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Review

Regenerating proteins and their expression, regulation, and signaling

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Abstract

The regenerating (Reg) protein family comprises C-type lectin-like proteins discovered independently during pancreatitis and pancreatic islet regeneration. However, an increasing number of studies provide evidence of participation of Reg proteins in the proliferation and differentiation of diverse cell types. Moreover, Reg family members are associated with various pathologies, including diabetes and forms of gastrointestinal cancer. These findings have led to the emergence of key roles for Reg proteins as anti-inflammatory, antiapoptotic, and mitogenic agents in multiple physiologic and disease contexts. Yet, there are significant gaps in our knowledge regarding the regulation of expression of different Reg genes. In addition, the pathways relaying Reg-triggered signals, their targets, and potential cross-talk with other cascades are still largely unknown. In this review, the expression patterns of different Reg members in the pancreas and extrapancreatic tissues are described. Moreover, factors known to modulate Reg levels in different cell types are discussed. Several signaling pathways, which have been implicated in conferring the effects of Reg ligands to date, are also delineated. Further efforts are necessary for elucidating the biological processes underlying the action of Reg proteins and their involvement in various maladies. Better understanding of the function of Reg genes and proteins will be beneficial in the design and development of therapies utilizing or targeting this protein group.

Keywords: cancer; diabetes; Reg expression regulation; regenerating (Reg) proteins; signaling.

Introduction

The first regenerating (Reg) protein was identified in pancreatic stones and was named pancreatic stone protein (PSP) (1). As later studies revealed, PSP could undergo cleavage by trypsin resulting in its insoluble form named pancreatic thread protein (PTP) and an 11-amino acid fragment (2, 3). PSP was also referred to as lithostathine due to its potential role in inhibiting pancreatic stone formation (4, 5). The protein was re-discovered independently in regenerating rat islets after pancreatectomy (6), and the term ‘regenerating protein’ was coined, although it was reported subsequently that Reg (now known as Reg1), PSP, and lithostathine refer to the same gene product.

In a similar manner, a related protein, the pancreatitis-associated protein (PAP) was independently found in acute pancreatitis samples (7), rat pituitary gland (peptide 23) (8, 9), and in hepatocellular carcinomas (hepatic intestinal pancreatic protein (HIP)) (10). Other members of the Reg family include Reg2, detected in the mouse genome (11), and the islet neogenesis-associated protein (INGAP), normally expressed in the acinar cells of hamsters and mice (12). Despite their initial association with pancreas, most Reg proteins are expressed in multiple organs. The latest addition to the Reg group is Reg4, which is overexpressed in drug-resistant colon cancer cells (13). All genes encoding for Reg proteins are approximately 3 kb in size, contain six exons/five introns and are on the same chromosomal locus (2p12) except for the Reg4 gene (1p11-3).

The findings, thus far, categorically show that Reg genes/proteins comprise a versatile group with a multitude of significant activities in various cell types in normal and diseased states. Although the first member of the Reg family was discovered over three decades ago, many aspects of the functions of Reg proteins and the mechanisms they utilize are still unclear. In this review, the various – sometimes confusing – conventions followed in the literature for Reg gene/protein names are presented first. Then, the expression of different Reg members in the pancreatic endocrine and exocrine compartments, along with their reported roles, is discussed. This discussion is extended to extrapancreatic tissues where several Reg members have been identified. The actions of Reg proteins in various contexts are also described. Subsequently, several factors are summarized, which have been identified as modulators of different Reg isoforms in different cell types. An important and largely uncharted area is the signaling pathways utilized by Reg ligands in conferring their observed

effects on cells including differentiation, proliferation, and apoptosis. An account of the signaling events documented to date, in conjunction with Reg actions, is given. Finally, a brief outlook is provided regarding research on Reg members and pertinent challenges.

Nomenclature of Reg family members

The discovery of various Reg family members independently has contributed, in large part, to the nomenclatural redundancy of the corresponding genes/proteins. For instance, PSP, lithostathine, PTP, P19, and Reg1 refer to the same gene product (14). The terminology for Reg genes/proteins becomes more perplexing with the indiscriminate use of Arabic/Roman numerals and Greek letters (e.g., Reg3a, RegIIIa, RegIIIα, or Reg3α) making more challenging the survey of pertinent findings in the literature. Efforts to standardize the names of different Reg proteins/genes across species led to their classification into four subfamilies based on DNA sequence and protein structure similarities (11, 15–17) (Table 1). According to the nomenclature suggested by Unno et al. (11) and Abe et al. (18): (i) Type 1 includes the mouse Reg1, rat Reg1, and human REG1α and REG1β (PSPα and PSPβ), (ii) Type 2 contains only the mouse and hamster Reg2, (iii) Type 3 encompasses the mouse Reg3α, Reg3β, Reg3γ and Reg3δ, the rat PAP/peptide 23 (p23), Reg3/PAP II and PAP III, and human HIP/PAP, INGAP-related protein (rp). Another member discovered recently that human REG4 appears to be homologous (19) to both PAP/HIP/p23 and PSP/lithostathine/REG1 but was originally classified in a separate subfamily (type 4).

Based on amino acid sequence homology, Reg family members from the same species, e.g. human, mouse and rat, are highly conserved (16). The coding region of the human REG1α and REG1β cDNA, for example, share 78% homology with each other (14) but only 38% and 39%, respectively, with REG4 (15). Interspecies (human, monkey, rodent, bovine, canine and porcine) homology is also evident for several Reg members (20). For instance, the rat Reg1 amino acid sequence is 68% homologous to the human REG1 (21) and 87% to the mouse Reg1 (11, 16). The Reg genes are considered to have evolved from a common ancestral species and also by duplication and divergence from common genes (17, 22, 23) given the high homology, similar intron-exon organization of the family members within and across species, and the presence of Reg members in the same chromosomal region in tandem order (except for Reg4). An evolutionary dendrogram created by Abe et al. (18) lists the evolutionary distance of the Reg family members from the gene of origin. Human, mouse, and rat tissues or organs where Reg genes have been identified are listed in Table 1. Additional information on Reg proteins/genes, such as their amino acid length and their chromosomal location can be found elsewhere (16).

Reg proteins in the pancreas

Type 1 Reg (Reg1, also known as PSP) is expressed in acinar cells under normal and pathological conditions including cancer and acute or chronic pancreatitis (11, 24–26). Immunoreactivity studies show the coexpression of Reg1 and pancreatic cell differentiation markers suggesting that Reg1 may play a role in the transdifferentiation of acinar cells to islets in patients with

Table 1 Reg family members in the mouse, rat, and human.

Name	Other names	Related tissue or organ
Mouse		
Reg1	Reg, PSP, PTP (cleaved form), Lithostathine	Pancreas, gallbladder
Reg2	PTP2, PSP2, Lithostathine 2	Pancreas, Schwann cells, motor neuron cells
Reg3α	PAP II	Pancreas, intestinal tract
Reg3β	PAP I, PAP, HIP	Pancreas, small intestine, liver
Reg3γ	PAP III	Pancreas, intestinal tract
Reg3δ	INGAP-rp, INGAP	Pancreas, stomach, duodenum, skeletal muscle
Reg4	RELP	Small intestine, gastrointestinal mucosa
Rat		
Reg1	Reg, PSP, PTP (cleaved form), Lithostathine	Pancreas, stomach, duodenum
Reg3α	Reg3, PAP II	Pancreas
Reg3β	PAP, PAP I, HIP, Reg2,Peptide23	Pancreas, small intestine, motor neurons
Reg3γ	PAP III	Pancreas, small intestine
Reg4	RegIV	Small intestine
Human		
REG1α	PSP, Lithostathine, PTP	Pancreas, gastric mucosa, colorectal cells, neurons
REG1β	REGH, REGL, Lithostathine, RS	Pancreas
REG3β	REG3A, PAP, HIP, PAP I, Reg2, PTP	Pancreas, liver, hepatocellular carcinoma
REG3γ	Reg3, PAP IB, PAP II, PAP III	Pancreas, liver, hepatocellular carcinoma
REG4	REGIV	Intestine, liver, pancreatic adenocarcinoma

RELP/REGL, Reg-like protein; REGH, Reg gene homologue; RS, Reg-related sequence; other abbreviations are described in the text.

chronic pancreatitis (27). Reg1 was also proposed to inhibit pancreatic stone formation (4), but subsequent studies rejected this hypothesis (5, 28). When Reg1 is digested by trypsin, it forms insoluble fibrils (referred to as PTP) that are resistant to further cleavage, but their physiological role remains unclear. It has been hypothesized that the PTP is generated in an attempt to protect the pancreas from further damage, but the resulting protein plug deposits contribute to pancreatic stone formation and chronic calcifying pancreatitis (20). A receptor of rat Reg1 was identified as homologous to a human multiple exostoses-like (EXTL) gene with previously unknown functions (29) (see 'Signaling pathways and Reg proteins').

Probably the best-documented effect of Reg1 is on the proliferation of acinar and islet cells (30). Reg1 is absent from healthy islets, as evidenced by the negative Reg1 immunoreactivity of normal rat islets (31, 32) and the lack of Reg1 mRNA expression in normal mouse islets (11). Diabetes-prone rats also do not exhibit Reg1 immunoreactivity in their inflamed islets (33). However, the expression of Reg1 (6, 11, 31, 32) increases in regenerating or hyperplastic islets. The augmented Reg1gene expression (34, 35) during β-cell replication points to a role of Reg1 as a marker for distinguishing replicating from differentiating β-cells. Besides β-cell proliferation, the expression of Reg1 only during islet regeneration or hyperplasia may suggest a possible involvement in islet cell ontogeny and maturation. In regenerating islet cells, Reg1 is detected in the central core of secretory granules (31). The mitogenic effect of Reg1 has also been confirmed in transgenic rodent models where the enhanced Reg1 expression or exogenous Reg1 administration causes islet proliferation and the amelioration of diabetes (21). The islets of $Reg^{-/-}$ mice display the same morphology as normal islets but decreased [^{3}H]thymidine incorporation. In contrast, transgenic mice with islet-specific overexpression of the Reg gene under the rat insulin promoter II (Ins-Reg) exhibit higher [^{3}H]thymidine incorporation (36). Furthermore, non-obese diabetic (NOD) mice carrying the Ins-Reg transgene show a significantly delayed development of diabetes compared to non-transgenic NOD mice, most likely due to the increased regeneration of β-cells. Similar to these *in vivo* observations, growth factors (see 'Factors regulating Reg expression') inducing Reg1 gene expression in cultured islets increase cell proliferation (37). Exogenous Reg1 is also mitogenic (30, 38) for pancreatic cell lines and rat gastric epithelial cells (39). Despite promoting islet cell proliferation, Reg1 is not present in proliferating β-cells immediately after birth as immunostaining studies of rat islets show (33). Thus, it appears that Reg1 is expressed only during proliferation of β-cells after damage. Moreover, Reg1 colocalizes with islet differentiation markers (e.g., insulin, chromogranin A) in transdifferentiating cytokeratin 19 $(CK19)^+$ acinoductular cells from human patients with chronic pancreatitis (27). Despite its islet proliferation- and regeneration-promoting effects, the tumor-promoting activity of Reg1 may hinder its clinical use for diabetes treatment (40,41). Overall, the roles of Reg1 members in islet regeneration and pancreatic cancer are still under intense investigation.

Reg2 has only been found in mice and hamsters (21). Nonetheless, mouse Reg2 exhibits 76% amino acid sequence homology with mouse Reg1 and 63% homology with both human REG1α and REG1β. Based on this comparison, Reg2 has been categorized as a member of the Reg1 subfamily in some reports (16, 42, 43). This may create confusion since a reportedly rat Reg2 protein has been considered a member of the type 3 subclass of the Reg gene family given its homology with mouse *Reg3*β (44, 45). Mouse *Reg2* mRNA is strongly expressed in the normal pancreatic acini and weakly in liver. *Reg2* expression is also evident in hyperplastic but not normal islets (11).

The third subclass of the Reg family consists of Reg3α, Reg3β, Reg3γ, and Reg3δ. These are expressed in the pancreas but not in hyperplastic islets (18, 22). Similar to Reg1, Reg3β – also known as PAP – can be cleaved by trypsin to form insoluble fibrils, and both Reg1 and Reg3β are coordinately secreted as stress proteins during pancreatic inflammation (25, 26). The nuclear factor kappa B (NF-κB)/Rel, which is involved in inflammatory disease, upregulates Reg3β to protect acinar cells during infiltration based on *in vivo* evidence (46). In cultured acinar cells, NF-κB-mediated upregulation of Reg3β during oxidative stress reduces apoptosis but not necrosis (47). Inhibition of acinar cell apoptosis by Reg3β is triggered by the addition of tumor necrosis factor-α (TNF-α) through an antiapoptotic mechanism involving NF-κB and mitogen-activated protein kinases (MAPK) (48). The transcription cofactor p8 is also implicated in upregulating Reg3β, which acts as an anti-inflammatory factor to improve pancreas resistance to acute pancreatitis inducers (49).

The mouse *reg3*δ mRNA has been detected only in normal pancreas but not in other tissues, such as the pituitary gland (unlike other Reg3/PAP members) despite the presence of Pit-1 binding sites on its promoter (18). In the mouse pancreas, Reg3δ is detected in exocrine cells and endocrine non-β-cells predominantly located at the islet periphery (18, 50, 51). When human, rat, and hamster genomes were subjected to Southern blot hybridization with the mouse *reg3*δ gene probe, bands appeared with sizes different than those of other Reg family members leading to the discovery of Reg3δ orthologs in these species. It should be noted that Reg3δ shows 72% amino acid sequence homology to the peptide-23/INGAP-rp (18, 52). Moreover, the *reg3*δ and INGAP cDNA sequences are 77% homologous suggesting that INGAP is the hamster ortholog of mouse *reg3*δ.

INGAP is detected in the healthy pancreas, duodenum, stomach, and skeletal muscle (52). Hamster acinar cells express INGAP during islet neogenesis after cellophane wrapping of the pancreatic duct (12). Protein extract isolated from these hamsters contains INGAP and reverses diabetes in hamsters treated with streptozotocin. Sucrose administration to hamsters also increases the expression of INGAP and islet cell mass, concomitantly (53). In fact, the total number of $INGAP^+$ cells rises mostly at the islet periphery (96%) as well as in the exocrine (3%) and ductal areas (<1%). Moreover, transgenic mice with a sustained INGAP expression in the acinar cells are resistant to diabetogenic doses of streptozotocin and have improved islet function (51, 54).

Interestingly enough, INGAP has been hypothesized to stimulate the differentiation of putative pancreatic stem cells (55) (including ductal cells (27)) to islet cells in certain pancreatic pathologies, especially diabetes (12, 56, 57). Administration of INGAP or its pentadecapeptide fragment (INGAP104–118) leads to increased β-cell mass in diseased and healthy pancreases. For instance, islet neogenesis has been observed in healthy dogs after intramuscular injection of INGAP (58). Even islet cells induced to differentiate into duct-like epithelial cells expressing ductal/progenitor cell markers, such as CK19, carbonic anhydrase, Ngn3, and nestin, can be coaxed back to functional islet cells in the presence of the INGAP104–118 (59). In addition, INGAP has been suggested to regulate islet ontogeny. Stem cell subpopulations positive for INGAP and pancreatic and duodenal homeobox-1 (PDX-1) at an early stage of development are highly activatable during neogenesis (60). Because of its induction of islet neogenesis, INGAP may be utilized in therapies for diabetes, where the β-cell mass is reduced (51).

Besides its role in islet cell proliferation and differentiation, INGAP may participate in other cellular processes as well. In cultured rat islets, the release of insulin stimulated by glucose or other amino acids is enhanced in the presence of the INGAP peptide (61). It is therefore possible that INGAP may act on islet genes involved in β-cell metabolism and insulin secretion. INGAP may also act in other organs outside the pancreas, although such roles are still largely unexplored.

The last subfamily, type 4, of Reg proteins contains only one member. Mouse Reg4 has 65% and 89% amino acid sequence similarities to the human and rat Reg4, respectively (16). Reg4 is expressed in rat acinar cells (62) and human insulin-producing β-cells (63). Overexpression of REG4 has been linked to the initiation and progression of pancreatic cancer leading to the notion of utilizing the protein as a marker for screening pancreatic adenocarcinoma (63, 64). Also, Reg4 has been suggested as a target for adjuvant therapy for pancreatic cancer (65).

Reg proteins in extrapancreatic tissues and cells

Reg family proteins are detected in several organs under normal and pathological conditions. At least one of the REG1α, REG1β, and REG3β is detected in human fetal and adult pancreas, brain, stomach, intestine, and pituitary gland (66), but the expression patterns and relevance to diseases and/or tissue homeostasis remain unclear. Members of the Reg3 subclass are expressed in the intestinal tract (Reg3α, Reg3β, Reg3γ) (18, 22) and duodenum of mouse (Reg3δ) and hamster (INGAP) (52) as well as the columnar epithelial cells of rat ileum, jejunum, and duodenum (Reg3β) (67). The expression of multiple Reg members in the same cell/tissue may indicate a compensatory mode of action, although direct supporting evidence is still lacking.

Type 1 Reg members are detected in tissues from the duodenum, gallbladder (11), and human brain (68). The expression of rat Reg1 in the duodenum is augmented by the growth hormone-releasing hormone (69). Moreover, Reg1 regulates gastrin-induced cell growth (70) in the fundic mucosa, where its expression coincides with that of the putative Reg1 receptor exostosin-like 3 (EXTL3) (29) (also known as exostosin-related 1 (EXTR1)) (71). In inflammatory bowel disease (IBD), the human REG1α is processed and exported, reducing cell apoptosis and contributing to colonic mucosal regeneration (72). Reg1-deficient mice have normal gastric development, but Reg1 promotes gastric mucosal growth and restoration synergistically with gastrin (73). The loss of Reg1 is dispensable for the development of gastric tumors as shown in the $gp130^{F/F}$ mouse model of gastric cancer (74). The deficiency in Reg1 of $gp130^{F/F}/Reg1^{-/-}$ mice is concomitant with the altered expression of other Reg family members, namely, the decrease in Reg3β levels. However, Reg1 is also found in enterochromaffin-like cells of hypergastrinemic patients with mutations preventing its secretion (75) and may act as a paracrine/autocrine tumor suppressor.

Human REG1α, -1β, and -3β are also upregulated in colorectal cancer tissues (76, 77). Similar to hyperplastic or regenerating cells, REG is expressed in human colon cancer cell lines during proliferation but is downregulated once enterocytic differentiation transpires (78). The expression of REG3β in human hepatocellular carcinoma samples, but not in fetal or adult normal cells, suggests its involvement in the proliferation or differentiation of liver cancer cells (10). This finding conflicts with observations of structural abnormalities in the liver and alimentary tract in Reg1 knockout mice (79) supporting a role for Reg1 in development. Reg3β (PAP) has also been implicated in lung inflammation during acute pancreatitis. Rats receiving PAP exhibit overexpression of hepatic TNF-α leading to lung inflammation, which is curtailed upon injection of anti-TNF-α antibodies (80).

Reg proteins are also expressed in cells of the central nervous system. PTP-like proteins (cleaved insoluble fibrils) referred to as neuronal thread proteins (NTPs) are found in the brain and have been associated with neuronal differentiation (20). They are also detected in primitive neuroectodermal tumor cell lines. The PTP/Reg1 is found in normal neurons, while its expression is higher in those of Alzheimer patients (68). Moreover, Reg2 is found in developing and regenerating rat motor and sensory neurons. Livesey et al. (81) showed that Reg2/PAP may cause Schwann cell proliferation during regeneration of motor neurons. Reg2 also has anti-apoptotic effects because cytokines related to the motor neuron survival ciliary neurotrophic factor (CNTF) induce its expression (45). The INGAP104–118 peptide promotes neurite outgrowth in cultured mouse dorsal root ganglia (82) and ameliorates nerve damage due to streptozotocin-induced diabetes (83).

Another member of the Reg family, Reg4, is prominently expressed in the human gastrointestinal tract, and its mRNA is significantly upregulated during mucosal injury due to active Crohn's disease or ulcerative colitis (15). Reg4 expression is also linked to pancreatic cancer and malignancies of the stomach, intestine, colon, rectum, gallbladder, and urogenital tract (13, 63, 84–86). In experiments with colorectal tumor

cell lines, the expression of Reg4 was stronger than that of other Reg members (13). Whereas Reg4 expression is low in normal colon, its level in normal small intestine is on a par with that in colorectal tumor samples. In a comparative study (87), Reg4 expression was the highest in intestinal metaplasia tissues and gradually lower in adenoma, carcinoma, and gastritis samples subjected to immunohistochemistry and RNA-DNA *in situ* hybridization. Like in pancreatic adenocarcinomas, Reg4 has also been proposed as a marker for gastric cancer (88), but its relevance of predicting the disease and survival probability of patients is still unresolved (88, 89).

The first account of Reg expression in embryonic stem cells (ESCs) was provided by Jing et al. (90). When probed for different Reg genes, mouse ESCs (mESCs) showed expression of Reg1 and Reg3γ under non-differentiating conditions. The putative Reg receptor Extl3 was also detected suggesting that Reg may act as an autocrine/paracrine factor in mESCs. When mESCs were treated with wingless-related MMTV (mouse mammary tumor virus) integration site 3A (Wnt3a) or LiCl (an activator of canonical Wnt), Reg1 gene expression was upregulated leading to an increase in secreted protein. Interestingly enough, the study findings also pointed to a possible role for Reg in the adoption of an endodermal fate by differentiating mESCs. Cells treated with exogenous Reg1 or overexpressing Reg1 after transduction with a recombinant adenoviral vector (AdReg1GFP) displayed enhanced expression of the endoderm genes *sox17* and *foxa2* [by quantitative polymerase chain reaction (qPCR)], whereas the levels of genes characteristic of mesoderm and ectoderm lineages were not different from those in control cells. The adoption of endodermal fate was also corroborated by immunocytochemistry analysis. Whether Reg proteins can serve as factors for coaxing stem cells to particular lineages is still an open question. Moreover, there are only a few published studies (66, 69, 91) to date on the expression pattern of Reg family members during (primarily rodent) fetal development hampering efforts to address if these molecules stimulate *in vivo* differentiation. Nonetheless, Reg proteins are present in cells undergoing regeneration, differentiation, and proliferation in the pancreas, intestine, and other tissues under normal and/or stress conditions. Therefore, the prospect of Reg proteins acting as differentiation agents is intriguing and warrants further investigation.

Reg proteins and bacterial aggregation

Reg1 and Reg3β have been observed to promote bacterial aggregation, which is enhanced with Reg1 fibrils cleaved by trypsin. It has been postulated that these secretory stress proteins may control bacterial levels (92, 93). Owing to the homology between the Reg1 fibril and carbohydrate-binding, Ca^{2+}-dependent lectins (C-type lectins) (94–96), it was conjectured that bacterial aggregation is caused by putative carbohydrate-binding domains on Reg1 and Reg3β facilitating the attachment to carbohydrates on bacterial surfaces. However, this hypothesis was ruled out by the lack of (i) a functional carbohydrate-binding site in a 3D model of the Reg1 protein (20) and (ii) aggregation interference after carbohydrate addition (93).

Factors regulating Reg expression

The production of Reg proteins is observed during development and is induced after tissue damage or in various pathologies, such as diabetes and cancer. The expression of Reg1 in the human embryonic pancreas is detected as early as 16 weeks during gestation (97, 98). Mouse islets become positive for Reg1 after induction of diabetes with streptozotocin (99). Reg2 is also overexpressed following administration of cyclophosphamide and in NOD mice (100), where it is believed to act as a β-cell-derived autoantigen (101). Another Reg, the HIP/PAP/Reg3, is expressed in the islets and ductal epithelium of prediabetic and diabetic NOD mice (102). Both Reg1 (REG1α) and Reg3 (REG3A/REG3α) are detected in human primary liver tumors with β-catenin mutations (103, 104).

In addition, to identifying the Reg genes/proteins expressed during development, regeneration, and/or in disease, efforts concentrated on the discovery of factors regulating their expression. Genes from the Reg family are activated in rat islets by cytokines, hormones, and nutrients including glucose, amino acids, serum, insulin, growth hormone, and the platelet-derived growth factor (PDGF) (37). Still, multiple factors may regulate Reg proteins in a combinatorial fashion. Dusetti et al. (105) reported two interleukin-6 (IL-6) response elements (T^{-266}TCCCAG^{-260} and T^{-249}TCCCAG^{-243} relative to the transcription initiation site) in the rat PAP I (Reg3β) promoter. Although incubation of acinar AR42J cells with IL-6 or IL-1 alone did not increase Reg expression, IL-6, synergistically with dexamethasone, led to significant induction of PAP expression. Moreover, this activation was not completely abolished with mutations in both IL-6 response elements pointing to the presence of additional *cis*-regulatory elements. Similarly, lithostathin/Reg1 is upregulated in acinar cells by IL-6 and dexamethasone together but not by IL-6 or IL-1 alone or in combination (106). Besides acinar cells, activation of lithostathin/Reg1 by IL-6 and dexamethasone together was also observed in rat insulinoma cell line (RINm5F) β-cells (107) and was attributed to the presence of a promoter *cis*-element (T^{-81}GCCCCTCCCAT^{-70}). This is a binding site for the poly(ADP-ribose) synthetase/polymerase (PARP), and PARP inhibitors (e.g., nicotinamide) induce islet regeneration in depancreatized rats with concomitant increase in Reg expression and secretion. Another cytokine, IL-22 stimulates the PAP mRNA transcription in acinar cells (108). The action of IL-22 on PAP is via signal transducer and activator of transcription 3 (STAT3) activation and requires the IL-10 receptor β (IL-10Rβ), a common component of the IL-10 and IL-22 receptors. A hypothesis that IL-10 also activates PAP was proven in a subsequent study utilizing AR42J cells (109). In the same study, PAP stimulated its own mRNA expression indicative of the existence of a positive feedback mechanism.

Rat PAP/HIP/p23 and lithostathin/Reg1 are upregulated in pancreatic acinar AR42J cells treated with interferon gamma (IFN-γ) and TNF-α independently. Here, however, the addition of dexamethasone to INF-γ- or TNF-α-treated cells curtails the expression of lithostathin (108). Inhibitors of MEK1 also reduce the TNF-α-activated expression of PAP suggesting involvement of this pathway in PAP regulation (48). The proinflammatory and proapoptotic factor lipopolysaccharide (LPS) also boosts PAP/HIP expression both directly and indirectly via the upregulation of TNF-α and interleukins (110). Similarly, PAP levels are enhanced by other apoptosis-associated factors, such as troglitazone (111) [a peroxisome proliferator-activated receptor-gamma (PPAR-γ activator)], cycloheximide (112) [in conjunction with poly(ADP)-ribosylation], and arginine (113). Thus, the expression of PAP may be linked to the elicitation of apoptosis (and act as anti-apoptotic agent), but further studies are needed to elucidate the underlying mechanisms.

The transcription factors NeuroD, activator protein (AP)-1, STAT, and transcription factor 3 (TCF3) bind to the hamster INGAP promoter and stimulate expression of the gene (114). PDX-1, a major transcriptional regulator of pancreatic β-cell development and homeostasis, binds directly to the INGAP promoter and stimulates its activity. When, however, PDX-1 is combined with other inductive transcription factors, INGAP gene activation is suppressed. PDX-1 expression rises in duct cells when INGAP is administered, and islet neogenesis occurs (56). Thus, PDX-1 may be operating in a feedback loop with INGAP to block uncontrolled islet cell expansion.

Extrapancreatic cells share the regulation of Reg genes observed in pancreatic cells. Similar to its regulation in acinar cells, PAP/HIP (Reg3β) is induced in intestinal cells treated with cytokines, such as INF-γ and IL-6 linking directly the overexpression of Reg proteins to intestinal inflammatory maladies. Of note is the fact that Reg1 mRNA is expressed in the small intestine, and $Reg1^{-/-}$ mice exhibit fewer proliferating [$Ki67^{+}$ or proliferating cell nuclear antigen $(PCNA)^{+}$] intestinal cells (115). Lithostathin/REG1α mRNA is markedly upregulated in human gastric corpus cells (and AR42J acinar cells) after exposure to gastrin. This effect is weakened by the gastrin/cholecystokinin B receptor (CCKBR or CCK2R) antagonist L740093 (75). Targeting of Reg proteins by CCK2R activation was also demonstrated in an ElasCCK2 transgenic mouse expressing the human CCK2R under the control of the elastase I promoter in pancreatic acini (116). Reg stimulation by gastrin appears to be mediated by activation of protein kinase C (PKC) and RhoA. Exposure of gastric cancer AGS-G_R cells to the PKC inhibitor Ro-32-0432 and the Rho family GTPase RhoA inhibitor C3-transferase (*Clostridium botulinum* toxin), or transfection with a dominant negative form of RhoA suppresses Reg expression in the presence of gastrin (117). The Reg promoter contains a GATA site (–91 to –86) and C-rich sequence (–79 to –76). Mutation of the GATA site has little effect on the Reg response to gastrin, but mutating the sequence $G^{-80}CCCCTCCCA^{-71}$ reduces basal expression and inhibits the response to gastrin by 50–80%.

Along with gastrin, the expression of Reg in gastric cells is also modulated by bacterial infection. Patients with *Helicobacter pylori* infection show significantly increased expression of REG1α. Similarly, higher levels of Reg1 have been observed in a mouse model of gastric cancer (118) after infection with *Helicobacter felis* (119). Analysis of two promoter constructs (–2111 and –104 bp) revealed that distinct elements are required for Reg stimulation by *H. pylori* and *H. felis*. Furthermore, the gastrin-interacting site on the Reg promoter is different from the one responsive to *H. pylori* infection. When a C-rich region ($G^{-98}GCTTT^{-93}$) of the promoter was mutated, the expression of Reg was rendered insensitive to *H. pylori*, but responsiveness to gastrin was retained. Although the precise promoter elements regulating Reg gene expression require further elucidation, the link of *H. pylori* infection to Reg upregulation reveals an attractive target for therapeutic interventions.

As already mentioned, Reg proteins are also implicated in the proliferation of central nervous system cells. Leptin or IL-6 alone activates PAP (Reg3β) in PC12 cells (120) derived from a pheochromocytoma of the rat adrenal medulla. This is not surprising considering that the leptin receptor shares very high sequence similarity with the IL-6 receptor (121). The activation of PAP by leptin is mediated by STAT3 (similar to IL-22, see above) but not STAT1 (120). Yet, the adenylyl cyclase activator forskolin enhances the leptin-induced PAP expression but lessens IL-6 activation of PAP indicating potential differences in signaling mediating the actions of these stimuli. Rat Reg2/PAP I (Reg3β) (81) has been coined a Schwann cell or motor neuron mitogen facilitating regeneration after injury. Mouse embryos homozygous for a mutated leukemia inhibitory factor (LIF) receptor, do not express *reg2* (i.e., *reg3*β) in their motor and sensory neurons. Therefore, Reg2 expression may depend on LIF family cytokines acting through the LIF receptor complex (gp130 and LIF receptor β). Indeed, the IL-6-related neurotrophic cytokines of the LIF/CNTF family including CNTF, cardiotrophin-1 (CT-1) and LIF binding to the same receptor complex induce the expression of Reg-2/PAP I (Figure 1A) (45). In contrast, the glial cell line-derived neurotrophic factor (GDNF), neuro trophin-3, hepatocyte growth factor (HGF) and fibroblast growth factor-2 (FGF-2) do not alter Reg2/PAP I expression.

The expression of Reg proteins is also detected in other endocrine tissues, such as the pituitary gland and ovaries and is regulated by the aforementioned factors. Rat pituitary cells secrete PAP/HIP (Reg3β) when treated with growth hormone-releasing hormone, whereas exposure to somatostatin has the opposite effect (9). Additionally, Reg proteins (p23/PAP III, Reg3β, and Reg3γ) in the ovaries and uterus are regulated by chorionic gonadotropin-induced progesterone synthesis (122) and estrogens (123), respectively.

Lastly, HIP/PAP (Reg3β) is not expressed in normal hepatocytes, but its production is stimulated after partial hepatectomy or other liver injury (124). Transgenic mice with human HIP/PAP overexpression have higher fractions of $BrdU^{+}$ hepatocytes than wild-type littermates. The $BrdU^{+}$ cell fraction is also higher in epidermal growth factor (EGF)-treated cultures of HIP/PAP overexpressing hepatocytes compared to normal liver cells. Hepatocyte apoptosis due to exposure to TNF-α and actinomycin D is also ameliorated by HIP/PAP.

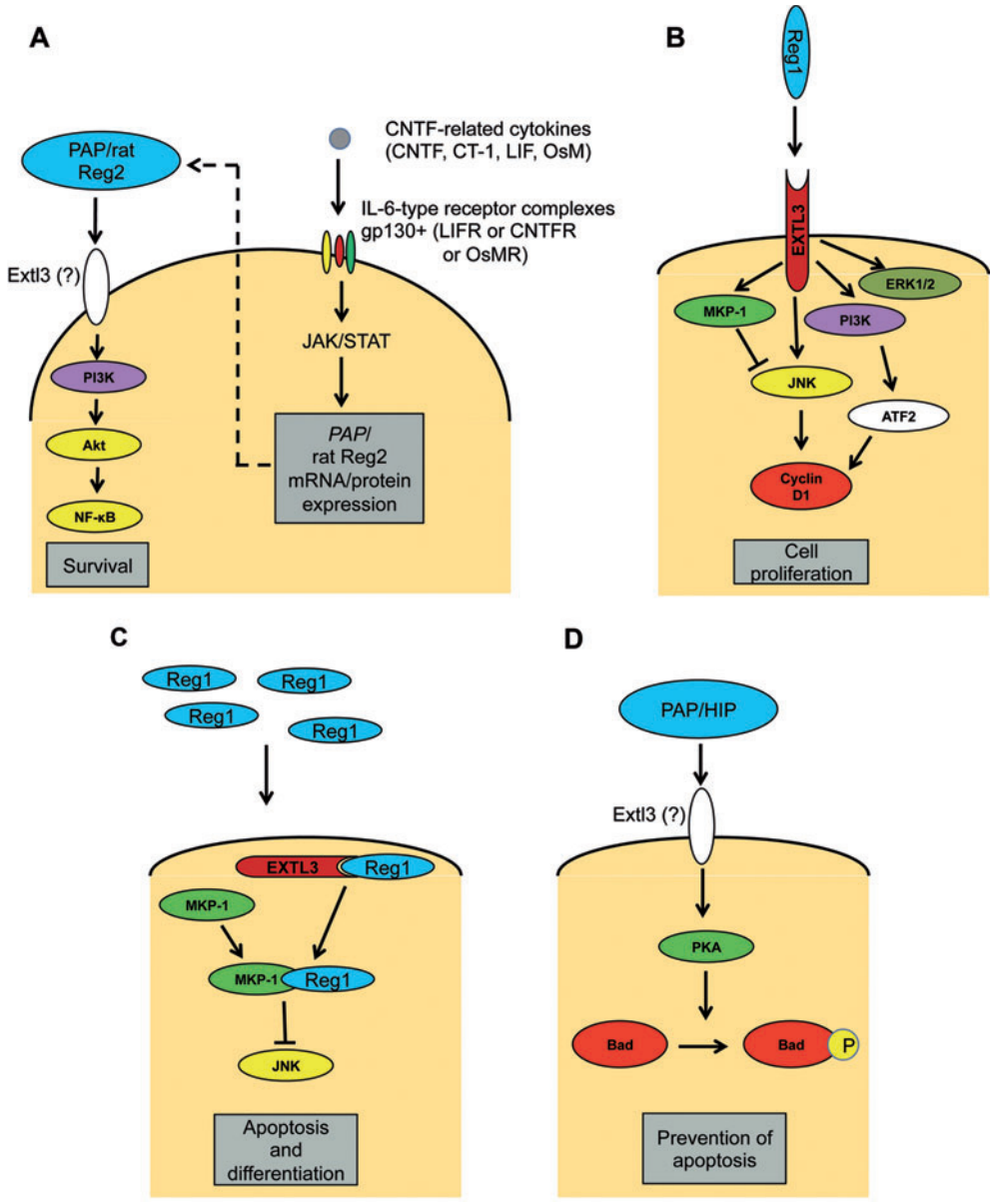

Figure 1 Potential mechanisms of Reg protein signaling.
(A) PAP/rat Reg2 (Reg3β) mRNA and protein expression is stimulated by CTNF-related cytokines through the respective gp130 receptor complex and acts in a paracrine/autocrine manner, possibly *via* the Extl3 receptor activating (→) the PI3K/AKT/NF-κB cascade enhancing cell survival (45). The (?) next to the Extl3 denotes that this receptor is a tentative mediator of Reg signaling. (B) Reg1 stimulates PI3K, which targets ATF-2 and the expression of cyclin D1 downstream (131). Alternatively, the Reg1 signal may be relayed *via* ERK1/2 (134). Signals triggered by extracellular Reg may cause direct activation of JNK and its indirect suppression (—|) *via* MKP1 (133). The net effect is promotion of cell proliferation. (C) At high concentrations of exogenous Reg, the ligand may form a complex with Extl3 or other internal receptors and interact with MKP-1 to inhibit JNK activity resulting in apoptosis and differentiation (133). (D) PAP/HIP (Reg3β) activates PKA, which promotes the phosphorylation of Bad, thus preventing apoptosis (125).

Signaling pathways and Reg proteins

Despite extensive evidence linking Reg proteins to cell differentiation, proliferation, and protection from apoptosis under normal and pathologic conditions, little is known about the pathway(s) involved in relaying Reg signals. The putative receptor of Reg protein (29) exhibits over 97% homology to the human multiple exostosis-like gene 3 (EXTL3) (125). This gene encodes the α1,4-*N*-acetylglucosaminyltransferases I and II enzymes, which participate in heparan sulfate biosynthesis. Both the rat and human Reg proteins bind with high affinity to the rat Extl3 receptor upon overexpression of its cDNA in CHO cells. In the adult mouse, *Extl3* transcripts are detected in the spleen, liver, testis, stomach, and heart, whereas the strongest expression is noted in the pancreas and brain (126). In E11.5–E18.5 mouse embryos, *Extl3* mRNA is present in the neurons, gastrointestinal

tract epithelia, liver, kidney, lung, and pancreas. Particularly in the pancreas, Extl3 expression is substantial in the adult acini and exhibits a biphasic pattern of expression during development (E11.5–E18.5). Yet, developing and mature islets have a weaker expression of Extl3. In humans, the *EXLT3* gene is expressed in skeletal muscle, liver, placenta, heart, brain, and pancreas (125) similar to other EXT family genes (127).

The details of the interaction between Reg and its putative receptor are still unclear, but overexpression of EXTL3 enhances the activity of NF-κB induced by the TNF-α (128). Activation of NF-κB, possibly through the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) kinase pathway, is suggested in regenerating motor neurons (81) after exposure to Reg2/PAP (Reg3β) (Figure 1A) (45). When these cells are incubated with the PI3K inhibitor LY294002, the Reg2-induced increase in phosphorylated Akt is abolished. NF-κB is a target of Akt kinase (129), but its direct activation by Reg2 has not been shown yet. It should be noted that Reg3β upregulation, which limits the death of pancreatitis-inflamed mouse acinar cells and rat acinar AR42J cells (46, 48), has also been linked to the action of NF-κB/Rel factors.

The involvement of PI3K and other pathways in Reg activation in pancreatic and extrapancreatic cells has also been reported. Takasawa et al. (130) probed several pathways in pancreatic β-cells stimulated with mouse Reg (most likely Reg1). Cyclin D1 was upregulated by Reg in RINm5F rat insulinoma cells through the activating transcription factor-2 (ATF-2) cascade (Figure 1B) but not *via* cAMP response element-binding (CREB), Jun proto-oncogene (cJun), E twenty-six (ETS)-like transcription factor 1 (Elk1), C/EBP homologous protein (CHOP), and FBJ murine osteosarcoma viral oncogene homolog (cFos). To that end, the levels of phospho-ATF-2 and cyclin D1 were suppressed in islets from $Reg^{-/-}$ mice. The addition of PI3K inhibitors wortmannin and LY294002 decreased the Reg-induced ATF-2 phosphorylation/activation in RINm5F β-cells, whereas inhibition of p38 MAPK, extracellular signal-regulated kinases 1/2 (ERK1/2), calmodulin (CaM) kinases II and IV, and protein kinase A (PKA) did not result in significant changes in the activity of the cyclin D1 promoter.

These findings, however, are not entirely aligned with results from other studies demonstrating the activation of MAPK and PKA by Reg proteins. The human Reg protein and a bioactive Reg fragment promoted the proliferation of rat ductal (ARIP) cells while activating p38 MAPK (131). Later, Mueller et al. (132) reported the activation of cyclin D1 by rat Reg1 in ARIP and rat insulinoma (RIN) cells. Microarray data analysis revealed increased expression of the MAPK phosphatase (MKP-1) after treatment with Reg1. At high concentrations of extracellular Reg, the protein was hypothesized to form intracellular complexes with its putative receptor and MKP-1 (Figure 1C) (133). There were also increased levels of phosphorylated ERK1/2, stress-activated protein kinase/cJun N-terminal kinase (SAPK/JNK), and p38 MAPK unlike the findings in the study by Takasawa et al. (130). This disagreement may be due to the different time frames utilized in the two studies (30 min to 4 h (132) vs. 15 min (130)), although the exact reasons are undetermined. The mitogenic effect of Reg1 via the MAPK/ERK1/2 pathway was also demonstrated in human gastric adenocarcinoma cells (133), which produce Reg boosting their proliferation.

In primary hepatocytes *in vivo* and in culture, HIP/PAP (Reg3β) confers its mitogenic and anti-apoptotic effects via activation of the cyclic AMP (cAMP)/PKA pathway (124). The addition of forskolin to EGF-treated cultures increased the synthesis of DNA in hepatocytes from transgenic mice overexpressing HIP/PAP. In contrast, wild-type mouse hepatocytes (with no HIP/PAP expression) did not experience a change in DNA synthesis in response to forskolin pointing to a cooperative effect of HIP/PAP with cAMP. Furthermore, inhibition of PKA by KT5720 was counterbalanced by enhanced PKA activity due to its induction by HIP/PAP in transgenic mice overexpressing this protein. This led to reduced apoptosis of cells treated with TNF-α. The results showed that HIP/PAP activates PKA and subsequently induces the PKA-dependent phosphorylation of Bad (Figure 1D), a proapoptotic target in the PKA pathway.

The overexpression of REG4 was recently reported in pancreatic cancer biopsies and cell lines with upregulated glioma-associated oncogene 1 (GLI1), a transactivator of Hedgehog signaling (134). There was a positive correlation in the change of expression of REG4 and GLI1. Analysis by chromatin immunoprecipitation (ChIP) revealed that GLI1 binds to the REG4 gene promoter at the $G^{-528}ATCATCCA^{-520}$ site, which is highly similar to the nucleotide GLI1-binding sequence (GATCATCCA) reported previously (135). These results were verified by electrophoretic mobility shift assay (EMSA) showing the GLI1 binding *in vivo* to the aforementioned site of the REG4 promoter. Despite these findings, further investigation is required to answer if REG4 is transcriptionally regulated by GLI1. Moreover, it is unclear whether signaling pathways, which are linked to both REG4 and Hedgehog, such as the EGF receptor/Akt/AP1 cascade (136), take part in this regulation.

Links between Reg and Wnt signaling have also been reported. Human primary hepatocellular carcinoma cells with β-catenin mutations subjected to subtractive hybridization with normal liver cells were found to overexpress REG1α and REG3α (103). Furthermore, hepatoma cells (Huh7) exposed to LiCl, which activates canonical Wnt signaling by inhibiting GSK-3β, exhibit increased *REG3*α mRNA. This increase was curtailed when the cells were transfected with siRNA against β-catenin prior to LiCl treatment. In the same experiments, the levels of *REG1*α mRNA were unaffected. Nonetheless, analysis of hepatocellular carcinomas from 42 patients by qRT-PCR showed overexpression of REG1α and REG3α correlating with β-catenin mutations. These findings were corroborated by immunostaining of biopsy sections. In a separate clinical study (104), 265 hepatocellular carcinoma samples with β-catenin mutations displayed high levels of REG1α and REG3α compared to normal biopsies. Synchronous upregulation of REG1α and REG3α has also been reported in colorectal carcinogenesis and colon adenomas with inactivated adenomatous polyposis coli (APC) gene (77, 137, 138). Collectively, these studies suggest that Reg genes serve as downstream targets of the canonical Wnt pathway.

As already mentioned, discovery of Reg expression in ESCs was recently reported (90). Considering the key roles of β-catenin/Wnt signaling in stem cell self-renewal and differentiation, we investigated whether a link between canonical Wnt and Reg exists in mESCs, similar to that in various cancer cells. Treatment with Wnt signaling activators such as LiCl or purified Wnt3a increased the expression of Reg1 but not Reg3γ. The increase in Reg1 was abrogated when mESCs were transfected with a plasmid encoding a dominant negative TCF4 prior to Wnt3a treatment. Other Reg genes were not detectable before or after Wnt activation. The observed upregulation in Reg 1 gene expression upon Wnt activation translated to increased amounts of secreted Reg1. These data support the association of Reg with Wnt signaling and merit further studies to unravel the underlying mechanisms and participating molecules.

Concluding remarks

Despite their initial discovery in pancreas inflammation and islet regeneration, Reg proteins have been found to be involved in the growth and differentiation of cells from various organs under normal and diseased states. In the pancreas, Reg proteins induce cell (trans)differentiation, especially to islet cells, and proliferation, while they reduce apoptosis due to damage or inflammation. The proliferative and anti-apoptotic effects of Reg members have also been documented in non-pancreatic tissues, mainly of the gastrointestinal tract. Reg expression is modulated by a diverse array of inducers including nutrients, hormones, growth factors, and bacterial infection. Cytokines, in particular, are major regulators of the expression of Reg proteins making them integral in inflammatory responses. Cellular responses to Reg ligands are transduced *via* several pathways including the classical MAPK and canonical Wnt cascades. Although further elucidation is necessary of their biological roles and mechanisms of action, Reg proteins are emerging as a group with great potential for uses as screening markers and targets for therapies against diseases such as diabetes and various forms of cancer.

Outlook

The majority of Reg-related studies have been performed in the context of diabetes or cancer, leaving the contribution of Reg proteins to homeostasis or malignancies of other organs to be unveiled. Reg expression has been associated with islet regeneration, making these proteins attractive candidates for diabetes or pancreatic cancer therapies, even though the tumorigenic potential of Reg proteins should be considered as well. It should also be noted that Reg proteins are the smallest members of the superfamily of C-type lectins. Hence, there are opportunities for gaining a deeper insight of the biological roles and interactions of Reg proteins from function-structure studies and recent advances in glycobiology. Moreover, a mounting body of literature exists on factors controlling Reg expression, but there are significant gaps in our knowledge of the signaling cascades involved in conferring the cellular responses triggered by Reg ligands. Detailed analysis of the pathways involved will lead to better understanding of the roles (and actions) of Reg proteins and will facilitate their therapeutic use.

Such studies will be expedited as antibodies with specificity against particular, highly homologous Reg members become widely available. Research in this field will also be aided by the generation of transgenic mice for the overexpression/knockdown of various Reg genes [besides Reg1 (40, 139)]. To that end, coordinated efforts should be undertaken toward establishing and adopting a unified nomenclature system for Reg genes/proteins. This will lower the threshold for researchers interested in the field, promoting progress at a faster pace.

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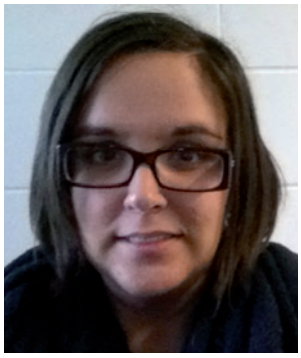

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