Crystal Structure of the Rac Activator, Asef, Reveals Its Autoinhibitory Mechanism*

Received for publication, September 5, 2006, and in revised form, December 15, 2006
Published, JBC Papers in Press, December 26, 2006 DOI 10.1074/jbc.C600234200
Kazutaka Murayama 1, 2, Mikako Shirouzu 2, Yoshihiro Kawasaki 3, Miyuki Kato-Murayama 2, Kyoko Hanawa-Suetsugu 2, Ayako Sakamoto 1, Yasuhiro Katsura 1, Atsushi Suenaga 1, Mitsutoshi Toyama 2, Takahiro Terada 2, Makoto Taiji 3, Tetsu Akiyama 2, and Shigeyuki Yokoyama 4

From the 1Tohoku University Biomedical Research Organization, Sendai 980-8575, the 2RIKEN Genomic Sciences Center, Yokohama Institute, Yokohama 230-0045, the 3Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113-0033, and the 4Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Tokyo 113-0033 Japan

The Rac-specific guanine nucleotide exchange factor (GEF) Asef is activated by binding to the tumor suppressor adenomatous polyposis coli mutant, which is found in sporadic and familial colorectal tumors. This activated Asef is involved in the migration of colorectal tumor cells. The GEFs for Rho family GTPases contain the Dbl homology (DH) domain and the pleckstrin homology (PH) domain. When Asef is in the resting state, the GEF activity of the DH-PH module is intramolecularly inhibited by an unidentified mechanism. Asef has a Src homology 3 (SH3) domain in addition to the DH-PH module. In the present study, the three-dimensional structure of Asef was solved in its autoinhibited state. The crystal structure revealed that the SH3 domain binds intramolecularly to the DH domain, thus blocking the Rac-binding site. Furthermore, the RT-loop and the C-terminal region of the SH3 domain interact with the DH domain in a manner completely different from those for the canonical binding to a polyproline-peptide motif. These results demonstrate that the blocking of the Rac-binding site by the SH3 domain is essential for Asef autoinhibition. This may be a common mechanism in other proteins that possess an SH3 domain adjacent to a DH-PH module.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Selenomethionine-labeled Asef (residues 66–540) with an N-terminal histidine tag was expressed in the cell-free expression system (5). The protein was purified by chromatography on a HisTrap column (GE Healthcare) and was subjected to tobacco etch virus protease digestion. The Asef protein was subsequently purified by MonoQ and Superdex75 gel filtration chromatography steps (GE Healthcare). The protein was concentrated in 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 2 mM dithiothreitol to a final concentration of 9.3 mg ml⁻¹. Decomposition of the sample was detected after a few months, and therefore, to monitor the hydrolysis of the sample during crystallization, matrix-assisted laser desorption/ionization-time of flight mass spectrometry was conducted for the crystallized samples. The spectrum indicated that the sample was intact (residues 66–540).

Crystallographic and Structure Determination—Crystals of Asef were grown in 20% polyethylene glycol 3350, 0.2M MgCl₂, and 0.1M HEPES-HCl buffer (pH 7.5) at 20 °C by the hanging drop vapor diffusion method. The crystals belong to the space group C2, with unit cell dimensions a = 100.93 Å, b = 79.82 Å, c = 68.00 Å, and β = 123.6°. Three sets of x-ray diffraction data at different wavelengths (peak, 0.978 Å; edge, 0.979 Å; and high remote, 0.964 Å) were collected at beamline BL44B2 of SPring-8 (Harima, Japan) with an ADSC Quantum-315 CCD
All MD simulations were carried out with the Amber 8.0<sup>5</sup> program on a personal computer equipped with an MDGRAPE-3 board (2 Tflops) (15, 16). The parm99 force field (17) was adopted, and the time step was set to 2.0 fs. All non-bonded interactions were calculated accurately using the MDGRAPE-3 board. All bond lengths were constrained to equilibrium lengths by the SHAKE method (18). The temperature of each system was gradually heated at a rate of 6 K/ps and was kept constant by coupling to a temperature bath at 300 K, with a coupling constant of 1.0 ps (19).

**Cell Culture and Transfection and Antibodies**—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Plasmids were transfected into COS-7 cells using Lipofectamine 2000 (Invitrogen). The rat monoclonal antibody against the HA tag (3F10) was obtained from Roche Diagnostics, and the mouse monoclonal antibody against the Myc tag (9E10) was from Santa Cruz Biotechnology.

**Immunoblotting**—Cell lysates in Laemmli’s SDS sample buffer were separated by SDS-PAGE, electrophoretically transferred to a polyvinylidene difluoride membrane filter (Immobilon P; Millipore), and analyzed by immunoblotting using an alkaline phosphatase-conjugated goat antibody against mouse IgG or rat IgG (Promega) as a second antibody.

**Rac Activity Assay**—To determine the cellular activation state of Rac, transfected cells were washed once with ice-cold phosphate-buffered saline and immediately lysed in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM MgCl<sub>2</sub>, 0.3 mM NaCl, and 2% IGEPAL. After centrifugation, the supernatants were mixed with 20 μg of bacterially produced GST-PAK-CRIB fusion protein bound to glutathione-Sepharose beads for 1 h. The beads and the proteins bound to the fusion protein were washed three times with 25 mM Tris-HCl buffer (pH 7.5) containing 30 mM MgCl<sub>2</sub> and 40 mM NaCl, and then the bound proteins were separated by SDS-PAGE before the immunoblotting analysis.

**RESULTS AND DISCUSSION**

In the present study, we determined the crystal structure of Asef, consisting of the ABR and the SH3, DH, and PH domains (Fig. 1b). The present Asef structure reveals how the DH and

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<sup>5</sup> D. A. Case, T. A. Darden, T. E. I. Cheatham, C. L. Simmerling, J. Wang, R. E. Duke, R. Luo, K. M. Merz, B. Wang, D. A. Pearlman, M. Crowley, S. Brozell, V. Tsui, H. Gohlke, J. Mongan, V. Hornak, G. Cui, P. Beroza, C. Schafmeister, J. W. Caldwell, W. S. Ross, and P. A. Kollman, personal communication.

<sup>4</sup> W. L. DeLano, personal communication.
PH domains interact with the SH3 domain (Fig. 1b). The SH3 domain of Asef adopts the typical fold of SH3 domains, with five antiparallel β-strands packed to form two perpendicular β-sheets and one turn of a 3_10 helix with the conserved Pro-173. The region connecting the SH3 and DH domains is disordered. The DH domain is composed of elongated helical bundles with six major helical segments (α1–α6) (Fig. 1b). The DH and PH domains are connected in the conventional way, which is widely conserved in many structures of GEFs for Rho family GTPases (20). In the Asef structure, the last α-helix (α6) of the DH domain, consisting of 37 amino acid residues, is longer than those of other DH domains. Furthermore, this helix is characteristically bent in an arched shape with a curvature radius of 55 Å. Consequently, the relative location of the DH and PH domains in the Asef structure is different from those in other GEF structures.

The intramolecular interactions of the SH3 domain with the DH and PH domains in Asef are shown in Fig. 2a. The SH3 domain associates mainly with the DH domain, with a contacting surface area of 350 Å². The RT-loop (the loop between the first and second β-strands) and the C-terminal part of the SH3 domain are involved in the interactions. The main chain of the RT-loop parallels the α6 helix, with many hydrophilic interactions (Fig. 2b). In the Asef structure, the interdomain interaction surface of the SH3 domain is perpendicular to its proline peptide (PXXP)-binding groove and thus blocks one end of the groove. Trp-132, Trp-160, and Phe-176 of the Asef SH3 domain correspond to the three aromatic residues involved in the canonical PXXP binding. Trp-132 interacts with the α6 helix, whereas the other two are exposed to the solvent region.

In the crystal structure of the p53-53BP2 complex (21), the PXXP-binding groove and some other residues in the RT-loop of the SH3 domain of 53BP2 interact with a non-PXXP peptide of the p53 L3 loop. The intermolecular interaction surface of the 53BP2 SH3 domain (Supplemental Fig. 1a) is different from the intradomain interaction surface of the Asef SH3 domain (Fig. 2a). Furthermore, recent NMR studies revealed the binding surfaces of the p67-phox, Pex13P, and Kalirin SH3 domains for non-PXXP motifs (22, 23) (Supplemental Fig. 1a). These non-PXXP-binding surfaces are located on a different side than the PXXP-binding groove and partially overlap each other (Supplemental Fig. 1, a and b). Therefore, the interdomain interaction surface of the Asef SH3 domain is unique.

The DH and PH domains constitute a unit responsible for the guanine nucleotide exchange activity in Dbl family proteins. Asef is a Rac-specific GEF (3). The common Rho family GTPase-binding site has been identified on the DH domain in the structures of DH-PH units complexed with RhoA, Cdc42, and Rac (20, 24–28). In the present Asef structure, the Rac-binding site of the DH domain is occupied by the SH3 domain (Supplemental Fig. 2). Therefore, the present Asef structure represents the autoinhibited form, with respect to the GEF activity for Rac. We created a model structure by superimposing the DH domain of Asef on that in the Rac-Tiam1 complex (27) (Fig. 3). The model revealed that the α6 helix of the Asef DH domain causes steric hindrances with the switch 2 region and the helix α3b C-terminal region of Rac. Thus, we speculated that the α6 helix may assume a straight form, as in the

FIGURE 2. Interaction networks and in vivo assay. a, interdomain interactions in Asef. The DH-PH unit and the SH3 domain are aligned to show the interaction surface. The amino acids involved in specific interactions (hydrogen bond, salt bridge, cation–π) are depicted in cyan. The molecular surface model of the SH3 domain, drawn in the same direction and color as the ribbon model, is also shown. b, the interacting amino acid residues in panel a are drawn in stick models. Interactions are shown by dashed lines. The main chain is traced by a wire model, colored as in Fig. 1. The DH domain was omitted from the figure. c, GEF activities of Asef mutants. COS-7 cells were transfected with the indicated constructs and HA-tagged Rac1. After 24 h, the cells were lysed and subjected to a pull-down assay, using GST-PAK-CRIB fusion protein coupled to glutathione-Sepharose beads (upper panel). The amount of GTP-bound Rac1 was detected by immunoblotting with an anti-HA antibody (lower three panels). The total amounts of HA-tagged Rac1 in the lysates and the expression levels of HA-tagged Asef mutant proteins and MYC-tagged constitutively active Tiam1 (Tiam1-C1199) are shown. mock, mock-transfected.
E365A mutations are slightly greater than that of the D133A/Asef structure are important for the autoinhibition of full-length Asef protein or the truncated form (Fig. 2 bottom right) for the autoinhibition. Therefore, the SH3 domain plays the crucial role in autoinhibiting the Rac1 GEF activity of the DH domain in Asef.

In proteins with the DH and PH domains following the SH3 domain(s), such as mouse/human collybistin I (14, 28) and intersectin-1 (12), the GEF activity is negatively regulated by the SH3 domain. Intriguingly, a mutant intersectin-1, with the proximal SH3 domain mutated so as to abolish the PXXP binding ability, revealed that the PXXP-binding groove is not involved in inhibiting the GEF activity. These findings are consistent with our Asef structure, thus suggesting that the structural mechanism of the intramolecular GEF inhibition by the SH3 domain is conserved in these proteins.

FIGURE 3. Docking model of the Asef DH domain and Rac. In the model, Rac (green) is located on the structure of the MD-simulated (2 ns) Asef (yellow), with the straight α6 helix, by superimposing the DH domains of Asef and the Rac-Tiam1 complex. The PH domain was omitted from the figure for clarity. The bent α6 helix in the crystal structure of Asef is superimposed in red, and its movement is indicated by the curved black arrow. The switch-2 region, which clashes with the bent α6 helix, is indicated with a black arrow.

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Acknowledgments—We thank Y. Nabeshima for GST-PAK-CRIB, H. Sugimura for Myc-tagged Tiam1-C1199, and Hiroko Uda-Tochio for purification of the protein. We also thank Dr. Nobuo Kamiya, Taiji Matsu, and Dr. Hisashi Naito for help in data collection at RIKEN beamline BL44B2 of Spring-8.
ACCELERATED PUBLICATION: Crystal Structure of the RhoGEF Protein Asef

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