys residues either at position 25, 94, or 189, respectively, with 1,5-IAEDANS was carried out as described previously (19). Briefly, the protein (50–70 μM) in 50 mM Tris (pH 7.5), 100 mM NaCl, and 1 mM EDTA and label were mixed at a molar ratio of 1:40 and incubated at 30 °C for 2 h and then at 4 °C overnight in the dark. The free unreacted label was removed by gel filtration in buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, and 1 mM EDTA. The incorporation stoichiometry was determined by the absorbance at 336 nm using an extinction coefficient of 5700 M⁻¹ cm⁻¹ (Molecular Probes, Eugene, OR).

Mass Spectrometry Analysis—MALDI-TOF mass spectrometry was used to check amino acid sequences of 14-3-3 protein constructs with mutated cysteines and verify the modification of cysteine residues by the AEDANS moiety. Samples were first separated by 12% SDS-PAGE, mutated cysteines and verify the modification of cysteine residues by the AEDANS moiety. Samples were first separated by 12% SDS-PAGE, mutated cysteines (Cys25 or Cys189) in used 14-3-3

Fluorescence Peptide Binding Assay—Fluorescence resonance energy transfer was observed between the single tryptophan residue Trp242 and the AEDANS moiety covalently attached to Cys25 or Cys189. Fluorescence intensity decays of Trp242 were measured on an apparatus as described by Obisola et al. (16). Fluorescence decays were acquired under “magic angle” conditions, where the measured intensity decay I(t) was independent of a rotational diffusion of the chromophore and provided unbiased information about lifetimes. The apparatus response function was done at the excitation wavelength with a diluted Ludox solution. The samples were placed in a thermostat holder, and all experiments were performed at 22 °C in buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA. 14-3-3 protein concentration was 20 μM, and ligand (pRaf-259 or Raf-259 peptide) concentration was 50 μM. Fluorescence data processing was performed as described previously using the singular value decomposition maximum entropy method (16, 23–25), and the mean lifetimes were calculated. The average efficiency of energy transfer E was calculated from the mean donor lifetime in the presence (τD) and absence of acceptor (τ0).

The average distance between the donor-acceptor pair R can be calculated from Equation 3,

\[ R = R_0 \cdot \sqrt{\varepsilon D E - 1} \]  

where \( R_0 \) is the Förster critical distance (the distance at which the energy transfer occurs with 50% efficiency). \( R_0 \) is given by (26, 27),

\[ R_0 = \frac{8.8 \times 10^{-21} \text{n}^2 \text{Q}_D \lambda^{1/6}}{[\text{cm}^3 \text{M}^{-1}]} \]  

where \( n \) is the refractive index of the medium, \( Q_D \) is the quantum yield of the donor in the absence of the acceptor, and \( \lambda^{1/6} \) is the orientation factor that accounts for relative orientation of the donor emission and acceptor absorption transition dipole. The spectral overlap integral \( \lambda(\lambda) \) of the donor fluorescence and acceptor absorption was calculated from Equation 5 (26, 27),

\[ \lambda(\lambda) = \int \frac{F_D(\lambda)}{\lambda^4} d\lambda \times \int \frac{\lambda F_A(\lambda) d\lambda}{\lambda^4} \left[ \frac{\text{cm}^3 \text{M}^{-1}}{\text{nm}^{-4}} \right] \]  

where \( F_D(\lambda) \) and \( \epsilon_n(\lambda) \) represent the fluorescence intensity of the donor and the molar extinction coefficient of the acceptor, respectively, at the wavelength \( \lambda \) expressed in centimeters. The orientation factor was assumed to be equal to 0.5, which corresponds to randomly oriented dipole moments of donor-acceptor pairs. A value of 1.5 was used for the refractive index of the aqueous solution. Quantum yields of the donor, which is the Trp242 in the single Trp mutant of the 14-3-3 protein, were calculated relative to the \( Q_0 \) of L-Trp in water (28).

Molecular Modeling—Molecular dynamics (MD) simulations were performed according to the protocol published previously (16). The GROMACS version 3.1 (www.gromacs.org) molecular dynamics software package was used with the parameter set ffg43a1p (29). All MD trajectories were analyzed using GROMACS software. The cluster analysis was performed using the GROMOS software method (30). A model of the 14-3-3 protein with attached AEDANS moieties at residues Cys25 and Cys189 was built using a representative conformation of 14-3-3 protein (obtained using a cluster analysis of simulated trajectories with a root mean square deviation cutoff of 1 Å) and AEDANS coordinates obtained from the crystal structure of labeled RNase (Protein Data Bank accession code 1RAS) (31). The geometry of the resulting complex was optimized by energy minimization using the molecular modeling package Gschemic (32) and Desktop Molecular Modeler version 4.2 (Polyhedron Software).

Results

Construction of 14-3-3 Protein Mutants for Förster Resonance Energy Transfer—To provide evidence for the physical location of the C-terminal stretch using FRET, we have constructed three 14-3-3 protein mutants containing a single Trp residue within the C-terminal stretch at the position 242 (residues Trp259 and Trp228 were mutated to Phe) and a single Cys residue located at three different positions: Cys25 (this mutant was named 14-3-3-Sw259C25), Cys94 (14-3-3-Sw242C94), and Cys189 (14-3-3-Sw242C189). The Trp residue served as an energy transfer donor, and the Cys residues were selectively labeled with...
In the presence of 1,5-IAEDANS, Trp 242 fluorescence intensity of AEDANS/mol of these two 14-3-3 be completely modified by 1,5-IAEDANS. The stoichiometries of both single cysteine-containing mutants were found to be -1 (0.95 and 1.06, respectively). The third cysteine, Cys94, was modified partially by 1,5-IAEDANS (labeling stoichiometry was found to be only -0.4) and therefore was not used for FRET measurements.

Modification of 14-3-3 protein mutants by 1,5-IAEDANS has been verified using MALDI-TOF mass spectrometry. A fluorescence binding assay was used to test the binding abilities of both 14-3-3 protein mutants (Fig. 1). These experiments revealed that both 14-3-3 protein mutants modified with 1,5-IAEDANS bind phosphorylated pRaf-259 peptide with comparable affinity as wild-type protein, which binds pRaf-259 peptide with KD value of 116 nM (5). No significant binding of unphosphorylated Raf-259 peptide within the used concentration range has been detected (data not shown).

Investigation of the C-terminal Stretch Location Using FRET Steady-state Fluorescence Measurements—FRET measurements between Trp242 and AEDANS moiety attached to a cysteine residue of 14-3-3 protein mutants were used to investigate the physical location of the C-terminal stretch of the 14-3-3 protein. First, we tested the presence of the energy transfer between Trp242 and AEDANS moieties using the steady-state fluorescence. Tryptophan fluorescence emission spectra of 14-3-3 protein mutants, unmodified and modified with 1,5-IAEDANS, are shown in Fig. 2. When excited at 295 nm, Trp242 has a fluorescence emission maximum near 350 nm. In the presence of 1,5-IAEDANS, Trp242 fluorescence intensities of both single cysteine-containing mutants were significantly reduced compared with unmodified proteins, indicating the presence of FRET. The sensitized fluorescence emission of AEDANS (induced by the nonradiative transfer of energy from Trp242) was observed as a peak at 485 nm. In the case of the 14-3-3w242c25 mutant, the donor–steady-state fluorescence was quenched by -24% compared with the emission of unlabeled protein (Fig. 2A). Higher steady-state FRET efficiency was observed for the 14-3-3w242c189 mutant, with -50% reduction of the donor fluorescence (Fig. 2C), indicating that AEDANS attached to Cys189 is closer to Trp242 than in the case of Cys25. Next, we tested the effect of the peptide binding on steady-state FRET efficiency between Trp242 and AEDANS. The presence of the unphosphorylated peptide Raf-259 had no effect on steady-state FRET efficiency, irrespective of AEDANS position within the 14-3-3 protein molecule (data not shown).

On the other hand, the addition of the phosphorylated peptide pRaf-259, which contains optimal 14-3-3 protein binding motif and binds to 14-3-3 protein with high affinity, was found to affect steady-state FRET efficiency. The effect was dependent on the position of AEDANS. In the case of the 14-3-3w242c25 mutant, the pRaf-259 binding slightly reduced FRET efficiency (compare Fig. 2, A and B), whereas for the 14-3-3w242c189 mutant, the FRET remained practically unchanged (compare Fig. 2, C and D).

Investigation of the C-terminal Stretch Location Using FRET Time-resolved Fluorescence Measurements—To quantitatively investigate the physical location of the C-terminal stretch, we employed time-resolved intensity decays of Trp242 to measure distances between residue Trp242 and an AEDANS moiety attached at different positions within the 14-3-3 protein molecule. We chose the time-resolved fluorescence approach, because, unlike fluorescence intensities, lifetimes do not depend on the excitation intensity, excited sample volume, protein concentration, or photobleaching, etc. Consequently, time-resolved data are more reliable than and superior to fluorescence intensities for quantitative FRET measurements. To use FRET to measure distances between Trp242 and AEDANS, the Förster critical distance R0 of this donor-acceptor pair must be determined first. The R0 is a distance between the donor and acceptor at which the energy transfer is 50% efficient. The R0 was determined from Equations 4 and 5. The spectral overlap J(\lambda) between Trp242 and AEDANS (obtained by numerical integration of a product of an area-normalized emission spectrum of 14-3-3 protein containing a single tryptophan at position 242 and the absorption spectrum of AEDANS) was found to be 5.876 × 10^{-15} cm^3 sr^{-1}. The quantum yield of Trp242 fluorescence of 14-3-3 protein mutants at 22 °C was determined to be 0.074 ± 0.002 in the absence of the ligand, 0.071 ± 0.002 in the presence of 25 μM pRaf-259 peptide, and 0.073 ± 0.003 in the presence of 25 μM Raf-259 peptide, respectively. The quantum yields were determined relative to a standard solution of tryptophan in water (Q0 = 0.140) (28). This resulted in the calculated R0 of 19.7 Å in both the absence of the ligand and the presence of unphosphorylated peptide Raf-259. In the presence of the phosphorylated peptide pRaf-259, the R0 is 19.5 Å. These R0 distances are somewhat lower compared with the value of 22 Å reported elsewhere (19), presumably because of the lower quantum yield of Trp242 fluorescence.

Time-resolved fluorescence intensity decays were analyzed using a singular value decomposition maximum entropy method as described previously (16). The intensity decays of
Trp<sup>242</sup> for all three 14-3-3<sub>3</sub> protein mutants can be adequately described by a lifetime distribution containing three lifetime components (data not shown). Mean excited-state lifetimes (ε<sub>mean</sub> of Trp<sup>242</sup>) in the absence and presence of acceptor and the efficiency of the energy transfer are presented in Table I. Upon labeling by 1,5-IAEDANS for both mutants, the ε<sub>mean</sub> of donor was reduced as a result of the energy transfer (Fig. 3). Values of the FRET efficiency were used to calculate the average distances between the donor and the acceptors. Distances between Trp<sup>242</sup> and AEDANS attached at Cys<sup>25</sup> and Cys<sup>189</sup> were calculated to be 30.4 Å and 23.8 Å, respectively. These distances strongly indicate that, in the absence of the ligand, the C-terminal stretch of 14-3-3<sub>3</sub> protein is physically located within the ligand binding groove (see “Molecular Modeling” for details).

Next, we tested the effect of phosphopeptide binding on the location of the C-terminal stretch. The addition of phosphorylated peptide pRaf-259 significantly decreased the FRET efficiency and thus increased the distance between Trp<sup>242</sup> and AEDANS attached to Cys<sup>25</sup> from 30.4 Å to 36 Å. On the other hand, the distance between Trp<sup>242</sup> and AEDANS-Cys<sup>189</sup> remained practically unchanged (Table I). These data were supported by observations obtained from the steady-state measurements and indicate that the binding of the phosphorylated peptide pRaf-259 induces structural rearrangement of the C-terminal stretch. This conformational change caused an increase in distance between Trp<sup>242</sup> and Cys<sup>25</sup>, whereas the distance between Trp<sup>242</sup> and Cys<sup>189</sup> remained unaltered. The presence of the unphosphorylated peptide Raf-259, which did not interact with the 14-3-3 protein had no effect on FRET efficiency as was documented in the case of the 14-3-3<sub>3</sub> protein (Table I). These data were consistent with the steady-state fluorescence measurements where no effect of unphosphorylated Raf-259 peptide on FRET efficiency was found.

Molecular Modeling of Full-length 14-3-3<sub>3</sub> Protein—Molecular dynamics was used to investigate interactions between C-terminal stretch and the ligand binding groove of 14-3-3<sub>3</sub> protein and to confront intramolecular experimental distances obtained from FRET measurements. Recently, we have reported a 3-ns-long molecular dynamics simulation of full-length 14-3-3<sub>3</sub> protein with the C-terminal stretch located within the ligand binding groove (16). To better sample the conformational space of the C-terminal stretch, the 3-ns-long MD simulation was extended to a trajectory of 20 ns. The time course of the root mean square deviations of backbone-heavy atoms during the production run shows that the 14-3-3<sub>3</sub> protein structure was stable during the simulation (Fig. 4A). Fig. 4B shows time evolution of distances between Cys atoms of residue pairs 25—242 and 189—242. Average distances were calculated to be 23.9 ± 1.1 Å for the Cα<sup>25</sup>—Cα<sup>242</sup> pair and 16.1 ± 0.9 Å for the Cα<sup>189</sup>—Cα<sup>242</sup> pair. Representative conformation of the 14-3-3<sub>3</sub> protein has been calculated as an average conformation of the most populated cluster obtained using a cluster analysis of simulated trajectory with a root mean square deviation cutoff of 1 Å (30).

For better comparison with proteins used in our FRET experiments, we built a model of full-length 14-3-3<sub>3</sub> protein with AEDANS moiety attached at Cys<sup>25</sup> and Cys<sup>189</sup> (Fig. 5A). This model revealed that distances between the dansyl group and the indole group of Trp<sup>242</sup> in the absence of the phosphopeptide are ~30.0 and 23.5 Å for AEDANS moiety attached at Cys<sup>25</sup> and Cys<sup>189</sup>, respectively (Fig. 5A). These values are in an excellent agreement with experimental distances provided by FRET measurements (Table I), suggesting that conformation with the C-terminal stretch located within the ligand binding groove is an appropriate model for the full-length 14-3-3<sub>3</sub> protein structure. Next we attempted to model the position of the C-terminal stretch with bound phosphopeptide. The crystal structure of the 14-3-3<sub>3</sub> protein with bound phosphopeptide containing the sequence around phosphoserine 259 from Raf-1 kinase (7) has been used to position the phosphopeptide into the ligand binding groove. To meet average distances obtained from FRET measurements (Table I), the Trp<sup>242</sup> has to be located somewhere above the helix H9 as suggested in Fig. 5B. Only in this conformation, will the
model fulfill the measured distances 23.5 and 36 Å. Taken together, our data indicate that the C-terminal stretch is physically located within the ligand binding groove of the 14-3-3 protein molecule and is displaced when phosphorylated peptide binds.

Possible contacts between the C-terminal stretch and the ligand binding groove of the 14-3-3 protein and their comparison with the crystal structures of the 14-3-3 protein-phosphopeptide complex (6) are shown in Fig. 6. The main chain of the C-terminal stretch adopts an extended conformation. On the basis of our MD simulation the 11-residue sequence, DEAEAGEGGEA, from Asp235 to Asn245, constitutes the region of the C-terminal stretch interacting with the ligand binding groove of the 14-3-3 protein. The key interactions of the C-terminal stretch binding seem to be contacts between side chains of negatively charged residues Glu241 and Glu244 and several residues located within helices H3, H5, and H7 (Arg127, Tyr128, and Asn173). Residues Arg127 and Tyr128 are known to participate in the coordination of the phosphate group of 14-3-3 protein binding partners, whereas the side chain of Asn173 makes contact with the ligand main chain (Fig. 6B) (6). The second acidic residue within the C-terminal stretch, which is predicted to interact with the ligand binding groove of 14-3-3 protein is Glu244 (Fig. 6A). Other possible contacts between the ligand binding groove of the 14-3-3 protein and the C-terminal stretch involve residues Asn42, Ser214, and Asn224.

**DISCUSSION**

**Location of the C-terminal Stretch in the Absence of the Ligand**—To fully understand the role of the C-terminal stretch in the regulation of 14-3-3 protein binding properties, we decided to investigate the localization of the C-terminal stretch and its changes upon the ligand binding. For this purpose, we used FRET measurements and MD simulations. FRET reports on distances in the range of ~10–100 Å and its efficiency depends on the sixth power of the distance between the energy donor and energy acceptor, where the FRET efficiency is 50%.

![Fig. 3.](image3.png) **Fluorescence energy transfer in 14-3-3w242 protein.** Comparison of fluorescence decays of Trp242 in the absence (open circles) and in the presence (filled circles) of the AEDANS acceptor positioned at Cys25 (A) and Cys187 (B). The time scale is 36.6 ps/channel.

![Fig. 4.](image4.png) **Molecular dynamics simulation of full-length 14-3-3 protein.** A, time course of the root mean square deviations (RMSD) of backbone-heavy atoms during the production run. B, time evolution of distances between Cα atoms of residue pairs 25–242 and 189–242. Average distances were calculated to be 23.9 ± 1.1 Å for the Cα25–Cα242 pair and 16.1 ± 0.9 Å for the Cα189–Cα242 pair.

**TABLE I**

| 14-3-3ζ Mutant | Label | Ligand | τ<sub>mean</sub><sup>a,b</sup> | E<sup>c</sup> | R<sub>0</sub><sup>d</sup> | R |
|---------------|-------|--------|----------------|-------|------|-------|
| 14-3-3w242c25 | Donor only | None | ns | % | Å | Å |
| 14-3-3w242c25 | Donor + AEDANS | None | 2.93 | 6.8 | 19.7 | 30.4 |
| 14-3-3w242c25 | Donor only | pRaf-259 | 2.90 | 7.2 | 19.5 | 36.0 |
| 14-3-3w242c25 | Donor + AEDANS | pRaf-259 | 2.92 | 7.2 | 19.5 | 30.1 |
| 14-3-3w242c189 | Donor only | None | 3.04 | 24.0 | 19.7 | 23.8 |
| 14-3-3w242c189 | Donor + AEDANS | None | 2.31 | 24.6 | 19.5 | 23.5 |
| 14-3-3w242c189 | Donor only | pRaf-259 | 2.97 | 24.6 | 19.5 | 23.5 |
| 14-3-3w242c189 | Donor + AEDANS | pRaf-259 | 2.24 | 24.6 | 19.5 | 23.5 |

<sup>a</sup> Mean lifetimes were calculated as τ<sub>mean</sub> = ∑<sub>i</sub>f<sub>i</sub>τ<sub>i</sub>, where f<sub>i</sub> is an intensity fraction of the i<sup>th</sup> lifetime component τ<sub>i</sub>.

<sup>b</sup> Standard deviation is 0.01 ns.

<sup>c</sup> E, energy transfer.

<sup>d</sup> Förster critical distance represents the distance between energy donor and energy acceptor, where the FRET efficiency is 50%.

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**FIG. 3.** Fluorescence energy transfer in 14-3-3w242 protein. Comparison of fluorescence decays of Trp242 in the absence (open circles) and in the presence (filled circles) of the AEDANS acceptor positioned at Cys25 (A) and Cys187 (B). The time scale is 36.6 ps/channel.

**FIG. 4.** Molecular dynamics simulation of full-length 14-3-3ζ protein. A, time course of the root mean square deviations (RMSD) of backbone-heavy atoms during the production run. B, time evolution of distances between Cα atoms of residue pairs 25–242 and 189–242. Average distances were calculated to be 23.9 ± 1.1 Å for the Cα25–Cα242 pair and 16.1 ± 0.9 Å for the Cα189–Cα242 pair.
donor and the energy acceptor (26, 27). Therefore, FRET is a very sensitive “ruler” for measurements of distances on the molecular scale. To study the localization of the C-terminal stretch by FRET we created two 14-3-3/H9256 protein mutants containing a single energy donor Trp 242 and a single Cys residue labeled by energy acceptor 1,5-IAEDANS (Fig. 5). Average distances between Trp242 and AEDANS bound to Cys 25 and Cys189 obtained from FRET measurements (Table I) indicate that, in the absence of the ligand, the C-terminal stretch is located within the ligand binding groove of the 14-3-3/H9256 protein. Molecular modeling showed that only in this conformation both measured distances are fulfilled and that any different conformation of the C-terminal stretch would increase either one of the measured distances or both. Molecular dynamics simulation of full-length 14-3-3/H9256 protein revealed that the C terminus stayed bound within the ligand binding groove during the whole simulation and adopted an extended conformation. If we consider the size of the indole ring of the Trp residue and the AEDANS moiety attached at the Cys residues, the calculated average distances between C atoms of residues used in this study (Fig. 4B) fit well with experimental distances obtained from FRET measurements. MD simulation of full-length 14-3-3/H9256 protein also suggests the possible mode of the C-terminal stretch interaction with the ligand binding groove (Fig. 6A) and shows that it can share some similarities with the binding of the phosphorylated peptides (5, 6). Crystal structures of several 14-3-3 protein complexes demonstrated that residues Lys49, Arg56, Arg127, and Tyr128 are involved in the coordination of the phosphate group of 14-3-3 binding partners (Fig. 6B), whereas residues Asn52, Asn173, Glu180, and Asn224 provide additional contacts between the 14-3-3 protein ligand binding groove and the binding partner (5–10). Our MD simulation suggests that negatively charged residues Glu241 and Glu244 mimic the negative charge of the phosphate group, and together with residues Ser45, Lys49, Arg127, Tyr128, and Asn173.
located within helices H3, H5, and H7 could be responsible for the interaction between the C-terminal stretch and the ligand binding groove of the 14-3-3α molecule.

Location of the C-terminal Stretch in the Presence of the Ligand—Next, we tested whether C-terminal stretch changes its localization upon the binding of the phosphorylated peptide. In the presence of the phosphopeptide, one of the measured distances (Trp242-AEDANS-Cys25) increased significantly, whereas the second distance (Trp242-AEDANS-Cys189) remained practically unchanged. Residue Cys25 is located in the middle of the helix H2 (Fig. 5A), which is tightly packed with helices H1 and H3. In addition, helix H1 interacts with helices H3 and H4 of the second monomer and thus forms a dimer interface (5–10). The 14-3-3 molecule is very rigid because of an extensive number of interactions between the α-helices (5–10). Comparison of 14-3-3 protein structures in the absence of bound ligand (7, 8) and 14-3-3 protein complexes with bound phosphopeptides (5–7, 10) or serotonin N-acetyltransferase (9) revealed a negligible amount of movement of the α-helices. Therefore, any conformational change involving the helix H2, which can also (in theory) increase the average distance between Trp242 and AEDANS attached at Cys25, is very unlikely. Because both labeled 14-3-3α protein mutants are able to bind phosphorylated peptide (Fig. 1), we interpret the increase in the average distance between Trp242 and AEDANS attached at Cys25 for ~6 Å as a displacement of the C-terminal stretch from the ligand binding groove induced by phosphopeptide binding. The second Cys residue used as a site of attachment of AEDANS fluorophore, Cys189, is located at the beginning of the helix H8. Phosphopeptide binding had no effect on average distance between Trp242 and AEDANS attached at Cys189 (Table I). We suggest that the C-terminal stretch upon its displacement from the ligand binding groove by peptide binding adopts a new conformation just above the helix H9 (Fig. 5B). Only in this conformation, will our model fulfill the measured distances 23.5 and 36 Å obtained from FRET measurements.

In conclusion, our results indicate that, in the ligand-free form, the C-terminal stretch of 14-3-3 protein occupies the ligand binding groove. Binding of the phosphopeptide to the 14-3-3 protein displaces the C-terminal stretch from the ligand binding groove. In addition, molecular dynamics simulation suggests some similarities in the binding of the C-terminal stretch and phosphorylated peptides.

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14-3-3 Protein C-terminal Stretch Occupies Ligand Binding Groove and Is Displaced by Phosphopeptide Binding
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