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The Role of Reg Proteins, a Family of Secreted C-Type Lectins, in Islet Regeneration and as Autoantigens in Type 1 Diabetes

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1. Introduction

This chapter reviews literature regarding the role of Reg, a family of small C-type lectins, with a focus on the endocrine pancreas and introduces novel findings generated in our laboratory on the role of Reg as autoantigen in T1DM. It is not intended as a comprehensive literature review, but rather discusses a selection of articles in more detail in order to illustrate aspects that are important for the understanding of this protein family. In the author's view the scientific challenge consists in the development of a theory of Reg action that unifies seemingly disparate roles played by members of this family and provides the field of Reg research with a defined framework allowing unification of the many, to date, unconnected observations. Thus, although we focus on Reg and the endocrine pancreas, we also discuss findings obtained in other Reg research areas that appear to have little connection with the endocrine pancreas and T1DM. We choose this approach because we consider that the effects of Reg in the endocrine pancreas need to be understood in the context of a broader view of this protein family. The reader may easily find a large volume of additional literature on the effects of Reg referenced in the presented reports.

2. Nomenclature and classification of the Reg proteins and the origin of the family name

To act as a guide though the confusing nomenclature of this protein family we have prepared a phenogramm of the family incorporating Reg proteins from 5 different species (Fig. 1). In the text - as in the phenogramm- we use 'Reg', followed by a roman numeral designating the subfamily and a Greek letter that designates the member within the subfamily. Alternative names are used for those members where no Reg nomenclature exists and are otherwise indicated in brackets behind the standard name. 'Reg' is used without further classification when referring to the entire family. Reg stands for 'regenerating islet-derived', a name, which is not ideal since many Reg proteins are not or not solely associated with islet regeneration but function in different tissues and under quite diverse physiological conditions, as will be discussed in more detail later. In fact the first members of this family were described as a component of pancreatic stones in chronic pancreatitis or as constituent of normal pancreatic juice involved in the control of calcium

carbonate crystal growth (Keim, Rohr et al. 1984; Multigner, De Caro et al. 1983; Sarles, Dagorn et al. 1990). Hence the names PAP or PSP (pancreatitis associated protein or pancreatic stone protein) or lithostathine remain in use for some of these proteins. Additional designations include 'pancreatic thread protein' (PTP), a name given to a bovine
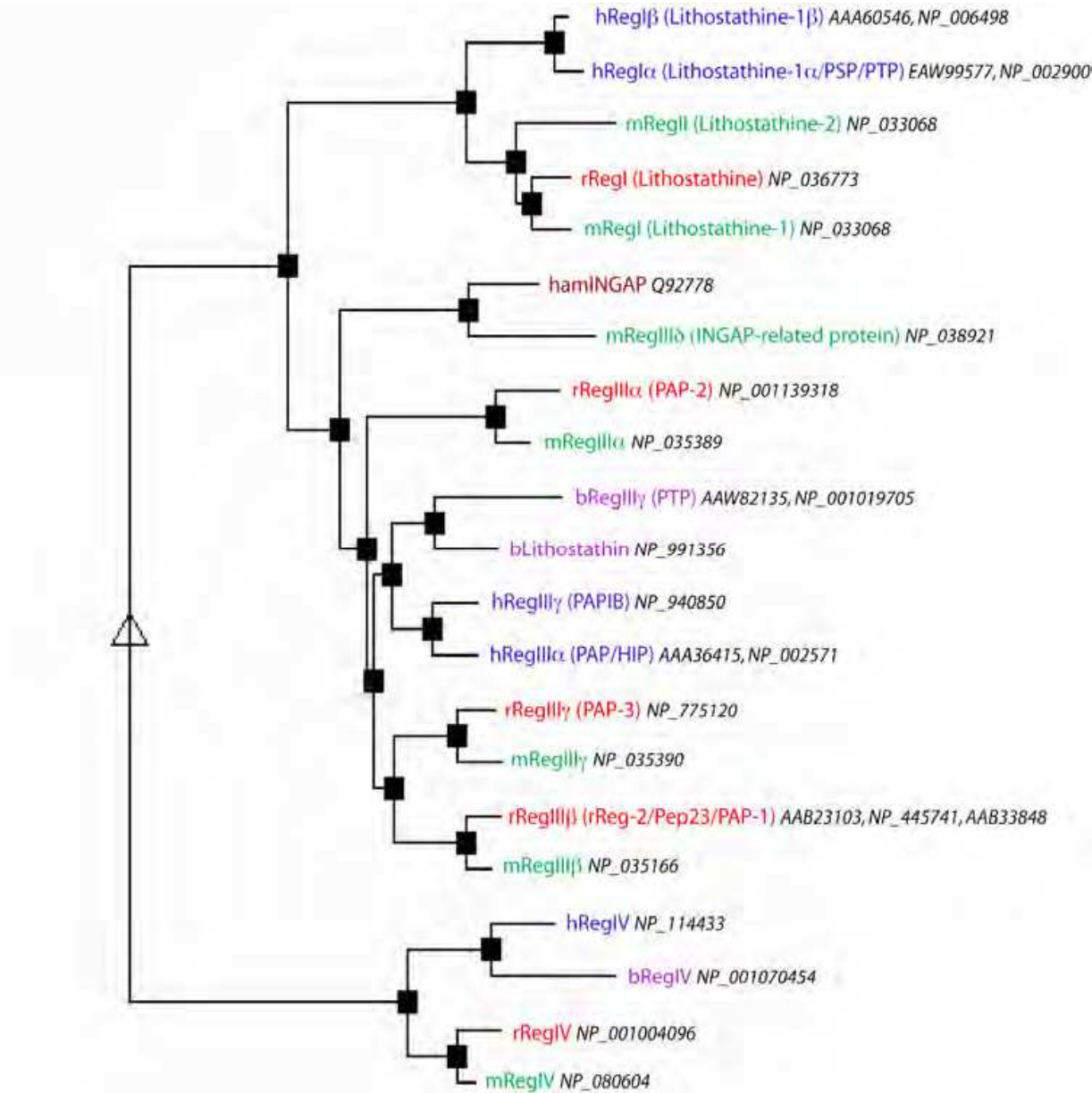

Fig. 1. Phenogramm of human (h), mouse (m), rat(r), hamster (ham), and bovine (b) Reg family members. 'Reg' followed by roman numerals and Greek letters was chosen as standard protein name whenever this nomenclature was found in the protein data bank. Otherwise the alternative names were used. These are indicated in brackets for members, which are found under both the standard and the alternative name in the data banks. Protein data bank accession numbers are given after each member. Abbreviations: Reg=regenerating islet-derived; INGAP=islet neogenesis associated protein; PTP=pancreatic thread protein; PAP=pancreatitis associated protein; HIP=hepatocellular carcinoma/intestine/pancreas; PSP=pancreatic stone protein

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mRegI     .....MARNAYFILLSCLIVLSPSQGQEAEED.......LPSARISCPEGSNAYSSYCYYFTEDRLTWADADLFCQNMNSG.YLVSVLSQAE
rRegI     .....MTRNKYFILLSCLMVLSPSQGVKAEE.......LVKARITCPEGSNAYSSYCYYFMEDHLSWAEADLFCQNMNSG.YLVSVLSQAE
mRegII    ....MAQNNVYLILFLCLMFLSYSQGCVAEEDFPLAEKDLPSAKINCPEGANAYGSYCYYLIEDRLTWGEADLFCQNMNAG.HLVSILSQAE
hRegII    ....MAQTSSYFMLISCLMFLSQSQGQEAQTE.......LPQARISCPEGTNAYRSYCYYFNEDRETWVDADLYCQNMNSG.NLVSVLTQAE
hRegI     ...MAQTNSFFMLISSLMFLSLSQGQESQTE.......LPNPRISCPEGTNAYRSYCYYFNEDPETWVDADLYCQNMNSG.NLVSVLTQAE
mRegIII   MLPHLVLNSISWMLLSCLLFVFQVQGEDFQKE.......VPSPRTSCPMGYKAYRSHCYALVMTPKSWFQADLVCQKRPSG.HLVSILSGGE
rRegIII   MLPRLSFNNVSWTLLY.YLFIFQVRGEDSQKA.......VPSTRTSCPMGSKAYRSYCYTLVTTLKSWFQADLACQKRPSG.HLVSILSGGE
mRegIII   MLPPTACSVMSWMLLSCLMLLSQVQGEDSLKN.......IPSARISCPKGSQAYGSYCYALFQIPQTWFDAELACQKRPGG.HLVSVLNSAE
rRegIII   MLHRLAFPVMSWMLLSCLMLLSQVQGEDSPKK.......IPSARISCPKGSQAYGSYCYALFQIPQTWFDAELACQKRPEG.HLVSVLNVAE
mRegIII   MLPRITITIMSWMLLSCLMLLSQVQGEVAKKM.......ATSSTSSCPKGSRAYGSYCYALFSVSKNWYDADMACQKRPSG.HLVSVLSGAE
rRegIII   MLPRVALTTMSWMLLSSLMLLSQVQGEDAKED.......VPTSRISCPKGSRAYGSYCYALFSVSKSWFDADLACQKRPSG.HLVSVLSGSE
hRegIII   MLPPMALPSVSMLLSCLMLLSQVQGKDPSRK.......LPSARIRCPKGSKAYGSHCYALFLSPKSWTDADLACQKRPSG.NLVSVLSGAE
INGAP     MMLPMTLCRMSWMLLSCLMFLSWVEGEESQKK.......LPSSRITCPQGSVAYGSYCYSLILIPQTWSNAELSCQMHFSG.HLAFLLSTGE
mRegIV    .........MASKGVRLLLLLSWVAGPEVLSD.........ILRPSCAPGWFYYRSHCYGYFRKLRNWSHAELECQSYGNGSHLASVLNQKE


mRegI     GNFVASLIKESGTTDANVWTGLHDPKRNRR.....WHWSSGSLFLYKSWATGSPNSSNRGYCVSLTSNTGYKKWKDDNCDAQYSFVCKFKG
rRegI     GNFLASLIKESGTTAANVWIGLHDPKNNRR.....WHWSSGSLFLYKSWDTGYPNNSNRGYCVSVTSNSGYKKWRDNSCDAQLSFVCKFKA
mRegII    SNFVASLVKESGTTASNVWTGLHDPKSNRR.....WHWSSGSLFLFKSWATGAPSTANRGYCVSLTSNTAYKKWKDENCEAQYSFVCKFRA
hRegII    GAFVASLIKESGTDDFNVWIGLHDPKKNRR.....WHWSSGSLVSYKSWGIGAPSSVNPGYCVSLTSSTGFQKWKDVPCEDKFSFVCKFKN
hRegI     GAFVASLIKESSTDDSNVWIGLHDPKKNRR.....WHWSSGSLVSYKSWDTGSPSSANAGYCASLTSCSGFKKWKDESCEKKFSFVCKFKN
mRegIII   ASFVSSLVNGRVDNYQDIWIGLHDPTMGQQPNGGGWEWSNSDVLNYLNWDGDPSSTVNRGHCGSLTASSGFLKWGDYYCDGTLPFVCKFKQ
rRegIII   ASFVSSLVTGRVNNNQDIWIGLHDPTMGQQPNGGGWEWSNSDVLNYLNWDGDPSSTVNRGNCGSLTATSEFLKWGDHHCDVELPFVCKFKQ
mRegIII   ASFLSSMVKRTGNSYQYTWIGLHDPTLGAEPNGGGWEWSNNDVMNYFNWERNPSTALDRAFCGSLSRASGFLKWRDMTCEVKLPYVCKFTG
rRegIII   ASFLASMVKNTGNSYQYTWIGLHDPTLGGEPNGGGWEWTNNDIMNYVNWERNPSTALDRGFCGSLSRSSGFLRWRDTTCEVKLPYVCKFTG
mRegIII   ASFLSSMIKSSGNSGQYVWIGLHDPTLGYEPNRGGWEWSNADVMNYINWETNPSSS.SGNHCGTLSRASGFLKWRENYCNLELPYVCKFKA
rRegIII   ASFVSSLIKSSGNSGQNVWIGLHDPTLGQEPNGGGWEWSNADVMNYFNWETNPSSV.SGSHCGTLTRASGFLRWRENNCISELPYVCKFKA
hRegIII   GSFVSSLVKSIGNSYSYVWIGLHDPTQGTEPNGEGWEWSSSDVMNYFAWERNPSTISSPGHCASLSRSTAFLRWKDYNCNVRLPYVCKVH.
INGAP     ITFVSSLVKNSLTAYQYIWIGLHDPSHGTLPNGSGWKWSSSNVLTFYNWERNPSIAADRGYCAVLSQKSGFQKWRDFNCENELPYICKFKV
mRegIV    ASVISKYITGYQRNLP.VWIGLHDPQKKQL.....WQWTDGSTNLYRRW..NPRTKSEARHCAEMNPKDKFLTWNKNGCANRQHFLCKYKT
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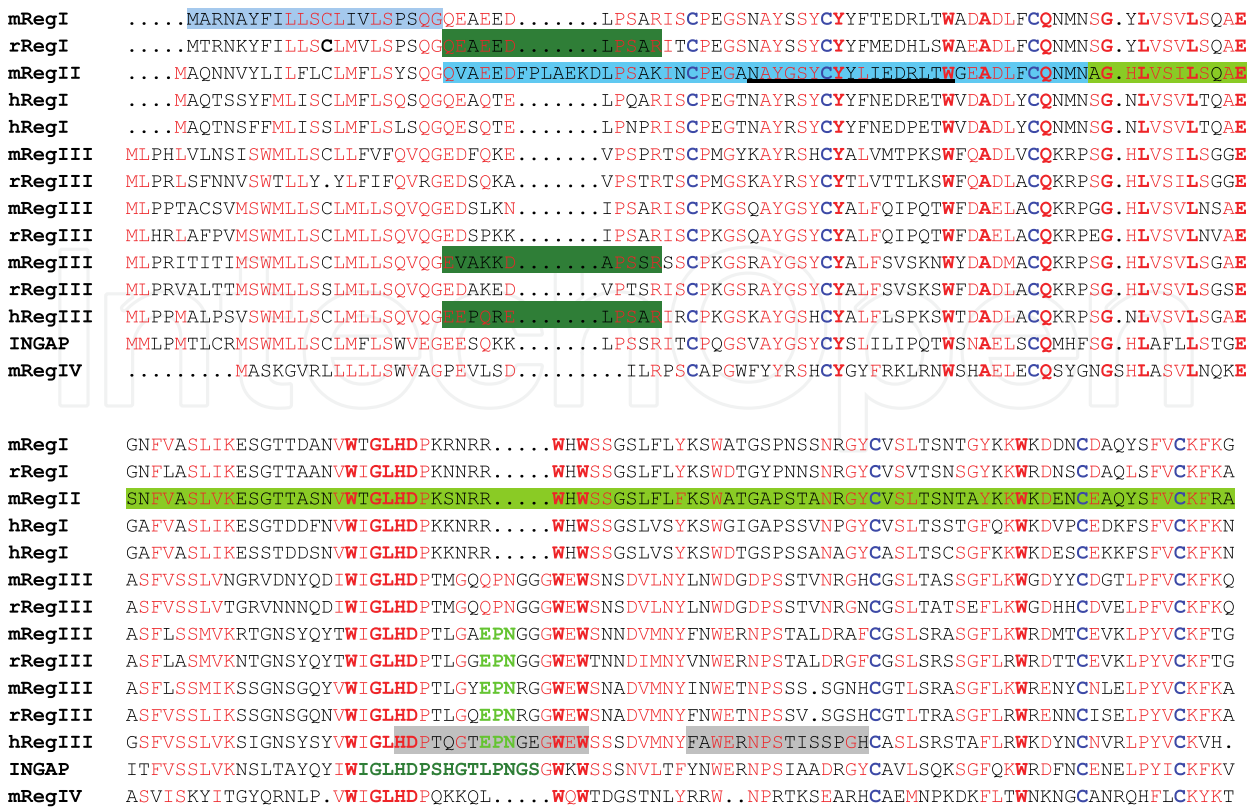
Fig. 2 Alignment of human, mouse, rat and hamster Reg family members. Features of Reg discussed in this review are highlighted. Blue background (mRegI): signal peptide; dark green background: prosegment (bactericidal effect/fibril formation. The arginine residue at the end of the prosegment is part of tryptic cleavage site); light blue background (mRegII): N-terminal fragment (acceleration of T1DM, activation of autoaggressive T-cells in NOD mice); lime green background: C-terminal fragment (delay of T1DM, T regulatory cells in NOD mice); light green letters: EPN motif (carbohydrate binding); gray background: loop 1 and loop 2 (carbohydrate binding); dark green letters: INGAP peptide (islet neogenesis/regeneration); blue letters denote the 6 cysteine residues that form the Reg C-type lectin domain.

pancreatic protein that precipitates at neutral pH in the form of double helical threads and was subsequently identified as a member of the Reg family (Gross, Brauer et al. 1985) and 'HIP' (for hepatocellular carcinoma, intestine, pancreas) – a name assigned to a human family member after Lasserre at al. found that it was overexpressed in a proportion of the hepatocellular carcinoma samples they had investigated (Lasserre, Christa et al. 1992). The designation 'Reg' was introduced because rat RegI (lithostathine) was rediscovered in a cDNA library constructed from *reg*enerating islets that had been isolated from the remaining pancreases of partially depancreatized, nicotinamide-treated rats (and was not expressed in normal islets) (Kobayashi, Akiyama et al. 2000). The investigators had previously shown that partial surgical removal of the pancreas followed by nicotinamide treatment induced a marked enlargement of the islets of Langerhans due to an increase in the number of β-cells. They write in their abstract: "The increase in expression of the gene was temporally correlated with the increase in size of regenerating islets…" and conclude "Thus the expression of the gene in regenerating and hyperplastic islets suggests a possible role for

this gene in replication, growth and maturation of β-cells". Looking back on the years of Reg research, especially that performed with a focus on islet regeneration the authors of this manuscript might, in hindsight, have taken the trouble to remind their readers of an important scientific rule, namely, that correlation does not necessarily imply causation.

Although 'Reg' may not be an ideal designation the family is structurally well defined and a given protein sequence can unequivocally be assigned to it. To qualify as a member of the family a protein has to fulfill only one structural requirement; it must consist of a single C-type lectin domain of the type found in the first discovered Reg member, without additional attached domains. Any newly discovered protein was assigned to the family if it fulfilled this requirement and was assigned to a subfamily by primary sequence comparison with already existing Reg family members. This classification method resulted in the depicted phenogramm in which the three major subfamilies RegI and II, RegIII and RegIV each arise from a separate root. To illustrate the features of individual Reg members discussed in this review a primary sequence alignment is also shown (Fig. 2).

3. Studies investigating the role of reg in islet regeneration

Most studies that have investigated the role of Reg proteins have restricted themselves to one particular Reg member. Their basic approach *in vivo* and *in vitro* has been to either increase or decrease the concentration of the member under study or to add or eliminate it. Results of studies that increased Reg concentrations *in vivo* by administration of exogenous Reg protein include the finding that rRegI treatment can ameliorate surgically induced diabetes in rats (Watanabe, Yonemura et al. 1994). The same study also found that *in vitro* incubation with rRegI increased incorporation of radioactive thymidine into isolated rat islets. A corresponding approach was tested in NOD mice by i.p, administration of hRegIα with or without co-administration of linomide (Gross, Weiss et al. 1998). This study showed that Reg treatment of NOD mice that were still glucose tolerant could significantly reduce the incidence of diabetes and, when combined with linomide treatment, led to a reversal of glucosuria in glucose intolerant mice. In this study area the hamster INGAP protein deserves special mention. It was isolated from hamster pancreas using the cellophane wrapping procedure, which induces formation of new islets from pancreatic ducts (Rafaeloff, Pittenger et al. 1997). The results reported in this study draw attention to the actual definition of the process termed 'islet regeneration'. Should this term define β-cell proliferation taking place in pre-existing islets or should it rather describe a process that generates 'new' islets or β-cells from precursor cells? Although β-cell replication must presumably also take place at some stage in 'new' islets generated from precursor cells the investigators who discovered INGAP place their emphasis on neogenesis and argue that INGAP is associated with this process. Their designation for this Reg member, **i**slet **neog**enesis **a**ssociated **p**rotein, reflects this standpoint as does their demonstration that isolated primary duct cells thought to contain potential islet/β-cell progenitors respond with increased cell proliferation upon incubation with INGAP. The whole area of 'islet regeneration' is a subject of controversy. There are investigations that establish β-cell proliferation in preexisting islets as the predominant path to islet regeneration in rodents (Dor, Brown et al. 2004) whereas in other studies this process is attributed to the de novo formation of β-cells from endogenous progenitors (Xu, D'Hoker et al. 2008). 'Islet regeneration' should perhaps best be understood as the outcome of a combination of several

processes including islet neogenesis, β-cell proliferation/size increase/maturation and islet destruction. As the cited studies show Reg seems to be at least involved in the first two processes and might also influence the third via its ability to protect from β-cell apoptosis (Bonner, Bacon et al. 2010; Simon, Pauloin et al. 2003).

INGAP is noteworthy from another aspect. The discoverers of this Reg member report in their initial publication that they were able to achieve duct cell proliferation not only by exposure to full length INGAP but also with a pentadecapeptide corresponding to amino acids 104–118 (Fig.2). The investigators seem to have derived this peptide without any hypothesis based on a possible effector mechanism of INGAP/Reg or any kind of peptide screening approach. We learn that "We included this region of the deduced protein because it differs from another related family of genes known to affect islet regeneration, Reg/PSP, in that it has a unique insertion of five amino acids and it precedes a potential N-glycosylation site situated at position 126, hence, a core of potential biological activity". Although the discovery of this peptide might have been taken as a peculiarity in the Reg field it has resulted in a string of subsequent investigations all showing some positive effect of a peptide with this sequence and corresponding peptides from other Reg members on diabetes and/or ductal cell proliferation. The findings finally led to a trial in humans, which again demonstrated positive effects in type 1 as well as in type 2 diabetic patients (Dungan, Buse et al. 2009; Levetan, Upham et al. 2008; Pittenger, Taylor-Fishwick et al. 2007; Rosenberg, Lipsett et al. 2004).

We have detailed the INGAP studies because they illustrate some important problems affecting the Reg field especially concerning diabetes/islet regeneration. No unequivocal effector mechanisms have been defined for this family and therefore it is not known if and how the differences between family members might manifest themselves clinically. It is not clear what process of 'islet regeneration' is preferentially impacted by Reg. Do all Reg members act as β-cell mitogens? Can all Reg members induce islet neogenesis and are all Reg members equally able to protect β-cells from apoptosis? Since these questions have not been answered we are left with a collection of observations lacking a basis (or a precise theory) from which they can be explained. As a consequence, it is difficult to generate testable hypotheses that would, for example, predict which cells should proliferate upon Reg stimulation and which should not, or that would allow improvement of the INGAP peptide sequence to produce a peptide with better mitogenic/anti inflammatory/anti-apoptotic/islet neogenesis-inducing properties.

Studies using overexpression or knockout mouse models to demonstrate Reg effects will be briefly considered here. As mentioned above there is no theory available that would predict which of the Reg members would be most promising for these studies and investigators have therefore tried different Reg candidates in these test systems. The first member to be tested in knockout experiments was RegI and this was also the first to be overexpressed in pancreatic islets under an insulin promoter (Unno, Nata et al. 2002). Overall, the results from these studies supported the observations obtained on exogenous administration of Reg. RegI ko mice had morphologically normal pancreatic islets and were euglycemic. However islets isolated from these mice incorporated less radioactive thymidine than islets from non-transgenic mice. Furthermore, stimulation with aurothioglucose, a method of inducing hyperplastic islets, failed to increase islet size in RegI ko mice to the same extent as it did in non transgenic mice, again supporting a mitogenic effect. A group that reported results on a knockout of RegIIIβ found that the mice were more sensitive to caerulein-

induced pancreatitis but the report did not mention any deleterious effect of this genotype on islet size, insulin or glucose levels, suggesting that there may have been no difference between knockout mice and normal littermates (Gironella, Folch-Puy et al. 2007). Expression of transgenic mRegI under the insulin promoter in β-cells did not affect the size or morphology of islets compared to non-transgenic mice. However, when mRegI transgenic mice were crossed onto NOD mice the incidence of T1DM in the NOD RegI transgenic mice was significantly reduced compared to normal NOD mice (Unno, Nata et al. 2002). Subsequent mouse models tested the effects of transgenic expression of hamINGAP and mRegIIIβ targeted to islets via an insulin promoter (Chang, Weaver et al. 2010; Xiong, Wang et al. 2011). These studies found enhanced glucose tolerance, partial protection from streptozotocin (STZ)-induced diabetes and alterations in gene expression profiles when compared to non-transgenic mice. Overexpression of mRegII targeted to the exocrine pancreas with an elastase-1 promoter failed to provide protection from streptozotocin-induced diabetes (Li, Wang et al. 2010), whereas hamINGAP targeted to the exocrine pancreas with the same promoter did confer resistance to STZ-induced diabetes and increased the β-cell mass (expressed as insulin positive cells and the total pancreatic insulin/protein ratio) as well as the number of smaller islets compared to non transgenic littermates (Taylor-Fishwick, Bowman et al. 2006). The findings obtained from the two transgenic models with exocrine targeting of mRegII or hamINGAP were interpreted to indicate that different Reg members could have different effects. None of the more recent transgenic Reg models has so far been crossed onto the NOD background to study the impact of Reg overexpression in a T1DM model. The general conclusion from these studies is that Reg-induced effects appear to be pleiotropic probably impacting at least the three known processes mentioned above that contribute to 'islet regeneration'.

In our discussion of the effects induced by Reg, as derived from the presented findings, we place emphasis on a feature that was **not** observed in mice treated with exogenous Reg or in Reg transgenic mice. It is noteworthy that in the studies presented above euglycemia was maintained in the treated mice, hypoglycemia or excessive serum insulin levels were not observed and increases in islet mass were limited. This implies that the effects of exogenous (or transgenic) Reg as β-cell mitogen or stimulator of islet neogenesis did not lead to an expansion of the islet mass beyond what is physiologically normal. This feature is especially apparent in the transgenic models, where Reg is expressed from a constitutive promoter leading to sustained Reg overexpression from an early time-point. In this situation it is tempting to argue that the absence of documented effects in these studies suggests that the Reg proteins do not have any effects under normal physiological conditions. However, a different argument can be presented. The findings might indicate that the effects of Reg on 'islet regeneration' are tightly regulated and do not exceed the limits imposed by the target system, be it β-cells/islets or ductal precursor cells. In other words, when normal and transgenic mice are compared under normal, non-experimental conditions clinical differences are not found because the effect of the additional experimentally provided Reg input remains marginal. If this situation were expressed in the form of a hypothetical Reg effect curve where the X-axis represents the concentration and the Y-axis the effect, addition of exogenous or transgenic Reg would occur close to or at the point where the curve levels off i.e. adding more Reg at this stage would only have a marginal effect. In this model the system is kept close to the point of saturation due to the presence of endogenous Reg members and additionally due to the overlapping effect range of the various Reg members where one member can compensate at

least to some extent for the loss of others. Thus knockout or overexpression of one Reg member shows little effect under normal conditions because the reduction/increase in the overall Reg concentration is small compared to the basal concentration that is provided by endogenous Reg members and keeps the system close to saturation. According to this interpretation it is possible that the effects of Reg are extremely important in the generation and maintenance of islet mass under normal conditions with an entire family of closely-related Reg members with overlapping functions rather than a single protein being necessary as a fail-safe mechanism to protect this function in a system that has to be kept at or close to saturation. Consequently this view would predict that, in order to better reveal the effects of Reg, a pronounced reduction in the Reg concentration by knocking out more than one and possibly all Reg members would be necessary.
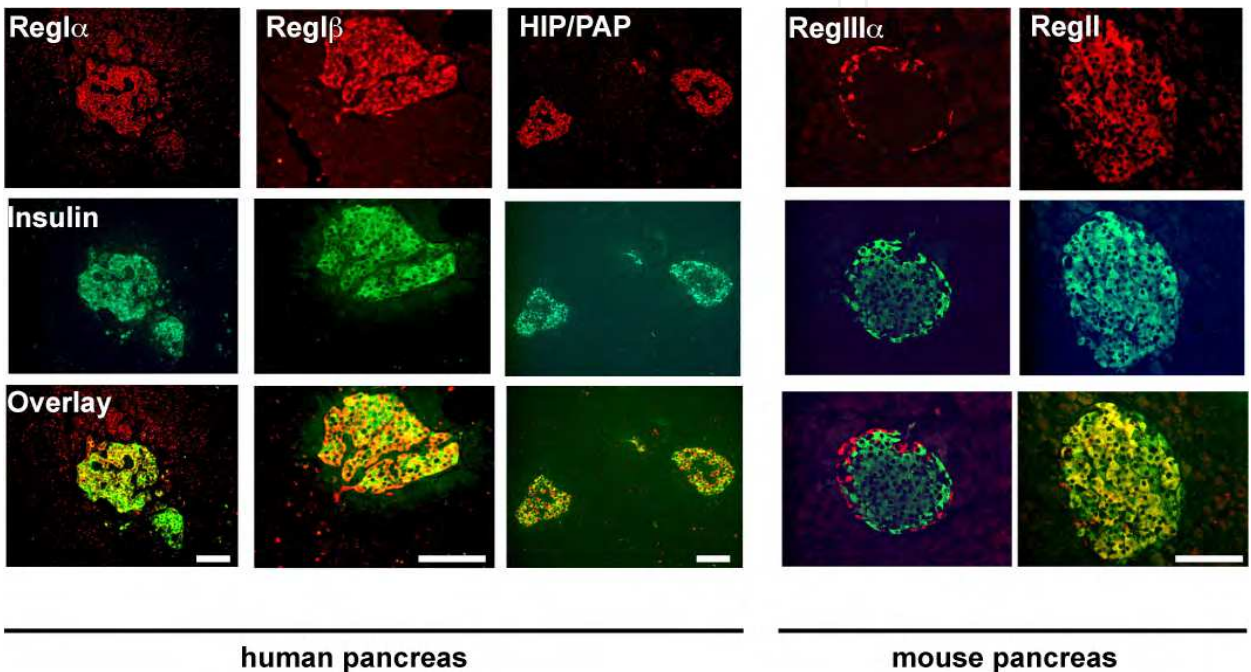
Fig. 3. Reg expression in human and murine islets. RegIα, RegIβ and RegIIIα (HIP/PAP) are expressed in human endocrine pancreas whereas RegII and RegIIIα are expressed in murine islets. (scale bar =50μm)

Such a multimember knockout model would be within the current technical capabilities because in the mouse all Reg family members except RegIV are located on one contiguous 75kb stretch of DNA (Kobayashi, Akiyama et al. 2000), and could therefore be knocked out as a whole. In such a model the effects of exogenous administration of Reg even under normal conditions might be quite spectacular or absolutely necessary for the survival of the animals with this genotype. Although this prediction might be considered a little extreme there are a few observations, which might support it. As we and others have shown, individual Reg family members seldom appear on their own. Wherever one family member is expressed others are likely to be found as well (see Fig. 3). Most importantly, the individual Reg members all contain the Reg C-type lectin domain, which is a strong indication that some of their functions may overlap and therefore generate redundancy. In terms of the hypothetical Reg effects graph mentioned above this means that the X-axis Reg concentration is unlikely to be derived from one single Reg member but is rather a result of the cumulative concentrations of all Reg members present at this site.

4. Studies in search of a reg receptor

The potentially pleiotropic effects of Reg emphasize the importance of defining if, where, when, and how the Reg members bind. A receptor for Reg, which are secreted proteins, might act as focal point for Reg action and might enable us to place the events taking place upon receptor binding and the subtype specific characteristics of the Reg family within a theoretical framework. There has only been one investigation that specifically set out to isolate an islet Reg receptor (Kobayashi, Akiyama et al. 2000). In this study an expression library of rat pancreatic islets was probed with rRegI. This led to the isolation of a cDNA that had significant sequence homology to those "of multiple exostoses (EXT) family genes especially to human EXT-like gene 3 (EXTL3)/ EXT-related gene 1 (EXTR1) (over 97% amino acid identity), indicating that the cDNA encodes a rat homolog to human EXTL3/EXTR1". EXTL genes are homologs of the EXT genes, which have been linked to hereditary multiple exostoses (HME), a rare medical condition in which multiple bony spurs or lumps (also know as exostoses or osteochondromas) develop on the bones of a child. However, EXTL genes are not linked to HME. EXT1 and 2 are thought to form Golgi-located hetero-oligomeric complexes and EXTL 1 and 3 contain a putative transmembrane domain with a short (31 amino acid for EXTL3 cytoplasmic domain) (Busse, Feta et al. 2007). All EXT family members are glycosyltransferases, which are involved in the biosynthesis of heparan sulfate and its analog heparin. EXT1 and 2 are essential for chain polymerization of heparan sulfate whereas EXTL3 most likely is involved in both chain initiation as well as elongation of heparan sulfate (Kim, Kitagawa et al. 2001). Heparan sulfate is a glycosaminoglycan found abundantly on the surface of most cells and in the extracellular space as proteoglycan. Heparan sulfate (HS) proteoglycans are involved in a wide range of biological processes such as cell adhesion, morphogenesis, cytokine effects and regulation of growth factors (Bernfield, Gotte et al. 1999). Regulation of HS proteoglycan biosynthesis would therefore be a good starting point to explain the pleiotropism of the Reg effects. However, the effects of Reg binding to EXTL3 are not known. Does this disrupt or enhance HS proteoglycan biosynthesis? How and where on the EXTL3 molecule does Reg bind and do different Reg members have different affinities? We do know that PANC-1 cells when incubated with a human version of the INGAP pentadecapeptide mentioned above respond with an accelerated translocation of the EXTL3 protein to the nuclear subcellular fraction (Levetan, Upham et al. 2008). Unfortunately, in this study, no alanine scans (mutated peptides each of which has an alanine replacing the naturally occurring residue at a different position in the sequence) or truncation or comparison studies with corresponding pentadecapeptides from other Reg members were performed. It is also known that islets contain HS (proteoglycans) and that heparinase treatment of isolated islets reduces their glucose responsiveness (glucose stimulated insulin secretion, GSIS). Furthermore islets of mice with a β-cell specific ablation of EXTL3 obtained with a rat insulin 2 promoter-Cre/loxP-system had reduced GSIS, a reduced number of insulin positive cells and, until the age of four weeks, fewer cells positive for the proliferating cell nuclear antigen. These mice also responded to glucose challenge with increased blood glucose levels and their plasma insulin levels were reduced. These findings demonstrate that β-cell specific EXTL3 knockout can affect the regulation of postnatal islet maturation via ablation or reduction or alteration of HS proteoglycan biosynthesis (Takahashi, Noguchi et al. 2009).

These encouraging data might lead to the formation of a basis from which the effects of Reg could be explained. However, large gaps in our knowledge remain. It is not clear if Reg binding to EXTL3 changes HS proteoglycan synthesis and if so how. Even if Reg binding to

EXTL3 were to cause an alteration in HS proteoglycan synthesis it is not clear if this would be sufficient to explain the effects seen in the models with transgenic expression or exogenous administration of Reg. There is therefore room for consideration of other potential Reg receptors. The methodology used in the experiments that discovered EXTL3 as Reg receptor involved screening of an islet cDNA library expressed in bacteria with rRegI. However proteins in a bacterial expression system are not subject to the same glycosylation that is introduced in a eukaryotic cell. Due to these limitations the screening assay could not detect Reg binding to carbohydrate structures found on glycoproteins or proteoglycans in eukaryotic cells. Reg-carbohydrate interaction however is an area that should definitely be covered by a search for potential Reg receptors because the family-defining structural feature of the Reg proteins is the C-type lectin domain. Lectins are proteins that bind to carbohydrate structures and C-type lectins do this better in the presence of calcium ions or their binding to carbohydrates is dependent on the presence of these cations. Surprisingly, despite the presence of a canonical C-type lectin fold, Reg-carbohydrate binding in the context of islet regeneration has so far not received any attention at all. A reason for this may possibly be that Reg proteins lack conserved amino acid residues that support Ca^{2+}-dependent carbohydrate binding in other C-type lectins (Drickamer 1999). However this in no way rules out the possibility that Reg proteins recognize carbohydrate epitopes. To uncover what is known about this property of Reg it is necessary to leave the area of islet regeneration and explore other fields of Reg research.

5. Reg effects in areas other than islet regeneration

5.1 Reg as a carbohydrate-binding bactericidal lectin and fibril-forming protein in degenerative processes afflicting the brain

Researchers studying the Reg family of proteins often work with a test system that involves the application of an artificial noxious stimulus thought to mimic a naturally occurring stress event. In a screen performed after application of the stimulus overexpression of one or more Reg members is observed. This was the case with the study that described the discovery of rRegI in regenerating pancreas and of INGAP. Recently a group of scientists investigating how gut microbial flora is maintained and controlled performed a study that compared gut epithelial cells from germ-free mice with those of mice that had been reconstituted with an intestinal microflora from conventional mice. They write "…To gain new insights into how intestinal surfaces cope with microbial challenges, we used DNA microarrays to identify Paneth cell antimicrobial factors whose expression is altered by bacteria. [….] The results of our screen revealed 149 transcripts whose expression was changed 2- to 45-fold by microbial colonization. One of the most prominent responses uncovered by our analysis was a 31-fold increase in the abundance of RegIIIγ transcripts in Paneth cells from conventionalized as compared with germ-free mice". Subsequently this group demonstrated that mRegIIIγ and hRegIIIα were bactericidal for Gram-positive bacteria. The bactericidal effect could be fully blocked by addition of chitotetraose ($GlcNAc_4$) and somewhat attenuated by the addition of soluble peptidoglycan fragments, chitobiose ($GlcNAc_2$), or N-acetylglucosamine. Interestingly, this assay revealed clear differences in susceptibility to glycan blocking between the mRegIIIγ and hRegIIIα. Precipitation experiments and binding studies revealed that both mRegIIIγ and hRegIIIα had affinity for peptidoglycans as well as mannan and chitin (Cash, Whitham et al. 2006). In a subsequent publication this group demonstrated that a short N-terminal peptide controlled the antibacterial activity of mRegIIIγ and hRegIIIα. This peptide spans the region

between the end of the leader sequence and a canonical trypsin cleavage site that is conserved in all but the RegIV subfamily where the conserved arginine residue is followed by a proline thus blocking tryptic cleavage (see Fig. 2). The group found that removal of the prosegment by tryptic cleavage drastically increased the antibacterial properties of the two Reg members. They also showed that removal of the prosegment did not interfere with peptidoglycan binding, which is essential for the antibacterial effects and suggest that the prosegment exerts its blocking activity via a mechanism different from interference with Reg-peptidoglycan binding. The authors propose that this regulatory mechanism has evolved to give the host control over antibacterial activity and that the activation of Reg by cleavage of the small inhibitor peptide likely occurs in the lumen of the gut by two trypsin isozymes, which are expressed in gut epithelial cells (Mukherjee, Partch et al. 2009). It should be added that, as mentioned before, some Reg members are secreted by the exocrine pancreas under normal physiological conditions and this might therefore be another site of activation of Reg for release of antibacterial effects. A third publication of this group reports on the molecular basis for peptidoglycan recognition by mRegIIIγ and hRegIIIα. In this study the researchers used solution nuclear magnetic resonance to identify residues of hRegIIα involved in peptidoglycan binding. The three-dimensional structure of Reg is the C-type lectin fold containing a characteristic 'long loop' structure. In this long loop two subdomains can be distinguished which the scientists have designated loop 1 and loop 2. In other C-type lectins the loop 2 is involved in Ca^{2+}-dependent carbohydrate binding via specific amino acid residues (the EPN motif, Fig. 2). However the corresponding residues in hRegIIIα (HIP/PAP) are not found in loop 2 but in loop 1 and, as the authors of the study show, participate in carbohydrate binding. Also missing in hRegIIIα are two conserved residues in the β4 strand of the C-type lectin fold, which are necessary for carbohydrate binding in other C-type lectins. Thus although these Reg family members lack the canonical C-type lectin carbohydrate binding motifs they are nevertheless lectins. Interestingly, based on the presence of the EPN motif in loop 1 scientists were able to predict that mRegIIIβ should bind peptidoglycans whereas mRegIIIα, which lacks this motif should not. They were then able to confirm this prediction experimentally (Lehotzky, Partch et al. 2010). What conclusions can be derived from this? First, it should be appreciated that a C-type lectin domain can apparently bind carbohydrates via more than one mechanism. The domain functions as a three-dimensional scaffold within which changes affecting fine specificity are possible, allowing generation of diversity in carbohydrate binding. It is therefore quite conceivable that other Reg members also possess these properties despite lacking many or all of the residues that constitute the canonical C-type lectin carbohydrate binding motifs. These findings additionally give rise to new questions. All Reg members (except RegIV) contain the trypsin cleavage site, necessary for removal of the blocking peptide from hRegIIIα and mRegIIIγ. Would this mean that all Reg members are potentially bactericides or only those containing the EPN motif in loop 1? This would make RegIIIβ a bactericidal protein, while, as mentioned above, also protecting islets from STZ-induced diabetes when overexpressed (Xiong, Wang et al. 2011). What is the actual bactericidal mechanism of such Reg proteins? In studies on mRegIIIγ Mukherjee, et al. discovered that carbohydrate binding per se is insufficient to explain this effect. Removal by tryptic cleavage of the short N-terminal blocking peptide does not interfere with carbohydrate binding yet drastically increases the bactericidal effect of the two investigated RegIII members (hRegIIIα and mRegIIIγ). Is there a Reg effect that can manifest itself as bactericidal in the gut but as protective in pancreatic β-cells? Or can Reg proteins act in different ways depending on

whether they are 'trypsin-activated' or not? It should also be noted that RegIV, which has a blocked tryptic cleavage site and can therefore not undergo the unblocking process leading to an active bactericidal Reg member and has a deleted EPN motif nevertheless does bind carbohydrates (Ho, Lou et al. 2010). Perhaps the carbohydrate-binding step is common to all Reg members, with effector mechanisms diverging downstream of this event.

In this context it is noteworthy that trypsin cleavage of Reg, which was discovered some years ago was reported to convert the previously soluble protein into a form that precipitates above pH6.5 and forms oligomeric fibrillar structures that combine into quadruple-helical filaments (QHF). It was also suggested that the small Reg prosegment inhibits formation of these fibrils. It was furthermore observed that rRegI is actually cleaved at the conserved trypsin site under conditions that rule out the presence of trypsin and other proteases thus suggesting that Reg may be subject to self-proteolysis at this site (Cerini, Peyrot et al. 1999) (Schiesser, Bimmler et al. 2001). Reg fibril formation may have profound consequences in degenerative processes in the central nervous system. These fibrils were found to be present in the pathological lesions of Alzheimer's disease and hRegIα was found to be overexpressed during very early stages of the disease before clinical signs appear (Duplan, Michel et al. 2001). Furthermore hRegIα (lithostathine-1) deposits were also observed in the blood vessels and in focal and multicentric plaques in the cerebellum of patients with Creutzfeldt-Jacob disease (Laurine, Gregoire et al. 2003). The authors of these studies suggest that Reg fibrils might represent a common component of amyloid deposits and may be a component of the material that coats amyloid fibrils observed clinically. This raises the question as to whether Reg could play a role as a link between brain injury and the later onset of neurodegenerative diseases. If indeed this protein possesses the ability to undergo a process of self-proteolysis promoting the tendency to fibril formation then overexpression of Reg members in response to traumatic brain injury, which is well-documented as discussed below, might under certain circumstances contribute or even act as trigger for subsequent degenerative processes.

To return to the bactericidal properties of Reg: fibril formation might actually be a process that contributes to its antibacterial effect. In such a scenario binding of Reg to bacterial carbohydrates would provide an anchorage and would be followed (or accompanied) by tryptic activation, which then leads to the formation of a Reg fibrillar coat on the surface of the bacteria. This coat could either inhibit bacterial movement or 'plug' channels in the bacterial cell wall or possibly cause perforation of the cell wall. A way to investigate this hypothesis would be to test if drugs that can dissociate Reg fibrils into individual protofilaments can actually reverse the bactericidal effect of Reg. Two remarks should end this section. The loop 1 of the C-type lectin domain that contains the EPN motif in mRegIIIγ and hRegIIIα, described above, overlaps to a large extent with the peptide of INGAP reported to stimulate islet neogenesis (although there is no EPN motif in INGAP, see Fig. 2). Thus the intuition of the discoverer of this peptide was correct in so far as the loop 1 indeed corresponds to "a core of potential biological activity". Although it should be added that a loop that has been cut at both ends no longer corresponds to a loop. These findings, together with the discovery by Christa et al. in 1994 that hRegIIIα (HIP/PAP) acted as a C-type lectin that bound to the disaccharide lactose (Christa, Felin et al. 1994) indicate that Reg proteins can bind carbohydrates despite the absence of the canonical binding motifs found to be essential for this task in other C-type lectins. This underlines the consideration that the rules governing carbohydrate binding in Reg C-type lectins are more complex than we currently understand.

5.2 Reg as regenerative factor in the nervous system and modulator of inflammatory responses

Involvement of the Reg family of proteins in nerve cell regeneration was observed while screening for genes overexpressed in an rat model that investigates motor neuron regeneration after crushing of the sciatic nerve. The authors write: "Within 24h of crushing the sciatic nerve, all motor neurons and a subpopulation of sensory neurons express high levels of rReg-2 (rRegIIIβ/PAP-1) mRNA and protein. This again exemplifies the standard process of discovery for this family. The authors observed that migration of rReg-2 along growing motor and sensory axons occurs via orthograde transport and that rReg-2 acts as mitogen to Schwann cells, the cells that create the support on which neuronal regrowth can take place. Intraneural injection of an antiserum, which blocks rReg-2 greatly reduced regeneration of neurons. Interestingly, the authors found that recombinant rReg-2 alone had only a weak mitogenic effect on Schwann cells. However this effect could be enhanced by addition of forskolin, an activator of adenylate cyclase. It should be noted that the discovery of rRegI in a model of islet regeneration mentioned before involved the treatment of rats with nicotinamide, an inhibitor of 3'-5' cAMP phosphodiesterase. Thus forskolin and nicotinamide both increase the intracellular levels of cAMP leading to an amplification of Reg-induced mitogenic effects. The authors mention that enhancement of the mitogenic effect by forskolin is also seen with other Schwann cell mitogens such as glial growth factors and platelet derived growth factor. There it is attributed to cAMP-induced increase in receptor expression for those factors. In an attempt to trace the signal that induced overexpression of rReg-2 the authors investigated the effects of peripheral axotomy on Reg-2 expression in the neonatal period when motor neurons are particularly sensitive to this intervention. The sensitivity to axotomy is ascribed to the interruption of retrograde transport of peripherally-derived neurotrophic factors. Indeed they found that the intervention greatly reduced constitutive Reg-2 expression levels compared with uninjured motor neurons. This finding indicated that Reg-2 expression in developing motor neurons was dependent on contact with peripheral targets, which act as source of neurotrophic factors. The authors argued that these factors might be leukemia inhibitory factor or ciliary neurotrophic factor (LIF/CNTF) because these proteins are related to the IL-6 family of cytokines and rReg-2 and other Reg gene family members have IL-6 responsive elements in their promoter region. They could show that targeted disruption of gp130, which is a common component of the receptors for IL-6, LIF, CNTF and CT-1 indeed abolished Reg expression in developing motor and sensory neurons of the mutant mice thus demonstrating that the expression of the mouse Reg protein(s) at this site is dependent on the cytokines of the LIF family acting through the LIF receptor (Livesey, O'Brian et al. 1997).

This study was expanded subsequently by another group of scientists who showed that CNTF-related cytokines could induce rReg-2 expression in cultured motor neurons, which in turn acts as an autocrine/paracrine neurotrophic factor for a subpopulation of motor neurons by stimulating a survival pathway involving phophatidylinositol-3-kinase, Akt kinase and NF-κB. They also demonstrated that rReg-2 expression *in vivo* is controlled on a cell-by-cell basis and that not all neurons that have the LIF receptor express Reg-2. Since, by the time this report was prepared, the EXTL3 islet Reg receptor had already been identified the authors also performed rtPCR on motor neurons cultured with CNTF but failed to find mRNA for EXTL3. This resulted in the conclusion that "the Reg-2 receptor in motor neurons

probably remains to be identified" (Nishimune, Vasseur et al. 2000). Over a decade later this receptor has still not been identified. In our opinion this is related to the little-appreciated fact that Reg proteins are lectins and therefore bind carbohydrates. Both studies presented again show pleiotropic effects of Reg impacting on the one hand Schwann cell mitogenesis and on the other hand motor neuron survival.

Before leaving this area two additional studies on the effects of Reg in the nervous system should be mentioned. The first provides an example of the observation that Reg family members are seldom found alone while the second introduces yet another role for mRegIIIβ, which has been identified as a potential bactericidal lectin, an islet regeneration factor and an inflammatory modulator in pancreatitis. The first study used a model that induces traumatic brain injury (TBI) by weight drop and then studied the expression of rat Reg members by quantitative real-time PCR and in situ hybridization in the cerebral cortex. This study reported a low level of rReg-2 (rRegIIIβ/PAP-1) and of rRegIIIγ (PAP-III) mRNA in normal animals. Upon surgery and TBI the mRNA levels of both rat Reg members increased dramatically. First rReg-2 mRNA increased 12hrs post TBI and remained elevated until day 5 post TBI. This was followed, at 24 hrs post TBI, by rRegIIIγ mRNA with a sharper temporal peak persisting until day 3 post TBI. The mRNA level of rReg-2 was also somewhat elevated in the cortex contralateral to TBI and the sham-operated ipsilateral side (Ampo, Suzuki et al. 2009). Two points here are relevant. First, low levels of Reg mRNA are detected in normal cortex, and as mentioned above, more than one Reg member is expressed. As shown in Fig. 3 this also correlates to the situation in pancreatic islets and would suggest redundancy due to overlap in the effector mechanisms mediated by different Reg members and a role for these proteins not only in response to stress but also under normal physiological conditions.

The second study described the generation of a mouse model in which the exons 2-5 of the gene encoding mRegIIIβ had been deleted and replaced with a lacZ reporter selection cassette. These mice were phenotypically indistinguishable from wild-type or heterozygous littermates. Interestingly, the knockout mice exhibited marked elevation of expression of mRegIIIα suggesting that the Reg system is designed to contain redundancies where one Reg member to some extent compensates for loss of another. The authors of this study provide evidence that, as in the case of rReg-2, expression of mRegIIIβ is restricted to certain neuronal populations and is also developmentally regulated. Peak RegIIIβ expression was observed on postnatal day 4 and had disappeared from all areas of the brain and spinal cord by postnatal day11. mRegIIIβ deletion did not affect the total number of motor neurons in the facial motor nucleus, suggesting that no neuronal cell death had resulted from the loss of mRegIIIβ. As in the aforementioned knockout studies, it was the application of a noxious stimulus that clearly revealed the effect of the knockout. When the scientists severed the facial motor nerve at postnatal day 3.5 and applied either saline or CNTF to the cut end of the nerve, more CNTF-treated motor neurons survived in the wild-type mice than in the knockout mice where CNTF treatment showed no improvement in survival over saline (Tebar, Geranton et al. 2008). In the latter case, as in pancreatic islets, knockout of a single Reg member causes no or only mild effects under normal physiological conditions. Only when a noxious stimulus is applied do the effects of the knockout become visible. As described above we attribute this to the presence of compensatory effects induced by other Reg members leading to saturation of the system under normal conditions, hence our

advocacy of a multimember Reg knockout model and experiments designed to demonstrate the redundancies predicted to exist within the Reg family.

The neuronal role of Reg also extends to effects on macrophages. We previously mentioned publications that investigated the role of rReg-2 (rRegIIIβ/rPAP-1) and of mRegIIIβ in the neuronal context. As was to be expected more than one Reg member is involved in this process. rRegIIIγ is not only overexpressed in the cerebral cortex in response to traumatic brain injury as briefly mentioned above but also in the Schwann cells upon nerve injury (Namikawa, Fukushima et al. 2005). Injuries to axons in the peripheral nervous system induce the degeneration of distal axons, a process, which is accompanied by cellular responses. Among these responses the most striking is the invasion of the degenerating nerve by macrophages. They clear debris consisting of myelin components that inhibits axonal growth and secrete a number of soluble factors that can stimulate axonal growth. The influx of macrophages is ascribed to attractants that are released from the Schwann cells, which have undergone dedifferentiation as a consequence of loss of axon-Schwann cell contact. A number of such attractants have been described and include LIF and the monocyte chemoattractant protein-1 (MCP-1). Using a two-chamber cell migration assay Namikawa et al. could show that rRegIIIγ is equally effective as MCP-1 in promoting macrophage migration. The scientists produced a dose-response curve that showed an increase in the macrophage migration index at concentrations of rRegIIIγ between 0.1 and 10ng/ml. Doses above 10ng/ml did not further increase this index but rather led to a decrease with the effect completely disappearing at 500ng/ml, therefore revealing an effective concentration range typical for cytokines and chemokines. The authors also demonstrated that rRegIIIγ knock-down achieved with siRNA introduced into rat nerves by adenoviral gene transfer reduced the capacity of explanted nerve segments distal to the injury to attract macrophages (Namikawa, Okamoto et al. 2006). However, attraction is not the only effect on macrophages ascribed to Reg. rRegIIIα (PAP-2) is also able to modulate the inflammatory response in these cells. Viterbo et al. showed migration of macrophages to beads coated with rRegIIIα with subsequent agglutination. They also demonstrated that this Reg member binds to macrophages, but do not reveal whether this occurs via EXTL3 the islet Reg receptor, or via a different receptor. (We suggest that it occurs via carbohydrate binding). They then measured, by real-time PCR and ELISA, a range of cytokine responses in the macrophages during exposure to rRegIIIα. They reported upregulation of mRNAs for IL-1β, IL-6, TNFα and IL-1α most likely via the NF-κB pathway (Viterbo, Bluth et al. 2008).

Considering the roles of Reg introduced here the general conclusion may be drawn that Reg proteins are functionally placed well within the large family of endogenous lectins of which C-type lectins are a subgroup (Mascanfroni, Cerliani et al. 2011; Toscano, Ilarregui et al. 2007). These endogenous glycan-binding proteins have been implicated in a wide range of functions including first-line defense against pathogens (bactericidal effect of Reg) cell-trafficking (macrophage attraction of Reg), immune regulation (cytokine responses induced by Reg) neoplastic transformation (Reg expression in liver cancer). Reg is unique among the C-type lectin family in that it consists of a secreted lectin domain only. This feature makes its distribution wider than that of C-type lectins, which constitute part of receptors in the cell membrane. It is therefore possible that Reg can also act as a modulator of the interactions between lectins in receptors and their carbohydrate ligands (van Vliet, Garcia-Vallejo et al. 2008).

6. Reg as autoantigen in T1DM

Returning to the endocrine pancreas we now focus on studies performed in our laboratory that investigate the possibility that Reg might act as an autoantigen in T1DM. The insight that the pathogenesis of T1DM is driven by a disorder of the immune system has been well established by numerous animal experiments and clinical observations. That certain MHC alleles are associated with an increased risk of developing T1DM, that antibodies are present against β-cell self antigens and that lymphocyte infiltrates are observed in the endocrine pancreas all point to the adaptive immune system, i.e. B- and T-cells, as necessary elements in the process leading to islet damage/destruction and thus insulin dependency. Although insulin has received much attention in the search for potential cellular targets of the autoimmune process it has become evident that it is not the only β-cell component to which self-reactivity exists in T1DM. Our initial interest in the Reg proteins arose following our observation that one of its members, hRegIIIα, (HIP/PAP) was overexpressed in the remaining pancreatic islets of a patient who had died shortly after onset of the disease. This raised the question as to whether a vicious circle could become operational in a situation where a protein that becomes overexpressed as a result of the inflammatory process in the pancreatic islets, subsequently functions as an autoantigen (Gurr, Yavari et al. 2002). We therefore investigated if such a situation could occur during the pathogenesis of T1DM using the NOD mouse as model. We first defined Reg members present in mouse islets and found RegIIIα as well as RegII and, in more heavily infiltrated islets, also RegIIIδ. RegIIIα expression was restricted to non β-cells of the islets while RegII was found throughout the endocrine pancreas (Fig. 3). In the NOD model of T1DM autoantigens such as insulin and glutamic acid decarboxylase have been used as vaccine antigens to prevent or delay the onset of the disease. If a Reg member acted as autoantigen it might also have these properties and we therefore tested both RegII and RegIIIα as vaccines. While we could not prevent or even delay T1DM with RegIIIα we found a clear preventive effect with RegII vaccination. To ascertain that this effect was due to an immune-mediated process and not to the potential effects of Reg on islet regeneration we cleaved RegII into two fragments. The N-terminal fragment (NtfrII, residues 22-75) contained the first three cysteine residues of the Reg C-type lectin domain and the C-terminal fragment (CtfrII) contained the remainder of the protein (see Fig. 2). This, we argued, would inactivate possible direct effects of RegII on islet regeneration while retaining the function of Reg as autoantigen. On testing the two fragments we found that vaccination with the N-terminal fragment actually accelerated disease whereas vaccination with the C-terminal fragment delayed disease. This delay was more pronounced than that achieved with the full-length protein. In addition, it could be induced with late stage vaccinations, that is, delaying vaccination until shortly before onset of T1DM in NOD mice. We next demonstrated by adoptive transfer experiments that the clinical effects were mediated by T-cells. Both CD4+ and CD8+ T-cells from donors vaccinated with the N-terminal fragment could transfer disease to NOD-SCID mice - a strain that lacks B and T-cells and therefore does not develop T1DM. Similar experiments with T-cells obtained from donors vaccinated with CtfrII showed that CD4+ T-cells from these mice could delay the onset of T1DM induced by diabetogenic T-cells. Taken together these experiments clearly demonstrated that the clinical effects seen after RegII vaccination were in fact immune-mediated and not a result of a direct effect of RegII on islet regeneration. They also identified the N-terminal fragment of RegII as the part of the protein that contained T-cell epitope(s) able to activate autoreactive T-cells, whereas it appeared that vaccination with the C-terminal fragment had

activated T-regulatory cells. We had thus established that RegII could act as an autoantigen in T1DM of NOD mice (Gurr, Shaw et al. 2007). Another group had demonstrated in the meantime that 24.9% of patients with T1DM, 14.9% of patients with type 2 diabetes and 2.7 % of control subjects had autoantibodies against RegIα, thus confirming that Reg can also become a target of the autoimmune response in humans (Shervani, Takasawa et al. 2004). To add the second component of the vicious circle postulated above we had also shown that isolated human islets, when incubated with IL-6, secreted increased amounts of hRegIIIα (PAP/HIP). In a pancreatic islet under autoimmune attack this cytokine might be released by infiltrating macrophages leading to overexpression of Reg. Reg in turn then, since it acts as autoantigen, and as attractant for macrophages, as discussed above, enhances the inflammatory process thus leading to further overexpression and the operation of a vicious circle. However, we wanted to know if this process actually occurred *in vivo*. Autoantigens drain from the islets to the pancreatic lymph nodes where they are processed by antigen-presenting cells to generate peptides for display in the context of MHC molecules. Self-reactive T-cells are activated by these complexes, proliferate and migrate to the islets where they exert their deleterious effects. Since NtfrII activated self-reactive T-cells it had to contain one or more T-cell epitope(s). If at any time during the pathogenesis of T1DM a vicious circle operated then an increase in the NtfrII peptide MHC complexes should occur in the pancreatic lymph nodes. This should be followed by an increase in proliferation of T-cells specific for NtfrII. NOD mice only have one classII MHC molecule, namely I-A^{g7}. We therefore generated an antibody, termed D9 that was able to detect an NtfrII-derived peptide in context of I-A^{g7}. This antibody was phage-displayed, that is it was expressed, fused to a coat protein, on the surface of bacteriophages. The DNA strand carried by these phages encodes not only the antibody but also an antibiotic resistance gene. When bacteria are infected the phage DNA is transmitted and renders the bacteria resistant to the antibiotic. This allows estimation of the number of phages (colony forming units) in a given sample by infecting non-resistant bacteria with an aliquot of the phage-containing sample, spreading them out on an antibiotic containing agar plate and then counting the colonies that have grown after overnight incubation. This unique feature of phage-display allows the isolation of an antibody with a given specificity by selection of an antibody library constructed from appropriate source material on a given antigen. Non-binding phages are washed away while binding phages are retained and then eluted. Eluted phages serve to infect bacteria, producing a new batch of phages, which is then subjected to a new round of selection further amplifying specific binders. We used this technology to isolate antibody D9. In this case the antigen used for selection consisted of NOD antigen-presenting cells pulsed with NtfrII. The sequence of the NtfrII peptide recognized by D9 in context of I-A^{g7} was identified by pulsing antigen- presenting cells of NOD mice with short peptides covering NtfrII and analyzing if the pulsed cells could be stained with D9. We also determined that the complex formed by this peptide in context of I-A^{g7} NtfrII could activate autoreactive CD4^{+} T-cells. The complex therefore represented the link connecting Reg overexpression with the Reg specific immune attack on islets.

In order to establish a temporal profile of the I-A^{g7} NtfrII peptide complexes we collected pancreatic lymph nodes of NOD mice at different ages, prepared single cell suspensions and exposed them to the antibody. After washing, bound phages were detached, bacteria were infected and a colony count was obtained. As a control we used a mutated version of the antibody D9, which had a deletion in its heavy chain and only marginally bound to NOD antigen-presenting cells pulsed with NtfrII. The numbers of T-cells in the pancreatic lymph
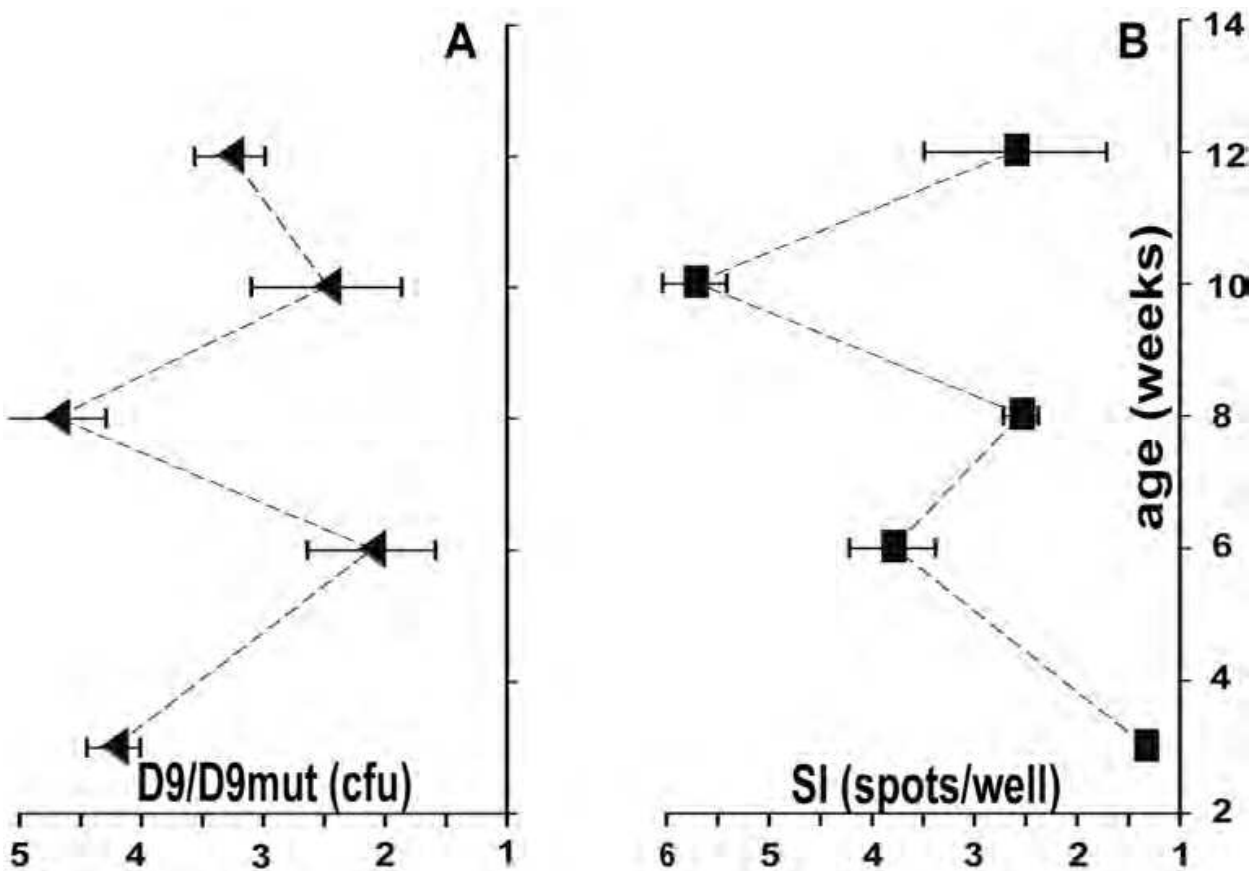
Fig. 4. Temporal profile of I-A^{g7} NtfrII peptide complexes (A) and spontaneous T-cell responses (B) in the pancreatic lymph nodes of NOD mice during the pathogenesis of T1DM. The y-axis in (A) displays the ratio of colony forming units (cfus) obtained with a binding antibody (D9) to colony forming units obtained with a mutated, non-binding antibody (D9mut); the y-axis in (B) displays the ratio (SI=stimulation index) of the number of spots obtained when cells of the pancreatic lymph nodes were incubated with NtfrII to the number of spots obtained when cells of the pancreatic lymph nodes were incubated without antigen (ELISPOT assay for IL-2). Both profiles show the same biphasic pattern but the T-cell pattern is shifted by two weeks i.e. the T-cell response in the pancreatic lymph nodes follows the temporal pattern of the I-A^{g7} NtfrII peptide complexes.

nodes that responded to stimulation with NtfrII were also counted. On plotting both curves (NtfrII peptide I-A^{g7} complexes and number of T-cells reactive for NtfrII) we observed that both curves had a biphasic profile and that the 'T-cell' curve followed the 'complex' curve with a delay of about two weeks (Fig. 4 A and B). This biphasic profile observed would clearly rule out the continuous operation of a vicious circle but suggests a temporary operation between 6 and 10 weeks of age. It also hints at the tight control of Reg expression. Reg does appear to become overexpressed in response to islet inflammation as manifested by the increase of I-A^{g7} NtfrII peptide complexes in the pancreatic lymph nodes. However, beyond a certain degree (or duration) of the inflammatory process (at ages >8 weeks Fig. 4A) this response ceases and the islets appear to actually reduce Reg expression. The existence of such control mechanisms can also be inferred from the observation that Reg acts as a macrophage chemoattractant. In this case an additional vicious circle may be constructed, involving IL-6 released from macrophages, which leads to up-regulation of Reg

and more influx of macrophages. However, as demonstrated by the discoverers of the chemoattractant properties of Reg, the dose response curves do not level off but the attractant effect disappears above a certain Reg concentration. These observations exemplify two main levels of control that must be considered for the understanding of Reg action. One of the control levels is represented by the limits, which the target system imposes on Reg action - chemoattractant effect on macrophages - and the other by the limits, which the source system imposes on Reg production or release – I-A^{g7} NtfrII peptide complexes in pancreatic lymph nodes of NOD mice.

7. Concluding remarks and future research directions

The Reg family of proteins has been studied for over 25 years and today all mouse and human Reg members have been identified. However to date there is no unifying theory of Reg action and therefore the large body of work on the effects of Reg in different physiological systems remains a collection of unconnected observations. While it is important to accumulate more information on the role of individual Reg members, a framework needs to be developed within which this information can be placed and correlated. Such a unifying theory should be able to explain the seemingly very different effects induced by Reg such as, for example, Schwann or β-cell mitogenesis and bactericidal effects. Currently no such theory exists but its basic components should involve carbohydrate binding based on the fact that the unifying structure of the Reg family is a C-type lectin fold. It should additionally encompass fibril formation and possibly EXTL3 binding as well as up-regulation of Reg under a wide range of noxious stimuli (cytokine-mediated or other). Extracellular matrix remodeling might also play a role. These components then need to be placed and understood within the framework of regulatory constraints imposed by the systems impacted by Reg as well as by those that generate Reg. In this review we have drawn attention to areas of Reg research that introduce and exemplify some of these components. Development of a theory of Reg action would require a change in experimental approach away from 'what particular function is conveyed by a particular Reg member in a specific system?' to comparative studies concerned with 'what is common to all Reg members, how do they differ and what is the origin of these differences?' implemented with consideration of the basic components of the theory as outlined above. Consequently we argue that a model in which not one particular member but an entire subfamily or all Reg members have been knocked out would greatly further the development of this unifying theory. Such models might allow the characterization of the effects of Reg under normal conditions by eliminating the 'masking effects' of the likely redundancies that occur within the family. How the genomic organization of the Reg family would facilitate generation of these models is mentioned above. We are confident that they would also reveal that Reg proteins are necessary for islet (re)generation.

As far as the properties of Reg as autoantigen are concerned we are in the process of performing studies that evaluate if the antibodies specific for the Reg peptide MHC complexes generated in our laboratory may be useful as therapeutic agents for the treatment or prevention of T1DM. An important aim in the prevention and treatment of T1DM is the development of a specific immunotherapy, that is an intervention that targets and blocks/eliminates specifically self-reactive T-cells but does not affect T-cells, which are necessary for the normal functioning of the immune system. This aim can be achieved by intervening with the process that generates specificity in the immune system, namely the

binding of the T-cell receptor to its antigen, which is the complex formed by the T-cell epitope and the presenting MHC. An antibody that recognizes this complex, such as D9 introduced here, might interfere with the activation of the self-reactive T-cell recognizing the same complex and could represent a potential means to achieve specific immune suppression. A variant of this approach is the active vaccination with the receptor of a self-reactive T-cell in order to generate an immune response that targets only T-cells with this receptor but not others. This approach is known as anti-idiotypic vaccination and has been tested with some success in models of other autoimmune diseases. Which of these approaches might actually be practicable and effective in the treatment of T1DM will be the focus of future studies in our laboratory.

A successful therapy for T1DM will likely have two main components; one designed to support or enhance islet protection/regeneration and the other to control the aberrant immune response. Although more widely studied autoantigens like insulin or glutamic acid decarboxylase might be more suitable for addressing the control of the aberrant immune response, Reg is unique in that it represents an islet component that can play a role in relation to both therapeutic main approaches through its impact on islet regeneration on the one hand and its role as autoantigen on the other hand.

8. Acknowledgment

9. References

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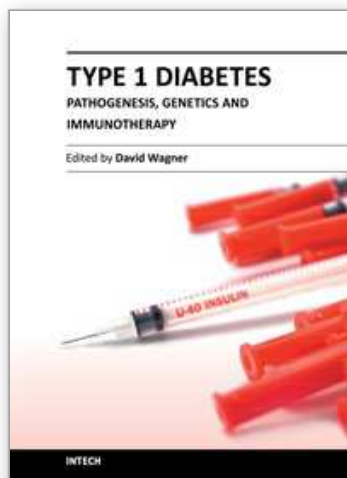
Type 1 Diabetes - Pathogenesis, Genetics and Immunotherapy

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This book is a compilation of reviews about the pathogenesis of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes. Areas including T cell development, innate immune responses, imaging of pancreata, potential viral initiators, etc. are considered.

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