

## **Proteins of the 14-3-3 family and cytoskeletal regulation**

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**Abstract.** Phosphorylation of serine, threonine, and tyrosine residues catalyzed by protein kinases is one of the widespread types of posttranslational modifications that affect the structure and properties of a protein. The transfer of the phosphoric acid residue is accompanied by a change in charge, which often leads to significant conformational rearrangements that affect the structure, properties, and functional activity of phosphorylated proteins. There are several families of proteins in the cell that recognize and specifically interact with certain regions of phosphorylated proteins. One of these families is represented by proteins 14-3-3, which were discovered more than 40 years ago during the systematic classification of nerve tissue proteins, where their content exceeds 1% of the proteome [1, 2]. To date, more than 300 different target proteins capable of interacting with 14-3-3 have been described [3]. Representatives of the 14-3-3 family are practically ubiquitous and act as regulators of apoptosis, cell cycle, division, transcription, replication, functioning of ion channels and transporters, and cytoskeleton organization. In addition, the number of reports on the involvement of 14-3-3 in the development of a number of neurodegenerative and oncological diseases has increased recently. All this aroused an increased interest in the proteins of this family. This review is devoted to the description of the structure, properties, and mechanisms of regulation of the activity of proteins of the 14-3-3 family, as well as the analysis of their interaction with substrate proteins and their possible participation in the regulation of the cytoskeleton.

**Keywords:** 14-3-3 proteins, cytoskeletal regulation

### **STRUCTURE AND PROPERTIES 14-3-3**

Proteins of the 14-3-3 family were first discovered in 1967 during the systematic classification of nerve tissue proteins [4]. These proteins got their somewhat strange name from the number of the fraction during ion-exchange chromatography of the cattle brain extract, the most enriched in the studied proteins, and from the position of these proteins during subsequent electrophoresis in starch gel. It was found that proteins of the 14-3-3 family have a low isoelectric point (4.0-4.5), that the molecular weight of monomer 14-3-3 is about 30 kDa, and that these proteins are expressed in large amounts in the brain [ five].

It was originally thought that 14-3-3 are mainly involved in the regulation of neurotransmitter synthesis. In 1987, it was found that 14-3-3 activate the key enzymes for the synthesis of serotonin and catecholamines - tryptophan- and tyrosine monooxygenases [6]. Analyzing the protein composition of crude preparation 14-3-3,

a heterogeneous sample of protein isolated from the brain was subjected to fractionation using the method of reverse phase liquid chromatography, and the obtained isoforms were designated with Greek letters (from  $\alpha$  to  $\sigma$ ) in accordance with the position of the peaks on the profile elution [7]. This separation revealed 9 isoforms 14-3-3 -  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ,  $\tau$  (or  $\theta$ ),  $\sigma$ . Later, it was found that  $\alpha$  is a  $\beta$  isoform phosphorylated at Ser184, and  $\delta$  is indistinguishable from a protein isoform phosphorylated at Ser184 (in this work, the numbering of amino acid residues corresponding to the numbering of human  $\zeta$  isoform 14-3-3 was used) [8]. It should be noted that in addition to the classic designation "14-3-3" and the corresponding Greek letter denoting the isoform, representatives of this family received additional names that reflected the peculiarities of their discovery. For example, Leonardo (14-3-3 $\epsilon$  Drosophila), Bilardo (14-3-3 $\zeta$  Drosophila), Stratifin (14-3-3 $\sigma$ ), BAP-1 (14-3-3 $\tau$  /  $\theta$ ), CBP (Cruciform-Binding Protein), KCIP-1 (protein kinase C inhibitor-1), Exo1 (stimulator of Ca<sup>2+</sup>-dependent exocytosis), GF14 (G-box binding factor of plants), etc. [five]. In the early 1990s, it was found that 14-3-3 can undergo phosphorylation [9], participate in the regulation of the activity of some protein kinases [10], and are expressed in many tissues of various eukaryotes [11-13]. After that, 14-3-3 proteins became the focus of attention of many laboratories, and the period of their intensive study began.

In 1995, the three-dimensional structure of the  $\zeta$  and  $\tau$  isoforms 14-3-3 in mammals was established [14, 15]. It turned out that 14-3-3 exist in the form of dimers, and 14-3-3 monomers can easily exchange, which leads to the formation of both homo- and heterodimers of the indicated protein [16]. Later it was found that the dimer structure is necessary for the normal functioning of 14-3-3, and the destabilization of dimers weakens their interaction with target proteins (Raf kinase and p53 protein) [17, 18].

Advances in the study of the structure and properties of 14-3-3 have led to an increased interest in members of this family and to an avalanche-like accumulation of data on substrate proteins and processes in the regulation of which proteins 14-3-3 can participate. In a short review, it is impossible to list all substrate proteins or to discuss in any detail the possible involvement of 14-3-3 in various processes in the cell. For this reason, we will restrict ourselves only to a brief description of the structure and properties of proteins of the 14-3-3 family and a discussion of some aspects of the regulation of the cytoskeleton and the contractile apparatus by proteins 14-3-3.

### ***ISOFORMS AND PHYLOGENY OF REPRESENTATIVES OF THE FAMILY 14-3-3***

Proteins 14-3-3 have been found in almost all eukaryotic organisms, with at least two 14-3-3 isoforms found in almost every representative. Probably, only in two cases - in the fungus *Candida albicans* and the simplest *Dictyostelium discoideum*, one isoform was found. It is interesting to note that initially only one isoform 14-3-3 was found in *Drosophila* [19], but later it was found that *Drosophila* also has at least two isoforms,  $\epsilon$  and  $\zeta$  [20].

Members of the 14-3-3 family are highly conservative. For example, the homology of 14-3-3 $\zeta$  of the clawed frog *Xenopus tropicalis* and humans according to the alignment of primary structures using the BLAST program

(<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) is about 90% ... Human 14-3-3 $\zeta$  coincides by 82% in sequence with its homologue from the silkworm *Bombyx mori* and by 81% with the analogous isoform 14-3-3 of *Drosophila* [21]. 14-3-3 $\omega$  from the *Arabidopsis thaliana* plant and its closest human homologue 14-3-3 are 75% identical. It should be noted that the homology of 14-3-3 isoforms isolated from tissues of the same species is even higher, and this creates significant difficulties in obtaining antibodies specific to specific isoforms [19]. As already mentioned, 7 isoforms 14-3-3 were found in human tissues: beta ( $\beta$ ), gamma ( $\gamma$ ), epsilon ( $\epsilon$ ), eta ( $\eta$ ), sigma ( $\sigma$ ), tau / theta ( $\tau / \theta$ ) and zeta ( $\zeta$ ) [7]. The previously described alpha ( $\alpha$ ) and delta ( $\delta$ ) isoforms are phospho related derivatives of the beta ( $\beta$ ) and zeta ( $\zeta$ ) isoforms, respectively [8]. Isoforms 14-3-3 differ in the structure of short variable regions, although they are not products of alternative splicing, and each isoform is encoded by a separate gene [19]. The genes for the different isoforms 14-3-3 are usually located on different chromosomes. For example, the 14-3-3 $\eta$  gene is located on the 22nd, and 14-3-3 $\beta$  - on the 20th human chromosome [22]. This is in good agreement with the data obtained by Aitken in the analysis of the Human Genome Database, according to which the  $\beta$  and  $\eta$  isoforms are encoded by genes located on the 20th and 22nd chromosomes, respectively, and the  $\sigma$  genes are located on 1- oh,  $\tau / \theta$  - on the 2nd,  $\gamma$  - on the 7th, and  $\epsilon$  - on the 17th chromosomes. The main gene giving the complete product 14-3-3 $\zeta$  is localized on chromosome 8 [19]. In addition to “working” genes, a large number of pseudogenes that are not involved in the expression of functional proteins have been found in the structure of human chromosomes [5]. So, for the  $\epsilon$  isoform, pseudogenes were found located on the 2nd and 7th, and for the  $\zeta$  - on the 5th, 6th, 9th, 11th and 15th chromosomes. The sequence corresponding to the  $\sigma$  isoform 14-3-3 also occurs more than once in the human genome [62]. The search for the main “working” genes for different isoforms 14-3-3, which we carried out using the BLAT program of the Human Genome Browser Internet resource ([http:// genome.ucsc.edu/cgi-bin/hgBlat?command=start](http://genome.ucsc.edu/cgi-bin/hgBlat?command=start)), confirms the presence of the human genome of several pseudogenes. Probably, such forms of sequences were formed during the incorporation of regions into chromosomes.

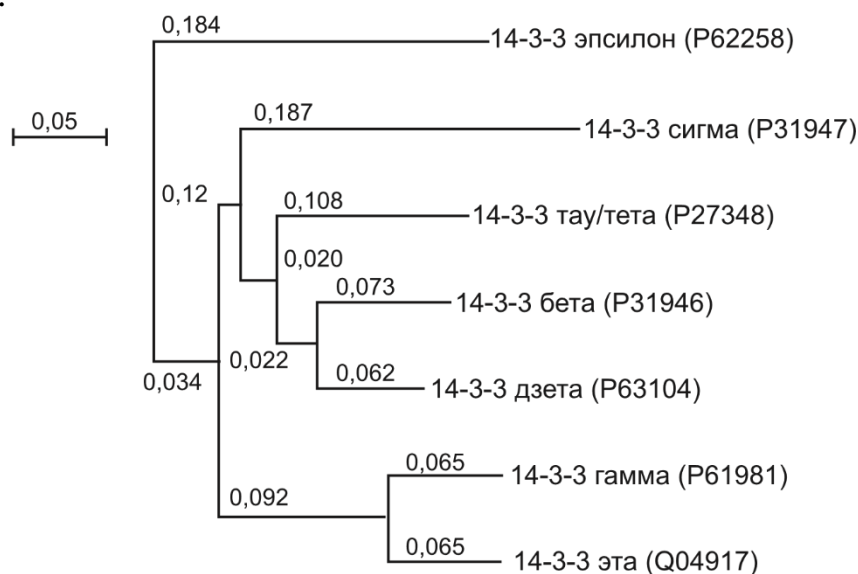


Fig. 1. Phylogenetic tree of seven human isoforms 14-3-3, constructed using the ClustalW algorithm.

The identification numbers of the corresponding isoforms in the UniprotKB database are indicated in brackets. The scale of 0.05 denotes the number of substitutions in the evolution of the ancestral polypeptide sequence per amino acid residue.

DNA obtained by reverse transcriptase of retroviruses from mRNA 14-3-3 present in the cell. In fig. 1 shows a phylogenetic tree, reflecting the degree of homology of the primary structures of various isoforms 14-3-3 humans. The tree is built on the basis of multiple alignment of amino acid sequences using the program

ClustalW v.1.83, available on the Internet (<http://www.ebi.ac.uk/Tools/clustalw/index.html>). The alignment was carried out on the basis of the gon net and blosum matrices with the parameters gap open = 10 and extension gap = 0.05 and gave the same results for both matrices, which are presented as a tree in the NJplot program (<http://pbil.univ-lyon1.fr/software/njplot.html>). As can be seen from the diagram, the most similar are the primary structures  $\eta$  and  $\gamma$ ,  $\beta$  and  $\zeta$ , as well as  $\sigma$  and  $\tau$  /  $\theta$  isoforms. The presented data, most likely, indicate the presence of a common ancestor, which gave rise to six isoforms 14-3-3. At the same time, the  $\varepsilon$  isoform belongs to the evolutionary branch, which is strongly separated from the common trunk  $\eta$  /  $\gamma$  /  $\beta$  /  $\zeta$  /  $\sigma$  /  $\tau$ . It is believed that the 14-3-3 $\varepsilon$  sequence underwent the greatest changes in the course of evolution and is most different from other isoforms 14-3-3 in humans [23].

#### ***DISTRIBUTION OF PROTEINS 14-3-3 IN HUMAN TISSUE***

Proteins of the 14-3-3 family are expressed locally in the human body. The radioimmunochemical method made it possible to establish a high level of their expression in extracts of the brain, testes and intestines, where the concentration of 14-3-3 exceeds 0.5% of soluble proteins and in the brain can reach 1.3% of soluble proteins (i.e., about 40  $\mu$ M per monomer) [1]. At the same time, in the kidneys and hemolysate, the content of 14-3-3 was minimal and amounted to only about 0.008% of soluble proteins [1]. The presence of all 7 isoforms of 14-3-3 in the cornea and mucous membrane of the eye, as well as  $\zeta$  and  $\gamma$  isoforms in human lacrimal fluid, was established by the method of immunofluorescence and Western blotting [61].

Currently, 14-3-3 is considered as a promising marker of various neurodegenerative and oncological diseases [25, 26]. Indeed, it turned out that the concentration of 14-3-3 in the cerebrospinal fluid is greatly increased in various types of dementia, encephalopathy and tumors of the central nervous system. An increase in the concentration of 14-3-3 in the cerebrospinal fluid was also noted in Creutzfeldt-Jakob disease [23, 27]. In tuberculous meningitis and multiple sclerosis, the concentration of 14-3-3 in CSF can reach 100-130 ng / ml [1]. The concentration of 14-3-3 is also increased in some forms of cancer [28–30]. All this makes it possible to consider 14-3-3 as a promising marker protein for various human diseases.

#### ***STRUCTURAL ORGANIZATION 14-3-3***

To date, the structure of all seven human 14-3-3 isoforms has been determined [31, 32]. It was found by X-ray diffraction analysis (XRD) that the 14-3-3 subunits form a dimer resembling a somewhat oblate horseshoe with an internal channel about 20

Å deep and about 35 Å wide (Fig. 2) [14, 15]. The linear dimensions of such a dimer structure, calculated in the PyMOL v.1.1 program. based on X-ray diffraction data, 14-3-3ζ (identification number PDB 1QJB) are about 55-60 Å in width, about 75-80 Å in length and about 35-40 Å in height. The main element of the 14-3-3 structure is α-helices. According to theoretical predictions made with the Psipred and Sable2 programs, α-helices account for up to 65–75% of the 14-3-3 structure.

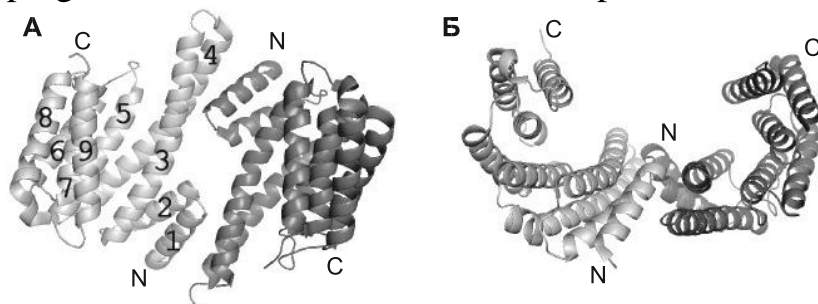


Fig. 2. α-Helical structure of the 14-3-3 dimer.

Built in the PyMOL program based on the X-ray structural data of 14-3-3ζ (PDB 1QJB). A - top view of the dimer, B - side view of the dimer. The two subunits are marked in dark and light gray. The numbers indicate the numbers of the α-helices in the composition of the monomer 14-3-3.

In conclusion, the experimental data obtained by the circular dichroism method [33, 34] and the data of X-ray diffraction analysis (PDB 1QJB) are in good agreement, indicating that about 70% of the 14-3-3 structure is represented by α-helices. Each monomer 14-3-3 is built of 9 α-helices numbered with Arabic numerals from N-terminal α1 to C-terminal α9 (Fig. 2). The α5 – α9 helices form the side walls of the bowl-shaped dimer. The amino acid residues of the α1 – α4 helices form the “bottom” of the dimer and the contact area of the subunits, while the α1 and α2 helices of one polypeptide chain contact the α3 and α4 helices of the other chain, so that the subunits in the 14-3-3 dimer are located antiparallel. The α3 and α4 helices are the longest, their length is about 55 Å, while the α1 and α2 helices are the shortest and do not exceed 25 Å in length (Fig. 2). As noted, a dimer structure is required for 14-3-3 to function properly. The stabilization of the dimeric state of 14-3-3ζ, in all likelihood, is carried out by three salt “bridges” - Arg18-Glu89, Glu5-Lys74, Asp21-Lys85 (there are 6 such “bridges” in the dimer) and several weakly polar or hydrophobic interactions, in which residues Leu12, Ala16, Ser58, Val62, Ile65, and Tyr82 (Fig. 3) [62]. The first salt “bridge” of Arg18-Glu89 and all the weak contacts mentioned above are present in the structure of homodimers of all 14-3-3 human isoforms, the second “bridge” Glu5-Lys74 is absent in the σ, η, ε and γ isoforms. The third “bridge”, Asp21-Lys85, participates in the formation of dimers of all isoforms, except for ε. For the σ isoform.

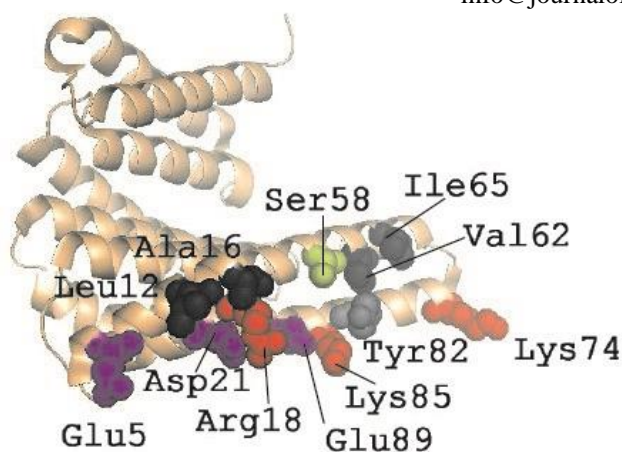


Fig. 3. Area of contact of subunits 14-3-3 $\zeta$ .

Built in the PyMOL program based on the X-ray structural data of 14-3-3 $\zeta$  (PDB 1QJB). Monomer 14-3-3 is shown in beige spirals, uncharged amino acid residues involved in dimerization are marked in black and gray, and serine-58 residue involved in the regulation of the dimeric state 14-3-3 is marked in green. Residues that form salt "bridges" between monomers 14-3-3 are marked in red (positively charged) and violet (negatively charged) colors.

an alternative salt "bridge" Lys9-Glu83 [32]. The X-ray diffraction data were confirmed by the results obtained using point mutagenesis. Thus, it was found that mutations of residues 5 and 12 in  $\alpha$ 1-, as well as residues 82, 85, and 87 in  $\alpha$ 4-helices of 14-3-3 $\zeta$  lead to disruption of intersubunit contacts in this protein [35]. Similar results were obtained when analyzing the structure of the  $\sigma$  isoform. It turned out that mutation of residues 5 in  $\alpha$ 1-, 20 in  $\alpha$ 2-, and 55 in  $\alpha$ 3- and 80 in  $\alpha$ 4-helices leads to a change in the inter subunit contacts for this isoform 14-3-3 and a change in the interaction of the  $\sigma$  isoform with other isoforms of this protein [35]. According to many researchers, an important role in the formation of 14-3-3 dimers is played by the Ser58 residue (Fig. 3) located on the  $\alpha$ 3-helix of each monomer  $\zeta$ ,  $\beta$ ,  $\epsilon$ ,  $\gamma$ , and  $\eta$  isoforms ( $\sigma$  and  $\tau$  isoforms in alanine have this position). It was shown in different laboratories that phosphorylation of this residue can strongly affect the state of the 14-3-3 dimer [18, 36, 60].

It has been suggested that one of the factors influencing the specificity of 14-3-3 isoforms upon interaction with certain substrates is the ability of 14-3-3 proteins to form homo- and heterodimers of various compositions [16, 59]. Moreover, it turned out that only certain isoforms 14-3-3 are capable of forming heterodimers. For example, in the region of dimerization of the 14-3-3 $\sigma$  molecule, non-conserved residues Ser5, Glu20, Phe25, Gln55, and Glu80 were identified, which ensure the formation of homodimers, but prevent the formation of heterodimers. This structural feature leads to the uniqueness of the 14-3-3 $\sigma$  properties [35, 58].

There is a hypothesis according to which the ability of 14-3-3 to form heterodimers from monomers of various isoforms significantly expands the range of potential partner proteins of 14-3-3. It is possible that the formation of heterodimers from  $\epsilon$  and  $\zeta$  or from  $\tau$  and  $\zeta$  isoforms is necessary for the binding of certain substrates or opens up the possibility for the interaction of such partner proteins that do not in themselves

interact with each other. For example, Liang et al. showed that for the binding of the Nedd4-2 protein, which has several binding sites 14-3-3 and is involved in aldosterone-mediated regulation of sodium channels, it is necessary to form precisely the heterodimers of  $\beta$  and  $\epsilon$  isoforms 14-3-3 [59]. The formation of 14-3-3 heterodimers is also necessary for the binding of cruciform DNA structures, which are often located near the origin of replication and partially determine the level of replication [57].

Analysis of primary structures in order to search for similar domains, carried out by us using the InterProScan program (<http://www.ebi.ac.uk/InterProScan>), did not reveal any significant similarity of proteins of the 14-3-3 family with domains of other proteins available in InterPro's extensive compound database. However, Ostrerova et al. it has been shown that members of the 14-3-3 family have some homology with the protein synuclein, which is abundantly present in the brain. Analysis of the primary structure of 14-3-3 and synucleins revealed two short regions with a homology of about 40% [61]. In addition, it was found that synuclein and 14-3-3 interact with each other and are able to bind the same target proteins, such as protein kinase C and Bad. Based on these data, it was suggested that 14-3-3 and synucleins have similar functions [56].

In a certain contradiction with this are the results of Korean researchers [52], who found similarities in the structure of completely different regions of synuclein and 14-3-3. The sequences of 14-3-3 and synuclein revealed in this work have a certain similarity with the  $\alpha$ -crystallin domain of small heat shock proteins. This is probably the reason why both synuclein and 14-3-3 can have chaperone-like activity [55].

Recently, it was shown that in the structure of 14-3-3 GF14 $\omega$  (general factor 14-omega) of plants homologous to human 14-3-3 $\zeta$  there is a sequence reminiscent of the canonical "EF-hand", which effectively binds divalent metal ions (Ca<sup>2+</sup> and Mg<sup>2+</sup>) [43]. When comparing the primary structures of these proteins using the BLAST program in 14-3-3 $\zeta$ , we also found the -ELDTLSEESYKD- sequence, which coincides with the sequence in GF14 $\omega$  and vaguely resembles the "EF-hand". Nevertheless, experimental data obtained in the Aitken group indicate that 14-3-3 proteins are unable to bind calcium ions [53].

When comparing the structures of 14-3-3, p53, and I $\kappa$ B $\alpha$ , it was found that the  $\alpha$ 9-helix located at the C-terminus of the molecules of all 14-3-3 isoforms in humans contains a 13-membered peptide, the primary structure of which corresponds to the canonical NES ( Nuclear Export Sequence) sequence that is the signal for export from the kernel [54]. It was suggested that the presence of this signal determines the ability of 14-3-3 to bind other proteins in the cytoplasm [54, 53]. However, it was later found that members of the 14-3-3 family do not contain the functional sequence of NES, and the regulation of protein transport between the nucleus and the cytoplasm, mediated by 14-3-3, is carried out only through shielding or exposure of the corresponding signals on the surface of substrate proteins [52, 56]. Searches in structure 14-3-3 for a characteristic sequence NLS (Nuclear Localization Sequence), which determines the movement of proteins into the nucleus, were unsuccessful [47]. Some authors believe that the ability of 14-3-3 to penetrate into the nucleus is due to the formation of strong complexes of 14-3-3 with substrate proteins that have

an NLS sequence and are easily transferred by the protein-importing system of the nucleus. On the other hand, one cannot exclude the possibility that the structure of 14-3-3 contains a certain nonstandard sequence responsible for the movement from the cytoplasm into the cell nucleus, which has not yet been detected [58].

The search for domains similar to certain regions of 14-3-3 in terms of tertiary structure revealed the so-called TPR-domain (Tetratricopeptide Repeat Domain) in the C-terminal region of 14-3-3 [51]. Such modules in the composition of different proteins are distinguished both on the basis of similarity

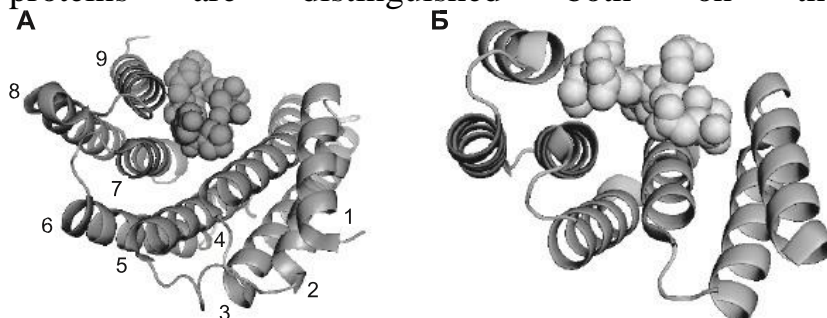


Fig. 4. Comparison of the tertiary structures of the monomer 14-3-3, which binds phosphopeptide (A), with the TPR domain of the Hop protein interacting with the Hsp70 fragment (B).

For construction, the PyMOL program and X-ray diffraction data 14-3-3 (PDB 1QJB) and Hop (PDB 1ELW) were used.  $\alpha$ -helices 14-3-3 (numbered from 1 to 9), as well as protein Hop, are shown as a ribbon model; the spheres indicate the ligands bound.

Primary and secondary and tertiary structures. The elementary unit of TPR domains, as a rule, are 34-amino acid repeats, which form pairs of antiparallel  $\alpha$ -helices separated by a turn. The presence of a large number of such repeats can lead to the formation of several  $\alpha$ -helices, which turn out to be rotated relative to each other at a certain angle. In fig. 4 shows, as an example, a comparison of the structure of monomer 14-3-3 with a fragment of the TPR domain of the p60 protein, or Hop, which binds the chaperones Hsp70 and Hsp90. Such tertiary structure elements are ideal for binding a wide variety of ligands and have been found in more than 800 proteins that perform various functions ([http://pawsonlab.mshri.on.ca/index.php?option=com\\_content&task=view&Itemid=64&id=183](http://pawsonlab.mshri.on.ca/index.php?option=com_content&task=view&Itemid=64&id=183)) ... Apparently, TPR domains 14-3-3, built by the C-terminal  $\alpha$ -helices of interacting subunits, take part in the positioning of target proteins when they bind in the internal channel 14-3-3 /

### ***MECHANISMS OF INTERACTION 14-3-3 WITH TARGET PROTEINS. SEQUENCES RECOGNIZED 14-3-3***

Numerous studies have shown that phosphorylation of various proteins at Ser and Thr residues leads to an increase in their binding to 14-3-3. To date, proteomic studies have identified more than 300 14-3-3 partner proteins [43]. It turned out that the interaction of 14-3-3 with other proteins occurs on the basis of recognition of certain consensus sequences in the composition of ligand molecules. Members of the 14-3-3 family firmly bind to a wide variety of proteins, the structure of which contains at least one of two motifs containing phosphoserine (pS) - RSXpSXP (motif I) or RX



(Y / F) XpSXP (motif II) ... The serine residue in these motifs can be replaced by a phosphorylated threonine residue [50]. In addition, the so-called motif III, recognized by proteins 14-3-3, has been described. The structure of this motif can be represented as pSX1-2-COOH, where X is any amino acid, except proline, located at the C-terminus of some 14-3-3 partner molecules [57]. It is suggested that if the binding site 14-3-3 is located in the middle of the substrate molecule, then at position +2 relative to the phosphorylated serine / threonine residue there should be a proline (or glycine) residue (motifs I and II). The presence of proline (or glycine) residues at these positions provides a kink (or sufficient mobility) in the polypeptide chain, which is necessary for the phosphorylated site to fit into the 14-3-3 channel. In the case of motif III, the binding site is located at the C-terminus of the target protein and therefore does not require the presence of proline (or glycine) at position +2 from phosphorylated serine or threonine residues and significantly expands the repertoire of proteins interacting with 14-3-3 [49].

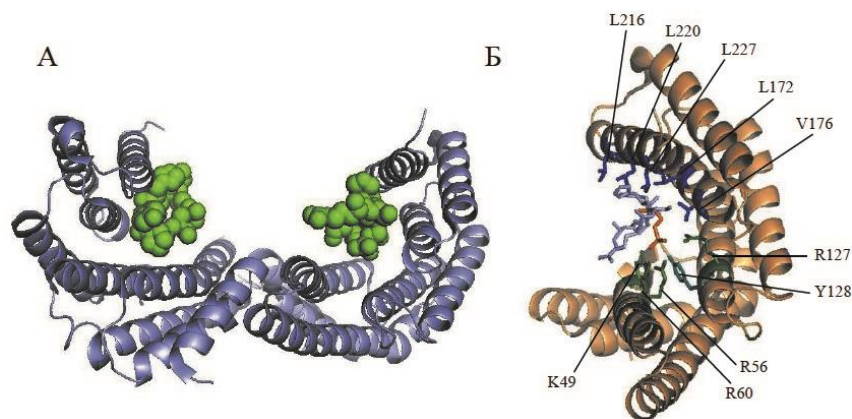


Fig. 5. Complex formation between 14-3-3 and phosphopeptides.

Built in the PyMOL program on the basis of X-ray structural data 14-3-3 $\zeta$  (PDB 1qQJB). A - helical model of the 14-3-3 $\zeta$  dimer complex (gray helices) with two bound phosphopeptides (marked in green). B - device of the amphipathic groove of the monomer 14-3-3 (beige spirals), inside which ligands are bound. Amino acid residues of the hydrophobic surface of the groove are marked in blue, residues of a positively charged "pocket" are marked in green. The numbering of the residues corresponds to the  $\zeta$  isoform 14-3-3. The phosphorylated residue in the ligand is shown in orange; the rest of the phosphopeptide is shown in gray.

It is interesting to note that in addition to the Ser / Thr residue, which occupies a central position in motifs I and II, the serine residue at position -2 can also be phosphorylated. Such a rather exotic way of regulation was described when studying the interaction of 14-3-3 with p53 and CDC25C, while phosphorylation of the residue at position -2 led to a weakening of the binding of 14-3-3 [48].

Thanks to the use of model phosphopeptides and point mutagenesis, not only the consensus sequences themselves, with which 14-3-3 interact preferentially, were determined, but also those amino acid residues 14-3-3 that are involved in their binding were determined (Fig. 5) [44, 46].

It turned out that the binding of the phosphopeptide involves the amino acid residues of the amphipathic groove (Fig. 5A), which is present in the structure of each L-shaped monomer 14-3-3. The main role in the retention of the substrate protein phosphopeptide is played by a special “pocket” built with the participation of positively charged amino acids K49, R56, R60, and R127, as well as the Y128 residue. These residues are involved in the coordination of phosphoserine -pS- in the bound peptide (Fig. 5B) [42, 43]. Zhang et al. demonstrated the necessity of these residues for the interaction of 14-3-3 with partner proteins by the example of Raf-1 and ExoS proteins [49]. Hydrophobic residues of  $\alpha$ -5, 7, and 9 helices located on the inner surface of the walls of dimers 14-3-3 (Fig. 5B) are responsible for the positioning of the substrate molecule and for the stabilization of the formed complex [45]. Mutation of the residues Leu172, Val176, and Leu220 belonging to the  $\alpha$ 9-helix leads to a strong weakening of the interaction of 14-3-3 with Raf-1 [41]. Substitutions Leu216 and Leu227 also lead to a weakening of the binding of Raf-1 to 14-3-3, but to a lesser extent [38]. Amino acid residues lining the inner surface of 14-3-3 dimers are highly conserved [39]. Apparently, this indicates the general principle of ligand recognition by proteins of the 14-3-3 family [37].

As already noted, 14-3-3 forms a stable dimer containing in its structure two potential binding sites for substrate proteins. This allows 14-3-3 to simultaneously bind two different substrate proteins or to cooperatively bind one protein-substrate having two binding sites with 14-3-3. Yaffe et al. found that the presence of two sites simultaneously containing phosphoserine and capable of interacting with 14-3-3 in the structure of the ligand to be bound increases the strength of the complexes by more than 30 times [36]. Similar results were obtained by Obzil et al., Who showed that the fragment of the transcription factor FOXO411-213 phosphorylated at residues Thr28 and Ser193 binds to the 14-3-3 dimer with very high affinity (Kd about 30 nM), while the affinity phosphorylated at only one of the indicated FOXO411-213 residues to 14-3-3 is at least an order of magnitude lower (Kd 290-650 nM) [11]. Phosphorylation of two residues Ser346 and Ser368 in the composition of protein kinase C (PKC $\epsilon$ ) leads to a significant increase in the binding to 14-3-3, as compared to the binding of the enzyme phosphorylated only at one of the indicated centers [42].

Partner proteins may contain one or more regions recognized by 14-3-3. It has been suggested that the presence in the structure of the target protein of one of the centers with a structure similar to the aforementioned consensus sequences provides for the recruitment of 14-3-3 (the so-called gatekeeper phosphorylation site). In this case, a not very strong complex is formed, in which 14-3-3 does not have a sufficiently strong effect on the structure and properties of the partner protein. If the structure of the partner protein contains the second binding site 14-3-3, then the strength of the complex increases sharply and, in parallel, the effect of 14-3-3 on the structure and properties of the partner protein increases [34]. Most proteins interacting with 14-3-3 have the above (or at least similar to them in primary structure) motifs containing phosphoserine. At the same time, quite a few proteins have been described in the literature that do not contain the motifs described above, and even do not contain phosphorylated Ser or Thr residues, and nevertheless form strong complexes with 14-

3-3. An example of such an interaction is the toxic exoenzyme S (ExoS) (ADP-ribosyltransferase) from *Pseudomonas aeruginosa*, which binds to 14-3-3 via the 424DALDL428 sequence [28], which is not similar to the consensus binding site. Other examples include phosphatase CDC25B [32] and protein kinase SGK1 activated by serum and glucocorticoids [31], the binding of which to 14-3-3 does not depend on phosphorylation.

The 14-3-3 structure apparently contains several centers that take part in the binding of target proteins. For example, it turned out that the 14-3-3 molecule has an additional region located in the 7th  $\alpha$ -helix (residues 163-187), which is involved in the interaction with Raf-kinase [27]. Binding of phosphorylated tryptophan hydroxylase is mainly due to contacts formed by residues 171-213 of the  $\eta$  isoform 14-3-3 [29]; due to residues remote from the above-described traditional binding site for phosphopeptides. Finally, the interaction of 14-3-3 $\zeta$  with the von Willebrand factor platelet receptor (GPIb-IX complex and the cytoplasmic domain GPIb $\alpha$ ) occurs in the region of the  $\alpha$ 9-helix 14-3-3 (residues 202-231), which is significantly different from that which determines the binding consensus sequences containing phosphoserine / phosphothreonine [26].

Thus, in the structure of 14-3-3 there are several different binding sites for substrate proteins, and the principles of binding in these sites can be quite noticeably different from each other. All this may be due to the multipoint character of protein – protein interactions and a significant difference in the structure and properties of substrate proteins interacting with 14-3-3.

#### ***PARTICIPATION OF 14-3-3 IN THE REGULATION OF MOTOR PROTEINS ASSOCIATED WITH ACTIN FILAMENTS***

The contractile activity of smooth muscles and many nonmuscular cells is regulated by phosphorylation of myosin regulatory light chains [14, 26]. In smooth muscle and non-muscle cells, the productive interaction of myosin with actin and the contraction process become possible only after phosphorylation of the regulatory light chain of myosin under the action of a specific kinase of myosin light chains (MLCK). Relaxation requires dephosphorylation of myosin light chains, which is provided by a specific myosin light chain phosphatase (MLCM). Thus, the contractile activity of smooth muscles and non-muscle cells is under the coordinated control of two antagonist enzymes - myosin light chain kinase and phosphatase.

Myosin light chain phosphatase consists of 3 subunits. A subunit with a molecular weight of 105–120 kDa provides attachment of the enzyme to myosin filaments; the catalytic subunit (molecular weight of about 36–37 kDa) provides proper dephosphorylation of myosin light chains; a subunit with a molecular weight of 20–21 kDa performs some not completely understood regulatory function [25]. It turned out that the largest subunit of myosin light chain phosphatase can be phosphorylated by several protein kinases (in particular, Rhokinase) and this leads to inhibition of the enzyme activity [15]. A detailed study made it possible to establish that phosphorylation of the largest phosphatase subunit of myosin light chains at the Ser472 residue, catalyzed by Rho kinase, provides strong binding of this subunit to 14-3-3 $\beta$ , which results in the dissociation of phosphatase from the myosin filament and inhibition of phosphatase activity [ 17]. Thus, 14-3-3 not only removes

phosphatase from myosin filaments, but also inhibits its activity. Both of these processes lead to an increase in the degree of phosphorylation of myosin regulatory light chains and an increase in contraction or retardation (or prevention) of relaxation.

As noted earlier, 14-3-3 effectively interacts and regulates the activity of a large number of different protein kinases [8, 21]. In particular, it has long been known that 14-3-3 is able to influence the activity of protein kinase C [19, 22]. One of the isoforms of protein kinase C is capable of phosphorylating the heavy chains of myosin of *Dictyostelium discoideum* and thus regulating the ability of myosin to form ordered filaments. It was found that 14-3-3 forms a strong complex with the indicated protein kinase C isoform and thus can indirectly participate in the regulation of the contractile activity of *Dictyostelium* [10]. Summarizing this section, we can conclude that, although proteins of the 14-3-3 family, in all likelihood, do not directly interact with myosin, they are able to efficiently regulate the activity of this protein.

### ***INTERACTION OF 14-3-3 WITH SMALL G-PROTEINS AND PROTEINKINASES INVOLVED IN CYTOSKELETAL REGULATION***

Small G proteins are involved in the transmission of hormonal signals from receptors into the cell. Among the small G-proteins belonging to the Ras family, a small group of proteins united in the RGK family is distinguished, named after the first letters of the names of the four proteins belonging to this family - Rem (also referred to as Rem1 or Ges), Rem2, Rad and Gem / Kir [12, 27]. Proteins belonging to the RGK group are quite different from other proteins of the Ras family, and, unlike other proteins of the Ras family, do not contain fatty acid residues that ensure stable attachment to the membrane, have low GTPase activity and unique N- and C-terminal sites [6]. Proteins of the RGK group are polyfunctional and can interact with a large number of partner proteins [3]. Thus, proteins of the RGK group interact with the  $\beta$ -subunit of voltage-dependent calcium channels and inhibit the functioning of these channels. The GTP binding sites and the sites involved in the binding of the  $\beta$ -subunit of the calcium channel overlap, and the completeness of inhibition depends on the presence of a guanyl nucleotide in the active center of RGK, as well as on the binding of calcium-saturated calmodulin in the site located in the C-terminal part of the RGK molecule (Fig. . 8) [29, 30]. In addition, proteins of the RGK group can be phosphorylated at several sites, while two serine residues located at the very N- and at the very C-terminus are recognized by proteins 14-3-3 [21]. Thus, after phosphorylation, a strong bidentate interaction between 14-3-3 and proteins of the RGK group becomes possible [32]. Interaction with 14-3-3 can lead to a redistribution of proteins of the RGK family between the nucleus and the cytosol, an increase in their stability, and conformational changes in the GTP-binding site of these proteins [16, 30]. RGK proteins (both in free form and in a complex with 14-3-3) interact and affect the activity of Rho-dependent protein kinases [29]. The  $\beta$  isoform of Rho kinase phosphorylates and activates myosin light chain kinase, as well as LIM kinase, which is involved in the phosphorylation of cofilin. In addition, Rho kinase phosphorylates and inhibits the activity of myosin light chain phosphatase (Fig. 8) [19]. Apparently, the  $\alpha$ -isoform of Rho kinase is also involved in the regulation of these enzymes. If shown in Fig. Scheme 8 is correct, the proteins of the

RGK group are able to influence the formation of stress fibrils, focal contacts, and changes in cell shape. Considering the fact that 14-3-3 affects the stability of proteins of the RGK family and their intracellular distribution, it can be concluded that 14-3-3 can have a multifaceted indirect effect on both the formation of the cytoskeleton and various forms of cell motility. Previously, we analyzed in detail the interaction of 14-3-3 with various isoforms of integrins and the role of 14-3-3 in signal transduction from integrins to adapter proteins such as filamin, talin, or tensin. In all likelihood, the picture is further complicated by the fact that focal contacts contain not only adapter proteins, but also small G-proteins and certain protein kinases. It seems to us that by now many questions concerning the role of 14-3-3 in signal transmission in the field of focal contacts remain insufficiently clear. Nevertheless, we will try to discuss some of the existing hypotheses. Insulin and many growth factors, by binding to their receptors, lead to the activation of phosphoinositide 3-kinase, which ensures the synthesis of phosphatidylinositol-3,4,5-trisphosphates (PIP3). The formation of PIP3 promotes membrane transition and activation of protein kinase Akt, which can phosphorylate a wide variety of substrate proteins, including integrin subunits, the Guanine nucleotide Exchange Factor (GEF) of the small G protein Rac-1, and Pak protein kinase [133, 134]. The sites phosphorylated by Akt and having the primary structure RXXRXX (S / T) [5] are often binding sites for various isoforms 14-3-3.

RGK proteins can interact with calcium-calmodulin and bind GTP, and this can lead to a change in the intracellular distribution of RGK and inhibition of the voltage-gated calcium channel. In addition, RGK proteins can undergo phosphorylation, and this leads to their strong interaction with 14-3-3. Proteins of the RGK family affect the activity of Rho kinases, which are capable of phosphorylating and increasing the activity of myosin light chain kinase (MLCK), phosphorylating and inhibiting the activity of myosin light chain phosphatase (MLCK), and phosphorylating and increasing the activity of LIM kinase (LIMK) involved in the phosphorylation of cofilin. By controlling the activity of Rho kinases, the RGK-14-3-3 complex affects the formation of stress fibrils and focal contacts, as well as the shape and mobility of cells. In particular, in the region of focal contacts, the small G-protein Rac1 [4] is bound, which is in an activated state, since its activating factor GEF is present in the same place. Activated Rac1 in some incompletely understood way can activate protein phosphatases, which, by dephosphorylating cofilin, promote the disassembly of actin filaments, thereby increasing cell motility. On the other hand, Rac1 in a complex with 14-3-3 $\beta$  increases the activity of p21 activated protein kinase (PAK), which can also lead to rearrangement of the cytoskeleton [7]. In addition, recently published data indicate that the Kank protein may be located in the region of focal contacts [1]. This protein can inhibit actin polymerization and can be phosphorylated by the Akt protein kinase. Phosphorylated Kank, interacting with 14-3-3, competes with other target proteins of 14-3-3 in the region of focal contacts, which can also lead to rearrangement of the actin cytoskeleton [34]. Concluding this section, we can conclude that 14-3-3 is capable of interacting with many G-proteins and protein kinases, participating to one degree or another in the rearrangement of the cytoskeleton and thus exerting an indirect effect on intercellular interactions, cell attachment to the extracellular matrix and cell motility.

### ***14-3-3 AND INTERMEDIATE PROTEINS***

It is generally accepted that the cell cytoskeleton is formed by three systems of filaments - microfilaments (actin filaments with a diameter of about 8 nm), microtubules assembled from tubulin heterodimers (a diameter of about 25 nm), and so-called intermediate filaments with a diameter of about 10–12 nm [53, 24]. There are six classes of intermediate filament proteins. Including, two classes of cytokeratins (the so-called acidic and alkaline keratins); proteins of muscle, mesenchymal and glial intermediate filaments (desmin, vimentin); neurofilament proteins; lamins that form the membrane of the nucleus, and proteins of the so-called "orphan" intermediate filaments of the lens of the eye [33, 14].

The proteins of intermediate filaments have a similar structure, consisting of a highly conserved central part, prone to the formation of supercoils (structures of the coiled-coil type) and N- and C-terminal domains of different length and structure [55]. Dimers of intermediate filament proteins (stabilized by the formation of coiled coil structures) assemble into mature intermediate filaments, in which contacts between adjacent dimers are provided by variable N and C terminal sequences. Despite the fact that intermediate filaments have very high stability and mechanical strength, these elements of the cytoskeleton are characterized by a constant and dynamic process of subunit exchange [34]. Subunit exchange, as well as the processes of assembly and disassembly of intermediate filaments, depend on the phosphorylation of residues located in the N- and C-terminal variable regions of proteins of intermediate filaments [46], while, as a rule, phosphorylation destabilizes protein – protein interactions and promotes disassembly of filaments. In early studies, it was found that in the S / G2 / M phases, some cytokeratins (cytokeratins K8 / K18) undergo hyperphosphorylation (it is possible that the protein kinase cdc2 plays an important role in this) [27]. Phosphorylated cytokeratin K8 / K18 interacts with 14-3-3 and the formation of such a complex prevents the incorporation of cytokeratin into intermediate filaments. Further studies showed that phosphorylation of Ser33 of cytokeratin K18 is a prerequisite for binding 14-3-3 [8]. It should be noted that the sequence near Ser33 (RPVSSAAS33VY) is quite different from the consensus sequence recognized by 14-3-3 in other substrate proteins. It was suggested that the binding of 14-3-3 can affect both the intracellular distribution of cytokeratins and their ability to form intermediate filaments [5]. At the same time, data appeared indicating that treatment of cells with a protein phosphatase inhibitor, caliculin A, is accompanied by an increase in the proportion of phosphorylated vimentin. After phosphorylation of the sites located in the N-terminal part of the protein (residues 1–96), vimentin is able to interact with 14-3-3 [30]. Due to the fact that the formed complex is very strong, phosphorylated vimentin effectively competes with other proteins-substrates 14-3-3, and, as a result, it becomes possible to redistribute 14-3-3 in the cell and change the intensity of various processes, adjustable 14-3-3 [10].

Recently, additional information has appeared on the interaction of 14-3-3 with intermediate filament proteins. Glial fibrillary acidic protein (GFAP) together with vimentin forms intermediate filaments and plays an important role in the formation of the cytoskeleton of astrocytes. It turned out that both in the cytoplasm and in the glial fibrillar protein, which is part of the intermediate filaments, is capable of

interacting with 14-3-3. The formation of the complex depends on the phosphorylation of Ser8 in GFAP [41]. Interestingly, the primary structure near Ser8 (RRITS8AR) is quite different from the primary structure of the consensus regions recognized by 14-3-3 in other substrate proteins, and from the region recognized by 14-3-3 in the case of cytokeratins. As in the case of cytokeratins, binding of 14-3-3 complicates the incorporation of glial fibrillar protein into intermediate filaments and thus significantly affects the dynamics of assembly and disassembly of these filaments and their integrity [11]. It should be emphasized that the share of intermediate filament proteins accounts for at least 1% of cellular proteins [18]. Considering this fact, it can be assumed that phosphorylation of proteins of intermediate filaments can lead to the binding of large amounts of 14-3-3 and a change in the activity of protein phosphatases (cdc25 protein phosphatase) and protein kinases (Raf, Akt), which in one way or another depend on proteins of the family 14-3-3 and are regulated by them [13, 54]. Thus, interacting with proteins of intermediate filaments, 14-3-3 is able to regulate both the assembly and disassembly of important elements of the cytoskeleton and numerous processes that depend on the phosphorylation and dephosphorylation of various intracellular proteins.

### ***CONCLUSION***

Concluding the review, we can conclude that proteins of the 14-3-3 family are universal adapters capable of interacting with numerous and diverse proteins of the cell. The activity of 14-3-3 inside the cell can be regulated in several ways. First, it is possible to regulate both the synthesis and proteolytic degradation of 14-3-3. Second, the cell is capable of synthesizing several isoforms 14-3-3, which differ in their ability to form homo- and heterodimers, as well as in their interaction with target proteins. Third, proteins of the 14-3-3 family can undergo various post-translational modifications (such as phosphorylation or acetylation), which significantly affect their structure and properties. Fourthly, both homo- and heterodimers of 14-3-3 contain at least two regions providing the binding of phosphorylated ligands with a definite primary structure. This allows proteins 14-3-3 with a particularly high affinity to bind target proteins that have two phosphorylation sites in their structure, as well as to bind and bring together two different target proteins, each of which contains one phosphorylation site. Proteins of the 14-3-3 family are able to bind not only target proteins with specific phosphorylation sites, but also other proteins with unusual phosphorylation sites or proteins that do not undergo phosphorylation at all. The study of the mechanisms of recognition of target proteins has been and still remains the subject of detailed studies. The formation of complexes with target proteins can result in several consequences. Target proteins bound to 14-3-3 appear to be protected from the action of proteases and / or phosphatases. Due to the formation of strong complexes, the intracellular localization of the target protein can change and the convergence of two different target proteins can occur. The latter circumstance may be especially important if one of the target proteins is an enzyme (for example, protein kinase), and the other is its substrate. And, finally, taking into account the fact that 14-3-3 interacts with various target proteins, it can be assumed that a change in the concentration of one target protein under certain conditions can lead to the displacement of another target protein from its complex with 14-3-3. This

circumstance plays a particularly important role in the case of target proteins that are present in the cell at especially high concentrations. All of the above allows us to conclude that proteins of the 14-3-3 family are highly efficient regulators of numerous processes occurring in the cell, and therefore further study of their properties is extremely important. In a relatively small review, it is impossible to consider in detail the structure, properties and functions of proteins of the 14-3-3 family. The interested reader can gain important information concerning the history of the discovery and study of 14-3-3 [5], the mechanisms of functioning and regulation of the activity of 14-3-3 [12, 3], the specific functions performed by 14-3-3 in the nervous system [23], the role of 14-3-3 in apoptosis [54] and tumorigenesis [13] in excellent previously published reviews.



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