Characterization of the calcyclin (S100A6) binding site of annexin XI-A by site-directed mutagenesis

Toshiki Sudo*, Hiroyoshi Hidaka

Department of Pharmacology, Nagoya University School of Medicine, Showa-ku, Nagoya 466-8550, Japan

Received 27 November 1998; received in revised form 18 December 1998

Abstract Residues in annexin XI-A essential for binding of calcyclin (S100A6) were examined by site-directed mutagenesis. GST fusion proteins with the calcyclin binding site of annexin XI-A, GST-AXI 34–62 and GST-AXI 49–77 bound to calcyclin-Sepharose Ca^{2+}-dependently. The mutants GST-AXI L52E, M55E, A56E and M59E lost the binding ability, whereas GST-AXI A57E retained the ability. These results demonstrate that the hydrophobic residues L52, M55, A56 and M59 on one side surface of the α-helix are critical for the binding. Assays with GST fusion proteins and synthesized peptides corresponding to the calcyclin binding site indicated that other regions around the calcyclin binding site are important to stabilize the conformation.

Key words: Annexin XI; Calcyclin; S100A6; Hydrophobic interaction

1. Introduction

Ca^{2+} binding proteins are classified into two major groups, a EF hand family and an annexin family, on the basis of their Ca^{2+} binding structures [1]. The EF hand family has subfamilies with 1, 2, 3, 4, 6 and 8 EF hand structures and is represented by calmodulin and S100 proteins. These are considered to bind Ca^{2+} and then exert their Ca^{2+}-dependent actions in cells by binding to target proteins. The annexin family of phospholipid binding proteins with an endonexin fold has 10 members in mammals (annexin I–VIII, XI and XIII) which are present in a wide variety of cells and tissues. All members consist of a C-terminal core domain with either four or eight repeating units of about 70 amino acids, and an individually unique N-terminal regulatory domain. Due to the Ca^{2+}/phospholipid binding ability through the core domain, it has been shown that annexins can bind to biomembranes in a Ca^{2+}-dependent manner and aggregate and fuse them [2–5].

Many kinds of annexin binding proteins have been identified so far [6]. These include some Ca^{2+} binding proteins with EF hand structures [7–10]. In particular, three members of the S100 protein family, S100C (S100A11, calgizzarin), p11 (S100A10), and calcyclin (S100A6), specifically interact with annexins I, II and XI, respectively [8–10]. The S100 protein family are Ca^{2+} binding proteins with two EF hand structures. Since p11 forms heterotetramers with annexin II and affects its subcellular localization and affinity for Ca^{2+}, and stimulates the activity of plasmin generation on the endothelial cells [11–14], annexin-S100 protein interactions have been suggested to play important roles in intracellular and extracellular functions.

Annexin XI was first discovered as a calcyclin-associated protein (CAP-50) [10,15]. Calcyclin itself is overexpressed in tumor cells with mRNA levels specifically increased in the G1 phase of the cell cycle when stimulated by growth factors [16,17]. Thus it is possible that the annexin XI-calcyclin complex may play a role in cell proliferation and division. Calcyclin binding is an annexin XI-A isoform-specific property, the binding site being located in residues Q49–T62 in the N-terminal regulatory domain of annexin XI-A [18]. Interactions appear to be mediated by hydrophobic-hydrophobic bonding [19].

In the present study, we characterized the calcyclin binding site of annexin XI-A and established that hydrophobic residues in the calcyclin binding site of annexin XI-A play an important role in the annexin XI-calcyclin interaction.

2. Materials and methods

2.1. Materials

Restriction endonucleases and DNA modifying enzymes were from Takara Shuzo, Boehringer Mannheim Biochemica, New England Biolabs and Fermentas MBI. The pGEX-3X vector and glutathione-Sepharose 4B were purchased from Pharmacia Biotech. Phospholipids were from Doosan Serdary Research Laboratories. Peptides corresponding to Y43–T62 and N48–T62 of annexin XI-A (AXI 43–62 peptide and AXI 48–62 peptide) were synthesized and purchased from Peptide Institute Inc. (Osaka, Japan). Rabbit calcyclin protein was isolated from rabbit lung as described previously [15]. Calcyclin-Sepharose was prepared as detailed earlier [18]. Recombinant rabbit annexin XI-A protein was prepared with a baculovirus/Sf9 expression system as described previously [18]. All other materials and regents were of the highest quality available from commercial suppliers.

2.2. Preparation of glutathione S-transferase (GST) fusion proteins

GST fusion proteins with P34–T62 and Q49–P77 in the N-terminal domain of rabbit annexin XI-A (GST-AXI 34–62, GST-AXI 49–77) were prepared as previously reported [18]. GST-AXI 49–62 and site-directed mutants of GST-AXI 34–62 and GST-AXI 49–77 were prepared with oligonucleotides employed for forward and reverse primers. Their sequences were as follows (bold letters indicate the mutated nucleotides): GST-AXI 49–62, 5′-AAGGATCCAGGACTACCTCTCGGGAA-3′ and 5′-TGGAATTCATGTCCCAGACATGTTGGCCG-3′; GST-AXI L52E, 5′-AAGGATCCAGGACTAC**GAG**TCGGGAATGGCGGCC-3′ and 5′-GGGAATTCAGGGGGCACCAGGGTACAGGT-3′; GST-AXI M55E, 5′-AAGGATCCAGGACTACCTCTCGGGA**GA**GGCGGCCAAC-3′ and 5′-GGGAATTCAGGGGGCACCAGGGTACAGGT-3′; GST-AXI A56E, 5′-TAGGATGCCCATCGGGCTGGACAACGTG-3′ and 5′-TGGAATTCATGTCCCAGACATGTTGGCC**T**CCATTCCC-3′; GST-AXI A57E, 5′-TAGGATGCCCATCGGGCTGGACAACGTG-3′ and 5′-TGGAATTCATGTCCCAGACATGTT**CT**CCGCCATTCC-3′; GST-AXI M59E, 5′-TAGGATGCCCATCGGGCTGGACAACGTG-3′ and 5′-TGGAATTCATGTCCCAGAC**T**CGTTGGCCGCC-3′. A schematic illustration of the fusion proteins is shown in Fig. 1A. PCR products were verified by DNA sequencing. The constructs of GST-AXI were transformed into *Escherichia coli* XL-1 Blue cells. Overnight cultures

*Corresponding author. Fax: (81) (52) 744-2083.
E-mail: tsudo@tsuru.med.nagoya-u.ac.jp

PII: S0014-5793(99)00014-9

were grown, and fusion proteins were induced with 1 mM IPTG and isolated from the cell homogenates with glutathione-Sepharose columns. The fractions containing purified protein were collected and dialyzed against 30 mM Tris-HCl pH 7.5, 30 mM NaCl, and stored at −80°C.

2.3. Calcyclin-affinity Sepharose chromatography of annexin XI-A and GST fusion proteins

Binding capacities of annexin XI-A and GST fusion proteins for calcyclin were determined by calcyclin-affinity Sepharose chromatography as previously reported [18]. Briefly, purified annexin XI-A (18 μg) or GST fusion proteins (20 μg) were incubated in 40 mM Tris-HCl pH 7.5, 0.2 M NaCl, 0.2 mM CaCl2 with 300 μl of a 50% slurry of calcyclin-Sepharose. Following rotation for 1 h at 4°C, calcyclin-Sepharoses were washed three times in buffer containing 40 mM Tris-HCl pH 7.5, 1 M NaCl, 0.2 mM $CaCl_2$. Bound annexin XI or GST fusion proteins were specifically eluted from the Sepharose beads with buffer containing 40 mM Tris-HCl pH 7.5, 0.2 M NaCl, 5 mM EGTA and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie staining.

2.4. Liposome co-pelleting assays

Liposome co-pelleting assays were performed as described previously with slight modifications [18,20]. Briefly, 0.06 nmol (3 μg) of recombinant annexin XI-A and phosphatidylserine-containing vesicles were reacted in 50 μl of solution containing 0.2 M NaCl, 50 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$, 1 mM DTT and 2 mM $CaCl_2$ and were left to stand for 30 min at room temperature. 0.2 nmol (2 μg) of calcyclin protein and 10 nmol (final 100 μM) of the peptide were reacted in solution containing 2 mM $CaCl_2$. Their solutions were mixed, left to stand for 60 min at room temperature and then ultracentrifuged at 100 000 × *g* for 30 min at 25°C. The pellet was separated from the supernatant and resuspended in 100 μl of SDS-PAGE sample buffer. Aliquots of the supernatant and the suspended solution of the pellet were applied to SDS-PAGE.

3. Results and discussion

We previously demonstrated with GST fusion proteins that the calcyclin binding site of annexin XI-A is in residues Q49–T62 [18]. In order to identify essential residues for the binding, GST fusion proteins with the region including Q49–T62 of annexin XI-A and the site-directed mutants were constructed in the present study (Fig. 1A). Since the interaction of annexin XI-A with calcyclin has been suggested to be mediated by hydrophobic-hydrophobic bonding [19], we chose five hydrophobic residues, L52, M55, A56, A57 and L59, for the site-directed mutagenesis. Examination of the resultant GST fusion proteins for calcyclin binding with calcyclin-affinity Sepharose demonstrated GST-AXI 49–62, GST-AXI L52E and GST-AXI M55E to have no Ca^{2+}-dependent calcyclin binding ability, in contrast to full length recombinant annexin XI-A, GST-AXI 34–62 and GST-AXI 49–77 (Fig. 2). The site-directed mutants of GST-AXI 34–62, GST-AXI A56E and GST-AXI M59E, also lost the binding ability. In contrast, GST-AXI A57E retained the binding ability. This result demonstrates that the four hydrophobic residues, L52, M55, A56 and M59, of annexin XI-A are critical for calcyclin binding.

An alternatively spliced form of annexin XI-A, annexin XI-B, was earlier found not to bind to calcyclin [18]. The hydrophobic region of annexin XI-B corresponding to the calcyclin binding site of annexin XI-A is suggested to function as the Ca^{2+}-independent membrane-associating domain, not as a calcyclin binding site. Annexin XI-B lacks the second essential hydrophobic residue, M55, in the calcyclin binding site of annexin XI-A, replaced by a hydrophilic residue, threonine, that is, the four hydrophobic amino acid arrangement is not conserved. Therefore, the results in this study are also in agreement with the lack of calcyclin binding ability of annexin XI-B. The four essential hydrophobic residues are located in one side surface of the α-helix of the calcyclin binding site of annexin XI-A (Fig. 1B). A57 of annexin XI-A is located on the opposite surface and this is also in line with the finding that its amino acid substitution with a hydrophilic residue did not affect the interaction with calcyclin.

Annexin I and II have been shown to bind to other S100 proteins, S100C (S100A11, calgizzarin) and p11 (S100A10) by interactions involving the 13 and 12 N-terminal amino acids, respectively [21–24]. These bindings are also mediated by hydrophobic interaction [21,22,24,25]. Comparison of the primary structures of their S100 binding sites revealed a conserved amino acid arrangement consisting of four hydrophobic amino acids, residues V4, F7, L8, A11 of annexin I, and residues V4, I7, L8, L11 of annexin II [23]. Circular dichroism (CD) spectroscopy indicated the p11 binding site of annexin II to form an amphipathic α-helix [21]. In such α-helices, the hydrophobic residues are aligned on one side, whereas hydrophilic residues are aligned on the opposite side. Chou-Fasman secondary analysis has predicted that both the S100C binding site of annexin I and the calcyclin

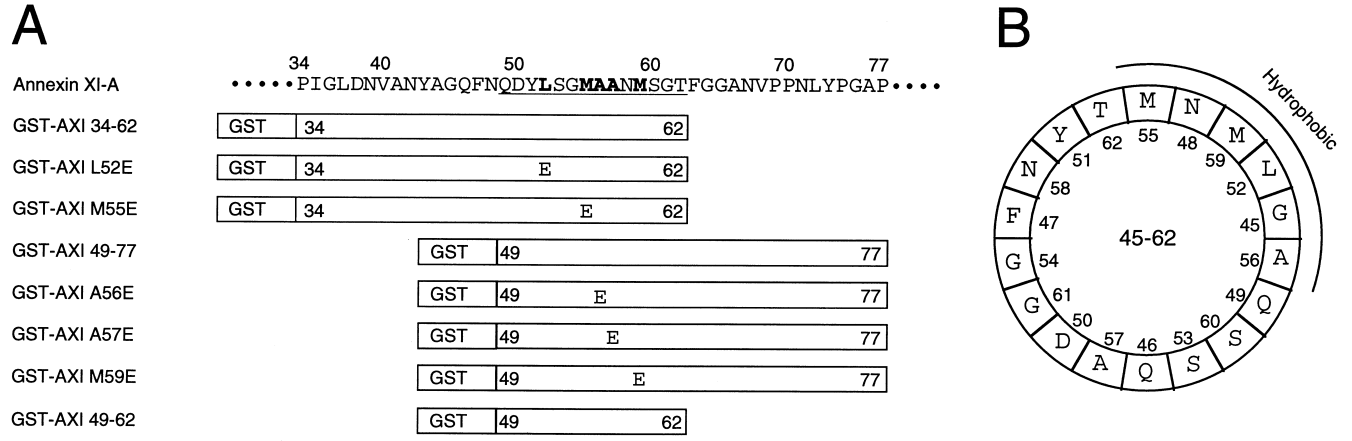

Fig. 1. Amino acid sequence of the calcyclin binding site of annexin XI-A. A: Schematic illustration of GST fusion proteins with the calcyclin binding site of rabbit annexin XI-A. The numbers in each construct refer to the amino acid positions for rabbit annexin XI-A. B: Helical wheel of the calcyclin binding site of annexin XI-A. The hydrophobic face of the amphipathic helix is indicated.

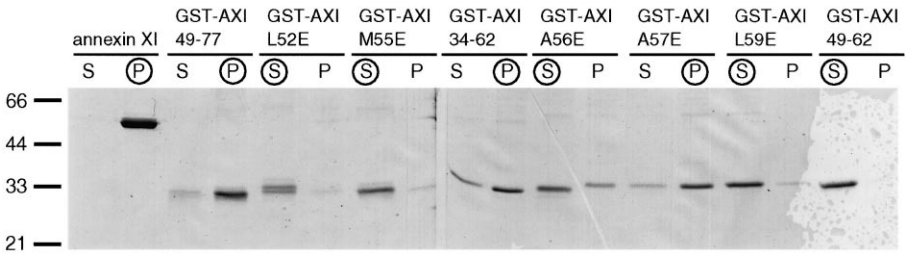

Fig. 2. Ca^{2+}-dependent binding of GST-AXI fusion proteins with calcyclin. Proteins were incubated with calcyclin-Sepharose in the presence of 0.2 mM $CaCl_2$ and the supernatant (unbound fraction, S) was then collected. The Sepharose was washed three times and eluted with 5 mM EGTA (bound fraction, P), as described in Section 2. Supernatants and EGTA-eluted fractions were subjected to SDS-PAGE (12.5% gel) followed by Coomassie staining. Molecular masses are indicated on the left (kDa).

binding site of annexin XI also form similar amphipathic α-helices. In the present study, the fact that the four hydrophobic residues in the hydrophobic surface of α-helix of the calcyclin binding site proved to be essential for the binding to calcyclin of annexin XI-A suggests that the secondary structures of S100 binding sites are conserved among annexins I, II and XI.

Among protein-protein interactions through hydrophobic faces within amphipathic helices, the best characterized are those of calmodulin with calmodulin-dependent enzymes such as myosin light chain kinase and phosphodiesterase 1 [26,27]. Calmodulin, a Ca^{2+} binding protein with four EF hand structures, similar to the two EF hands of S100 proteins, binds to specific peptide regions of many target proteins to regulate their activity in a Ca^{2+}-dependent manner. Formation of amphipathic α-helices has been demonstrated by CD spectroscopy and nuclear magnetic resonance analysis [27]. In the presence of Ca^{2+}, the hydrophobic accessible surface of calmodulin is exposed and binds to target proteins [27–29]. The three-dimensional structures of apo-calcyclin and Ca^{2+}-bound calcyclin have been investigated in detail [30–32]. In contrast to calmodulin and troponin C, there are only very small changes in the structure of the calcyclin subunit upon Ca^{2+} binding. The Ca^{2+}-bound structure is very similar to the closed conformation of calmodulin in the absence of Ca^{2+}, but clearly different from the open conformation of the Ca^{2+}-bound calmodulin [33]. In addition, the interaction of annexin II with p11 is Ca^{2+}-independent. This suggests that hydrophobic interaction of annexins with S100 proteins might be somewhat different from that of calmodulin with the target proteins.

We previously reported that a synthesized peptide corresponding to Y43–T62 of annexin XI-A (AXI 43–62 peptide) inhibits the interaction of annexin XI-A with calcyclin in liposome co-pelleting assays [18]. We have examined the effect of a shorter peptide, AXI 48–62, which is the calcyclin binding site, on the interaction of annexin XI-A with calcyclin (Fig. 3). The interaction was not inhibited by AXI 48–62 peptide. Also in the study with GST fusion proteins, in contrast to GST-AXI 34–62 and GST-AXI 49–77, the GST-AXI 49–62 fusion protein did not bind to calcyclin-Sepharose (Fig. 2). These results suggest that neighboring residues might contribute to the stabilization of the conformation to bind to calcyclin.

It has been reported that acetylation of N-terminal residues of annexins I and II is required for S100 binding [21,22,24]. For annexin II, the K_d for the binding of a non-acetylated peptide corresponding to residues 1–18 to a p11 dimer demonstrated at least a 1000-fold lower affinity than the acetylated peptide, indicating that the *N*-acetyl group on the N-terminal serine of annexin II is a functional part of the p11 binding site. For annexin I, the acetylated annexin I peptides, Ac1–18 and Ac1–13, are capable of competing with the annexin I-S100C interaction whereas their non-acetylated forms fail to show this effect. In addition, whereas porcine annexin I can bind to S100C, this is not the case with non-acetylated annexin I expressed in *Escherichia coli* [24]. Although the effect of the N-terminal acetylation on the interaction with S100 protein is difficult to evaluate, it seems to contribute the α-helical potential of S100 binding sites of annexin I and II [21]. The calcyclin binding site of annexin XI-A is within the N-terminal domain, which differs from the situation with annexin I and annexin II, and N-terminal acetylation is not required for the binding. Our studies with peptide competition and GST fusion proteins demonstrated that polypeptides consisting of only the calcyclin binding site, Q49–T62, were not able to interact with calcyclin. This suggested that the neighboring residues around the calcyclin binding site might contribute stability of the α-helical conformation to bind to calcyclin, like N-terminal acetylation of annexin I and annexin II.

Although the physiological function of annexins has still not been determined, the involvement of annexins in exocytosis and endocytosis has been demonstrated. Since calcyclin stimulates Ca^{2+}-induced insulin release from pancreatic β cells [34], the complex of annexin XI with calcyclin might play a

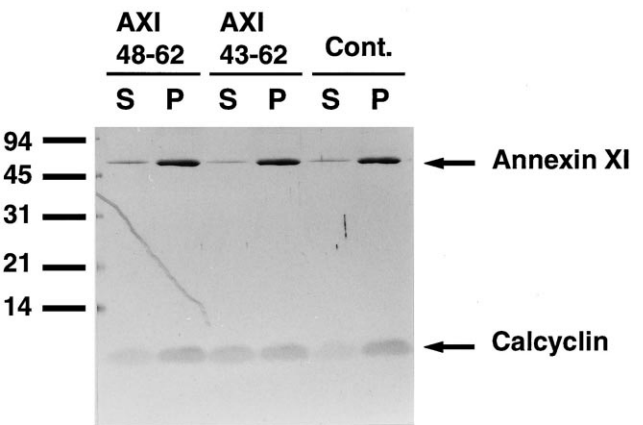

Fig. 3. Effects of synthesized peptides of calcyclin binding site on annexin XI-calcyclin interaction. Annexin XI-A and calcyclin proteins were mixed with PS liposomes in the presence or absence of AXI 43–62 or AXI 48–62 peptides. After centrifugation at 100 000 × *g*, supernatant (S) and pellet (P) fractions were subjected to SDS-PAGE (15% gel) followed by Coomassie staining. Molecular masses are indicated on the left (kDa).

role in the secretory process as well as cell proliferation and division.

Acknowledgements: This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (06044105, 06404019 and 06507001). We thank Dr. Malcolm Moore for critical reading of the manuscript.

References

[1] Niki, I., Yokokura, H., Sudo, T., Kato, M. and Hidaka, H. (1996) J. Biochem. 120, 685–698.
[2] Creutz, C.E., Pazoles, C.J. and Pollard, H.B. (1978) J. Biol. Chem. 253, 2858–2866.
[3] Blackwood, R.A. and Ernst, J.D. (1990) Biochem. J. 266, 195–200.
[4] Francis, J.W., Balazovich, K.J., Smolen, J.E., Margolis, D.I. and Boxer, L.A. (1992) J. Clin. Invest. 90, 537–544.
[5] Emans, N., Gorvel, J.P., Walter, C., Gerke, V., Kellner, R., Griffiths, G. and Gruenberg, J. (1993) J. Cell Biol. 120, 1357–1369.
[6] Sheldon, A. and Chen, J.M. (1995) in: Annexins: Molecular Structure to Cellular Function (Seaton, B.A., Ed.), pp. 133–141, Chapman and Hall, New York.
[7] Brownawell, A.M. and Creutz, C.E. (1997) J. Biol. Chem. 272, 22182–22190.
[8] Naka, M., Qing, Z.X., Sasaki, T., Kise, H., Tawara, I., Hamaguchi, S. and Tanaka, T. (1994) Biochim. Biophys. Acta 1223, 348–353.
[9] Gerke, V. and Weber, K. (1985) J. Biol. Chem. 260, 1688–1695.
[10] Tokumitsu, H., Mizutani, A., Minami, H., Kobayashi, R. and Hidaka, H. (1992) J. Biol. Chem. 267, 8919–8924.
[11] Powell, M.A. and Glenney, J.R. (1987) Biochem. J. 247, 321–328.
[12] Osborn, M., Johnsson, N., Wehland, J. and Weber, K. (1988) Exp. Cell. Res. 175, 81–96.
[13] Chasserot-Golaz, S., Vitale, N., Sagot, I., Delouche, B., Dirrig, S., Pradel, L.A., Henry, J.P., Aunis, D. and Bader, M.F. (1996) J. Cell Biol. 133, 1217–1236.
[14] Kassam, G., Choi, K., Ghuman, J., Kang, H., Fitzpatrick, S.L., Zackson, T., Zackson, S., Toba, M., Shinomiya, A. and Waisman, D.M. (1998) J. Biol. Chem. 273, 4790–4799.
[15] Tokumitsu, H., Kobayashi, R. and Hidaka, H. (1991) Arch. Biochem. Biophys. 288, 202–207.
[16] Calabretta, B., Battini, R., Kaczmarek, L., de Riel, J.K. and Baserga, R. (1986) J. Biol. Chem. 261, 12628–12632.
[17] Schäfer, B.W. and Heizmann, C.W. (1996) Trends Biochem. Sci. 21, 134–140.
[18] Sudo, T. and Hidaka, H. (1998) J. Biol. Chem. 273, 6351–6357.
[19] Watanabe, M., Ando, Y., Tokumitsu, H. and Hidaka, H. (1993) Biochem. Biophys. Res. Commun. 196, 1376–1382.
[20] Minami, H., Tokumitsu, H., Mizutani, A., Watanabe, Y., Watanabe, M. and Hidaka, H. (1992) FEBS Lett. 305, 217–219.
[21] Johnsson, N., Marriott, G. and Weber, K. (1988) EMBO J. 7, 2435–2442.
[22] Becker, T., Weber, K. and Johnsson, N. (1990) EMBO J. 9, 4207–4213.
[23] Mailliard, W.S., Haigler, H.T. and Schlaepfer, D.D. (1996) J. Biol. Chem. 271, 719–725.
[24] Seemann, J., Weber, K. and Gerke, V. (1996) Biochem. J. 319, 123–129.
[25] Kube, E., Becker, T., Weber, K. and Gerke, V. (1992) J. Biol. Chem. 267, 14175–14182.
[26] O'Neil, K.T. and DeGrado, W.F. (1990) Trends Biochem. Sci. 15, 59–64.
[27] Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B. and Bax, A. (1992) Science 256, 632–638.
[28] Meador, W.E., Means, A.R. and Quiocho, F.A. (1992) Science 257, 1251–1255.
[29] Meador, W.E., Means, A.R. and Quiocho, F.A. (1993) Science 262, 1718–1721.
[30] Potts, B.C., Smith, J., Akke, M., Macke, T.J., Okazaki, K., Hidaka, H., Case, D.A. and Chazin, W.J. (1995) Nature Struct. Biol. 2, 790–796.
[31] Potts, B.C.M., Carlstrom, G., Okazaki, K., Hidaka, H. and Chazin, W.J. (1996) Protein Sci. 5, 2162–2174.
[32] Sastry, M., Ketchem, R.R., Crescenzi, O., Weber, C., Lubienski, M.J., Hidaka, H. and Chazin, W.J. (1998) Structure 6, 223–231.
[33] Nelson, M. and Chazin, W. (1998) Protein Sci. 7, 270–282.
[34] Okazaki, K., Niki, I., Iino, S., Kobayashi, S. and Hidaka, H. (1994) J. Biol. Chem. 269, 6149–6152.