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Recent advances in physiological and pathological significance of NAD^+ metabolites: roles of poly(ADP-ribose) and cyclic ADP-ribose in insulin secretion and diabetogenesis

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Poly(ADP-ribose) synthetase/polymerase (PARP) activation causes NAD^+ depletion in pancreatic β-cells, which results in necrotic cell death. On the other hand, ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase (CD38) synthesizes cyclic ADP-ribose from NAD^+, which acts as a second messenger, mobilizing intracellular Ca^{2+} for insulin secretion in response to glucose in β-cells. PARP also acts as a regenerating gene (*Reg*) transcription factor to induce β-cell regeneration. This provides the new concept that NAD^+ metabolism can control the cellular function through gene expression. Clinically, PARP could be one of the most important therapeutic targets; PARP inhibitors prevent cell death, maintain the formation of a second messenger, cyclic ADP-ribose, to achieve cell function, and keep PARP functional as a transcription factor for cell regeneration.

Poly(ADP-ribose) synthetase/polymerase: Okamoto model for β-cell damage: Necrosis: Apoptosis: Regenerating gene

Introduction

The established biological roles of niacin are attributable to the function of its active metabolites, NAD and NADP, as redox coenzymes. NAD^+ is synthesized from tryptophan and also from preformed nicotinic acid and nicotinamide, and in addition to its coenzyme role can be further metabolized to poly(ADP-ribose) and cyclic ADP-ribose by poly(ADP-ribose) synthetase/polymerase (PARP) and ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase (CD38), respectively (Fig. 1). Our recent results indicate that these NAD^+ metabolites and enzymes are crucially involved in the death, regeneration, and functioning of the insulin-producing β-cells of the pancreatic islets of *Langerhans*.

In the present review, some of our studies will be described concerning the death of insulin-producing pancreatic β-cells by the activation of PARP. Second, a novel signal system, the CD38–cyclic ADP-ribose signal system for insulin secretion, will be presented. Finally, β-cell regeneration, in which PARP acts as a regenerating gene (*Reg*) transcription factor, will be described.

Poly(ADP-ribose) synthetase/polymerase activation and β-cell death

PARP was isolated from nuclei of mammalian cells at the end of the 1960s by three independent groups (Chambon *et al*. 1966; Nishizuka *et al*. 1967; Sugimura *et al*. 1967). PARP catalyses the formation of poly(ADP-ribose) from NAD^+. This enzyme is one of the best-known proteins with DNA damage-scanning activity and is activated by DNA damage (Ueda & Hayaishi, 1985).

Alloxan and streptozotocin are typical β-cytotoxic chemical agents and have been widely used to produce diabetes in experimental animals (Dunn *et al*. 1943; Rakieten *et al*. 1963). However, the mechanisms of action of these β-cytotoxins on pancreatic β-cells were not fully understood. In 1981, it was found that alloxan and streptozotocin induce DNA strand breaks in pancreatic islet cells (Okamoto, 1981; Yamamoto *et al*. 1981*a*,*b*). Islets isolated from rat pancreas were incubated with alloxan or streptozotocin for 5–20 min in Krebs–Ringer bicarbonate medium. After incubation, islets were layered over a linear sucrose gradient and centrifuged (Fig. 2). DNA from control islets was observed at a single peak near the bottom of the gradient, the position at which undamaged DNA sediments. However, after only 5–10 min incubation with alloxan or streptozotocin, a considerable amount of DNA sedimented as a broad peak in the middle of the gradient with a concomitant decrease in undamaged DNA; after the 20 min incubation, the DNA was almost completely fragmented.

Alloxan generates oxygen radicals during a reduction and oxidation reaction. In addition, the hydroxyl radical is

Abbreviations: CD38, ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase; IL-6, interleukin-6; IP_3, inositol 1,4,5-trisphosphate; PARP, poly(ADP-ribose) synthetase/polymerase; *Reg*, regenerating gene.

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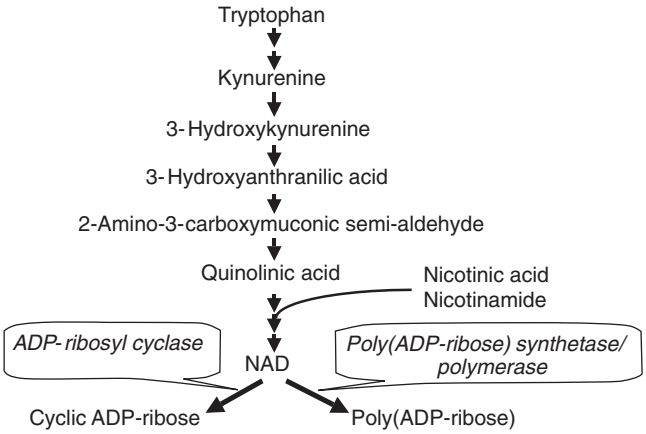

Fig. 1. Cyclic ADP-ribose and poly(ADP-ribose) formation from NAD^+.

produced by the interaction between superoxide and peroxide. When superoxide dismutase and catalase are present, the formation of the hydroxyl radical is reduced. Therefore, concerning the mechanisms of DNA strand breaks, alloxan yields oxygen radicals, especially hydroxyl radicals, that break islet DNA (Okamoto, 1981, 1985, 1990; Uchigata *et al*. 1982, 1983). On the other hand, the streptozotocin-induced DNA breaks are mainly associated with the alkylating activity of the agent itself (Okamoto, 1981, 1985, 1990; Uchigata *et al*. 1982, 1983; Cardinal *et al*. 2001).

The nuclear fraction from islets was then prepared and PARP activity was assayed. Both alloxan and streptozotocin induced a great increase in islet PARP activity with a peak at 10 min. NAD^+ is the substrate of PARP, and the islet NAD^+ level was greatly reduced by either streptozotocin or alloxan within 20 min of incubation (Yamamoto *et al*. 1981*a*). There was a striking temporal correlation between the decrease in the level of islet NAD^+ and the increase in the islet PARP activity. Picolinamide as well as nicotinamide inhibited the islet nuclear PARP activity in a dose-dependent manner (Yamamoto & Okamoto, 1980). Streptozotocin greatly decreased the islet NAD^+ level. Picolinamide and nicotinamide completely abolished the streptozotocin-induced decrease in the islet NAD^+ level. The same was true for the decrease in the islet NAD^+ level induced by alloxan. Nicotinamide is a precursor for NAD^+ synthesis and also an inhibitor of PARP. Picolinamide is an inhibitor of PARP, but incapable of acting as a precursor in NAD^+ synthesis. Therefore, the streptozotocin- or alloxan-induced decrease in NAD^+ is due to increased NAD^+ degradation to poly(ADP-ribose) by PARP activation rather than the inhibition of NAD^+ biosynthesis. Pro-insulin synthesis in pancreatic islets is a marker for the evaluation of the diabetogenicity of alloxan and streptozotocin. PARP inhibitors such as benzamides, nicotinamide, picolinamide, and methylxanthines reverse the inhibition of pro-insulin synthesis induced by alloxan and streptozotocin in a dose-dependent manner (Uchigata *et al*. 1982; Okamoto, 1985, 1990).

Therefore, a unifying model was proposed for the action of the diabetogenic agents streptozotocin and alloxan on pancreatic β-cells (Fig. 3). Central to the model are breaks in the nuclear DNA of β-cells caused by oxygen radicals or the alkylation of DNA. These breaks induce DNA repair involving the activation of PARP, which uses NAD^+ as a

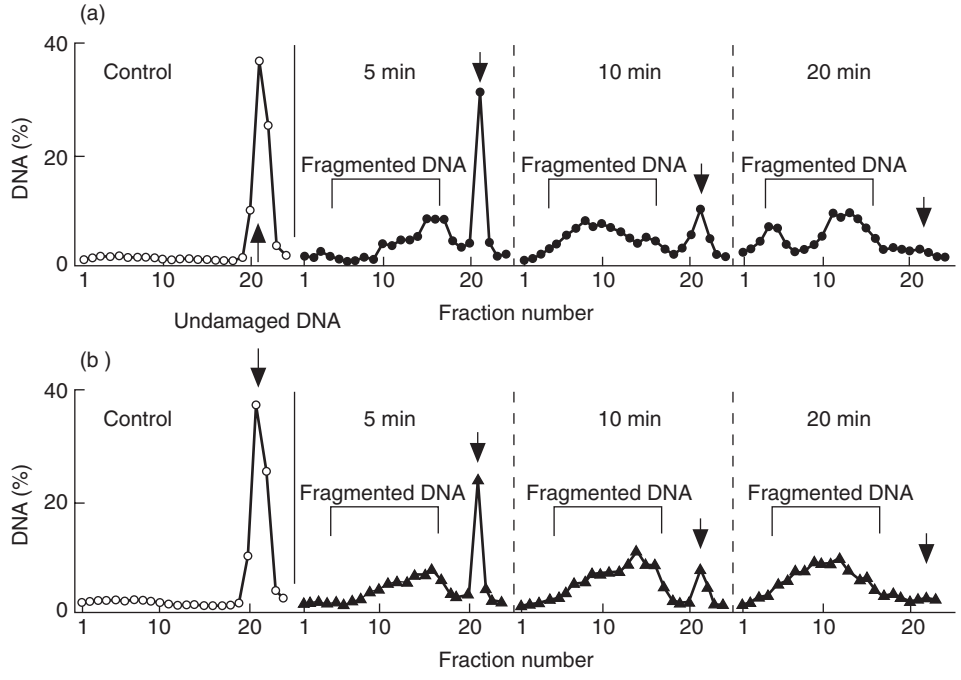

Fig. 2. Pancreatic β-cell DNA strand breaks by alloxan (1 mM) (a) and streptozotocin (2 mM) (b). Islets isolated from rat pancreas were incubated with alloxan or streptozocin for 5–20 min in Krebs–Ringer bicarbonate medium. After incubation the islets were laid on an alkaline 5–20 % sucrose gradient and centrifuged. (▲, ▼), Undamaged DNA. (Adapted from Yamamoto *et al*. 1981*a*.)

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substrate, resulting in cellular NAD^+ depletion. The fall in cellular NAD^+ decreases ATP and inhibits cellular functions including insulin synthesis and secretion, and thus the β-cell ultimately dies. Thus, this appears to be a suicide response for β-cells to DNA repair. The NAD^+ depletion and the decrease in β-cell functions were prevented by radical scavengers such as superoxide dismutase and catalase, and by the PARP inhibitors, nicotinamide and 3-aminobenzamide (Okamoto *et al*. 1988, 1997, 2000; Okamoto, 1990; Okamoto & Takasawa, 2002; Takasawa & Okamoto, 2002*a*,*b*). Recently, this model was supported by experiments using PARP-knockout mice by three independent groups in Germany, Japan, and the USA. In the pancreatic islets of wild-type mice, insulin-producing cells were almost completely destroyed by streptozotocin, but in PARP-deficient mice, the cells remained intact (Burkart *et al*. 1999; Charron & Bonner-Weir, 1999; Masutani *et al*. 1999; Pieper *et al*. 1999).

Interest in the model for the mechanism of action of alloxan and streptozotocin has been heightened by its possible extension to the effects of viruses, inflammation and radiation, especially immune-mediated events on β-cells. Thus, since the early 1980s, it has been proposed that, although type 1 (insulin-dependent) diabetes can be caused by many different agents such as immunological abnormalities, inflammatory tissue damage, and β-cytotoxic chemical substances, the final pathway for the toxic agents is the same (Fig. 4). Therefore, type 1 diabetes is theoretically preventable by suppressing immune reactions, scavenging free radicals, and inhibiting PARP by nicotinamide and 3-aminobenzamide (Okamoto, 1981, 1985, 1990; Yamamoto *et al*. 1981*a*; Uchigata *et al*. 1982; Okamoto *et al*. 1988, 1997, 2000; Okamoto & Takasawa, 2002; Takasawa & Okamoto, 2002*a*,*b*). Concerning NO, transgenic mice were produced expressing NO synthase constitutively in pancreatic β-cells and found that the β-cell mass was markedly reduced and that the transgenic mice developed severe diabetes (Takamura *et al*. 1998).

Recently, the cell death caused by the PARP activation has been recognized as providing the basis for necrotic cell death (Okamoto *et al*. 1988; Okamoto, 1990; Germain *et al*. 2000; Okamoto & Takasawa, 2002; Takasawa & Okamoto, 2002*a*,*b*). In apoptotic cell death, PARP is cleaved by caspases and inactivated. Therefore, PARP inhibitors can prevent necrosis but are ineffective for preventing apoptosis. Whether to die from necrosis or to die from apoptosis may depend on the severity and duration of the cell damage, differences in death signals, and the species of the cells. Furthermore, several lines of evidence indicate that macrophages and/or dendritic cells distinguish between the two types of cell death, with necrosis providing a control that is critical for the initiation of immunity (Sauter *et al*. 2000; Cocco & Ucker, 2001). Necrotic cells, when recognized, enhance the pro-inflammatory responses of activated macrophages and induce immune reactions. Therefore, immunological abnormalities, which are frequently observed in type 1 diabetes, may be triggered by the preceding necrotic cell death and then cause the apoptotic death of β-cells (Fig. 5). Recently, many other tissues and cells, such as those involved in diabetic endothelial injury and stroke, have been reported to die by the same mechanism as that involved in pancreatic β-cell death (Yamamoto *et al*. 1981*a*; Uchigata *et al*. 1982; Eliasson *et al*. 1997; Szabó *et al*. 1997, 1998; Zingarelli *et al*. 1998, 1999; Bowes *et al*. 1999; Burkart *et al*. 1999; Love *et al*. 1999; Mandir *et al*. 1999; Masutani *et al*. 1999; Oliver *et al*. 1999; Pieper *et al*. 1999, 2000; Stern *et al*. 1999; Tsao *et al*. 1999; Ducrocq *et al*. 2000; Jijon *et al*. 2000; Liaudet *et al*. 2000; Martin *et al*. 2000; Plaschke *et al*. 2000; Mabley *et al*. 2001; Soriano *et al*. 2001; Pacher *et al*. 2002). Recent human genome sequence projects have revealed the occurrence of similar DNA sequences to PARP. Until now, at least seven PARP-related genes (PARP-2, PARP-3, vaultPARP/PARP-4, Tankyrase/PARP-5, Tankyrase2, PARP-6, and PARP-7) in addition to the original PARP (PARP-1) have been isolated and revealed to constitute a multigene family, the PARP gene family (Shall, 2002). PARP-1 activation by extensive DNA damage is the major pathway in necrotic cell death, and therefore 'PARP' will be used from here onwards to indicate PARP-1.

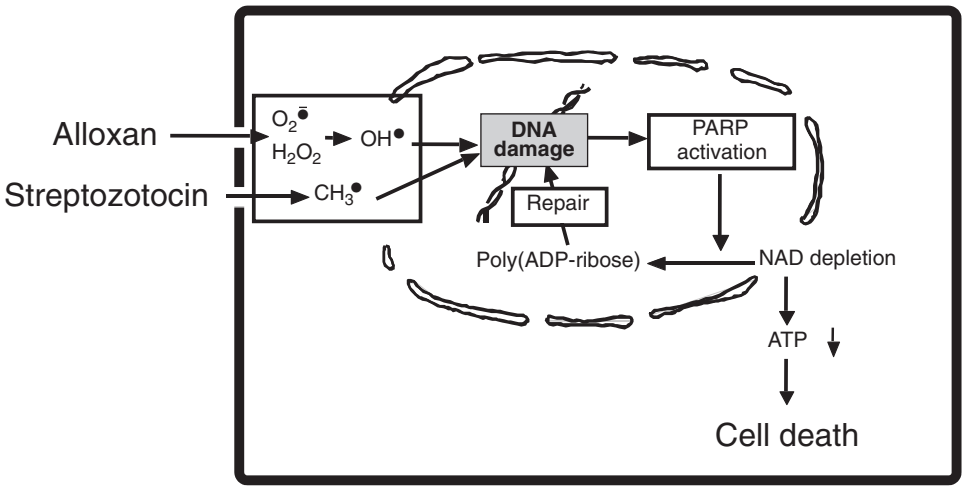

Fig. 3. A unifying model for the action of diabetogenic agents, streptozotocin and alloxan, on pancreatic β-cells. PARP, poly(ADP-ribose) synthetase/polymerase. (Adapted from Okamoto, 1981, 1985; 1990; Okamoto *et al.* 1988.)

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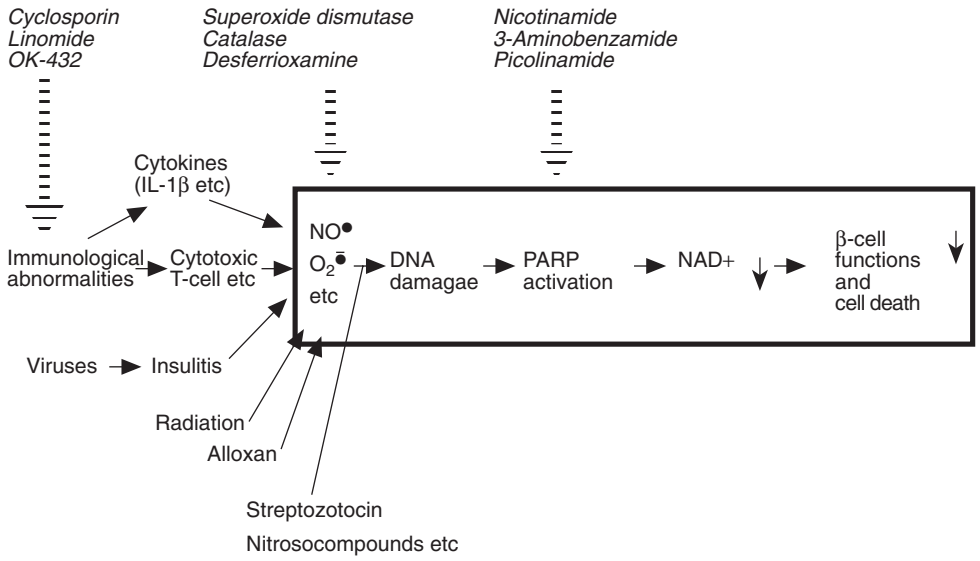

Fig. 4. A unifying model for β-cell damage and its prevention in toxin- or virus-induced diabetes and immune diabetes (the Okamoto model). The β-cell damage is theoretically preventable through inhibition of the serial reactions (⁂). One method is by inhibiting abnormal immune reactions with immunomodulators such as cyclosporin, linomide and OK-432. Others include scavenging the radicals, which break DNA, by superoxide dismutase and other radical scavengers and inhibiting the poly(ADP-ribose) synthetase/polymerase (PARP) by specific inhibitors such as nicotinamide, 3-aminobenzamide and picolinamide to prevent the decrease in the NAD^+ level. IL-1β, interleukin-1β. (Adapted from Okamoto *et al*. 1988; Okamoto *et al*. 1995; Okamoto *et al*. 1997; Okamoto *et al*. 2000; Okamoto, 1990; Okamoto & Takasawa, 2002; Takasawa & Okamoto, 2002*a*,*b*.)

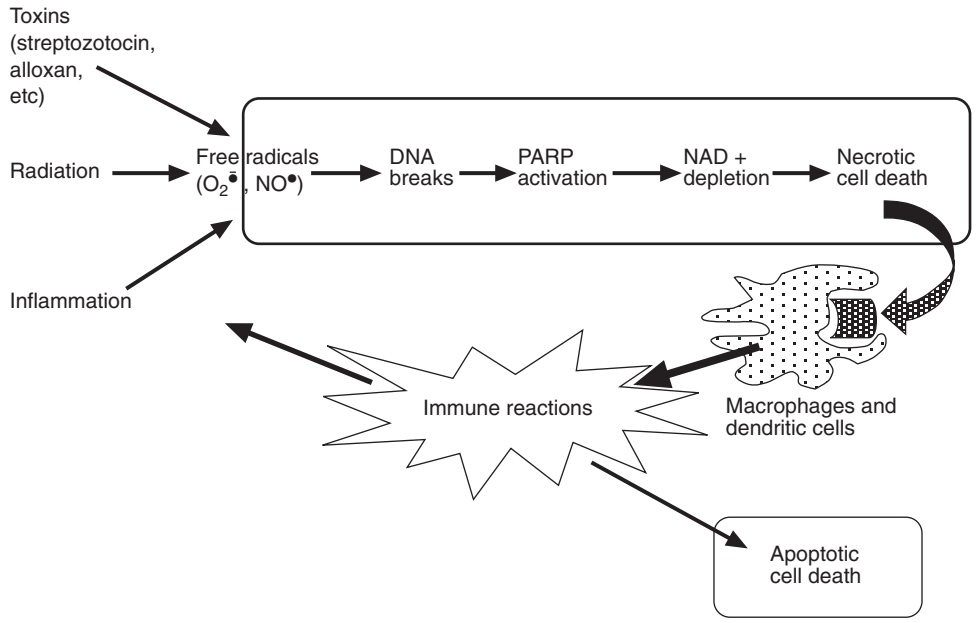

Fig. 5. The Okamoto model for necrotic cell death. The Okamoto model, originally proposed as a unifying model for β-cell damage and its prevention, explains well both how autoimmunity for β-cell necrosis is initiated and how necrotic cell death is involved in various diseases in many tissues other than β-cells. Under physiological conditions, apoptotic cell death constitutively occurs for renewal and maintenance in animal tissues, whereas necrotic cells initiate and enhance (auto)immune reactions under pathological conditions. On the other hand, apoptotic cells recognized by macrophages and dendritic cells inhibit phlogistic (auto)immune responses. For the initiation of massive and pathological apoptotic cell death, necrotic cell death, which triggers autoimmune responses in macrophages and dendritic cells, is required. The Okamoto model can explain the mechanism of necrotic cell death in many tissues and cells in various diseases. PARP, poly(ADP-ribose) synthetase/polymerase. (Adapted from Okamoto & Takasawa, 2002.)

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The ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase–cyclic ADP-ribose signal system for insulin secretion

Cyclic ADP-ribose is a cyclic compound synthesized from NAD^+. This compound was first found in 1987 by Dr Lee of Minnesota University when studying Ca^{2+} release in sea urchin eggs (Clapper *et al*. 1987). The physiological significance of cyclic ADP-ribose in mammalian systems was not understood at the time.

As shown in Fig. 4, decreases in the NAD^+ level under various circumstances can cause decreases in cyclic ADP-ribose in β-cells. In 1993, a model of insulin secretion by glucose was proposed, as shown in Fig. 6 (Takasawa *et al*. 1993*a*, 1994; Okamoto *et al*. 1997, 2000; Okamoto & Takasawa, 2002; Takasawa & Okamoto, 2002*a*,*b*). That is, ATP, produced in the process of glucose metabolism, inhibits the cyclic ADP-ribose hydrolase activity of CD38, causing the accumulation of cyclic ADP-ribose, which acts as a second messenger for Ca^{2+} mobilization from an intracellular Ca^{2+} pool, the endoplasmic reticulum, for insulin secretion. CD38 is a 300-amino-acid protein that catalyses the formation of cyclic ADP-ribose from NAD^+ and also the hydrolysis of cyclic ADP-ribose to ADP-ribose (Takasawa *et al*. 1993*b*; Tohgo *et al*. 1997). It was determined that glutamic acid-226 is essential for NAD^+ binding and that cysteine-119 and cysteine-201 are essential for the hydrolase reaction (Tohgo *et al*. 1994; Okamoto & Takasawa, 2001). It is of special importance that lysine-129 is the cyclic ADP-ribose binding site and that ATP competes with cyclic ADP-ribose for the binding site, inhibiting the cyclic ADP-ribose hydrolase of CD38 and increasing the cyclic ADP-ribose level (Tohgo *et al*. 1997). In fact, the cyclic ADP-ribose content of islets incubated with high-concentration glucose was increased within 5 min but not when incubated with low-concentration glucose (Takasawa *et al*. 1998).

Then, the Ca^{2+} release from islet microsomes was examined. Cyclic ADP-ribose caused the release of Ca^{2+} from islet microsomes. Inositol 1,4,5-trisphosphate (IP_3) did not cause the release of Ca^{2+}, and after the addition of IP_3 the islet microsomes were still responsive to cyclic ADP-ribose. In cerebellar microsomes, IP_3 caused the release of Ca^{2+} and cyclic ADP-ribose also caused the release of Ca^{2+}. Heparin, an inhibitor of the IP_3 receptor, inhibited the Ca^{2+} release by IP_3 but not that caused by cyclic ADP-ribose. Therefore, cerebellum microsomes respond to both cyclic ADP-ribose and IP_3, but cyclic ADP-ribose induces Ca^{2+} release via a different mechanism from that utilized by IP_3 (Takasawa *et al*. 1993*a*).

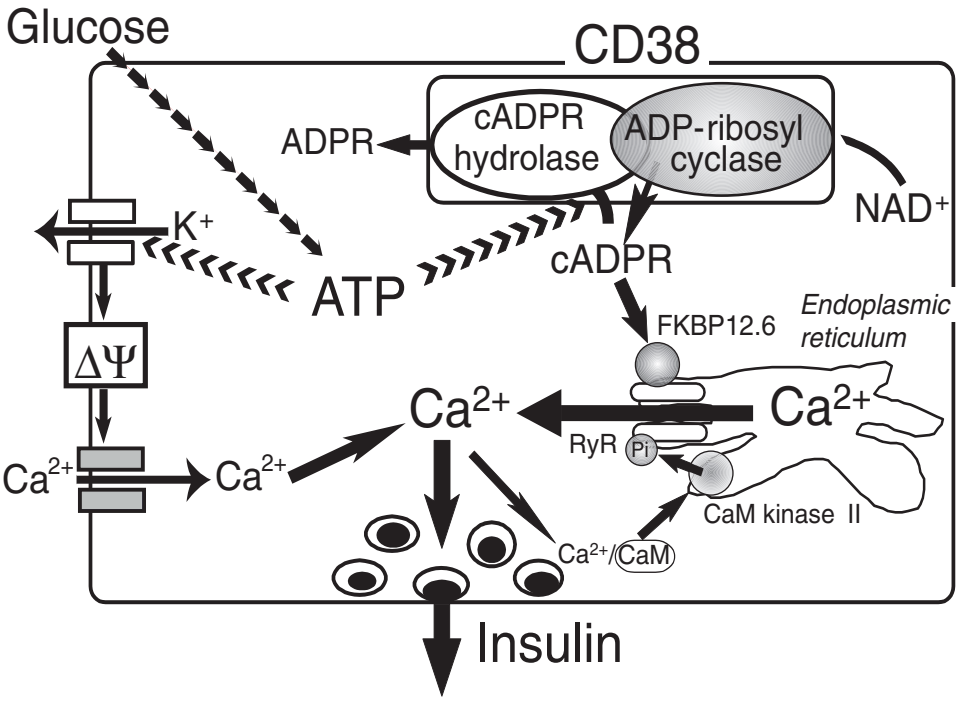

Fig. 6. The ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase (CD38)–cyclic ADP-ribose (cADPR) signal system for insulin secretion by glucose. In the process of glucose metabolism, millimolar concentrations of ATP are generated, inducing cADPR accumulation by inhibiting the cADPR hydrolase of CD38, and cADPR then acts as a second messenger for intracellular Ca^{2+} mobilization from the endoplasmic reticulum for insulin secretion through ryanodine receptor (RyR). Ca^{2+} and calmodulin (CaM)-dependent protein kinase II (CaM kinase II) phosphorylates RyR to sensitize and activate the Ca^{2+} channel (phosphorylation of RyR by CaM kinase II; Pi). Ca^{2+}, released from the RyR and influxed from the extracellular source, further activates CaM kinase II and amplifies the process (Takasawa *et al.* 1995). In this way, Ca^{2+}-induced Ca^{2+} release is explained (Okamoto *et al.* 2000; Takasawa & Okamoto, 2002*b*). cADPR binds to FK506-binding protein 12·6 (FKBP12·6) to release Ca^{2+}, dissociating FKBP12·6 from RyR (Noguchi *et al.* 1997). The conventional insulin secretion mechanism by Ca^{2+} influx from extracellular sources, proposed by Ashcroft *et al.* (1984), is also shown on the left. (Δ Ψ), Depolarisation of β-cell membrane. (Adapted from Okamoto *et al.* 1997; Okamoto & Takasawa, 2001, 2002; Takasawa & Okamoto, 2002*a*,*b*.)

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From these results and other available evidence, the CD38–cyclic ADP-ribose signal system for insulin secretion was proposed, as shown in Fig. 6. To verify our model of insulin secretion, CD38-knockout mice were created (Kato *et al*. 1999). In CD38-knockout mice, the ADP-ribosyl cyclase activity of the islet homogenate was almost undetectable. The cyclic ADP-ribose content in wild-type mouse islets was greatly increased when incubated with high-concentration glucose, but in CD38-knockout mouse islets the cyclic ADP-ribose content was not affected by high-concentration glucose. In the CD38-knockout mouse islets, the increase in the intracellular Ca^{2+} concentration by high-concentration glucose was much lower than that in wild-type mouse islets, and insulin secretion was severely decreased in knockout islets (Fig. 7).

The CD38–cyclic ADP-ribose signal system for insulin secretion is different from the ATP-sensitive K^+ channel theory proposed by Ashcroft *et al*. (1984). Furthermore, the CD38–cyclic ADP-ribose signal system is also different from the theory proposed by Berridge & Irvine (1984), in which IP_3 induces Ca^{2+} release from the endoplasmic reticulum. In this context, controversial results were reported by Swedish and Swiss groups (Islam *et al*. 1993; Rutter *et al*. 1994; Webb *et al*. 1996; Islam & Berggren, 1997). They used *ob*/*ob* mouse islets and RINm5F β-cells. Our group showed that the Ca^{2+} release responses of microsomes of diabetic β-cells such as *ob*/*ob* mouse islets and RINm5F β-cells were quite different from those of normal islet microsomes (Takasawa *et al*. 1998). Microsomes from normal C57BL mouse islets released Ca^{2+} in response to cyclic ADP-ribose but scarcely in response to IP_3. In contrast, *ob*/*ob* mouse islet microsomes released only a small amount of Ca^{2+} in response to cyclic ADP-ribose but released much in response to IP_3. The microsomes of RINm5F β-cells, which are a cell line derived from a rat insulinoma and show almost no insulin secretion response to glucose, responded well to IP_3 to release Ca^{2+} but did not respond to cyclic ADP-ribose. The mRNA expression of the type 2 ryanodine receptor, which is a Ca^{2+} release channel for cyclic ADP-ribose, was detected in normal islets but not in *ob*/*ob* islets. In contrast, IP_3 receptor mRNA was scarcely detectable in normal islets but was clearly detectable in *ob*/*ob* islets (Takasawa *et al*. 1998). This fits well with the observation that IP_3-induced Ca^{2+} mobilization preferentially acts in *ob*/*ob* islet microsomes.

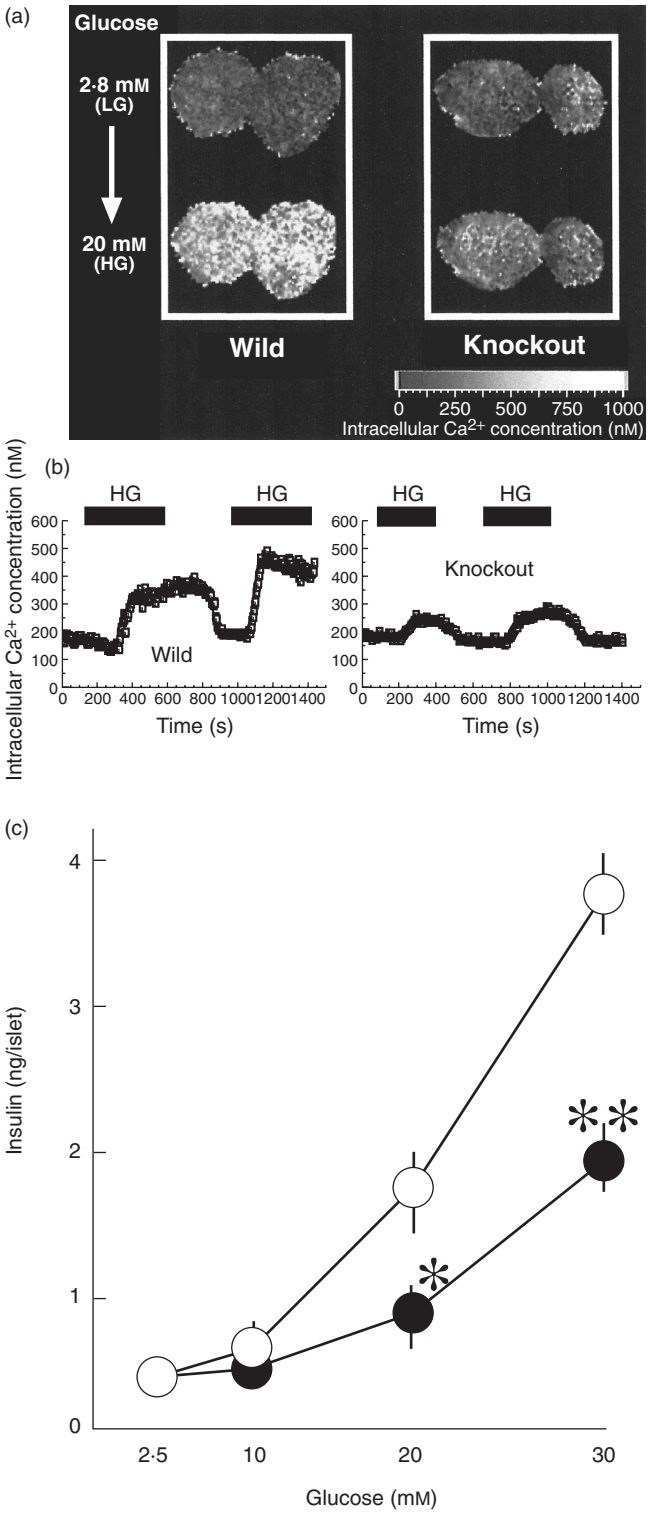

Fig. 7. Impaired Ca^{2+} signalling and insulin secretion in ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase (CD38)-knockout mouse islets. Digital imaging of intracellular Ca^{2+} concentration in the islets (a) and changes of intracellular Ca^{2+} concentration (b) in response to glucose stimulation are shown. Islets were loaded with fura-2 by a 30 min incubation at 37°C in Krebs–Ringer buffer containing 0·2 % (w/v) bovine serum albumin, 2·8 mM-glucose, and 25 μM-acetoxymethyl ester of fura-2. Islets were perfused with Krebs–Ringer buffer containing 2·8 mM-glucose (LG) or 20 mM-glucose (HG) at 37°C at a rate of 2·5 ml/min. Fura-2 excitation (340 and 380 nm) and fluorescence detection (510 nm) were accomplished, and the 340:380 nm fluorescence value was converted to intracellular Ca^{2+} concentration using QuantiCell 900. The interval between successive recording images was 4 s (Kato *et al*. 1999). Insulin secretion from isolated islets under various glucose concentrations is shown (c). Twenty islets were pre-incubated for 2 h at 37°C in RPMI1640 medium (1 ml) containing 10 % (v/v) fetal calf serum and 2·5 mM-glucose and then incubated for another 1 h in the same medium containing various concentrations of glucose. The medium samples were subsequently assayed by radioimmunoassay for insulin (Kato *et al*. 1995, 1999). (○), Wild-type mouse islets; (●), CD38-knockout mouse islets. Mean values were significanly lower than for the wild-type mouse islets: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$. (Adapted from Kato *et al*. 1999; Okamoto *et al*. 2000; Okamoto & Takasawa, 2002; Takasawa & Okamoto, 2002*b*.)

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Furthermore, the CD38 mRNA level was significantly decreased in *ob*/*ob* islets (Matsuoka *et al*. 1995), and in RINm5F β-cells CD38 was not expressed (Koguma *et al*. 1994). Therefore, the cyclic ADP-ribose signal system for insulin secretion is used under normal physiological conditions but is replaced by the IP_3 system in diabetic β-cells such as *ob*/*ob* mouse islets and RINm5F β-cells (Okamoto *et al*. 1997; Takasawa *et al*. 1998; An *et al*. 2001; Mitchell *et al*. 2001; Okamoto & Takasawa, 2002; Takasawa & Okamoto, 2002*b*; Varadi & Rutter, 2002). Cells can therefore utilize two second messengers, IP_3 and cyclic ADP-ribose, depending on the species of cells as well as differences in the cellular conditions, physiological or pathological (Fig. 8). Recently, a novel signal system, the CD38–cyclic ADP-ribose system, has been reported to function in various tissues and cells (Galione, 1993; Sasaki *et al*. 1993; Hua *et al*. 1994; Lee *et al*. 1994; Thorn *et al*. 1994; Allen *et al*. 1995; Gromada *et al*. 1995; Higashida *et al*. 1995, 1997; Kuemmerle & Makhlouf, 1995; Tanaka & Tashjian, 1995; Rakovic *et al*. 1996, 1999; Ebihara *et al*. 1997; Li *et al*. 1998; Mothet *et al*. 1998; Prakash *et al*. 1998; Yamaki *et al*. 1998; Guse *et al*. 1999; Inngjerdingen *et al*. 1999; Reyes-Harde *et al*. 1999; Sun *et al*. 1999; Han *et al*. 2000; Khoo *et al*. 2000; Fukushi *et al*. 2001; Partida-Sanchez *et al*. 2001; Chini *et al*. 2002; Tang *et al*. 2002). Therefore, the NAD^+ metabolism may control cellular functions via the novel metabolite, cyclic ADP-ribose.

Recently, a missense mutation was identified in the CD38 gene in Japanese diabetic patients (Yagui *et al*. 1998). The resulting CD38 protein showed altered catalytic activities with a decreased production of cyclic ADP-ribose. Furthermore, anti-CD38 auto-antibodies have been detected in 10–14 % of Japanese as well as Caucasian diabetic patients (Ikehata *et al*. 1998; Pupilli *et al*. 1999; Antonelli *et al*. 2001, 2002; Mallone *et al*. 2001). These results indicate that the CD38–cyclic ADP-ribose signal system functions in insulin secretion in man. Thus, both the CD38–cyclic ADP-ribose signal system and the ATP-sensitive K^+ channel system are important in insulin secretion in response to glucose (Okamoto *et al*. 1997, 2000; Okamoto & Takasawa, 2002; Takasawa & Okamoto, 2002*a*,*b*).

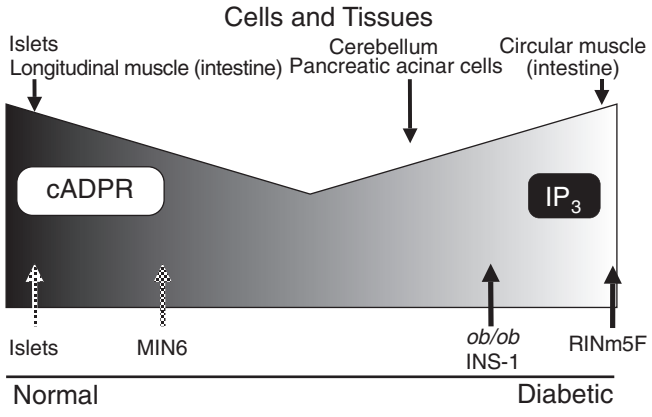

Fig. 8. Alternative use of the two Ca^{2+}-mobilizing second messengers depending on differences in the cell types and on physiological and pathological conditions. Various physiological phenomena from animal to plant cells become understandable in terms of this novel signal system. MIN6, INS-1 and RINm5F are β-cell lines. cADPR, cyclic ADP-ribose; IP_3, inositol 1,4,5-trisphosphate. (Adapted from Okamoto & Takasawa, 2002; Takasawa & Okamoto, 2002*b*.)

β-Cell regeneration and the regenerating gene

At the end of 19th century, von Mering & Minkowski (1890) in Strasbourg found that a dog became diabetic following pancreatectomy. This observation stimulated many workers to try to isolate the active pancreatic agent as a possible treatment for diabetes. In 1984, our group produced 90 % depancreatized rats and injected them with PARP inhibitors such as nicotinamide and 3-aminobenzamide intraperitoneally every day. Urinary glucose excretion was normalized in 90 % depancreatized rats treated with nicotinamide or 3-aminobenzamide, and the surgical diabetes was ameliorated. The islets in the remaining pancreases of the rats that had received the PARP inhibitors for 3 months were extremely large and almost the entire area of the enlarged islets stained for insulin (Yonemura *et al*. 1984). From the regenerating islets a novel gene, *Reg*, was isolated. The human *REG* gene was also isolated. Human and rat genes encoded 166 and 165 amino-acid proteins with signal peptides, indicating that Reg proteins are secretory proteins (Terazono *et al*. 1988). Three disulfide bonds were conserved in human and rat Reg proteins. A Reg protein receptor of 919 amino acids was also isolated. The Reg receptor-expressed cells showed increased 5′-bromo-2′-deoxyuridine incorporation upon the addition of Reg protein (Kobayashi *et al*. 2000). Thus, the Reg–Reg receptor system for β-cell regeneration was proposed (Fig. 9). Reg protein is synthesized in, and secreted from, pancreatic β-cells and acts on its receptor as an autocrine or paracrine growth factor (Watanabe *et al*. 1994; Gross *et al*. 1998; Unno *et al*. 2002). Our recent results indicate that the Reg receptor-mediated growth signal induces the cell cycle via cyclin (Okamoto & Takasawa, 2002).

More recently, it was found that the *Reg* gene is activated by interleukin-6 (IL-6), dexamethasone, and PARP inhibitors, and that the regeneration and proliferation of pancreatic β-cells are primarily regulated by *Reg* gene expression (Akiyama *et al*. 2001). The combined addition of IL-6 and dexamethasone induced *Reg* gene expression, and further addition of nicotinamide or 3-aminobenzamide increased the expression further. Progressive deletion of the 5′-flanking region of rat *Reg* gene revealed that the region between nucleotides −81 and −71 is essential for *Reg* gene promoter activity. In gel mobility shift assays using the *Reg* gene promoter, the DNA–protein complex was detected in the nuclear extracts of cells treated with IL-6, dexamethasone and/or nicotinamide, and the intensity was correlated with the promoter activity. The addition of NAD^+ to the nuclear extracts attenuated the complex. Nicotinamide and 3-aminobenzamide quenched the effect of NAD^+. These results suggest that PARP participates in the formation of the active transcriptional DNA–protein complex and that the formation of the active complex was inhibited by the poly(ADP-ribosyl)ation of nuclear proteins. The involvement of PARP in the active transcriptional complex was evidenced by the fact that the complex was stained by an anti-PARP antibody. The involvement of PARP in the

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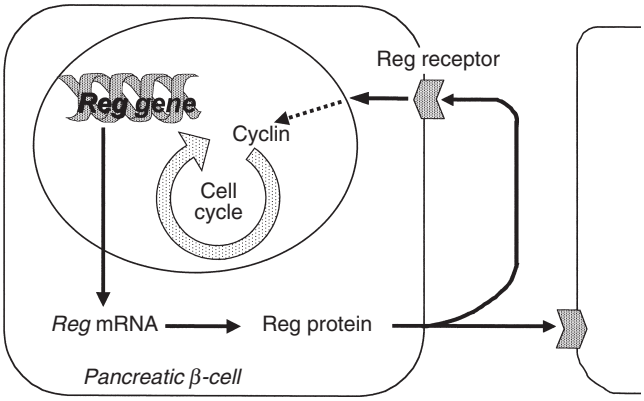

Fig. 9. The Reg–Reg receptor system for β-cell regeneration. Reg protein produced in β-cells acts as an autocrine and paracrine growth factor on β-cells via the Reg receptor. DNA replication and cell cycle progression in β-cells occurs via cyclin, and β-cell regeneration is achieved. *Reg*, regenerating gene. (Adapted from Okamoto & Takasawa, 2002.)

active complex was further evidenced by the immunodepletion of PARP with an anti-PARP antibody.

Thus, as shown in Fig. 10, inflammatory mediators IL-6 and glucocorticoids induce the formation of an active transcriptional complex for *Reg* gene, in which PARP is involved, and *Reg* gene transcription proceeds. On the other hand, during inflammation, superoxide and NO are produced and cause DNA damage. In this case, PARP is activated and poly(ADP-ribosyl)ates itself. The poly(ADP-ribose) chains on the PARP protein inhibit the formation of the active transcriptional complex, and *Reg* gene transcription is stopped. In the presence of PARP inhibitors, the PARP is not poly(ADP-ribosyl)ated, the transcriptional complex is stabilized, and *Reg* gene transcription proceeds. When the DNA is massively damaged, PARP is rapidly activated to repair the DNA, as has been mentioned earlier in the present review, and the complex for *Reg* gene transcription is not formed at all.

Recently, *Reg* and *Reg*-related genes have been isolated and shown to constitute a multigene family, the *Reg* gene family (Unno *et al*. 1993; Okamoto, 1999; Okamoto & Takasawa, 2002). Based on the primary structures of the Reg proteins, the members of the family are grouped into four subclasses; type I, II, III, and IV (Fig. 11). In man, four *REG* family genes, i.e. *REG Iα* (Watanabe *et al*. 1990), *REG Iβ* (Moriizumi *et al*. 1994), *REG*-related sequence (*RS*) (Watanabe *et al*. 1990), and *HIP/PAP* (genes expressed in hepatocelullar carcinoma, intestine, pancreas/gene encoding pancreatitis-associated protein), are tandemly ordered in the 95 kbp region of chromosome 2p12 (Miyashita *et al*. 1995), whereas *REG IV/RELP* (regenerating protein-like protein) locates on chromosome 1p12–13.1 (Hartupee *et al*. 2001; Kämäräinen *et al*. 2003). In the mouse genome, all the *Reg* family genes, i.e. *Reg I*, *Reg II*, *Reg IIIα*, *Reg IIIβ*, *Reg IIIγ*, and *Reg IIIδ*, were mapped to a contiguous 75 kbp region of chromosome 6C (Abe *et al*. 2000). Type I (and type II) Reg proteins are expressed in regenerating islets (Unno *et al*.

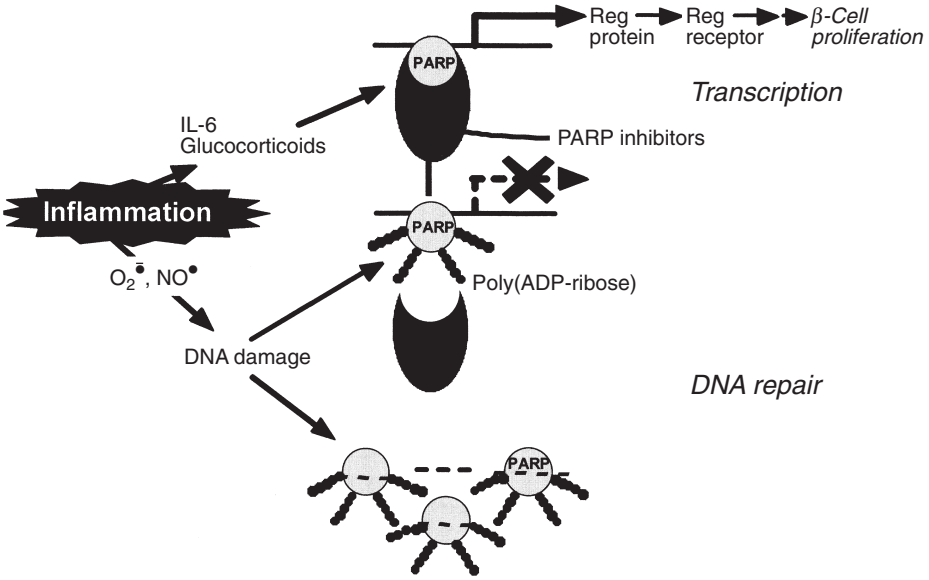

Fig. 10. Representation of the unified role of poly(ADP-ribose) synthetase/polymerase (PARP) in the regenerating (*Reg*) gene transcription and DNA repair. β-Cells are affected by many agents such as immunological abnormalities, virus infections, irradiation, and chemical substances (see also Figs. 3–5), leading to local inflammation in and/or around pancreatic islets. Inflammatory mediators such as interleukin-6 (IL-6) and glucocorticoids are produced in the inflammation process. IL-6–glucocorticoid stimulation induces the formation of an active transcriptional complex for *Reg*, in which PARP is involved. DNA-damaging substances such as superoxide ($O_2^{\bullet-}$) and nitric oxide (NO•) are frequently produced in inflammatory processes. When the DNA is damaged, PARP senses DNA nicks and auto-poly(ADP-ribosyl)ates itself for the DNA repair. Once PARP is self-poly(ADP-ribosyl)ated, the formation of the *Reg* gene transcriptional complex is inhibited, interfering with the interaction between PARP and other nuclear proteins necessary for the active complex, and therefore the transcription of *Reg* gene stops. When the PARP is not poly(ADP-ribosyl)ated in the presence of PARP inhibitors, the transcriptional complex is stabilized and the *Reg* gene transcription is maintained. When the DNA is massively damaged, PARP is rapidly activated to repair the DNA and the complex for *Reg* gene transcription is not formed at all. (Adapted from Akiyama *et al.* 2001; Okamoto & Takasawa, 2002; Takasawa & Okamoto, 2002*b*.)

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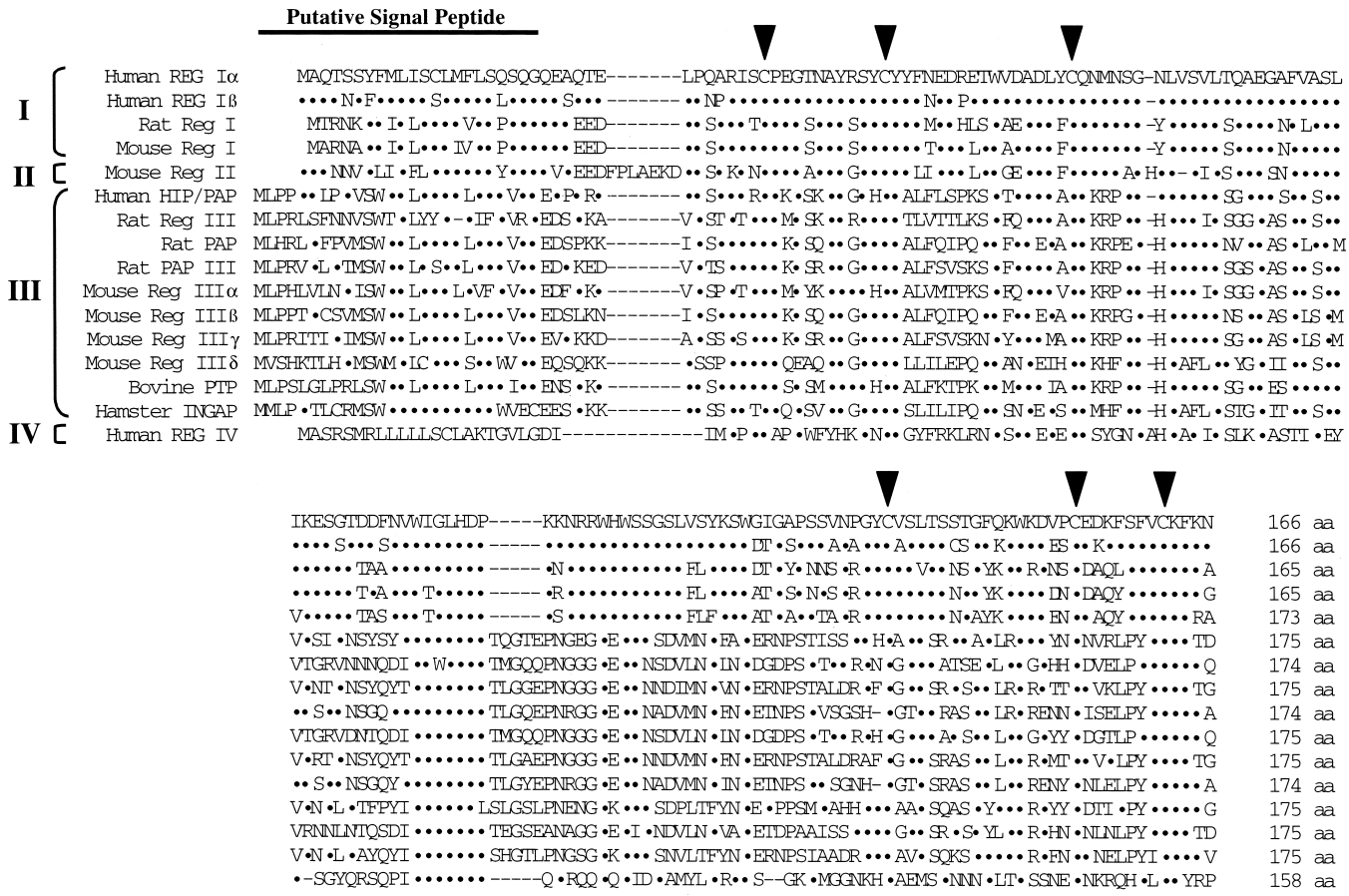

Fig. 11. Alignment of amino acid sequences of the regenerating (*Reg*) gene family. Amino acid sequences are depicted by single letter symbols. Based on the primary structures of the encoded proteins, the members of the *Reg* gene family are grouped into four subclasses; type I, II, III, and IV. (•), Amino acids identical to human REG Iα; (-), gaps for maximal alignment; (▼), six conserved cysteines in the mature proteins; aa, amino acids. (Adapted from Okamoto, 1999; Okamoto & Takasawa, 2002.) HIP, protein expressed in hepatocellular carcinoma, intestine, pancease; PAP, pancreatitis-associated protein; PTP, pancreatic thread protein; INGAP, islet neogenesis associated protein.

1993). Type III Reg proteins have been suggested to be involved in cellular proliferation in intestinal cells, hepatic cells, and neuronal cells. Importantly, mouse Reg III was shown to be a Schwann cell mitogen that accompanies the regeneration of motor neurons (Livesey *et al*. 1997), and Reg protein functions as a neurotrophic factor for motor neurons (Nishimune *et al*. 2000). Reg was also shown to mediate gastric mucosal proliferation (Asahara *et al*. 1996; Fukui *et al*. 1998; Kazumori *et al*. 2000; Alderman *et al*. 2003) and vascular cell viability (Kiji *et al*. 2003) in rats. The expression of Reg protein receptor mRNA has also been detected in liver, kidney, stomach, small intestine, colon, adrenal gland, pituitary gland, and brain (Kobayashi *et al*. 2000), suggesting that the Reg–Reg receptor signal system is involved in a variety of cell types other than pancreatic β-cells. In fact, our preliminary histopathological analyses of *Reg*-knockout mice showed that there are some structural abnormalities in tissue organization in the alimentary tract and liver.

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