Cyclic ADP-ribose (cADPR) serves as a second messenger for Ca\(^{2+}\) mobilization in insulin secretion, and CD38 has both ADP-ribosyl cyclase and cADPR hydrolase activities (Takasawa, S., Tohgo, A., Noguchi, N., Koguma, T., Nata, K., Sugimoto, T., Yonekura, H., and Okamoto, H. (1993) J. Biol. Chem. 268, 26052-26054). Here, we produced transgenic mice overexpressing human CD38 in pancreatic \(\beta\) cells. The enzymatic activity of CD38 in transgenic islets was greatly increased, and ATP efficiently inhibited the CD38 hydrolase activity. The Ca\(^{2+}\)-mobilizing activity of cell extracts from transgenic islets incubated in high glucose was 3-fold higher than that of control, suggesting that ATP produced by glucose metabolism increased cADPR accumulation in transgenic islets. Glucose- and ketoisocaproate-induced but not tolbutamide- or KCl-induced insulin secretions from transgenic islets were 1.7-2.3-fold higher than that of control. In glucose-tolerance tests, the transgenic serum insulin level was higher than that of control. In the present study, we produced transgenic mice overexpressing CD38 in islets and analyzed the subcellular localization of expressed CD38, changes in Ca\(^{2+}\) mobilizing activity, and glucose-induced insulin secretion.

**EXPERIMENTAL PROCEDURES**

Construction of Rat Insulin II Promoter/Human CD38 Hybrid Gene—A rat insulin II promoter previously reported to be active in pancreatic \(\beta\) cells of transgenic mice (16, 17) was employed. The 0.7-kilobase pair BamHI-X fragment (17) of the rat insulin II promoter (nucleotides –695 to +22 in Ref. 18), the 0.9-kilobase pair Xmal-Sall fragment of the human CD38 cDNA (11, 13) (nucleotides +50 to +980 in Ref. 13; Xmal and Sall sites were introduced by polymerase chain reaction), and the 1.6-kilobase pair Sall–EcoRI fragment of the SV40 intron and polyadenylation signal (19) (Sall site derived from plasmid sequence) were ligated at the Xmal and Sall sites in the correct orientation. The resultant hybrid gene (Ins-CD38; 3.2 kilobase pairs) was separated from the plasmid vector pBlueScript SK– (Stratagene) by KpnI and NotI and was microinjected into fertilized eggs as described (17).

Northern Blot Analyses—Northern blot analyses were carried out on total RNA extracted from various tissues as described (17) using a \(^{32}\)P-labeled human CD38 cDNA probe (EcoRI–EcoRI fragment of 528 base pairs; nucleotides +29 to +556 in Ref. 13). Hybridization signals were scanned with a bioimage analyzer, BAS 2000 (Fuji Photo Co., Ltd., Tokyo, Japan).

Immunohistochemical Analysis—Immunohistochemical analysis was carried out as described (17) using the diluted (1:20) monoclonal antibody to human CD38 (T16; CosmoBio, Japan) and an avidin-biotin peroxidase kit (Vector, Burlingame, CA).

Measurement of cADPR and ADPR Productions from NAD\(^{+}\)—Transgenic (lines 18 and 56) and nontransgenic islets were isolated in parallel from 6-10-week-old litters by the collagenase digestion method (20). Five hundred islets were sonicated at 4°C for 15 min in 0.1 ml of the homogenizing buffer (50 mM MES, pH 7.2, containing 1 mM MgCl\(_2\), 0.1 mM EGTA, and 10 \(\mu\)g/ml aprotinin). Formations of cADPR and ADPR from NAD\(^{+}\) were measured as described (11, 21); briefly, the islet cell homogenate (10 \(\mu\)g of protein) was incubated for 10 min at 37°C in 0.1 ml of phosphate-buffered saline (pH 7.4) with 0.2 mM NAD\(^{+}\) containing 5 \(\mu\)Ci of \(^{32}\)P-NAD\(^{+}\) (DuPont NEN). Reaction products were analyzed by high pressure liquid chromatography (HPLC) using a flow scinitillation analyzer (Flow-One Beta-525TR, Packard, Meriden, CT).

Subcellular Fractionation and Measurement of NAD\(^{+}\) Glycero- and Palmito-lipid Activity—The subcellular fractionation was performed according to the islet fractionation method of McDaniel et al. (22). One thousand islets were homogenized in 400 \(\mu\)l of homogenizing buffer (50 mM MES, 1 mM EDTA, and 0.25 mM sucrose, pH 7.2). The homogenate was centrifuged at 600 \(\times\) g for 5 min to yield a pellet containing nuclei (nuclear fraction). Centrifugation of the supernatant at 20,000 \(\times\) g for 20 min yielded a pellet containing the plasma membrane, secretory granules, and lysosomes.
Insulin Secretion in CD38-expressing Transgenic Mice

and mitochondria (membrane fraction). Centrifugation of the resultant supernatant at 150,000 × g for 90 min yielded a pellet containing the microsome (microsome fraction). The supernatant after 150,000 × g centrifugation yielded a soluble protein (cytosol fraction). NAD⁺-glycohydrolase activity in the fractionated protein was measured as described (23). Briefly, 1 μg of each fractionated protein was incubated with 22 μM NAD⁺ containing 50 Ci of [14C]NAD⁺ (Amersham Corp.) at 37 °C for 10 min. The mixture was applied on a column of Dowex-1 (Bio-Rad Laboratories). Nicotinamide was eluted with 20 mM Tris-HCl (pH 7.5), followed by scintillation counting.

Measurement of Insulin Secretion from Isolated Islets—Islets of transgenic mice (line 18) and control mice were isolated in parallel from 6- to 8-week-old littermates by collagenase digestion. Twenty islets were incubated for 1 h at 37 °C in 1 ml of RPMI 1640 medium containing 10% fetal calf serum and various concentrations of glucose. The medium samples were subsequently assayed for radioimmunoassay of insulin using the insulin radioimmunoassay kit (Amersham Corp.) and rat insulin standards. For the time course experiment, 20 islets were incubated at 37 °C in 1 ml of the medium containing 11.1 mM glucose, and medium samples (2 μl) collected at 10, 20, 30, and 40 min after the incubation were subjected to radioimmunoassay for insulin. For measurements of insulin secretion by other insulin secretagogues, 10 islets were incubated for 1 h at 37 °C in 0.5 ml of the medium containing the lowest concentration of glucose (2.5 mM) and then incubated for another 1 h in the same medium containing 10 mM tetracosapropic acid (KIC) (Sigma). The medium samples were subsequently radioimmunoassayed for insulin.

Assay of Ca²⁺ Mobilizing Activity—Five hundred transgenic islets (line 18) or control islets were incubated at 37 °C for 15 min in 5 ml of RPMI 1640 medium containing 10% fetal calf serum and 2.5 or 11.1 mM glucose. After the incubation, islet cell extracts (50 μl) were prepared as described (3). Release of Ca²⁺ was monitored by adding the islet extracts (15–20 μl) to 3 ml of intracellular medium (3) containing 3 μM Fluo 3, a fluorescent Ca²⁺ indicator, and the rat cerebellum microsome fraction (88 μg of protein) prepared as described (3). Assay of Ca²⁺ mobilizing activity using the mouse islet microsome fraction (5 μg of protein) was carried out with 0.6 ml of the intracellular medium (see Fig. 6). Fluorescence was measured at 490-nm excitation and 535-nm emission at 37 °C.

Measurement of cADPR Hydrolyase Activity in the Presence of Various Concentrations of ATP—The islet cell homogenate (10 μg of protein) of transgenic mice (line 18) was incubated for 20 min at 37 °C in 0.1 ml of phosphate-buffered saline (pH 7.4) in the presence of 0–6 mM ATP and 0.2 mM cADPR containing 5 μCi of [32P]cADPR, prepared enzymatically from NAD⁺ and [32P]NAD⁺ using Aplysia kurodai ADP-ribosyl cyclase. Reaction products were analyzed by HPLC (11, 21).

Measurement of Serum Insulin and Blood Glucose Levels in Glucose Tolerance Tests—Transgenic mice (lines 18 and 56) and their respective nontransgenic siblings were fasted 10 h and then subjected to glucose tolerance tests by intraperitoneal injection of 1 g of glucose/kg of body weight. Blood samples (100 μl) were taken from the tail vein at each point after glucose administration, and the serum samples (25 μl) were prepared by centrifugation after incubating blood samples overnight at 4 °C to complete coagulation. The serum insulin levels were determined by radioimmunoassay. Blood glucose determinations were made on fresh whole blood (15 μl) using the Accucheck II (Boehringer Mannheim). All statistical analyses were performed using Student's t test.

RESULTS AND DISCUSSION

The rat insulin II promoter/human CD38 hybrid gene (Ins-CD38; see "Experimental Procedures") was designed to direct the overexpression of CD38 in pancreatic β cells of transgenic mice. The linearized gene fragment was microinjected into the fertilized eggs of (C57Bl/6 × CBA/J) F₁ mice. 20 out of 94 newborn mice were found to carry the Ins-CD38 transgene, as detected by polymerase chain reaction analyses using primers for the insulin promoter and human CD38 CDNA. In the present study, the six transgenic lines, 18, 30, 49, 56, 60, and 72, were maintained on ICR background and analyzed.

Northern blot analysis using the human CD38 cDNA probe (11) showed that all lines of transgenic mice but not that of the nontransgenic mice expressed human CD38 mRNA in the pancreatic islets (Fig. 1A). Densitometric scanning indicated that the transgenic lines 18, 56, and 60 expressed relatively higher levels of human CD38 mRNA in islets, whereas lines 30, 49, and 72 expressed lower levels of human CD38 mRNA. The human CD38 mRNA expression was not detected in other tissues such as brain, lung, heart, stomach, small intestine, liver, kidney, spleen, and testis in the transgenic mice (Fig. 1, B and C), indicating that the expression of human CD38 is limited to islets. In immunohistochemistry, islets of the transgenic mice were densely stained for human CD38 (Fig. 2, B and C). On the other hand, islets of the control mice showed no immunoreactivity for human CD38 (Fig. 2A). In contrast to islets, the pancreatic exocrine cells showed no detectable staining for human CD38 in any of the transgenic and nontransgenic mice.

Next, the pancreatic islet homogenates prepared from islets of transgenic and nontransgenic siblings of lines 18 and 56 were incubated with [32P]NAD⁺, and the reaction products were analyzed by HPLC. The formation of cADPR in the transgenic mouse (for line 18, 1.5 nmol/min·mg protein; for line 56, 1.5 nmol/min·mg protein) was indeed much higher than in the controls (<0.05 nmol/min·mg protein). The formation of ADP-ribose by cADPR hydrolyase activities, and the overall reaction is classified as an NAD⁺-glycohydrolase reaction (14). To determine the subcellular distribution of expressed CD38, islet proteins were fractionated by centrifugation (22) and fractionated proteins were assayed for NAD⁺-glycohydrolase activity (Table 1). The NAD⁺-glycohydrolase activity in the membrane fraction was greatly increased in the transgenic mice, indicating that expressed CD38 was predominantly localized in this fraction. Significant activities were also detected in the nuclear, microsome and cytosol fractions of the transgenic islets. The distribution of the percentage of total activity in fractions of transgenic mice showed a similar tend-
We isolated islets from transgenic mouse line 18 and their nontransgenic littermates and measured secreted insulin after incubation in medium containing various concentrations of glucose (Fig. 3A). At 6.8–15.6 mM glucose, the transgenic insulin secretion was 1.7–2.3-fold higher than that of the control. Essentially similar results were obtained using transgenic line 56 (data not shown). Time course experiments at 11.1 mM glucose indicated that at 10 min after the exposure to glucose, the glucose-stimulated insulin secretion was significantly higher in the transgenic islets and progressively increased in a time-dependent manner (Fig. 3B).

We next investigated the effects of other insulin secretagogues on insulin secretion from transgenic and control islets. When the islets were exposed to 10 mM KIC, which, like glucose, generates ATP during the metabolism (24), the transgenic insulin secretion was 1.7-fold higher than that of the control (Fig. 4A). Tolbutamide blocks ATP-sensitive K⁺ channel and facilitates Ca²⁺ influx through voltage-dependent Ca²⁺ channels (24) without increasing the islet ATP concentration (25). When the islets were exposed to 0.2 mM tolbutamide, the transgenic insulin secretion was not altered as compared with the control (Fig. 4B). When the islets were exposed to 25 mM KCl, which directly induces cell membrane depolarization resulting in Ca²⁺ influx (24), the transgenic insulin secretion was not altered as compared with the control (Fig. 4C).

We prepared cell extracts from the islets incubated in 2.5 or 11.1 mM glucose. The extracts were assayed for the Ca²⁺ mobilizing activity from microsomes (3). The Ca²⁺ mobilizing activity of extracts of transgenic islets incubated in 11.1 mM glucose was 3-fold higher than that of the control extracts (Fig. 5). The Ca²⁺ mobilization by the islet extracts was abolished when the microsomes had been desensitized by previously releasing Ca²⁺ in response to authentic cADPR (3), indicating that Ca²⁺ mobilization by the extracts of transgenic islets after high glucose treatment is cADPR-derived. In contrast, at 25 mM glucose, the Ca²⁺ mobilizing activities of the transgenic and control islet extracts were lower, and there was no significant difference between the two extracts (Fig. 5). The effect of exogenous cADPR on Ca²⁺ mobilization from control and transgenic islet microsomes was essentially similar (Fig. 6), suggesting that microsome sensitivity to cADPR is not altered by human CD38 overexpression.

### Table 1

| Fraction | Control | Line 18 | Line 56 |
|----------|---------|---------|---------|
|          | Specific activity | Total activity | Specific activity | Total activity | Specific activity | Total activity |
| Homogenate | 21.9 | 100 | 181.1 | 100 | 155.6 | 100 |
| Nuclear   | 22.6 | 48.1 | 123.1 | 23.1 | 104.3 | 30.3 |
| Membrane  | 12.3 | 24 | 270.3 | 67.7 | 244.5 | 51.1 |
| Microsome | 16.6 | 3.4 | 101.5 | 3.9 | 191.7 | 8.4 |
| Cytosol   | 2.9 | 1.7 | 51.8 | 3.9 | 35.9 | 2.8 |

**Fig. 2. Immunohistochemical detection of human CD38 in mouse pancreas.** Human CD38 protein was detected in mouse islets of transgenic line 18 (B) and line 56 (C) but not in nontransgenic mouse (A).
We have previously shown that ATP, generated during glucose metabolism in islets, dose-dependently inhibits the cADPR hydrolase activity of CD38 expressed in COS-7 cells and increases the accumulation of cADPR (11). In fact, higher concentrations of ATP efficiently inhibited the cADPR hydrolase activity of the CD38 expressed in transgenic islets (Fig. 7). The cellular ATP concentration in transgenic islets increased from 2.3 (at 2.5 mM glucose) to 3.3 mM (at 11.1 mM glucose); this rise in cellular ATP concentrations would inhibit the cADPR hydrolase activity of CD38 and thereby increase the cADPR concentration. It is therefore reasonable to assume that the expressed ADP-ribosyl cyclase/cADPR hydrolase (CD38) in transgenic islets generates the enhanced cADPR accumulation upon stim-

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**Fig. 4.** Insulin secretion from isolated islets by KIC, tolbutamide, and KCl. Insulin secretion from islets of control mice and transgenic mice (line 18) stimulated by 10 mM KIC (A), 0.2 mM tolbutamide (B), or 25 mM KCl (C) was measured as described under “Experimental Procedures.” Open bars and shaded bars show levels from control mice and transgenic mice (line 18), respectively. n = 5 for each point. Vertical bars indicate S.E., *p < 0.01.

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**Fig. 5.** Ca²⁺ mobilizing activity in the transgenic and control islets. Release of Ca²⁺ from cerebellar microsomes by the islet extracts prepared after incubation with 2.5 or 11.1 mM glucose was measured as described under “Experimental Procedures.” Open bars and shaded bars show levels from control mice and transgenic mice (line 18), respectively. n = 3 for each mouse. Vertical bars indicate S.E., *p < 0.05.

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**Fig. 6.** Effect of exogenous cADPR on Ca²⁺ mobilization from islet microsomes. Islet microsomes were prepared from control mice and transgenic mice (line 18). The release of Ca²⁺ from the islet microsomes (5 μg of protein) in response to various concentrations (0, 0.2, 0.35, 0.5, and 1.0 μM) of cADPR was measured as described under “Experimental Procedures.”

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**Fig. 7.** Effects of ATP on cADPR hydrolase activity of expressed CD38. ADP synthesis from CD38 by islet cell homogenate of transgenic mice (line 18) in the presence of 0, 2, 4, or 6 mM ATP was measured as described under “Experimental Procedures.”
Insulin secretion in CD38-expressing transgenic mice

The present results also indicate that CD38 plays a regulatory role in the glucose-induced insulin secretion. In non-insulin-dependent diabetes mellitus, the glucose-induced insulin secretion is impaired (28) even when pancreatic islets retain significant amounts of insulin (29). Thus, it would be important to determine whether there are qualitative or quantitative differences in the CD38 (ADP-ribosyl cyclase/cADPR hydrolase) in non-insulin-dependent diabetes mellitus β cells.

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Regulatory Role of CD38 (ADP-ribosyl Cyclase/Cyclic ADP-ribose Hydrolase) in Insulin Secretion by Glucose in Pancreatic βCells: ENHANCED INSULIN SECRETION IN CD38-EXPRESSING TRANSGENIC MICE
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