Lysine 129 of CD38 (ADP-ribosyl Cyclase/Cyclic ADP-ribose Hydrolase) Participates in the Binding of ATP to Inhibit the Cyclic ADP-ribose Hydrolase

Communication

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CD38 catalyzes not only the formation of cyclic ADP-ribose (cADPR) from NAD$^+$ but also the hydrolysis of cADPR to ADP-ribose (ADPR), and ATP inhibits the hydrolysis (Takasawa, S., Tohgo, A., Noguchi, N., Koguma, T., Nata, K., Sugimoto, T., Yonekura, H., and Okamoto, H. (1993) J. Biol. Chem. 268, 26052–26054). In the present study, using purified recombinant CD38, we showed that the cADPR hydrolase activity of CD38 was inhibited by ATP in a competitive manner with cADPR. To identify the binding site for ATP and/or cADPR, we labeled the purified CD38 with FSBA. Sequence analysis of the lysylendopeptidase-digested fragment of the labeled CD38 indicated that the FSBA-labeled residue was Lys-129. We introduced site-directed mutations to change the Lys-129 of CD38 to Ala and to Arg. Neither mutant was labeled with FSBA nor catalyzed the hydrolysis of cADPR to ADPR. Furthermore, the mutants did not bind cADPR, whereas they still used NAD$^+$ as a substrate to form cADPR and ADPR. These results indicated that Lys-129 of CD38 participates in cADPR binding and that ATP competes with cADPR for the binding site, resulting in the inhibition of the cADPR hydrolase activity of CD38.

Cyclic ADP-ribose (cADPR)$^1$ induces the release of Ca$^{2+}$ from microsomes in a variety of tissues and cells including pancreatic β cells (2–10). cADPR is synthesized from NAD$^+$ by ADP-ribosyl cyclase, which was purified as a soluble 29-kDa protein from Aplysia ovotestes (11–13). The amino acid sequences of Aplysia ADP-ribosyl cyclases (14, 15) showed a high degree of homology with that of CD38 (16, 17), which was reported to be a surface antigen of human lymphocytes (18). From the experiments in which CD38 cDNA was expressed in mammalian cells, CD38 was shown to catalyze not only the formation of cADPR from NAD$^+$ but also the hydrolysis of cADPR to ADP-ribose (ADPR) (19–21). We have demonstrated that ATP inhibited the hydrolysis, resulting in the accumulation of cADPR (19). Furthermore, we produced transgenic mice overexpressing human CD38 in pancreatic β cells and demonstrated that ATP, produced in the process of glucose metabolism, increased the accumulation of cADPR to enhance the Ca$^{2+}$ mobilization from the intracellular stores for insulin secretion in the transgenic islets (22).

In the present study, we expressed human CD38 in Escherichia coli and purified it to homogeneity. Using the purified CD38, we found that Lys-129 of CD38 participated in cADPR binding and that ATP competed with cADPR for the binding site, resulting in the inhibition of the cADPR hydrolase activity of CD38.

EXPERIMENTAL PROCEDURES

Purification of Soluble CD38—We isolated a CD38 cDNA from an insulinoma of a Japanese patient by reverse transcriptase-polymerase chain reaction (PCR) (19) and used it for functional analyses of CD38 (17, 19, 22). The CD38 sequence was exactly the same as the CD38 sequence reported by Jackson and Bell (18) except for two base substitutions (nucleotide 213, A → C, and nucleotide 215, C → A) (19). The substitutions were also found in the corresponding region of the genome isolated from a Japanese patient (23). The CD38 cDNA (19) encoding amino acids 45–300 (17, 23) was amplified by PCR using two primers, one sense (5′-ctcctggAGGTGGCGCCAGTGCTGAG-3′) and one antisense (5′-gtctagaGCTCAGATCTCA-GATGTGCA-3′). Sequence analysis of PCR products was done using automated sequencers (3, 17, 19, 26) with CD38. Briefly, 50 ng of CD38 cDNA was amplified according to the procedure described by Reikofski and Tao (25) using an LA PCR In Vitro Mutagenesis Kit (Takara Shuzo Co., Ltd., Otsu, Japan). The synthetic oligonucleotides used for site-directed mutagenesis were as follows: 5′-AGCAGAATAGCAGATCTGGCC-3′ (K129A) and 5′-AGCAGAATAGCAGATCTGAAGTGGCGCCAGTGAG-3′ (K129R). The amplified CD38 cDNA product was inserted into the unique XhoI site of the pMAL-p2 vector (New England Biolabs), downstream and in-frame with the maltose-binding protein (MBP) coding sequence. The accuracy of the PCR-derived recombinant CD38 construct was confirmed by sequencing. After induction of the fusion protein with isopropyl-1-thio-galactoside in E. coli, the protein was purified by affinity chromatography on cross-linked amylose resin (New England Biolabs). To separate the CD38 from MBP, the fusion protein was cleaved by Factor Xa (New England Biolabs), and the CD38 was purified by gel filtration (Sephacryl S-100HR, Pharmacia Biotech, Uppsala, Sweden). To reconstitute the enzymic activity, the purified CD38 was refolded as described (24).

Site-directed Mutagenesis—The site-directed mutants were made according to the procedure described by Reikofski and Tao (25) using an LA PCR In Vitro Mutagenesis Kit (Takara Shuzo Co., Ltd., Otsu, Japan). The synthetic oligonucleotides used for site-directed mutagenesis were as follows: 5′-AGCAGAATAGCAGATCTGGCC-3′ (K129A) and 5′-AGCAGAATAGCAGATCTGAAGTGGCGCCAGTGAG-3′ (K129R). The amplified CD38 cDNA product was inserted into the unique XhoI site of the pMAL-p2 vector (New England Biolabs), downstream and in-frame with the maltose-binding protein (MBP) coding sequence. The accuracy of the PCR-derived recombinant CD38 construct was confirmed by sequencing. After induction of the fusion protein with isopropyl-1-thio-β-D-galactoside in E. coli, the protein was purified by affinity chromatography on cross-linked amylose resin (New England Biolabs). To separate the CD38 from MBP, the fusion protein was cleaved by Factor Xa (New England Biolabs), and the CD38 was purified by gel filtration (Sephacryl S-100HR, Pharmacia Biotech, Uppsala, Sweden). To reconstitute the enzymic activity, the purified CD38 was refolded as described (24).

Enzyme Assays—ADP-ribosyl cyclase and cADPR hydrolase assays were performed as described (17, 19, 26) with CD38. Briefly, 50 ng of CD38 was incubated for 5 min at 37 °C in 0.1 ml of phosphate-buffered saline (pH 7.4) (26, 27) with 0.2 mM NAD$^+$ containing 5 μCi of [32P]NAD$^+$ (DuPont NEN) for ADP-ribosyl cyclase or with 0.2 mM cADPR containing 5 μCi of [32P]cADPR, prepared as described previously (17, 19, 26) for cADPR hydrolase. Reaction products were analyzed by HPLC (17, 19, 26) using a flow scintillation analyzer (Flow-One Beta-525TR, Packard, Meriden, CT). The protein concentration was measured by the method of Bradford (28) using bovine serum albumin as a standard.

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Modification of CD38 with FSBA—The reaction of CD38 with FSBA (Boehringer Mannheim) (29, 30) was performed at 30 °C in phosphate-buffered saline (pH 7.4). The concentrations of CD38 and FSBA were 100 nm and 1 nm, respectively.

Detection of FSBA Modification—The amount of covalent modification of CD38 with FSBA was estimated by Western blot as described previously (5, 17, 19, 26) using an ECL detection system (Amersham Corp.). A polyclonal antibody against FSBA (Boehringer Mannheim) was used at a final concentration of 1 μg/ml diluted with 5% milk powder solution as a primary antibody. The band intensity of the FSBA-labeled CD38 was measured using NIH Image software.

Identification of FSBA-labeled Peptide—0.2 mg of CD38 was labeled with 1 mM FSBA for 4 h and subjected to proteolysis by lysylendopeptidase (Wako Pure Chemical Industries, Osaka, Japan) as described previously (31). The lysylendopeptidase fragments were separated by reverse-phase HPLC using a µRPC C2/C18 column (3.2 × 30 mm, Pharmacia Biotech) at a flow rate of 200 μl/min. An aliquot (10 μl) of each peak was blotted on a polyvinylidene difluoride membrane and analyzed for the presence of FSBA-labeled peptide by Western blot using an anti-FSBA antibody as described above. The peak containing the FSBA-labeled peptide was subjected to automated Edman degradation as described previously (31).

cADPR Binding Assay—1.5, 3, and 6 nmol of wild type CD38, K129A-CD38, K129R-CD38, or bovine serum albumin were incubated for 10–30 min at 4 °C in 0.1 ml of phosphate-buffered saline (pH 7.4) with 10 nmol of cADPR containing 5 μCi of [32P]cADPR. During the incubation, neither CD38 nor its mutants converted cADPR to ADPR. The incubation mixture was fractionated at 4 °C by a gel filtration column (PD-10, Pharmacia Biotech). 0.5-ml fractions were collected, and an aliquot (10 μl) of each fraction was analyzed for radioactivity. Another aliquot (50 μl) of each fraction was blotted on a polyvinylidene difluoride membrane and analyzed for the presence of CD38 by Western blot using a monoclonal antibody against human CD38 (T16, Cosmo Bio Co., Ltd., Tokyo, Japan) (17, 19, 26) as described above.

RESULTS AND DISCUSSION

Human CD38 was expressed in E. coli and purified as a soluble protein without the hydrophobic membrane domain (see “Experimental Procedures”). When NAD+ was used as a substrate, the purified CD38 catalyzed the formation of cADPR and ADPR (Fig. 1A). On the other hand, when cADPR was used as a substrate, the CD38 exhibited the cADPR hydrolase activity to form ADPR (Fig. 1B) as described previously (17, 19). We then examined the effect of ATP on the hydrolisis of cADPR by CD38. ATP inhibited the hydrolysis in a dose-dependent manner (data not shown), which was consistent with the previous result obtained by using the COS-7 cell membrane fraction, in which CD38 was expressed, as an enzyme source (19). Kinetic analysis was performed with various concentrations of cADPR in the presence or absence of ATP, and the data were plotted in a double reciprocal manner. As shown in Fig. 2, in the absence of ATP, CD38 had a Vmax value of 470 ± 58 nmol/min/mg of protein (n = 3) and a Km value of 53 ± 7.0 μM (n = 3) for cADPR. ATP inhibited the hydrolysis of cADPR in a competitive manner, with a Ki value of 4.8 ± 0.53 mM (n = 3). Competitive inhibition of the CD3PR hydrolysis by ATP suggests that ATP and cADPR bind to the same site of CD38.

To identify the binding site for ATP and/or cADPR, we then labeled the purified CD38 with FSBA (29, 30). As shown in Fig. 3A, the CD38 was labeled in a time-dependent manner, and cADPR and ATP completely inhibited the incorporation of FSBA into CD38, indicating that the binding site for ATP and/or cADPR was affected by FSBA. We next subjected the
their catalytic activity to form cADPR and ADPR from NAD. The activities of mutant CD38s. As shown in Table I, the mutants did not catalyze the hydrolysis of cADPR to ADPR, whereas the catalytic activity to form cADPR and ADPR from NAD$^+$ still remained. In addition, the FSBA-labeled CD38 catalyzed the formation of cADPR and ADPR from NAD$^+$ but not the hydrolysis of cADPR to form ADPR (Fig. 1). We next tested the mutant CD38s for their ability to bind to cADPR. As shown in

| Table I | Enzymic activities of CD38 and its mutants |
|---------|------------------------------------------|
| Substrate | cADPR | ADPR |
|-----------|-------|------|
| Wild type CD38 | 108.6 ± 3.26 | 9500 ± 160 |
| K129A-CD38 | 26.4 ± 9.32 | 1970 ± 240 |
| K129R-CD38 | 13.5 ± 1.81 | 1530 ± 50 |

* — below detection (<0.5 nmol/min/mg of protein).

We have proposed a model for insulin secretion by glucose via cADPR-mediated Ca$^{2+}$ mobilization (35). In the process of...
glucose metabolism, millimolar concentrations of ATP are generated (22), inducing cADPR accumulation by inhibiting the cADPR hydrolase activity of CD38, and cADPR acts as a second messenger for intracellular Ca\(^{2+}\) mobilization from the endoplasmic reticulum for insulin secretion (3, 5, 19, 22, 35, 36). In the present study, it has been shown that Lys-129 of CD38 participates in cADPR binding and that millimolar concentrations of ATP compete with the cADPR binding site, inhibiting the cADPR hydrolase activity of CD38. Therefore, when mutations at or around Lys-129 occur in CD38, cADPR metabolism cannot be regulated by ATP, which is generated in the process of glucose metabolism. Such a mutation in CD38 could be a predisposing factor for diabetes mellitus.

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